Duarte Nuno Vieira • Anthony Busuttil
Denis Cusack • Philip Beth
Editors

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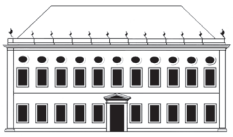

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MISCELLANEOUS

CLINICAL FORENSIC MEDICINE AND DOMESTIC VIOLENCE

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NOTE FROM THE PRESIDENT OF THE IALM

Founded in 1938, in Bonn (Germany), the International Academy of Legal Medicine (IALM) is the oldest and most prestigious scientific association with international scope in the sphere of Legal Medicine and the Forensic Sciences. Its membership is drawn from every continent, with professionals and academics who are directly and indirectly involved in the various areas of forensic expertise.

Its main aim is to further scientific progress in the field of Legal (Forensic) Medicine, especially by promoting cooperation and information exchange among specialists on an international level.

One of the many initiatives promoted by the IALM is the triennial scientific conference, a meeting point where professionals and academics can exchange experiences, engage in scientific discussion, share ideas and experiences, and keep in touch with the latest theoretical, technological and scientific advances. Many of the papers presented are subsequently published in the conference proceedings entitled *Acta Medicinae Legalis et Socialis*, consisting of one or more volumes.

The 21st IALM Conference took place in May 2009 in Lisbon. It was a resounding success, involving the participation of 1278 professionals and academics from 78 countries of the five continents. In addition to keynote lectures by some of the most highly-respected forensic specialists in the international scene, over 600 scientific works were presented in the form of conference papers and posters. Many of these papers have since been published in specialist journals, including the International Journal of Legal Medicine, the official organ of the IALM, published by Springer. Despite this, we would like to continue the tradition (which dates back to the very origins of IALM) of compiling and publishing a book containing papers by authors that wish to divulge their work in this way.

We would like to take the opportunity to extend our sincerest thanks to all those who participated in the 19th IALM Conference and presented there the results of

their professional experiences, research and reflections, and particularly to authors that decide to publish their work in this book.

We are sure that this volume will provide a stimulating read, which will not only contribute to the ethical principle of ongoing professional training, but will also encourage you to participate in forthcoming IALM conferences and to present there the fruits of your future work, hopefully to publish them in the *International Journal of Legal Medicine* and in further editions of the *Acta Medicinae Legalis et Socialis*.

Duarte Nuno Vieira
President of IALM

FORENSIC ANTHROPOLOGY

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PYROLYSIS GAS CHROMATOGRAPHY – MASS SPECTROMETRY ANALYSIS FOR THE ESTIMATION OF PIG BONE AGE FOR FORENSIC APPLICATION

Abstract: The potential of pyrolysis gas chromatography-mass spectrometry (Py-GCMS) as a tool for the estimation of post-mortem age of bones has been investigated. Pig bone specimens prepared under controlled burial conditions in soil were studied and the post-mortem ages ranged from 3 to 48 months. Notable differences were observed in the data produced for younger bone specimens (<1 year) compared to specimens of greater post-mortem age (>1 year). Py-GCMS also demonstrates a relationship of particular peak ratios with the age of bones. The ratios of peaks in the pyrograms were examined and it was demonstrated that the ratios of certain pairs of peaks increase as the bone age increases.

Introduction

Bone is a complex composite material consisting of approximately 10% water, 30% organic phase and 60% inorganic material. Although the chemistry of archaeological bones has been more widely studied, the structures of lesser aged bones such as those that are encountered in a forensic context, have not been as well studied. However, in a recent study, it was demonstrated that changes to relatively young bones can be detected using techniques that examine the decomposition of the organic phase of bone [1,2]. Thermogravimetric analysis (TGA) combined with mass spectrometry (MS) was used to examine young pig bones and demonstrated that TG-MS is a technique sensitive to the age of the bones and the environment in which the bones decomposed.

Pyrolysis gas chromatography – mass spectrometry (Py-GCMS) is established as an effective technique for the characterisation of complex organic molecules. This technique has the added attraction for forensic practitioners of being a cost-effective method that provides good discrimination, involves minimal sample preparation and is able to detect very small quantities of material. Py-GCMS is recognised as a useful tool in particular areas of forensic analysis, including automotive paints [3], tire trace analysis [4,5], adhesives [6,7], lubricants [8] and fingerprints [9]. One study that used GCMS to examine the pyrolysis products of animal bone found that the major decomposition products are nitriles, pyridines, pyrroles and amides [10].

In the current study, Py-GCMS was used to pyrolyse the organic phase of the bone and compare the pyrolysis products of the bone samples. A series of pig bones of different post-mortem ages were studied and changes to the data associated with age were investigated.

Materials and methods

Bone samples

Bone specimens with post-mortem ages ranging from 3 months to 7 years were obtained from pigs buried for different time periods. All samples were flat rib bones from female pigs (*Sus scrofa*) sourced from the same farm with an identical diet and weighing 40-45 kg. The carcasses were buried in soil at 60 cm below the surface for the designated amount of time. The average ambient temperature was 25°C and a soil pH 5. After exhumation the bones were stored in sealed in plastic bags at 4°C prior to analysis.

The compact bone specimens were cleaned by scraping of the surface with a scalpel to remove fatty bone marrow from the interior and any residues from the exterior of the specimens [11]. The bones were mechanically sliced using a Buehler IsoMet low speed diamond saw into 2 mm thickness slices. Each sample was dried in a vacuum oven at 50°C for 2.5 h to remove moisture. The slices were then cut in half using a scalpel.

Pyrolysis GCMS

Pyrolysis was carried out using a Shimadzu Furnace Pyrolyser-4a set at 450°C. The pyrolyser was mounted on the split injector of a Shimadzu GC-17A gas chromatograph coupled to a Shimadzu GCMS-QP5050A mass spectrometer. The chromatographic column used was a J & W Scientific DB-5MS column, 60 m in length with an internal diameter of 0.25 mm. Data analysis was performed using Shimadzu Class-5000 software and the mass spectra were identified using a NIST62 library database.

Results and discussion

A comparison of the results of replicates of the same sample and different bone samples of the same age was carried out in order to determine the reproducibility of the pyrograms. The retention times, peak patterns and the compound represented by each peak were compared and found to be very similar in each run of same age samples.

Figure 1 illustrates the pyrogram of a bone sample of age 3 months and shows the characteristic pattern observed by the younger bones studied. All pyrograms show a large peak at a retention time of 1 min. Prominent peaks are also observed in the 17-25 min region. In addition, the younger samples have significant peaks in the 29-30 min region. The peak at 20 min is a single peak in the younger samples.

A comparison of the peak patterns of the pyrograms of different bone age samples demonstrates a difference between the patterns of the younger bones (< 1 year) and those of the older bones (> 1 year). Figure 2 shows the pyrogram of bone of age 48 months and illustrates the pattern difference due to age. The older samples do not

have significant peaks in the 29-30 min region. The peak at 20 min is also split into two components for samples of greater age. Based on a comparison with the library database, the splitting peak appears to represent the presence of two organic compounds, pentanal and 1,1,3,4-tetramethylcyclopentane, eluting at very close retention times.

The data were normalised by calculating the ratios of significant peaks and any trends were observed. The peaks near 20 min were selected as it showed changes in its splitting pattern. The peaks at 19, 22 and 23 min demonstrate observable changes in peak heights with relation to age. In each pyrogram, the height of the peak at 19 min was divided by the height of the peak at 20 min to obtain a peak ratio. Similarly, the height of the peak at 22 min was divided by the height of the peak at 23 min to obtain a second peak ratio. The peak ratios with their corresponding post-mortem ages are presented in Figure 3. This plot demonstrates that there is a relationship between peak ratios and age, and as age increases, so do the peak ratios.

Conclusions

For all samples studied using Py-GCMS, the most prominent peaks occur in the 17-25 min region. Only the younger samples showed significant peaks in the 29-30 min region. A peak at 20 min is a single peak in the younger samples, but shows splitting in the older samples. A comparison of particular peak ratios with age demonstrates that as post-mortem age increases, so do the peak ratios.

There is potential for this technique to be used for the estimation of post-mortem age of bones. Further work is being carried out to develop the potential of this technique. A detailed investigation of the reproduction of the data is being undertaken. Analysis involving pattern recognition of the data is being carried out in order to establish a simple method of determining forensic bone age.

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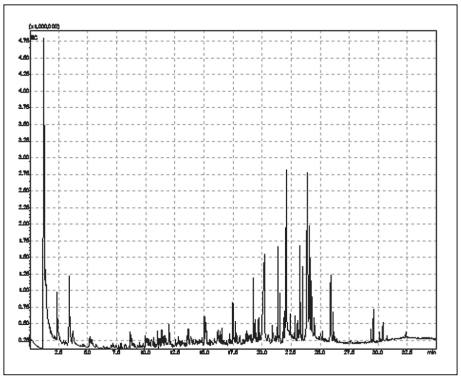

Figure 1 – Pyrogram of bone aged 3 months.

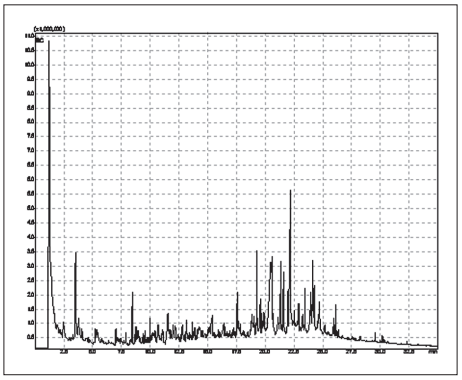

Figure 2 – Pyrogram of bone aged 48 months.

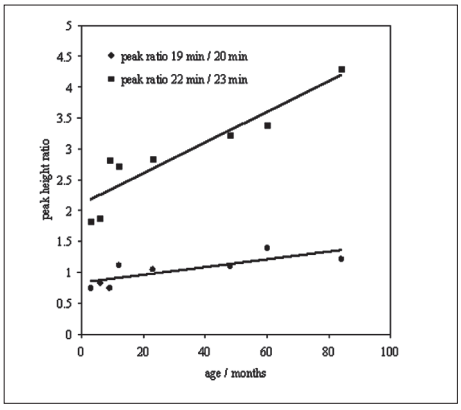

Figure 3 – Peak height ratios as a function of post-mortem age.

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ADIPOCERE FORMATION IN A RIVER ENVIRONMENT

Abstract: Adipocere is a late post-mortem decomposition product that consists of a mixture of fatty acids. The rate of formation of adipocere in a river water environment has been monitored. Adipocere formed from pig adipose tissue has been analysed using gas chromatography – mass spectrometry (GCMS) in order to determine the fatty acid composition, and hence, the degree of formation in the different environments. The study shows that the rate at which adipocere forms depends on the type of aqueous environment.

Introduction

Adipocere is a grey-white waxy substance that may be observed late post-mortem. The appearance of adipocere is of interest as it may slow the decomposition process or preserve human remains. Adipocere is observed in bodies found in aqueous environments and it is often noted that a moist environment is conducive to the formation of adipocere [1-4]. However, little work has been reported on the characterisation of adipocere formed in aqueous environments. An understanding of the composition of the adipocere formed can provide an indication of the extent of formation of this decomposition product and, thus, the effect of the aqueous environment on its formation.

Adipocere is mainly composed of a mixture of fatty acids, predominantly palmitic and stearic acids, as well as hydroxy fatty acids and salts of fatty acids [5-9]. Adipocere initially results from the conversion of adipose tissue into unsaturated fatty acids and then into saturated fatty acids. Previous studies have involved the analysis of the composition of adipocere formed in soil environments and demonstrated that the formation may be monitored by analysing the fatty acid composition of adipocere [10-14]. Gas chromatography – mass spectrometry (GCMS) has been utilised to identify the fatty acids present in adipocere. More recently, a modified GCMS method has been successfully employed to analyse samples formed in aqueous environments [15].

In the present study, an investigation into the formation of adipocere in pig tissue in a river environment has been carried out. GCMS was employed to determine the fatty acid composition, and hence, the degree of formation of each adipocere sample.

Materials and methods

Adipose tissue from pigs (*Sus scrofa*) was obtained from a retail butcher. A 10 cm x 10 cm piece of tissue collected from the abdominal region containing muscle and skin was used for each experiment.

For the laboratory model studies, 5L high density polyethylene sealed containers were used. The containers were acid-washed with 5 % v/v nitric acid, rinsed with distilled water and air dried. A basket of low density polyethylene was constructed to contain the adipose tissue and was suspended from the lid of the container to surround the tissue by solution. Three replicates of each set-up were prepared and stored at 20°C. 1 g of pig faecal material was smeared onto the skin surface of the tissue to ensure the necessary bacteria to promote putrefaction. Samples of pig adipocere were collected from random sections of the tissue over a period of 18 months at 3 monthly intervals. The collected samples were placed in sealed specimen containers, homogenised and stored at –18°C until analysis.

The field study site was a freshwater river located in Laggan, New South Wales, Australia. The sampling containers were similar to the model adipocere containers, but holes of approximately 0.5 cm in diameter were inserted throughout the surface and weight added to maintain submersion. The containers were covered in netting to exclude animals and the containers were secured to posts on the riverbank (Figures 1 and 2). Five repeats were created, which were spaced at regular intervals at the site. The containers were placed in the deepest parts of the river to ensure the containers were submerged for the entire sampling period. Samples were collected from the field trial over 18 months at 3 monthly intervals. The river laboratory model used water obtained from the field site.

For analysis, 5mg of each adipocere sample was placed in 5 ml hexane, sonicated for two 10 min sessions and centrifuged (2500 rpm, 5 min). The supernatant was aspirated and frozen until analysis. The samples were analysed using GCMS according to the procedure detailed by Notter *et al.* [15]. The fatty acids examined were myristic, palmitoleic, palmitic, linoleic, oleic, stearic and 10-hydroxy stearic acids.

Results and discussion

In order to observe any trends in the data, the ratios of the concentrations of the unsaturated (palmitoleic, linoleic, oleic acids) to saturated (myristic, palmitic, stearic, 10-hydroxy stearic acids) fatty acids were determined for each sample using GCMS. From such a ratio, an estimation of the stage of formation of adipocere can be made. An earlier investigation divided the process of adipocere formation into three stages based on the average % of saturated fatty acids present: early (40-60%), intermediate (70-90%) and advanced (>90%) [16]. The estimated unsaturated / saturated fatty acid concentration ratio for each stage used in this study was: early (1.17), intermediate (0.15) and advanced (0.05).

Figure 3 shows the fatty acid ratios for each collected specimen at the different sampling intervals. Inspection of the control values shows that the fatty acid ratio decreases with time and even at 3 months, the adipocere is in an intermediate stage of formation. A comparison of the control values with those of the model river system reveals that the decrease in ratio occurs earlier in the river water used, indicating that

adipocere is forming faster in the river water. The pig tissue placed in the field shows an even lower fatty acid ratio value at each sampling time than those determined for both the control and the model river systems. Thus, the adipocere formed by the pig adipose tissue forms faster in the field compared to the laboratory model systems.

As adipocere forms more readily in the river water, either in the laboratory model or in the field, there is the possibility that certain species contained in the river water may be responsible for the more advanced formation. Although a detailed chemical analysis of the river water used for these experiments is yet to be undertaken, such analyses should provide some insight into the mechanism of adipocere formation in river water. Other work carried out in our laboratory on model systems involving the presence of different cationic species and the modification of pH have indicated that these factors affect the rate of adipocere formation. The details of these studies will be reported in upcoming publications.

The notable increase in adipocere in the flowing river system compared to the still laboratory model system is significant. A possible explanation for the rate difference could be the removal of other decomposition products (e.g. glycerol) by the flowing environment. Such an action may disrupt other decomposition processes and so may allow the process of adipocere formation to more readily occur.

Conclusions

A study has been made of the rate of formation of adipocere for pig adipose tissue in river water environments. It has been demonstrated that adipocere forms faster in river water, in both model and field studies, compared to a control in distilled water. The natural flowing river system is also responsible for a more rapid formation of this decomposition product. Further work is being undertaken into the species present in the river water that may be responsible for the increased formation rate, as well as into the significance of the movement of the water.

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Figure 1 – Field study container.

Figure 2 – Placement of field study containers.

sampling time / months	control	model river	field river
3	0.15	0.14	0.04
6	0.15	0.07	0.03
9	0.05	0.01	0.01
12	0.06	0.01	0.03
15	0.05	0.01	0.01
18	0.03	0.00	0.01

Figure 3 – Unsaturated / saturated fatty acid concentration ratios for pig adipocere.

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ARCHAEOLOGY OF DEATH AND FORENSIC SCIENCES IN MOSTEIRO DA LUZ, SÃO PAULO, BRAZIL: THE EXCAVATION CAN MAKE A DIFFERENCE IN FORENSIC ANTHROPOLOGICAL STUDY OF MUMMIES AND SKELETONS

Abstract: This paper presents the preliminary results of the Archaeographic Plan of Action of the graves of religious in the Mosteiro da Luz, founded in 1774 and now in the central area of São Paulo, the largest and most urbanized city in Brazil. In the old cemetery inside a small chapel, 6 wall graves were found, and 1 soil grave with a funerary headstone. The procedures for preliminary archaeography were satisfactory to assist in mapping the spatial distribution of the human remains: use of burial terminology, subsuperficial scanning, photogrammetry, drawing and digital photography. Through the methods and techniques of forensic archaeology, there were identified, documented and preserved *in situ* 3 mummies and 6 skeletons. A major risk, that determined an emergencial intervention for the site preservation, was the building infestation by the exotic subterranean termite, *Coptotermes gestroi*, that imposed severe bioturbation to most corpses. Termite bioturbation can now be assigned as a major risk for the archaeological and anthropological patrimony in urban areas in Brazil.

Introduction

The Mosteiro da Luz (Figure 1), founded in 1774 and built between the years 1774 and 1802 by Fray Antonio de Sant'Anna Galvão (elected the first Brazilian Saint in 2007), is located in the neighborhood of "Luz", the city center of São Paulo and in 1988 was declared a monument "Cultural Heritage of Humanity" by UNESCO. Its historical value as representative of sacred buildings of the State of São Paulo of the eighteenth and nineteenth centuries resulted in the toppling by the Institute of National Historical and Artistic Heritage (IPHAN) in 1943 and the Council of Defense of Cultural Heritage, Artistic and Architectural of the State of São Paulo (Condephaat) in 1979 (BOVE *et al.*, 1996).

Currently residing in the monastery there are thirteen closed Concepcionists nuns, belonging primarily to the Collection of Our Lady of Divine Providence which in 1929 was added to the Order of Concepcion by Dom Duarte (also known by Order of the Immaculate Conception), a religious institute founded by Santa Beatriz da Silva in Toledo, Spain, in the year 1484 (BOVE *et al.*, 1996).

The monastery houses, yet, the Museum of Sacred Art of São Paulo, which holds a collection of about 4,000 pieces related to the religious cult, and the chapel of Nossa Senhora da Luz.

In February 2008, the previously unexplored and old cemetery that exists inside the building (Figure 2) was evaluated due to a severe infestation of the exotic subterranean termite, *Coptotermes gestroi*, which is the most important pest termite in the southeast region of Brazil and is continuously expanding its geographic distribution in recent decades (FONTES & MILANO, 2002). Emergencial control measures also included the opening of a carnarium, in order to identify termite invasion and nesting inside the closed burial cavity, and to evaluate de occurrence of termite bioturbation in the human remains.

A mummified body was found, with signs of termite bioturbation either of the bones as of the mummified soft tissues, and the skeleton of a second individual also exhibited severe signs of termite damage (Figure 3); the latter was most probable also mummified untill recently, but the mummy was virtually destroyed by termites. Then, an exhaustive search for historical documentation of the monastery was initiated, in order to obtain data of the mortuary tombs. The data obtained, however, are imprecise and generated doubts about the location of all the graves described, which probably contains the remains of the founder and/or other pioneering nuns.

This work was signed with the IPHAN, with participation of the Institut of Legal Medicine (IML), Police Academy (ACADEPOL), Museum of Archaeology and Ethnology of the University of São Paulo (MAE/USP) and Museum of Sacred Art of São Paulo (MAS).

Material and methods

The methods of identifying archaeological sites included documentary sources, as old record and chronicle manuscript books, printed books concerning the monastery, taphonomic evidences and rescue archaeology (RENFREW & BAHN, 1993; DUPRAS *et al.*, 2005).

The archaeological intervention was initially designed to be restricted to the internal area of the cemetery. The operation aimed at maximizing the information with minimal direct intervention for the control of the termite infestation. No chemical products (insecticides) were applied at the archaeological site, but evidently this action does not excluded the possibility of past contamination of the archaeological material by chemicals, due to unreported actions for the control of chronicle termite infestation in other areas of the building; — in the last two decades, the building was submitted to control actions based on concepts that are now proscribed or ineffective.

The multidisciplinary team or researchers work with the purpose of producing a general knowledge about the funerary practices of the church from the XVIII century to the first decades of the XX century, concerning especially the Order of the Concepcion nuns and the degradation biological processes operating in this type of historical archaeological site with the presence of human remains (MORAIS *et al.*, 2008).

The archaeological site was mapped through GPS (Global Positioning System; UTM 23 K, 333481 E, 7396828 N 23rd 31 ‘49 “South, 46, 37’ 52” West) (MORAIS *et al.*, 2008).

A prospective screening trial for subsurface anomalies was performed with a GPR (ground penetrating radar), by the company Geopesquisas®. This non-destructive and non-invasive technique enable the detection of geophysical anomalies in the soil through the use of various energies (RENFREW & BAHN, 1993). The survey revealed voids in the soil grave and wall graves, contributing to the archaeological research design.

The systematic archaeological interventions were initiated by the archaeological investigations of the architectural structure of the complete room. It was later followed by the removal of the facades of the tombs in the wall, layer by layer from the outermost (layers of painting) to the inner brick wall that gave access to the human remains. These were not visible at once, since they the bodies were covered with a thick layer of earth (Figure 4).

The disclosure of the contents of the tombs was made through the use of methods and techniques of traditional archaeology (ROSKAMS, 2001; BALME & PATERSON, 2006; COX *et al.*, 2009), which prove the reliability of the data and provides the most accurate information that most interest to archeologists (RENFREW & BAHN, 1993). The excavations were performed in vertical and horizontal plans in small areas by artificial levels within predefined grid, field methods that proved to be adequate and more suitable for the illustration of the human remains (Figure 5).

Results

The archeological work revealed six tombs in the walls, five of which are being explored. Human remains of two individuals were found inside each of four tombs (Figures 6-8), and one individual inside one tomb. Three bodies are mummified and 6 are represented by skeletons; 2 skeletons are disconnected and thus are considered remains of secondary deposition. Most of the human remains exhibit severe degradation due to an urban pest subterranean termite, of the species *Coptotermes gestroi* (Figures 7-12).

The systematic withdrawal of ground that covered the bodies showed macro and micro traces of plants and animals, lime and coal. Everything has been systematically collected and documented by photos and drawings.

Discussion

From the perspective of Archaeology of the Death, of Gender, Forensic Archaeology and Forensic Anthropology (PICKERING, 1997; DUPRAS *et al.*, 2005), the preliminary archaeographic procedures and the results were satisfactory and help in mapping the spatial distribution of the human remains (skeletons and mummies): use of mortuary terminology (SPRAGUE, 2005), ground penetrating radar/GPR, photogrammetry, planialtimetry, sketches and digital photography.

The methods and techniques, also used in forensic archaeology, proved to be adequate to documentation and study of the bodies in the historical context.

The mortuary data will be analyzed in order to understand the dynamic behavior of the funeral in the Monastery and the living conditions of the nuns of the Order

of Concepcion in the century XIX. These data are related to the temperature of the internal environment of each carnarium, the relative humidity, the type of soil and the funerary practices of the religious order. The condition and characteristics of corpses are of interestd, including sex, age, stature, ancestry, diseases, causes of death and also to the recent changes promoted by termite disturbance (Figures 7-12). Termite bioturbation can now be assigned as a major risk for the archaeological and anthropological patrimony in urban areas.

Conclusions

The methods employed have been fundamental to the preservation and documentation of the archaeological material and human remains. The termite turbation imposed severe damage to many human remains that, without a careful exploration under rigorous archaeological technique, would be simply lost and unavailable for future complementary studies.

Our results are also important in the course of future studies of urban archaeology, an incipient field of research in Brazil.

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Figure 1 – Mosteiro da Luz, São Paulo, Brazil. Front view. All photos by L. R. Fontes.

Figure 2 – Mortuary chapel with six burials, a hole in floor, central altar and bow.

Figure 3 – Stage of disclosure of the contents of the burial 1. Earth layer that cover the body was partially removed.

Figure 4 – Burial 1 during the archaeological intervention.

Figure 5 – Excavation of the contents of burial 2. Note termite tunnels at the wall, at the right, and the skull with signs of termite damage.

Figure 6 – Bodies of individuals 1 (mummified) and 2 (skeletized), burial 1. Severe termite bioturbation.

Figure 7 – Detail of excavation and partial exposure of individuals 1 and 2, burial 1. Note a large termite tunnel in the head of the mummified individual 1. In the same individual, the right ear and the integument of the chin were destroyed by the termites. Individual 2 shows overall signs of termite activity.

Figure 8 – Individuals 5 (mummified) and 6 (skeletonized), burial 3. Severe termite bioturbation.

Figure 9 – Side view of the mummified body, individual 5, burial 3. The mummy was severely damaged by termites, and the thoracic and abdominal cavities, full of carton termitic structures, were virtually transformed into a termite nest.

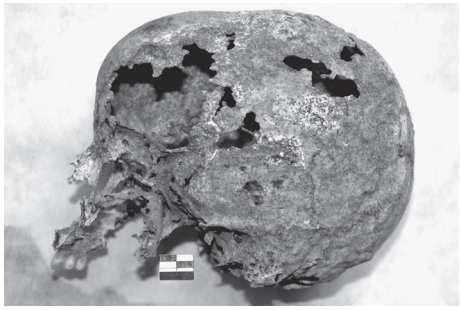

Figure 10 – Skull of individual 3, burial 2, left view. Severe termite bioturbation.

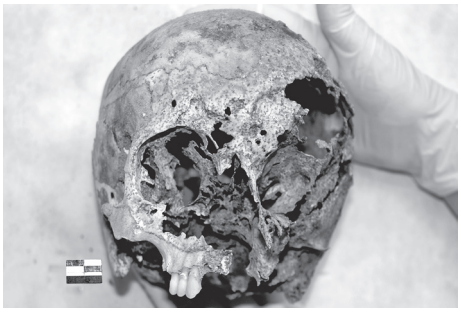

Figure 11 – Skull of individual 3, burial 2, front view. Severe termite bioturbation.

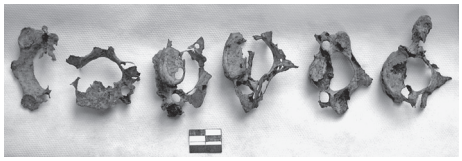

Figure 12 – Vertebrae of individual 4, burial 2. Severe termite bioturbation.

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RECENT ADVANCES IN FORENSIC ANTHROPOLOGY

Abstract: Forensic Anthropology is a dynamic field that continues to evolve with new research and augmented experience through case applications. The primary components of forensic anthropology (recovery, determination of human status, age at death, sex, ancestry, stature, and time since death, identification and assessment of evidence of foul play) all have advanced in recent years. The nature of this advancement reflects sustained research initiatives, growing numbers of scholars involved in the field, professionalization and the close link between forensic anthropology and the larger academic field of physical anthropology.

Introduction

In early 2009, the National Academies of Science in Washington D.C. released a lengthy report "Strengthening Forensic Science in the United States: A Path Forward" that critically examined the scientific basis and practice of forensic science. Although many areas of forensic science were targeted in this report, especially those involving pattern analysis, forensic anthropology received little attention. Since forensic anthropology currently enjoys considerable exposure in the forensic arena, the minimal treatment received likely does not reflect lack of awareness of the discipline. More likely it indicates that being a comparatively recent development in forensic science, forensic anthropology maintains close ties with the broader academic field of physical anthropology and a strong research base. Many practicing forensic anthropologists hold academic positions in universities and museums. Most fledging forensic anthropologists entering the field arrive with fresh academic degrees rather than on the job training. This strong academic base to the field ensures vitality and dynamic growth. Such growth is sustained in all of the major components of the field, although expressed in diverse ways.

Recovery

Recovery initiatives have been aided greatly by innovations in high technology. In particular, ground penetrating radar and electromagnetic approaches have proven their

worth in applications in some contexts. As this equipment evolves in sophistication, applications are enhanced but context largely drives the selection of survey and testing methodology. Ultimately, the classic archeological approaches usually are called for.

Major advancement in recovery efforts results from the increasing inclusion of forensic anthropologists, especially those trained in archeological techniques. With increasing frequency, forensic anthropologists are participating not only in recovery of potential criminal cases but also in situations involving mass casualties and investigations of human rights. Such inclusion greatly augments the quality of data recovered at scenes and improves the eventual interpretations that follow. Recovery efforts in human rights investigations represent a major new growth area in forensic anthropology. Those anthropologists with long-term participation in such efforts have developed unique skills and experience that translate into job opportunities and significant contributions to the overall projects.

Human or Not

Although qualified anthropologists can make most determinations of human status of recovered remains easily from morphological indicators, fragmentary and otherwise compromised evidence can be challenging. New techniques, driven by problems encountered in casework, have greatly augmented this endeavor. Databases developed using scanning electron microscopy/energy dispersive spectroscopy enable small particles of bone and tooth to be distinguished from other materials. Histological techniques, employing microscopic analysis, allow recognition of non-human bone patterns. Molecular analysis and a technique known as protein radio immunoassay (pRIA) not only can determine human/non-human status but also can determine what non-human animal is present if it is important to do so.

Age at Death

Advances in methodology in age at death assessment are driven by the general age status of the specimen, the material available for analysis and a growing appreciation of the nature of human variation in the aging process. Assessment of dental development continues to be the method of choice for immature individuals if teeth are present and can be identified. In their absence, bone size and morphology provide critical information, especially epiphyseal closure in older immature individuals. Research has demonstrated that attention must be given to sex differences and population variation in the aging process. The roles of nutrition, morbidity, genetics and other factors contribute to human variation in the aging process and their impact must be assessed in the various aging systems. Such assessment flows from the development of new collections and databases of individuals of known age at death in different regions of the world. Such diversity of research sampling contributes to greater understanding of population variation in the aging process and assists in sorting out the complex factors involved.

Sex

Research continues to provide diverse approaches to the estimation of sex from human remains. The methods of choice are largely shaped by what material is available for study. Of course, molecular approaches are now available to determine sex if sufficient DNA can be recovered and amplified. Techniques involving assessment of the pelvis remain the most accurate for sex estimation in adults. Sex estimation in immature remains continues to be problematic, especially for the very young.

Ancestry

Recent progress in assessment of ancestry is terminological. Word choice is important in this area of forensic anthropology since words such as "race" and "ancestry" mean different things to different people and can evoke strong individual reactions. Increasingly, anthropologists recognize the social dimensions of such assessments, especially in regards to the categories utilized and the dynamics of self-identification. Both metric and non-metric methodological approaches are available and continue to be improved through research. This area of forensic anthropology remains strongly dependent on studies of well-document remains from different regions of the world. Fortunately such collections and studies are growing in frequency. Although new emerging data on human variation in bone and tooth morphology strengthen interpretations of ancestry, they also define the limits of what can be reported and the probabilities involved.

Stature

Although some recent research has strengthened methodological approaches to stature estimation, most progress relates to new databases that document population variation in body proportions. Applications have brought new attention to the accuracy of so-called "known" statures among the missing. These studies note the difference between measured stature among the living and the more common "estimated" stature that dominates records of missing persons.

Time Since Death

Research and case experience have documented the many variables involved in postmortem change in human remains. Although much has been learned about the impact of seasonality, exposure to insects and foraging mammals and birds, ground water, soil ph and many other factors, the major lesson is that it is very difficult to estimate time since death using morphological indicators alone. Although regional patterns can be discussed, the exceptions are numerous and sometimes extreme.

Recent research has called attention to the value of radiocarbon analysis, with special reference to the bomb-curve to determine approximate time since death

and even approximate birth date. Atmospheric testing of thermonuclear devices from the 1950's through the 1960's produced high levels of atmospheric artificial radiocarbon, which, through the food chain have been incorporated into the bones and teeth of individuals living in that period of time. While levels have diminished since international test ban treaties were in place, they remain today above their 1950 levels. Thus if radiocarbon analysis detects modern high levels in human remains, it means the person was alive during the bomb-curve period. This determination alone represents a major advancement in assessing if recovered remains are of medico-legal significance. Careful analysis of particular tissues can offer more detail on both the death date and even the birth date for some individuals who were alive after 1950.

Identification

Identifications within forensic anthropology primarily result from comparisons of antemortem and postmortem radiographs that reveal unique details of skeletal and dental anatomy. Anthropologists are challenged to distinguish presumptive from positive identifications and to properly assess the probabilities and error rates involved. Advances in this area consist of growing awareness of the importance of the above stated issues and new research that seeks to document aspects of the related probabilities. Novel research aimed at testing expert judgment on radiographic comparison has provided useful information needed to validate identification approaches. The limits of identification procedures in forensic anthropology also are challenged as some expertise overlaps that found in odontology and forensic pathology.

Evidence of Foul Play

Progress in this area of forensic anthropology continues to be driven by growing case experience supplemented with experimental research. Evaluations are enhanced by increased knowledge about taphonomic factors, natural variation in anatomical structure and antemortem conditions. Key research targets minimal remodeling indicators to more tightly define the perimortem window. Considerable recent research has clarified issues of perimortem trauma, especially details of sharp force trauma and thermal alterations.

Conclusions

The above discussion provides ample evidence of the research and experience dynamic that feeds the sustained growth and maturity of the field of forensic anthropology. Growing numbers of highly qualified students are attracted to this endeavor, ensuring the continued growth and quality of the science. As suggested by the comparative lack of attention focused on forensic anthropology in the report by the National Academies of Science, the field remains firmly wed to the larger field of physical anthropology and nourished by rich research initiatives.

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ESTIMATING AGE AT DEATH FROM THE SIZE OF THE GROWING EPIPHYSES AND METAPHYSES OF THE FEMUR AND TIBIA AT THE KNEE

Abstract: The main purpose of the present study is to evaluate the utility of the width of the epiphyses and metaphyses of the femur and tibia at the knee in estimating age at death in subadult skeletons. The study sample was taken from the Lisbon documented skeletal collection (NMNH, Lisbon, Portugal) and it comprises 84 individuals (males, 47; females, 37) between 9 months and 18 years. Simple linear regression models and r^2 values were calculated for the relationship between chronological age and epiphyseal and metaphyseal width for the sex-pooled sample. Linear regression models were calibrated to obtain prediction equations. Mean standard error (MSE) and mean 95% confidence interval (MCI) were calculated for all equations. Results show that epiphyseal widths correlate better with chronological age (femur: r^2=0.91; tibia, r^2=0.93) than metaphyseal widths (femur: r^2=0.87; tibia, r^2=0.89). At the knee, the distal femoral metaphysis is the most accurate site for age estimation (MSE: 1.52 years; MCI: ±3.05 years).

Introduction

The lower limb bones are frequently the most common and best preserved elements in skeletal samples. In addition, both distal femoral epiphysis and proximal tibial epiphysis are present at birth and are the largest epiphyses in the human body. For these reasons, measurements of the osseous elements of the knee can be useful in estimating age at death in subadults.

There are several studies that correlate age with the linear growth of the skeleton using the diaphyseal length of long bones, including the femur and tibia (Maresh, 1970; Cardoso, 2005). Other studies focus on estimating age of death of fetal and perinatal ages from the length of long bones, also using the femur and tibia (Sheuer et al, 1980; Olsen et al, 2002). However, little attention has been given to the use of the epiphyseal and metaphyseal width of the tibia and femur in estimating age at death in subadult skeletons. Measurements of the growing epiphyses and metaphyses of the femur and tibia at the knee have been used to estimate ratios that are useful for maturation status assessment at young ages only (Pyle and Hoerr, 1955; Roche et al, 1975). Distal femoral metaphysis and proximal tibial metaphysis widths have also been used to indirectly estimate age at death by estimating complete diaphyseal length of fragmentary long bones (Hoppa and Gruspier, 1996).

This study wishes to assess the utility of the epiphyseal and metaphyseal widths at tibia and femur in estimating age at death from the osseous portions of the growing knee. Another goal of the present work is to determine which measurement (distal femoral epiphysis, distal femoral metaphysis, proximal tibial epiphysis or proximal tibial metaphysis) is the most suitable for age estimation.

Materials and Methods

Sample

The study sample comprises 84 individuals of known sex and age at death. All individuals were drawn from the Lisbon documented skeletal collection curated at the Department of Zoology and Anthropology (Bocage Museum) of the National Museum of Natural History, Lisbon, Portugal. Ages range from 9 months to 18 years, with a slight over-representation of male skeletons (male, 47; female, 37). The age and sex distribution are depicted in the Figure 1. Males at the age of 12, females under one and over 17 years of age are missing in the sample.

Measurements

The maximum width of the distal epiphysis and metaphysis of the femur and of the proximal epiphysis and metaphysis of the tibia were measured using a sliding calliper and recorded in millimetres. The maximum horizontal distance between the medial and lateral edges of the distal femoral and proximal tibial metaphyses were measured while holding the bone vertically, with the distal or the proximal end up, respectively. The maximum distance between the medial and lateral margins of the epiphyses of the femur and tibia were measured in anatomical position. The epiphyses of the femur were measured with the articular surface down, while the proximal tibial epiphyses were measured with the articular surface up. For each variable, measurements from the left and right sides were taken, whenever possible.

Analysis

Right and left side measurements were compared using paired t-tests in order to determine differences due to laterality. For each location, the sub-set used for testing only contained skeletons with bilaterally intact sites.

Intra-observer and inter-observer measurement errors were assessed in a random sub-sample of 19 individuals. Intra-observer error was estimated by comparing measurements of the same location in two different sessions, while inter-observer error was estimated by comparing measurements taken by two different observers. Both types of error were estimated by technical error of measurement (TEM), coefficient of reliability (R) and mean average difference (MAD) (Ulijaszek and Kerr, 1999).

Simple linear regression models and r^2 values were determined for the relation between chronological age (x axis) and measurements from the four locations (y axis): distal femoral epiphysis and metaphysis and proximal tibial epiphysis and metaphysis. Linear regression models for male and female subadults were compared in order to determine if they differed statistically (Zar, 1999).

Because these linear regression models are not suitable for age estimation from skeletal remains, they were calibrated in order to estimate age at death: the dependent variable (width) was fitted to the independent variable (age) (Lucy, 2005). Standard error associated with each point value and the mean standard error for point values (MSE) were calculated, for the four sites. A confidence interval for each point used to determine the linear regression model was calculated. The mean value of the confidence intervals was also calculated for each site. Mean 95% confidence intervals (MCI) were also calculated for each site (Lucy, 2005).

Results

Paired t-tests results showed statistically significant size differences due to laterality only at the distal femoral metaphysis in the male sample ($p=0.006$). However, these differences are negligible since the mean difference between sides (0.39mm) is smaller than the inter-observer error (MAD, see below). Therefore, right side measurements were used when bones from the left side were missing or damaged.

For intra-observer error, all variables had MAD values under 0.29mm, TEM values under 0.31mm and R values above 0.999. For inter-observer error, all variables had MAD values under 0.5mm, TEM values under 0.9mm and R values above 0.998 (Tab. 1).

Slope and elevation comparison between regression models for males and females (Zar, 1999) showed no differences at the distal femoral epiphysis, distal femoral metaphysis and proximal tibial metaphysis ($p>0,05$). Elevation for male and female linear regression models at the proximal tibial epiphysis was significantly different ($p<0.05$). At the proximal tibial epiphysis, slope and elevation differed significantly ($p<0.05$) and two different equations were calculated for males and females. Only at the distal femoral epiphysis did statistical tests show that a common model could be used for male and female subjects (Tab. 2). In a forensic context, however, sex cannot be easily determined from subadult skeletal remains. Therefore, linear regression models using sex-pooled data sets were also calculated for the other three locations: distal femoral metaphysis, proximal tibial epiphysis and metaphysis. Table 3 shows the linear regression equations and r^2 values calculated for the four locations for the sex-pooled sample.

Calibration of the linear regression models was performed. However, the inversion of the equation carries an implicit error that affects estimation. To assess the error, a 95% confidence interval for the points used to determine the linear regression model was calculated. Thus, we can evaluate the usefulness of the age estimate itself (Lucy, 2005). The location that shows greater MSE and MCI values is the proximal tibial epiphysis (2.13 years and ± 4.28 years, respectively) and the smallest MSE and MCI values is the distal femoral metaphysis (1.52 years and ± 3.05 years, respectively) (Tab.3).

Discussion

The present study wished to evaluate the utility of the measurements of the epiphyses and metaphyses of the tibia and femur at the knee in estimating age at death in subadult skeletons. Results showed that at the knee, there is a very high correlation

relation between chronological age and metaphyseal and epiphyseal width (r^2 values above 0.87), especially at the femoral and tibial epiphysis (r^2 values above 0.91). Another goal of this study was to determine which location (distal femoral epiphysis, distal femoral metaphysis, proximal tibial epiphysis or proximal tibial metaphysis) is most suitable for age estimation in subadult skeletons. For the sex-pooled sample, four models for age estimation were calculated for the relationship between age and the measurements taken at the various osseous portions of the growing knee. This provides alternative sites to estimate age at death. Nonetheless, the most suitable location for age at death estimation is the distal femoral metaphysis (MSE=1.52 years and MCI=±3.05 years) and should be preferably used.

Estimating age at death is not an easy task, especially when sex cannot be determined. This study provides models that can be used when estimating age at death in a forensic context. There are several methods that correlate the diaphyseal length of the long bones with age (Maresh, 1970; Sheuer et al, 1980; Olsen et al, 2002; Sheuer and Black, 2004; Cardoso, 2005). However in this study, a new approach was attempted in order to achieve a new reliable method to estimate age death from the developing epiphysis and metaphysis at the knee, which can be particularly helpful in fragmentary remains. This anatomical location was chosen for various reasons. First of all, the osseous portions of the knee are the largest of their kind in the human skeleton. Second, the epiphyses of the knee are present since birth and are well preserved in a variety of postmortem situations. Third, the epiphyses size and preservation increase the chances of their retrieval by investigators. Finally, the existence of multiple sites from which age estimates can be obtained renders this site particularly resilient to taphonomic phenomena, when accurate age estimation is concerned. Consequently, the size of the growing epiphyses and metaphyses at the knee could improve the accuracy of age estimation together with other well known measurement methods, such as the diaphyseal length of long bones. Age estimation based on metaphyseal size is useful when long bones are fragmented and until now, metaphyseal measurements have only been used to obtain indirect age estimates (Hoppa and Gruspier, 1996).

Conclusion

Estimating age at death in subadult individuals is particularly difficult when the skeleton is incomplete or badly preserved. Age indicators such as dental development or long bone lengths cannot be frequently used in those circumstances. Data in this study provides alternative techniques for age estimation of fragmentary remains. Size of the metaphyses and epiphyses at the knee, show a very high correlation with chronological age (r2 values above 0.87), and can be used with a high degree of accuracy. The most suitable location for age at death estimation is the distal femoral epiphysis (1.52 years and ±3.05 years, respectively) for the sex-pooled sample.

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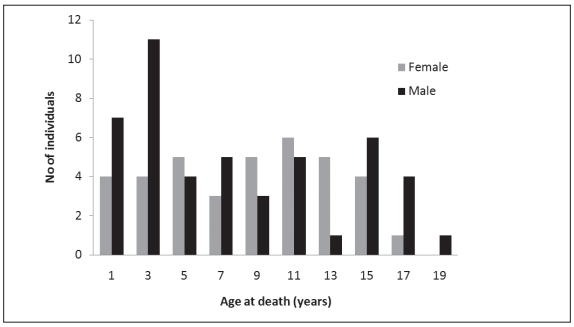

Figure 1 – Age and sex distribution of the sample (females, 37; males, 49).

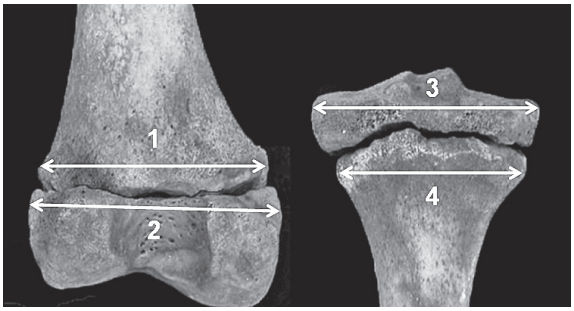

Figure 2 – Measurements of the distal epiphysis and metaphysis of the femur and of the proximal epiphysis and metaphysis of the tibia. 1- Maximum width of the distal femoral metaphysis; 2- Maximum width of the distal femoral epiphysis; 3- Maximum width of the proximal tibial epiphysis; 4-Maximum width of the proximal tibial metaphysis.

	N	MAD (mm)	TEM (mm)	R
Intra-observer measurement error				
Distal femoral epiphysis	19	0.17	0.17	0.999
Proximal tibial epiphysis	15	0.18	0.16	0.999
Distal femoral metaphysis	14	0.13	0.12	0.999
Proximal tibial metaphysis	13	0.28	0.31	0.999
Inter-observer measurement error				
Distal femoral epiphysis	19	0.33	0.45	0.999
Proximal tibial epiphysis	15	0.50	0.90	1.000
Distal femoral metaphysis	14	0.49	0.57	0.998
Proximal tibial metaphysis	13	0.27	0.23	0.999

Table 1 – Intra and inter-observer test results errors for the sex-pooled sample, calculated as mean absolute difference (MAD), technical error of measurement (TEM) and coefficient of reliability.

	Regression equation (*width in mm and age in years*)	R^2
Distal femoral epiphysis	width=3.59×age+22.02	0.91
Distal tibial epiphysis	width=3.42×age+17.12	0.93
Distal femoral metaphysis	width=2.14×age+34.99	0.87
Proximal tibial metaphysis	width=2.04×age+26.47	0.89

Table 2 – Regression equations and r^2 values for the sex-pooled sample.

	Age at death estimation equations	MSE (years)	Mean Confidence intervals (95%) (years)
Distal femoral epiphysis	$age = \frac{width - 22.02}{3.59}$	1.90	$age \pm 3.40$
Distal femoral metaphysis	$age = \frac{width - 34.99}{2.14}$	1.52	$age \pm 3.05$
Proximal tibial epiphysis	$age = \frac{width - 17.12}{3.42}$	2.13	$age \pm 4.28$
Proximal tibial metaphysis	$age = \frac{width - 26.47}{2.03}$	1.96	$age \pm 3.96$

Table 3 – Calibration of sex-pooled linear regression equations, mean standard error (MSE) and mean 95% confidence intervals (MCI).

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ESTIMATING AGE AT DEATH IN ADULTS FROM DEGENERATIVE CHANGES IN THE STERNAL END OF THE CLAVICLE – PRELIMINARY RESULTS USING A BAYESIAN APPROACH

Abstract: Age estimation of adult skeletal remains is still a very uncertain procedure. This study represents an attempt to decrease the estimation error associated with adult age estimation in a forensic context, by using alternative bone indicators and statistical approaches. A sample of 91 known-age adult skeletons (47 females and 44 males) from the Spitalfields skeletal collection housed at the Natural History Museum in London was used. Five age-related variables were assessed on the sternal end of the clavicle: microporosity (<1mm); macroporosity (>1mm), sclerosis; new bone formation and marginal lipping. Ages where categorized in ten-year intervals and multinomial regression was used to estimate the probability of an individual being in a certain age interval conditional on the observed data (skeletal indicators). Results presented here are considered preliminary and illustrative of alternative skeletal indicators for age estimation in adults using a Bayesian approach.

Introduction

Estimating age at death of adult skeletal remains has been one of the greatest challenges in anthropological research. Most existing methods fail to provide an accurate estimate, particularly in individuals over 40-50 years of age (eg. Brooks and Suchey, 1990). In addition, the various methods have been developed over a limited number of anatomical locations, such as the pubic symphysis, the auricular surface or the sternal end of the ribs. Alternative sites have potential to provide additional age-related changes. The only two studies that uses the clavicle to estimate adult age at death were conducted by De Palma (1957), which described changes in the articular discs of the sternal end; and Miles (1999) who established a five grade categorization for age related changes for the acromial end. In this study we test the accuracy of age estimation using the degenerative process occurring at the sternal end of the clavicle. The results presented here are only preliminary, and are part of a larger study that comprises several collections of identified skeletons. The remaining data is still being collected, and it is hoped that the larger sample allows us to achieve more satisfying results in order to obtain a new and more reliable methodology to estimate age at death.

Goals

This study comprises two main goals. The first is to assess the association between age and degenerative changes in the sternal end of the clavicle. The second goal is to test the adequacy of a Bayesian approach (Multinomial regression) to model our data for predictive purposes.

Materials and Methods

Ninety one skeletons (47 female and 44 male) from the Spitalfields Collection of Skeletons, housed at the Natural History Museum in London, between the ages of 30 and 91(Figure 1). All the clavicles were observed macroscopically using a 5x magnifying glass, and all were photographed. Five age-related variables were assessed: microporosity (<1mm); macroporosity (>1mm), sclerosis; new bone formation (NBF) and marginal lipping. The first four variables were recorded according to the percentage of surface affected (0 – 0%; 1 – 1-24%; 2 – 25-49%; 3 – 50-74%; 4 – 75-100%) and marginal lipping was recorded according to the size of the osteophytes. Spearman correlation and Multinomial Regression was undertaken using the statistical package SPSS 17.

Data Processing

Age was transformed in 10-year classes (starting at 30 years old). Observations in percentage were coded as explained above, however, we were not able to use this initial recording because there were too many zero cells, *i.e.* dependent variable levels by subpopulation with zero frequencies, so we were forced to find a new coding that reduced the number of stages in each variable and combined them with others in order to be better suited with the reality of our data. For example for microporosity the final encoding was: 0 – no changes; 1 only microporosity (regardless of the percentage); 2 – 0-24% of micro along with any other change; 3 – over 25% of micro along with other change. A Score variable (Similar to the one used by Buckberry and Chamberlain, 2002) was also calculated as the sum of the codes for each variable. This variable was also encoded: 1- when sum was 0, 1 or 2; 2- when 3, 4 or 5; 3 – when 6, 7 or 8. This new variable was used to test if age progression could be better explained by a combination of variables. Figures 2 and 3 show that there is a tendency for higher scores to be associated with elderly individuals.

Results and Discussion

Table 1 shows that the variables are differently correlated with age. Although in females almost all variables (lipping is the exception) have a significant correlation with age, the three significant correlations in males (sclerosis, NBF and Score) have a stronger correlation coefficient, although in none of the cases the correlation is higher than 0,540. These results rule out lipping as an important variable.

Using Multinomial Regression three steps were taken:

The first step was to test whether the male and female models were statistically significant, our hypothesis were:

H0: The model is not statistically significant vs. H1: The model is statistically significant

The model obtained for males includes only one of the measured variables (sclerosis) and the score. Table 2 shows the statistical results that allowed us to accept the model. $G^2(25)= 31,821$; $p=0,007$, so we reject H0 and, therefore conclude that at least one independent variable has significant influence on age.

The model obtained for females includes three measured variables (microporosity, sclerosis and NBF) (Table 3). A parallel analysis to this model tells us that the it is also significant ($G^2(35)= 61,384$; $p=0,004$) (Table 2).

Looking at the male and female models we can see that the male model has lower AIC/BIC values which mean that the final model obtained is better adjusted than the null model in the male group.

A second step was to test whether the model fitted the data, in this case the htpothesis were:

H0: The model fits the data vs. H1: The model does not fit the data

H0 is not rejected neither in the male nor female model, meaning that it fits the data in both sexes, in both statistics calculated (Deviance and Pearson). (More detailed results are not shown).

A third and final step was to calculate the multinomial regression model for each sex and age class. We obtained 12 regression formulae. The formulae for females and age class 2 (40-50 years) is shown in figure 4.

Conclusion

Although the data collection is not complete, results indicate that there is some potential in this new method. Both models were found statistically significant, and the correlations were also significant (especially in females). At this stage the method seems to work better in females, which in our opinion, is to be expected, since we hypothesize that women in the sample experienced more homogenous activity levels (most were housewifes) than men, thus possibly reducing the effect of environmental factors related to activity. We expect that with the future three-fold increase in data, some of these difficulties will be overcome and the models will be more accurate.

Acknowledgements

This project was funded by the SYNTHESIS program (reference GB-TAF-4898). We want to thank the NHM of London for granting us access to the Spitalfields collection. We also wish to thank Robert Kruszinski, Louise Humphrey and Silvia Bello for all their help during the collection of data at the NHM.

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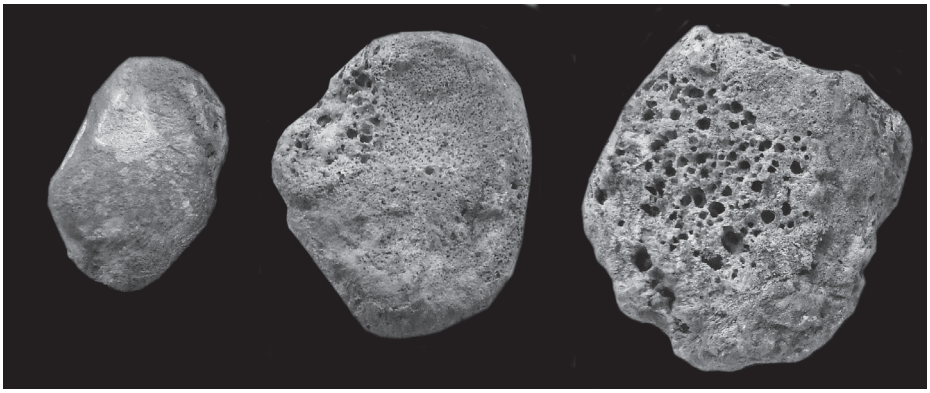

Figure 1 – Examples of observed clavicles. From left to right: specimen 2500 (female, age 37, almost no changes); specimen 2419 (male, age 63, some sclerosis clear in the upper left quadrant); specimen 2538 (male, age 81 sclerosis covers most of articular surface).

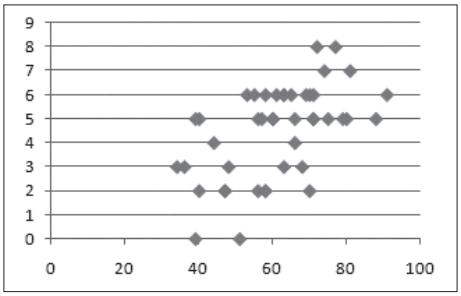

Figure 2 – Score vs. Age for males

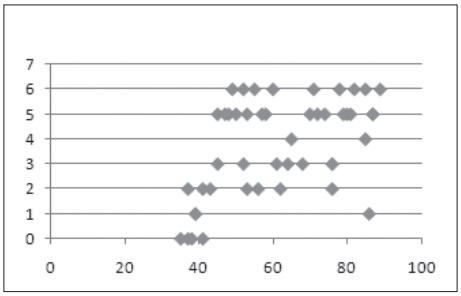

Figure 3 – Score vs. Age for females

$$P[Age = 40-50] = \frac{1}{1+e^{-14.36-12.54\,Microp(0)-25.00\,Microp(1)+2.21\,Microp(2)+12.44\,Sclerosis(0)+12.45\,NBF(0)-3.54\,NBF(1)+27.52\,NBF(2)} + \\ + e^{-12.00-28.36\,Microp(0)-9.60\,Microp(1)+1.97\,Microp(2)+10.44\,Sclerosis(0)+11.86\,NBF(0)+12.03\,NBF(1)+27.89\,NBF(2)} + \\ \ldots + e^{33.60-28.27\,Microp(0)-11.86\,Microp(1)+1.64\,Microp(2)-19.56\,Sclerosis(0)-16.56\,NBF(0)+13.54\,NBF(1)+12.32\,NBF(2)}}$$

Figure 4 – Model equation obtained for females in age class 2.

		Microporisity	Macroporosity	Sclerosis	New Bone Formation	Lipping	Score
Male	Correlation coefficient	0,241	0,241	0,414**	0,540**	0,065	0,415**
	Sig. (2 tailed)	0,200	0,115	0,005	0,000	0,675	0,005
	N	44	44	44	44	44	44
Female	Correlation coefficient	0,377**	0,385**	0,300*	0,296*	0,273	0,466**
	Sig. (2 tailed)	0,009	0,007	0,041	0,043	0,063	0,001
	N	47	47	47	47	47	47
	** - Correlation is significant at the 0.01 level (2-tailed)						
	* - Correlation is significant at the 0.05 level (2-tailed)						

Table 1 – Spearman correlation between each variable and age.

Model		Model Fitting Criteria			Likelihood Ratio Tests		
		AIC	BIC	-2 Log Likelihood	Chi-Square	df	Sig.
Males	Intercept Only	70,652	79,573	60,652			
	Final	68,831	104,515	28,831	31,821	15	0,007
Females	Intercept Only	106,606	115,857	96,606			
	Final	115,222	189,228	35,222	61,384	35	0,004

Table 2 – Model statistics

Effect		Model Fitting Criteria			Likelihood Ratio Tests		
		AIC of Reduced Model	BIC of Reduced Model	-2 Log Likelihood of Reduced Model	Chi-Square	df	Sig.
Males	Sclerosis	70,439	97,202	40,439	11,608	5	0,041
	Score	67,455	85,297	47,455	18,624	10	0,045
Females	Sclerosis	116,011	180,766	46,011	10,789	5	0,056
	NBF	108,375	154,629	58,375	23,153	15	0,081
	Microporosity	111,376	157,630	61,376	26,154	15	0,036

Table 3 – Variables in each model (male and female).

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JUXTACORTICAL OSTEOMA: AN HISTOPATHOLOGICAL DIAGNOSIS IN AN ANTHROPOLOGICAL SPECIMEN

Abstract: Deceased persons or human remains may be identified by the recognition of pathological structures, malformations or normal anatomical variants of organs and tissues. Throughout times, they have also contributed to historical and epidemiological studies of diseases and populations' characteristics. The case here reported concerns a femur presenting a mass-forming lesion, whose anthropological examination suggested to be a certain bone tumor and the histopathological evaluation provided a different and definitive diagnosis – that of a Juxtacortical (Parosteal) Osteoma –, thus emphasizing the value of cooperative work among forensic sciences, for any kind of medico-legal investigation.
Keywords: Forensic histopathology; forensic anthropology; bone tumors.

Introduction

The existence of pathological evidences/structures – like recent or signs of old bone fractures, tumors, etc –, of malformations – as femoral hypoplasia –, or even of normal anatomical variants of organs and tissues – as for example those of patella and trochlea at the patellofemoral joint –, may contribute, in a unique way, to medico-legal investigation. Yet, the characterization of some of them requires the input of various forensic areas, as illustrated by the present case.

Case report

A long bone was recovered from the countryside in the South of Portugal and brought to the Medico-Legal Institute for anthropological examination. This evaluation catalogued it as a femur belonging to human bone remains and pointed out an anomalous formation, which was described as: "an exuberant lesion located approximately 180mm from the great trocanter and corresponding to an ossified 'mass' lateraly joined to the diaphysis, with a length of 150mm and without external evidence of disorganization of the bone layers' architecture" (Figure 1). The hypothesis of a bone tumor – probably begnin, as for exemple an osteochondroma – was put forward, yet keeping in mind other possible etiologies. Imaging evaluation was then performed (Figure 2) and histopathological examination requested afterwards. In order

to do it, the bone was submitted to a particular variant of the usual tissue handling/ processing technique, named *Sandison's Technique*[1] (Figure 3), which is recommended for specimens with a long skeletization period; and whose crucial phase consists in an additional hydration procedure.

The results of the different methods were:

Plain X-ray disclosed reactive periosteal thickening and did not show bone infiltration by the lesion.

Histologically, the 'mass' was incorporated in the external femural surface, without evidence of malignant infiltration. It consisted of well-differentiated mature bone, with predominantly lamellar structure, presenting haversian system and architecture identical to the normal cortical bone (Figure 4).

Thus, the final diagnosis was *Juxtacortical (Parosteal) Osteoma***.**[2]

Discussion

Juxtacortical (Parosteal) Osteoma is a begnin bone tumor, rare, especially in this location (long bone), since it is more prone to involve the skull and facial bones.[3] It is a slow growing lesion, whose histogenesis is – for some authors – contoversial; since they consider it an hamartoma.[2] It may be asymptomatic or not, with a large range of possible dimensions.[3] Among its differential diagnosis, the parosteal osteosarcoma – its malignant counterpart – is of major relevance.[4] In the medico-legal setting, the presence of the tumor may represent an identification marker, either *per se* or in combination with other data and/or characteristics of a victim or his/her remains.

Conclusions

The present case reinforces the importance of the contribution from different forensic areas / sciences to, and their joint cooperation in, the identification of alive and dead individuals; the evaluation of their nosological conditions; as well as the characterization of diseases in ancient specimens[1,5], even from eventually disappeared cultures and civilizations. The multidisciplinary work definitely has medico-legal, epidemiological and/or historical relevance.

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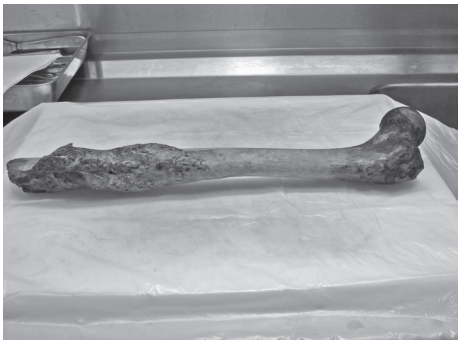

Figure 1

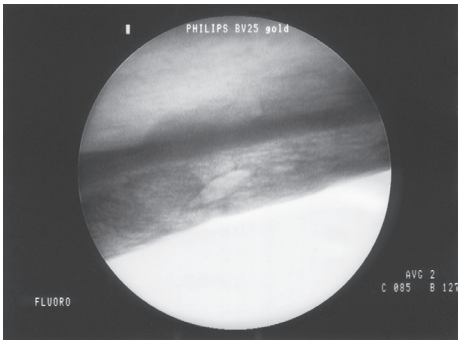

Figure 2

Figure 3

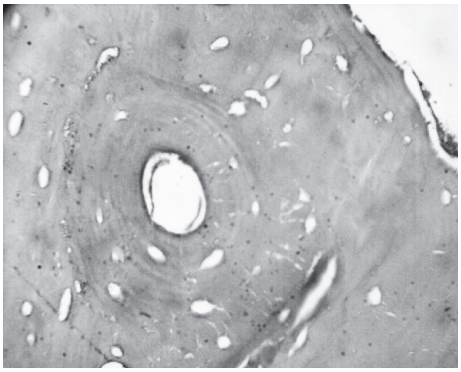

Figure 4

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EVALUATION OF SOFT TISSUE THICKNESSES WITH THE PURPOSE OF FACIAL RECONSTRUCTION IN BRAZILIAN

Abstract: The auxiliary technique of identification known as Facial Reconstruction makes possible to obtain face identification from the contours of the tissue around the skull, increasing the probabilities of recognition. The reliability of this technique depends on the evaluation of the thickness of the soft tissues that covers the skull. Those measurements were evaluated on a sample of studied cadavers in São Paulo state, Brazil. The thickness has been manually measured using the needle puncture technique in 10 anatomical landmarks of the skull located in the midleline and in 11 bilateral points of 40 cadavers of both sexes, aged between 17 and 90 years, classified by skin color and nutritional state. Descriptive statistics calculations were made accordingly to T-tests, ANOVA and Tukey tests. Those calculations, when compared with other populations studies, showed different results, that lead to the need of using a specific table with values of the local population to implement the technique of facial reconstruction in skulls without an attributable identity.

Introduction

Identification of corpses, bones or fragments is a fundamental in the field of Forensic Sciences and has legal and humanitarian implications. Data from post-mortem exams will be useless if it can't be correlated with those from someone with a previously known identity. In the past one hundred and five years, several studies have been performed correlating the external appearance of people with the soft tissue thickness over the skull base. Facial Reconstruction, as an auxiliary identification technique, allows the reconstruction of soft tissue contours over the skull and the face, thus increasing the probability of facial recognition. The reliability of this technique, however, depends on the evaluation of the mean values of soft tissue thicknesses observed in a given population. Existing studies for different ethnical groups, mostly from isolated populations with strong common characteristics, have produced models that led to questionable results when applied to mixed ethnical characteristics populations.

The purpose of this study is to assess the measurements of the thickness of soft tissue that covers anatomical landmarks of the skull using a population sample of corpses in Guarulhos, São Paulo, Brazil. Guarulhos is located in the metropolitan

area of Sao Paulo (Brazil's largest city), and is made up of a highly mixed population consisting of several different racial types. Thus the cadaver sample is comparable to the composition of the Brazilian population as a whole in terms of skin color.

This study was approved by the Committee on Ethics in Research of Faculdade de Odontologia da Universidade de São Paulo, Brazil (Protocol nº 21/07).

Materials and Methods

For an initial investigation, defining a depths standards to be applied in the Brazilian population, used a sample of 40 corpses (26 male and 14 female), with ages between 17 and 90 years, classified per skin color and nutritional state, autopsied in the "Seção Técnica de Verificação de Óbitos" (Technical Section of Death Cause Verification) in Guarulhos, São Paulo, Brasil.

Soft tissue thickness was measured by the needle puncture method in 10 craniometrical points and 11 bilateral points (used by Rhine and Campbell, 1980 [1]), described in Table I, by 2 examiners within 12 hours from death to avoid any post-mortem distortion effect.

Because Brazil has one of the most heterogeneous populations in the world [2,3], the Von Luschan chromatic scale was used to classify skin color with values of: 10 to 19 for leucoderms (alike Caucasian), 20 to 23 for xantoderms (native indians and immigrants of Asian origin), 24 to 29 for faioderms (descendents of interbreeding between black and white ancestors, with different skin tones), and 30 to 36 for melanoderms (alike Afro-Americans). The Von Luschan scale establishes different color tones that can be compared with a part of the skin unexposed to the sun. This classification was complemented by somatometric and somatoscopic observations for Brazilian anthropological types as suggested by Roquette Pinto [4].

Body mass index (BMI) was used to classify the sample's nutritional status. BMI was calculated using the following formula: kg/m^2. Height and weight were measured during the autopsies. The following classification was used: lean, when BMI < 20 (N= 11); normal, when 20 < BMI < 24.9 (N=13); overweight, when 25 < BMI < 29.9 (N=10); and obese, when BMI > 30 (N=6).

Measurements were taken by puncturing the skin with a thin stainless steel dental needle with a silicone marker stop. The needles were introduced in the previously located anatomical landmarks, perpendicular to the skin until they met bone resistance. The marker was then slid in to touch the surface of the skin, without pressing or deforming it. A caliper was used to measure the depth from the tip of the needle to the base at the skin.

Descriptive statistics were calculated using the Statistical Program for Social Sciences (SPSS) [5]. Student's T-test was used to compare two groups, ANOVA was used to compare more than two groups and Tukey's test, when there was indication of a statistically significant difference – $p < 0.05$. The averages were compared to those in the studies of Rhine and Campbell [1] and Rhine and Moore [6] for individuals with the same skin color and gender.

Results

A statistical model was established considering biological sex, age, skin color and nutritional state, between examiners measurements differences also being evaluated. Measurements from the two examiners had a very good statistical correlation for all points ($p<0.05$).

Measured thickness values were found greater for males, with 10 out of 32 measurements found statistically significant when tested with T- Student. ANOVA test of the sample showed significant differences due to nutritional state for 3 measurements in the mean line and 11 of the bilateral, proportional to fat quantity in the face, and that age was not significant.

Ethnical variable due to skin color, analyzed using ANOVA test showed a statistical significant characteristic between different groups only for the Nasion craniometrical point (Table II). However, these data showed to be different from other studies for different populations with the same skin color.

Discussion

The methodologies of measuring the thickness of soft tissues on the face have limitations, but the ease of obtaining them through the puncturing with needles at the points of the faces corresponding to those existing in the skull, has made this method withstand to the development of more technologically advanced methods , as can be seen in recent publications [7,8]. Non-invasive imaging diagnostic techniques will, undoubtedly, provide better accuracy and precision [9,10,11,12]. Such exams, however, are not always radiation hazard free and it is not possible to gather data for all necessary points to the Facial Reconstruction techniques without increasing radiation exposure time on patients being examined for any pathology. It may be also difficult to locate craniometric points and corresponding tissue depth.

Differences in the thickness of facial tissues related to sex, age, ethnicity and nutritional status have been singled out as the shortcomings of the Facial Reconstruction technique and have been studied by several authors and also in this work. The results indicate that the males present greater thickness of soft tissue than females, so those differences should be considered for the use of Facial Reconstruction.

Although several authors [13,14] showed that adult and child faces differ, it was not possible to correlate changes due to aging. In our study, only three out of thirty-two variables were found to be statistically different, those being above 55 years age. In the studied sample, 65% of the individuals were above 55 years age, so not allowing a statistical analysis of any aging effect. The chances that data related to age can pose some additional information for Facial Reconstruction Technique are limited to the determination of differences between children and adults.

The face will change due to different nutritional states because of fat found in Infraorbital, Zygomatic, and Malar regions and, when this information is needed, we agree with the suggestion of Starbuck and Ward [15] of creating different versions of a Facial Reconstruction considering several different nutritional states.

Data obtained with this study helped to realize that the highly mixed Brazilian population present statistically different results from those found in other populations that had different results between leucoderms and melanoderms people [1, 6, 7, 8, 9, 11, 12, 16].

We can see that even with no significant differences related to the color of the skin for this group studied in our population; these differences appear when compared to other populational groups for the same color of skin. This, Brazilians leucoderms have different thickness measures of soft tissues compared to the faces of American Caucasians [6]. Similar differences were also found for Brazilians melanoderms and African-Americans [1]. This study is based on the methodology described by Rhine and Campbell [1], who are the most cited and discussed in the available literature. The same craniometric points used for the measurements and the same measurement techniques indicate that the differences found are real. Afro-Americans in general have thicker soft tissues in the face when compared with Brazilian melanoderms of both genres and Caucasian Americans have the greater thickness of the facial bilateral points. Thickness tables produced for different populations will result in different faces being reconstructed over a single skull. These findings may corroborate with the current theories that there are no distinct races among humans, but morphological characteristics prevailing in different groups.

Conclusions

Our research indicates that the use of Soft Tissue Depth standards from other population groups, like North Americans, in the Brazilian population would result in an inaccurate representation of individuals while alive. Thus, we recommend to Facial Reconstruction in Brazil to use our published tissue depths (Table III). The use of values in our population is likely to perform more accurate Facial Reconstruction, increasing the chances of a Brazilian be recognized through the use of this technique.

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ANATOMICAL LANDMARKS	DESCRIPTION
MidlinePoints	
1. Supraglabella	Foremost point in the midline, above Glabella
2. Glabella	Most forward projecting point of the forehead in the midline at the level of the supraorbital ridges
3. Nasion	Midline of the nasofrontal suture
4. Rhinion	End of the nasal bone
5. Mid-philtrum	Midline of the intranasal depression
6. Supradentale	Center jaw, between the upper incisives
7. Infradentale	Center jaw, between the lower incisives
8. Supramentale	Most posterior midline point, above the chin in the jaw between the infradentale and the pogonion
9. Mental eminence	The most prominent point of the chin
10.Menton	Lowest point of the chin
Bilateral Points	
11.Frontal eminence	Bony projection of the ectocranial surface of the frontal bone
12.Supraorbital	Center upper part of the margin of the orbit
13.Suborbital	Center lower part of the margin of the orbit
14.Inferior malar	Lower part of the jaw
15.Lateral orbit	Line between the eye and the center of the zygomatic ach
16. Zygomatic arch	outermost point in the zygomatic arch from a vertical plan view
17.Supraglenoid	Above and forward the acoustic meatus
18. Gonion	The outer margin of the angle of the mandibule
19. Supra M2	Above the second upper molar
20. Occlusal line	Point in the jaw in the plane of dental occlusion
21. Sub M2	Bellow the second lower molar

Table I – Anatomical landmarks considered in present study

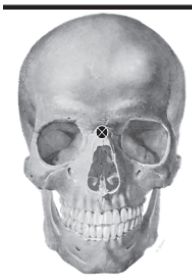
Nasion p=0,008		N	Means/mm
	faio	9	4.7
	leuco	**22**	**6.4**
	melano	8	4.7
	xanto	1	4.0
		40	

Table II – Means (mm) in Nasion craniometrical point for skin color

Localizacion	Means/mm		Localizacion	Means/mm	
	♂ (n=26)	♀(n=14)		♂ (n=26)	♀(n=14)
MidlinePoints			Bilateral Points		
Supraglabella	**5.0**	**4.3**	**Frontal eminence**	**4.9**	**3.9**
Glabella	5.5	4.6	SupraOrbital	6.9	5.8
Nasion	**5.9**	**5.0**	**SubOrbital**	**6.5**	**6.0**
Rhinion	5.2	4.2	Inferior malar	11.2	10.0
Filtro Médio	**10.6**	**7.7**	**Lateral orbit**	**9.1**	**9.2**
Supradentale	9.1	8.7	Zygomatic arch	9.2	8.8
Infradentale	**10.6**	**9.4**	**Supraglenoid**	**11.6**	**10.8**
Supramentale	11.0	9.1	Gonion	12.7	10.9
Mental eminence	**10.6**	**9.4**	**SupraM2**	**16.4**	**14.4**
Menton	10.4	8.7	Occlusal line	14.4	11.7
			SubM2	**14.6**	**11.3**

Table III – Facial soft tissues thickness in Brazilians (mm)

BLOOD PATTERNS

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BLOOD STAINS ANALYSIS ON CLOTHES: A CASE REPORT

Abstract: Some years ago a woman was found dead in her husband's factory, with multiple head injuries. The crime scene showed a lot of blood stains, different in form and dimension. The woman's husband was the first and only suspect of the murder. He was the first to reach the crime scene and a lot of blood was found on his clothes, while his shoes were clean. The suspect said he was dirty because of having trampled on the bloody floor and also because of having touched his wife's bloody body. An analysis of the blood stains on the man's clothes and their compatibility with the dynamic of the murder were requested by the judge during the trial. The possibility for the suspect of making himself dirty by embracing the dead corpse and/or by trampling the blood on the floor was investigated. The collected data were very useful for providing valuable information for the accurate reconstruction of the crime history.

Introduction

Few years ago a woman was found dead in her husband's factory. The cause of death was identified in multiple head injuries with skull fractures and neck vessel injury. The body was found lying on the floor, near a washing machine, and the crime scene showed many blood stains, different in form and dimension, on the floor, on the walls and on the washing machine.

The woman's husband was the first and only suspect of the murder. He was the first to reach the crime scene and a lot of blood was found on his clothes. In particular, a big blood stain was found on the front of his shirt and some smaller stains (round and oval drops) on its front, on its sleeves and collar. Besides, on the internal surface of the shirt, a large blood stain was also found. On the proximal side of the trousers some little drops of blood, almost all round shaped, were detected. The man's shoes were instead apparently clean, without macroscopic evidence of blood. The suspect explained he was dirty because of having trampled on the bloody floor and also because of having touched his wife's bloody body.

An analysis of the blood stains on the man's clothes and their compatibility with the dynamic of the murder were requested by the judge during the trial. As well the possibility for the suspect of making himself dirty by embracing the dead corpse and/

or by trampling the blood on the floor was investigated. The possibility of soiling the internal surface of the shirt was also evaluated, according to the suspect's statements.

Materials and methods

At the beginning, the stains on the clothes were submitted to the generic diagnosis, using TLC (Thin Layer Chromatography), to verify their bloody nature. On the shoes the search of secret bloody stains was performed, using "Luminol". Genomic DNA was extracted from the samples using Chelex® 100 method and typed using the commercial kit AmpFlSTR® Identifiler (Applied Biosystems, Foster City, CA, USA) in a GeneAmp® PCR System 9700 Gold Plate (Applied Biosystems, Foster City, CA, USA), according to the AmpFlSTR® Identifiler protocol. Alleles were separated by ABI PRISM 310 capillary electrophoresis using Gene Scan™-LIZ™ 500 (Applied Biosystems, Foster City, CA, USA) as internal standard and an allelic ladder to evaluate the sizes of the PCR products.

The electrophoresis results were analysed using the software GeneMapper® ID v3.2 (Applied Biosystems, Foster City, CA, USA).

Results and discussion

Some remarks on the production of the blood stains and their compatibility with the reconstruction of the crime were evaluated.

In particular, the bloody nature of the stains was demonstrated and a genetic female profile (victim's profile) was obtained by some blood stains typing.

On the shirt diffused blood stains were visible, some of them of moderate size, due to an absorption of bloody material on the tissue by direct contact and also by blood fallen from a short distance (Fig. 1). The right anterior portion showed a blood smear with characteristics of staining from the internal to the external surface of the tissue (Fig. 4). This element gives evidence for the hypothesis that the shirt had been stained in the same place or that the shirt, dressed entirely open and outside the trousers, had been reached on the internal surface by blood thrown in a moderate amount from a short distance, or that the shirt, not worn, had come in contact with a blood-stained surface (the victim's body or other surfaces).

On the shirt and on the trousers, bloody trails were also present (Figg. 2,3,5,6). These can be attributed to spatters (which are expression of blood from a source not in contact with the garment) of various dimensions, some of them with a pointed shape. These bloodstains can be classified mainly into medium-velocity impact spatters. The spatters can be considered as stains originating from the victim's body during the wounding, produced by the murder weapon. The different areas, the pattern and the variety of the spatters, show that they originated from different directions and in different moments. The small spatters located mainly on the left anterior side and on the right sleeve of the shirt and on the trousers also appeared to be compatible to this mechanism of production, attributing to medium-high velocity impact spatters. Another hypothesis was that such spatters originated after treading on a blood pool on

the floor. Such a hypothesis cannot be taken in consideration because of an absence of massive stains left either by shoes (Figg. 7,8) or by the extreme part of the trousers (Fig. 5); in particular, all those spatters, previously taken in consideration, and which take us back to medium-high velocity impact, cannot be compatible with such a hypothesis, in which blood drops take an anti-gravitational route.

The collected data were very useful for providing valuable information for the accurate reconstruction of the crime history.

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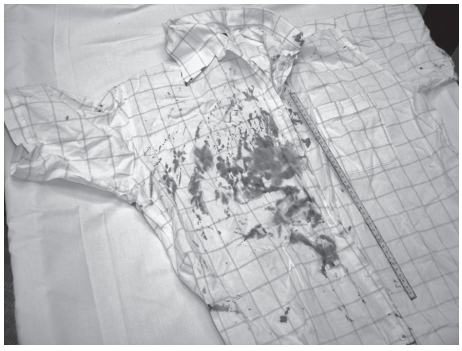

Figure 1 – External surface of the shirt. A large blood stain on its front and some little stains (round and oval drops) on its front, on its sleeves and on its collar.

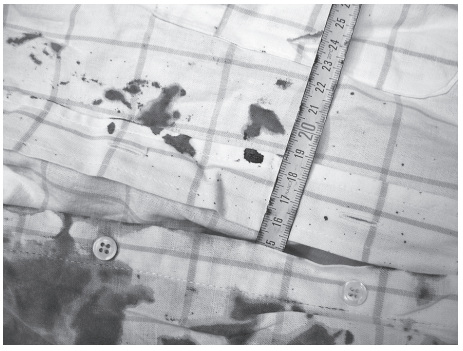

Figure 2 – External surface of the shirt. Particular of some little blood stains and part of the large stain on its front.

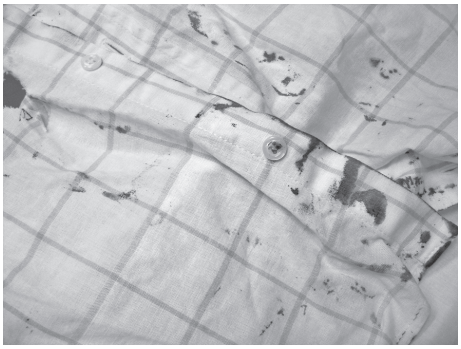

Figure 3 – External surface of the shirt. Particular of some little blood stains.

Figure 4 – Internal surface of the shirt. A large blood stain.

Figure 5 – Trousers. On the frontal side some little drops of blood, almost all round shaped.

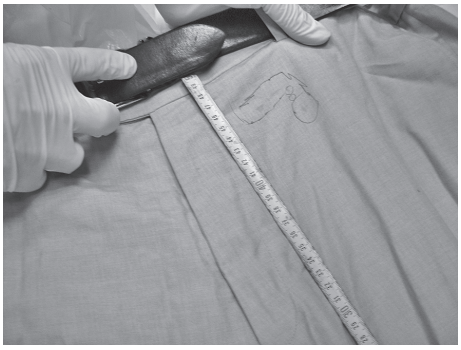

Figure 6 – Trousers. Particular of some little drops of blood, almost all round shaped, on the frontal side.

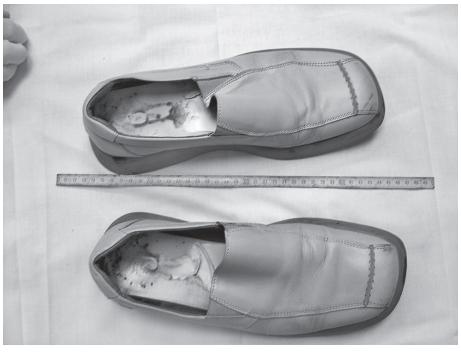

Figure 7 – Shoes. No macroscopic evidence of blood stains on the frontal side.

Figure 8 – Shoes. No macroscopic evidence of blood stains on the soles.

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EVALUATION OF BLOODSTAIN DETECTION TESTS FOR FORENSIC APPLICATIONS

Abstract: Recently the *Bluestar Forensic Kit*® a new luminol reagent based test was designed for blood detection. The aim of this study was to determine the followings parameters: time since collection; dilution and substrate. Samples of human and animal blood origin were tested according to those criteria. We verified that the *Bluestar*® *Forensic Kit* allows the detection of non-diluted and diluted bloodstains in different substrates. The maximum bloodstain dilution detected was 1/30 000 in denim jeans. False positive results due to the *Bluestar*® reaction with iron metallic particles present in everyday materials were frequent. The use of *Bluestar*® *Forensic Kit* on the samples does not interfere with DNA profile analysis. A non-diluted bloodstain was still detected although with less intensity, one month after reagent preparation. Two immunochromatographic tests (*Rapid Stain Identification–RSID*® and *Hexagon OBTI*) for confirmation of human blood were also compared. This study demonstrates that both are efficient but the *Hexagon OBTI* much quicker and essential to confirm the presence of human blood traces.

Introduction

Bloodstains may be present at crime scenes and can be found diluted as an attempt to conceal or hide evidence, by cleaning up blood-contaminated areas. These bloodstains can present different patterns on the floor and wall splashes. DNA analysis can determine the genetic profile of the crime perpetrator. We tested the *Bluestar*® *Forensic Kit* efficiency in different human and animal bloodstains. The *Bluestar*® *Forensic Kit* detects the presence of blood. This test contains two main chemicals, luminol and hydrogen peroxide, which react with the iron of blood cells producing a strong chemiluminescence blue light [1]. Two immunochromatographic tests – *Rapid Stain Identification (RSID®) and Hexagon OBTI* – were used as confirmatory tests for human blood origin. The *RSID*® test is based by the detection of glycophorin A present in red blood cells membrane, whereas the *Hexagon OBTI* detects human hemoglobin (hHb) [1, 2].

Materials and Methods

The effect of the luminol based reagent over DNA extraction and amplification was evaluated as well reagent performance over time. In this study whole blood contained in K3E/EDTA 3K tube was used to obtain several stains from different periods of time. Diluted bloodstains were also prepared and placed in various materials.

Samples of human and animal (duck, rabbit, pig, sheep, wild boar, fallow deer and otter) blood origin were tested for different criteria: time since collection (from 1 week old to 9 years); dilution – 1/2 to 1/100 000 and substrate (denim jeans, plants, aluminum, porcelain tiles, soil and metal supports). Some of the jean stains were submitted to a 5 % bleach treatment followed by normal machine wash, 40ºC plus commercial laundry detergent (*Skip* – composed of 15% – 30% zeolites, 5 – 15% bleaching agents based on oxygen, non–ionic surfactants, anionic surfactants; <5% soap, polycarboxylates, phosphonates, perfume, brighteners, enzymes, citronellol – Unilever). Negative controls (distilled milli–Q water) were also used.

The samples were submitted to a presumptive Luminol test using the *Bluestar® Forensic Kit* – Bluestar® to detect the presence of blood. In order to identify human blood origin samples, Immunochromatographic tests (*Rapid Stain Identification-RSID®* from Galantos Genetics GMBH and *Hexagon OBTI* from Bluestar®) were used. Bloodstains in denim jeans that tested positive with both *Bluestar®* and Immunochromatographic tests were submitted to DNA extraction, accordingly to the Chelex® 100 [3] and Phenol-Chloroform [4] protocols. The Qubit™ Fluorometer – Invitrogen was used for DNA quantification and the *PowerPlex® Y System Kit* – Promega for PCR amplification. The DNA profile detection was performed in the ABI PRISM® 310 Genetic Analyzer – Applied Biosystems.

Results

Substrates

After *Bluestar®* application in different substrates – plant, denim jeans, aluminum, porcelain tiles, soil and metal supports – non-diluted bloodstains were detected with low natural light conditions.

Dilutions

Diluted bloodstains ranging from 1/250 – 1/30 000 from animals and human were only visible after the application of the *Bluestar®* producing an intense blue chemiluminescence light. However, dilutions 1/750 – 1/30 000 were only detected in dark conditions.

Since the *Bluestar® Forensic Kit* is unable to distinguish human from animal blood, immunochromatographic tests – *Hexagon OBTI* and the *Rapid Stain Identification (RSID®)* had to be used. These two tests present similar results the only difference was the response time period. We were able to obtain results in 10 minutes with the *Hexagon OBTI*, whereas the *Rapid Stain Identification* takes approximately 2 hours to get the viable results (Figures 1a, 1b).

DNA profiles

Considering the positive results obtained through the immunochromatographic tests, DNA typing was performed on samples under the same conditions (volume, blood concentration and bloodstain period of time). The only difference was that some had been sprayed with the *Bluestar*® and others were not. The DNA typing revealed similar profiles with coincident genotypes (Figures 2a, 2b).

Discussion

The *Bluestar*® *Forensic Kit* is able to detect human and animal non–diluted and diluted bloodstains in different substrates – denim jeans, porcelain tiles, aluminum, soil, metal supports and plants. The presence of bloodstain was still detected with the luminol based reagent in denim jeans bloodstain treated with bleach and followed by machine wash. Bloodstains ranging from 1/250 – 1/30 000 dilutions in denim jeans were only visible after the application of the *Bluestar*® reagent. The highest dilution detected by *Bluestar*® was1/30 000. Furthermore, on the contrary of manufacturer instructions, our results emphasize the need of total darkness when detecting high diluted bloodstains (1/750 up to 1/30 000).

We observed strong cross reaction when applying *Bluestar*® over a variety of metal items. Due to the ubiquitous presence of iron metallic particles on ordinary materials, the possibility of false positive results must be taken in particularly consideration.

Despite the recommendation of using the reagent in 24 hours after preparation, we verified that a normal bloodstain was still detected one month later, although with less intensity. DNA typing revealed identical profiles in the samples treated and untreated with *Bluestar*®. Therefore we can infer that the *Bluestar*® does not interfere with DNA analysis.

As expected the *Bluestar*® *Forensic Kit* was unable to distinguish human from animal blood. Two immunochromatographic tests – *Hexagon OBTI* and the *Rapid Stain Identification (RSID®)* – were used. These tests gave also no cross–reactivity with animal samples (duck, rabbit, pig, sheep, wild boar, fallow deer and otter) and present similar results. On the other hand the *Hexagon OBTI* does not require consuming of the questioned bloodstain and is completed in less time (10 minutes against 2 hours of the *Rapid Stain Identification*).

Conclusions

The *Bluestar*® *Forensic Kit* allows the detections of non-diluted and diluted bloodstains (up to 1/30 000) in different substrates. False positive results must be taken into account due to *Bluestar*® reaction with iron metallic particles present in everyday materials. We confirmed that the luminol based reagent does not interfere with DNA profiling. The use of *Hexagon OBTI* for confirmation of human blood is advisable as it is efficient, does not waste sample and it is not time consuming.

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Figure 1a – *Hexagon OBTI* immunochromatographic test (Dilution 1/2 of human blood, Negative control and Duck blood – non human blood control).

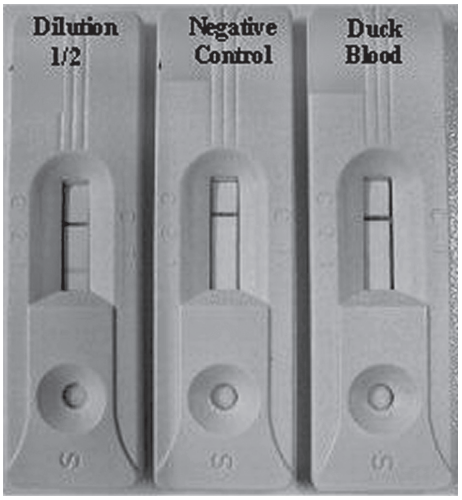

Figure 1b – *Rapid Stain Identification (RSID®)* immunochromatographic test (Dilution 1/2 of human blood, Negative control and Duck blood – non human blood control).

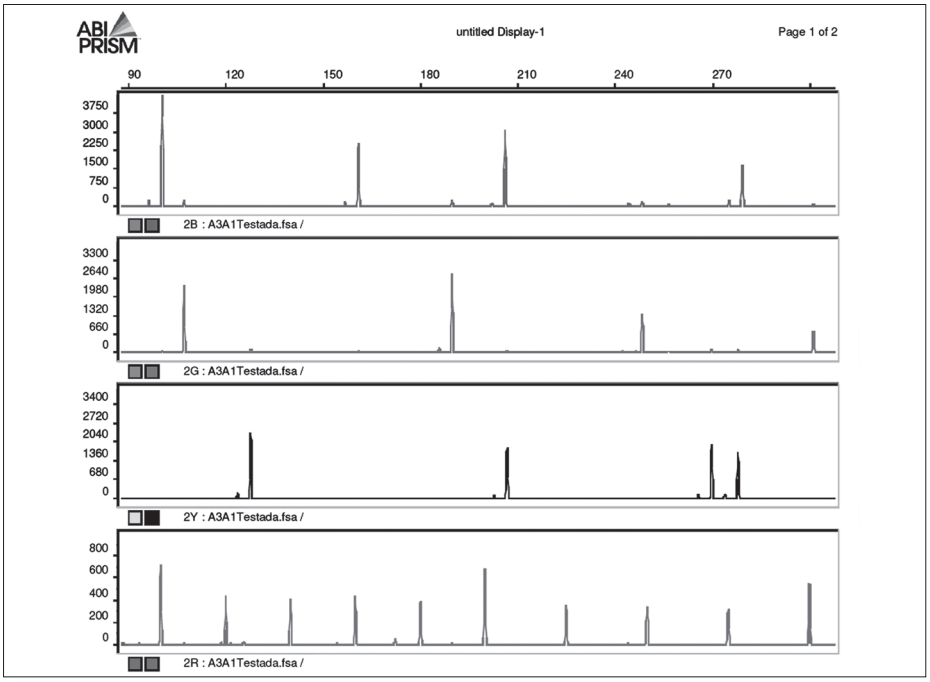

Figure 2a – DNA profile obtained from 1 month old bloodstain with *Bluestar*® reagent application.

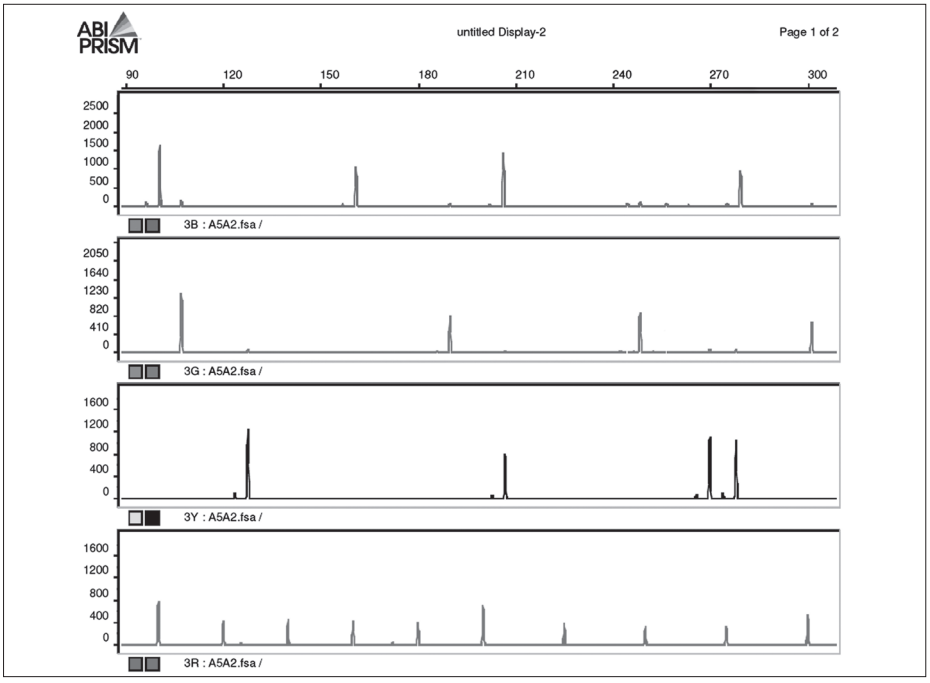

Figure 2b – DNA profile obtained from 1 month old bloodstain without *Bluestar*® reagent application.

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FORENSIC GENETIC

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MIXTURE DETECTION APPLYING MITOCHONDRIAL DNA SNPS VS TRADITIONAL MITOCHONDRIAL DNA SEQUENCING

Abstract: Mitochondrial DNA (mtDNA) analysis found an important role in forensic genetics, especially when nuclear DNA analysis does not give a conclusive response. In this study, mitochondrial DNA Single Nucleotide Polymorphisms (mtSNP) analysis was compared to traditional mtDNA sequencing in order to determine the most useful method for mix detection, a reality in forensic genetics routine. MtSNP analysis allowed to detect mixtures in samples sharing the same haplotype for HV1/HV2 regions.

Introduction

Mitochondrial DNA (mtDNA) analysis found an important role in forensic genetics, especially when nuclear DNA analysis does not give a conclusive response. One major advantage of mtDNA in forensic casework is the high copy number of mtDNA molecules within the cell contrary to nuclear DNA, and its resistance in highly degraded samples, allowing analysis of low level DNA evidence. A major problem in mixture detection through mtDNA sequencing is to distinguish between basal noise and minor components of a mix. By the other hand, mtDNA hypervariable regions typing shows a low power of discrimination, specially when common haplotypes are present. In such cases, mtDNA coding region SNP analysis can be useful in combination with HV1/HV2 analysis, to increase discrimination among individuals (1). In this study, mitochondrial DNA Single Nucleotide Polymorphisms (mtSNP) analysis was compared to traditional mtDNA sequencing in order to determine the most useful method for mix detection, a reality in forensic genetics routine.

Material and Methods

DNA extraction and quantification

DNA was extracted from blood samples using Chelex 100 protocol (2). DNA quantification was achieved with Quantifiler Human DNA Quantification Kit at an ABI Prism7000 Sequence Detection System (AB).

Mitochondrial sequencing

Two blood mixtures were prepared and analysed for mtDNA HV1 and HV2 regions. PCR amplification was made according to Wilson *et al*, 1995 (3) and segments sequenced using BigDye Terminator v1.1 Cycle Sequence Kit (AB) with BetterBuffer (MicroZone Limited), followed by a simple bead purification method (XTerminator) to remove unincorporated terminators. Electrophoresis was achieved in an ABI PRISM 3130 Genetic Analyser (AB) and analysis was done with ABI DNA Sequencing Analysis v5.2 and SeqScape v2.5.

MtSNPs

To test the ability to detect mixtures using mtSNPs, a set of five different mixtures in different ratios (9:1 to 1:1) were prepared using DNA control 9947A and 9948 (both Promega) as well as the blood mixtures previously described.

Sixteen mtSNP were amplified in two multiplex reactions using primers proposed by Brandstätter *et al*, 2003 (4) and amplification protocol published by Parson *et al*, 2008 (5). Previous to minisequencing reaction, PCR products were treated with ExoSAP-IT (USB) in order to remove unincorporated dNTPs and excess of primers. 5 µl of PCR product were incubated with 2µl of ExoSAP-IT for 90 minutes at 37ºC followed by 20 minutes at 80ºC for enzyme inactivation. Minisequencing reaction was achieved using SNaPshot kit (AB) according to Brandstätter *et al*, 2003 (4). Unincorporated ddNTPs were removed with a treatment with SAP (Roche Diagnostics Corporation). The minisequencing product was diluted 1:10 and the electrophoresis was undertaken on an ABI PRISM 3130 Genetic Analyzer using Liz-120 as size standard. Analysis was completed using GeneMapper ID v.3.2 software.

Results

In one blood mixture, HV1/HV2 analysis showed two bases at a large number of positions, indicating the presence of mixed samples and mtDNA SNP analysis confirmed the result (Figures 1 and 2).

For the other mixture, HV1 was CRS and HV2 analysis presented the 263G; 315.1C haplotype and no mixture was detected. Using mtSNP for the same samples, the mixture was detected, proofing the utility of this technology in forensic genetics since the observed HV1/HV2 haplotype is one of the most frequent in European populations. Although the samples shared the same haplotype, they belonged to different branches of haplogroup H, namely H1 and H3, since the mixture was only detected in positions 3010 (Mx 1) and 6776 (Mx 2) (Figure 3).

MtSNP analysis of the five mixture sets (9:1 to 1:1) using DNA controls 9947A and 9948, allowed full mixture detection in all ratios, as seen in Figure 4. In a 6:4 mixture, the mix positions are well detected and quite balanced, whereas in a 9:1 mix peaks appeared, as expected, extremely unbalanced but evident.

Discussion and Conclusion

In degraded samples without nuclear DNA, mtSNP analysis may allow mix detection even when HV1/HV2 haplotypes are identical. Comparing with traditional mtDNA sequencing, mtSNP analysis with SNaPshot technique presents a major advantage: each allele of a determined marker presents a slight deviation from one another. This deviation is due to mobility differences that occur depending on the incorporated terminator, which allows to better distinguish between basal noise and a minor component of a mixture.

Even in very unbalanced mixtures (9:1) it was possible to detect all SNPs in the studied mix ratios, showing the potentialities of this technique in forensics genetics.

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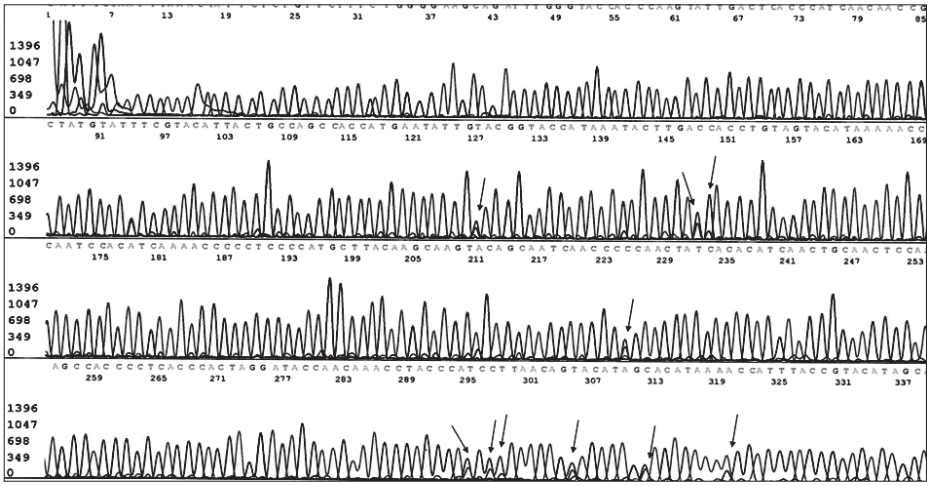

Figure 1 – Electropherogram of mitochondrial DNA sequencing using BigDye v1.1 from a blood mixture. The mixture is detected in several positions, signed with arrows.

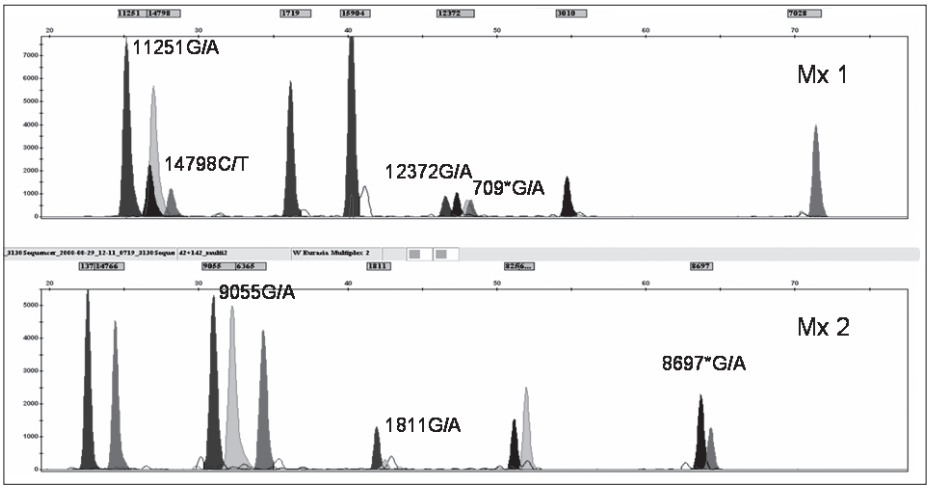

Figure 2 – Electropherograms of mtSNPs for multiplex 1 and 2 from a blood mixture. The mixture is detected in several markers. In the positions marked with * the true allele is the reverse of the detected one.

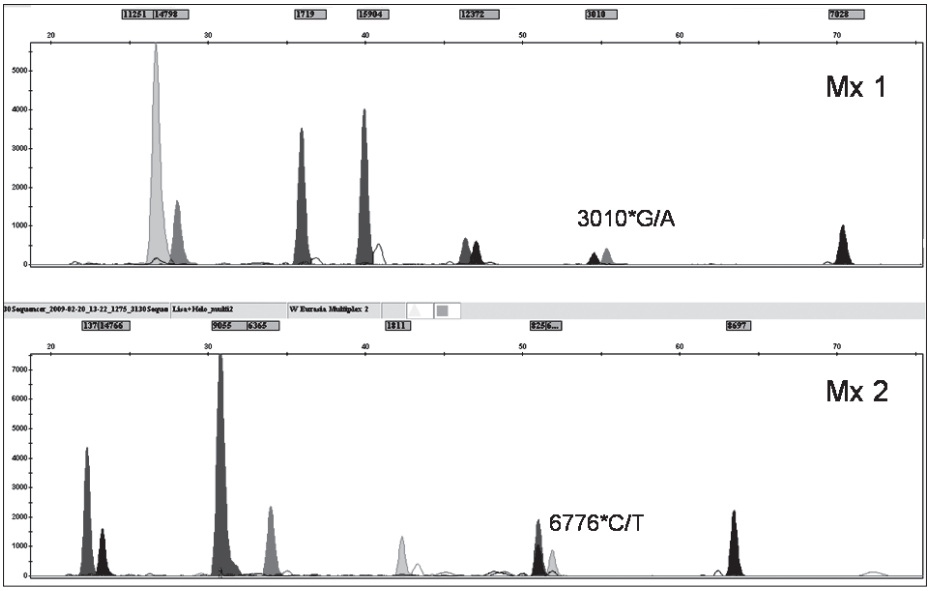

Figure 3 – Electropherograms of mtSNPs for multiplex 1 and 2 from a blood mixture. The mixture is detected in only two markers as both samples belong to haplogroup H (H1 and H3). In the positions marked with * the true allele is the reverse of the detected one.

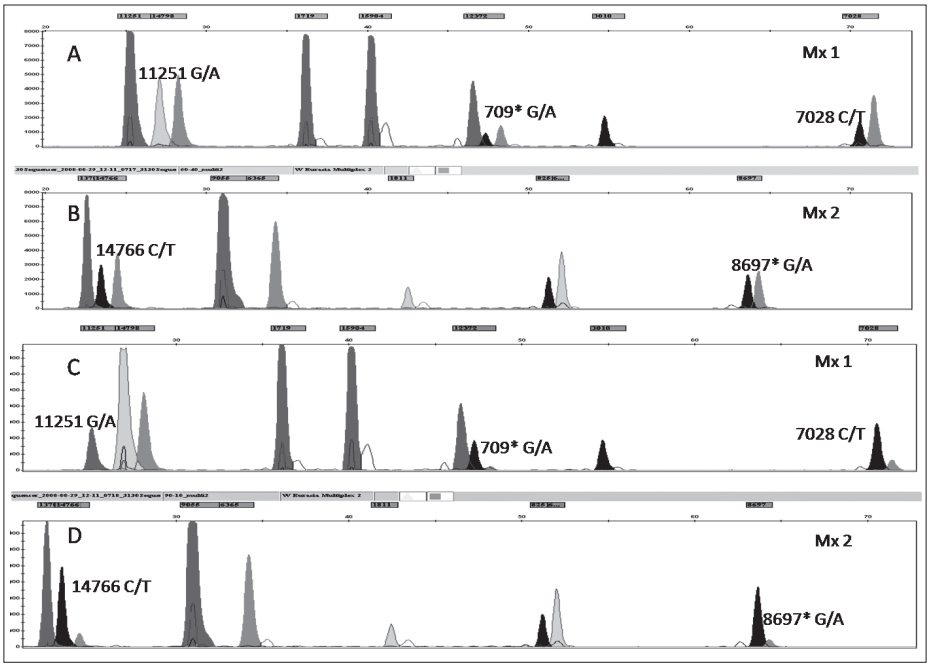

Figure 4 – Electropherograms of mtSNPs for multiplex 1 and 2 from mixture using DNA controls 9947A and 9948. A: multiplex 1 for a 6:4 mix; B: multiplex 2 for a 6:4 mix; C: multiplex 1 for a 9:1 mix; D: multiplex 2 for a 9:1 mix. The mixture is detected in several markers, as indicated by the positions. In the positions marked with * the true allele is the reverse of the detected one.

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COMPARATIVE STUDY OF 15 STR FORENSIC LOCI BETWEEN A BOLIVIAN POPULATION AND CENTRAL AND SOUTH AMERICA POPULATIONS

Abstract: A population of 117 individuals of La Paz was typed for 15 STR loci using the AmpFlSTR® Identifiler™ PCR kit (Applied Biosystems). Allelic frequencies and parameters of forensic interest (power of discrimination and probability of exclusion) were calculated. A comparative study with populations from Central and South America was done, using the calculation of genetic distances between them and the phylogenetic tree.

Introduction

The study of autosomal short tandem repeats (STRs) in a Bolivian population from La Paz is important since this is a country with a high genetic variability, according to previous studies made in mitochondrial DNA [1]. Allelic frequencies of 15 STR forensic loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818 e FGA) were calculated in a sample from La Paz and a comparative study with other populations from Central and South America [2-11] was done.

Material and Methods

DNA from 117 unrelated healthy individuals of La Paz, Bolivia was extracted, using Chelex100 method [12]. Samples were amplified using the AmpFlSTR® Identifiler™ PCR kit (Applied Biosystems) [13] according to manufacturer's instructions. Amplified products were separated and detected using an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems), analyzed with GeneScan software version 3.1. and typed using the reference sequenced ladder.

Allelic frequencies (see table 1), observed and expected heterozygosity and Hardy-Weinberg (HW) equilibrium were calculated using the Arlequin population genetics software v3.1 [14]. Matching probability, power of discrimination and power of exclusion were achieved using the PowerStats v1.2 software. Software *Phylip* v3.5c [15] was used to establish phylogenetic relationships between population of La Paz and other Central and South American populations, by calculating the genetic distances.

Neighbor-Joining method produced the phylogenetic tree, visualized by application of software *TreeView* v1.6.6 [16].

Results

Results are founded in figure 1, table 1 and table 2.

Discussion

This Bolivian population is in Hardy-Weinberg equilibrium for all markers, except for CSF1PO (P<0,05). The observed heterozygosity (Ho) is between 0.573 (D5S818) and 0,872 (FGA). The power of discrimination (PD) varies between 0.760 (TPOX) and 0.963 (FGA) and the combined power of discrimination for the 15 loci is 0.9999999. The probability of exclusion (PE) varies between 0.259 (D5S818) and 0.738 (FGA), and the combined probability of exclusion for all loci is 0.999987 (see table 2).

Phylogenetic analysis (figure 1) revealed that La Paz is distant from the remaining populations, mainly the Brazilians, with genetic distances greater than 0.1319. The population that reveals the smaller distance from La Paz population is Valley of Mexico (0.0381). Populations of El Salvador and Mexico have genetic distances of 0.0519 and 0.0439 respectively, Central region of Mexico (0.0719), Buenos Aires (0.0814), Caracas (0.1012) and Maracaibo-Venezuela (0.1031).

Conclusions

Our results are important to determine the genetic background of La Paz population and the combination of the studied 15 STR loci presents a powerful strategy for individual identification and parentage analysis.

The geographical situation of La Paz, namely the location at highlands around Lake Titicaca, could explain the higher genetic distances between other populations from Central and South American.

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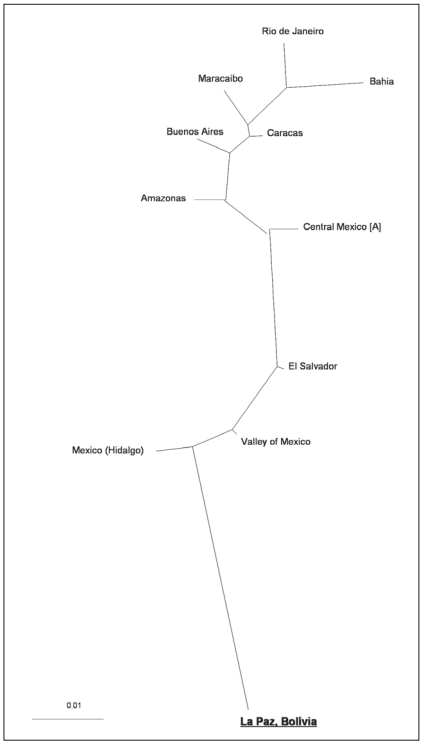

Figure 1 – Phylogenetic tree – genetic distances between Bolivia and other populations [1-10]; [A] Mestizos from the central region of Mexico.

Alleles	CSF1PO	D2S1338	D3S1358	D7S820	D8S1179	D13S317	D16S539	D19S433	D21S11	TH01	vWA	TPOX	D18S51	D5S818	FGA
6										0,1880					
7				0,0170						0,5170				0,1370	
8	0,0130			0,0210		0,0260	0,0090			0,0560		0,6030		0,0040	
9	0,0040			0,0380	0,0040	0,3590	0,2820			0,0340		0,0210		0,0850	
9.3										0,2010					
10	0,2260			0,3080	0,0510	0,0980	0,2480			0,0040		0,0300	0,0040	0,0040	
11	0,2610			0,3720	0,0680	0,1580	0,1790	0,0040				0,2090	0,0040	0,5850	
12	0,4060			0,2220	0,0980	0,1750	0,1880	0,0260				0,1320	0,1280	0,1410	
12.2								0,0170							
13	0,0810			0,0210	0,3420	0,1200	0,0810	0,1370			0,0040	0,0040	0,1410	0,0430	
13.2								0,1840							
14	0,0040		0,0260		0,2260	0,0640	0,0130	0,3500			0,0130		0,2560		
14.2								0,0260							
15			0,4700		0,1790			0,1710			0,0510		0,1410		
15.2								0,0510							
16		0,0170	0,3420		0,0260			0,0210			0,3550		0,1200		
16.2								0,0090							
17		0,1240	0,1150					0,0040			0,4020		0,1200		
18		0,0680	0,0470		0,0040						0,1150		0,0470		0,0130
19		0,3460									0,0560		0,0210		0,1320
20	0,0040	0,1450									0,0040		0,0040		0,0560
21		0,0170											0,0090		0,0680
22		0,0130											0,0040		0,0940
23		0,1880													0,1110
24		0,0510													0,1970
25		0,0260													0,1970
26		0,0040													0,1150
27									0,0040						0,0090
28									0,0470						0,0090
29									0,1880						
30									0,1670						
30.2									0,0090						
31									0,0510						
31.2									0,2090						
32									0,0040						
32.2									0,2440						
33.2									0,0600						
34.2									0,0090						
35.2									0,0090						

Table 1 – Allelic frequencies of 15 STR in a Bolivian population from La Paz

	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	D5S818	FGA
H_o	0,76923	0,80342	0,70085	0,68376	0,66667	0,70085	0,78632	0,86325	0,76923	0,73504	0,71795	0,58120	0,85470	0,57265	0,87179
H_e	0,78530	0,82657	0,71810	0,70867	0,64873	0,65544	0,79007	0,78798	0,80305	0,79707	0,69656	0,57661	0,85023	0,61208	0,86629
MP	0,079	0,060	0,127	0,136	0,199	0,168	0,075	0,097	0,075	0,070	0,149	0,240	0,046	0,191	0,037
PD	0,921	0,940	0,873	0,864	0,801	0,832	0,923	0,903	0,925	0,930	0,851	0,760	0,954	0,809	0,963
PIC	0,750	0,800	0,660	0,660	0,580	0,610	0,760	0,750	0,780	0,760	0,640	0,530	0,830	0,580	0,850
PE	0,543	0,621	0,430	0,404	0,379	0,430	0,574	0,721	0,543	0,485	0,457	0,269	0,704	0,259	0,738
P exact test for Hardy-Weinberg equilibrium	0,36616	0,41568	0,95814	0,03677	0,19950	0,96080	0,60668	0,05423	0,07985	0,37911	0,89536	0,10465	0,88244	0,13142	0,95495

H_o, observed heterozygosity; H_e, expected heterozygosity; MP, matching probability; PD, power of discrimination; PIC, polymorphism information content; PE, power of exclusion; *P*, probability value of exact test for Hardy-Weinberg equilibrium.

Table 2 – Forensic statistical parameters of La Paz population

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FORENSIC IMPLICATIONS OF A SINGLE DUPLICATION IN DYS448

Abstract: The Y- Chromosome is specific to males and therefore very helpful in sexual assault cases. Commonly forensic used Y-STRs alleles are single–copy loci, however it is known that Y –chromosome shows several structural variants such as copy duplications which can complicate evidentiary samples Y-STR profile analysis. With this work we focus on a DYS448 duplication obtained in a sexual assualt case evidence samples and the suspect reference sample matching.
Keywords: DYS448; duplication; AZFc region.

Introduction

The Y- Chromossome is specific to males and therefore very helpful in sexual assault cases where the perpretor's DNA needs to be identified in the presence of the male/female DNA mixtures.

Commonly used Y-STRs are single–copy loci, however it is known that in some Y-STR alleles, copy duplications could be observed in samples originated from a single individual. Large scale use of multiple Y-Chromosomal microsatellites in forensics can reveal such variants.

In this work we focus on a particular sexual assault case, whose victim's evidences – vaginal swab and a pair of undergarments– were in first step studied in search for male DNA contribution. These evidences reveal an allelic duplication at DYS448 lied on the proximal part of AZFc. In second step the suspect was also studied to compare with the evidences and the same duplication was detected on his profile. With this case study we pretend to show how a Y-chromosome duplication can reveal of whether or not a mixture of multiple allele DNA profile is present in an evidence sample.

Methods

Evidence's DNA was extracted with phenol/chloroform procedures and buccal swabs with Chelex method.

Purified DNA was quantified with Quantifiler™ Human DNA and Quantifiler™ Y Human Male DNA Quantification Kit (Applied Biosystems). All samples were

extracted in duplicated and amplified with AMPFlSTR® Yfiler® PCR (Applied Biosystems) amplification kit according to manufacturer's.

PCR products electrophoresis was carried out on an *ABI Prism 3130xl* (Applied Biosystems). DNA fragments, allele typing and peak areas were analysed using the Genemapper 3.2 (Applied Biosystems).

Results

Figure 1 a) represents Y-STRs results obtained in victim's vaginal swab, which matched with her undergarments profile. Note that all loci were single copy alleles, even the highly polymorphic locus DYS385a/b have one single allele with area approximately twice that of the other Y-STR peaks in same dye color. The results revealed a duplication in DYS448 allelic range (Fig.1 b) with almost equal peak area and only one repeat unit apart. We concluded that this profile should come from only one donor. Later suspect buccal swab analysis revealed the same duplication, as can be seen in the Fig 2 a) and b). The suspect was the only one included from matching the evidentiary profile.

Discussion

In evidentiary samples from sexual assault cases, differentiation between male DNA mixtures and allele duplications must be evaluated according to published rules. In this case, was particular easy, because we had only two allele copies in DYS448 locus. The greater the number of loci, with more than one allele, besides DYS385a/b, greater the possibility of having two or more contributors in that sample, unless an entire region of Y-chromosome have been duplicated. Y-STRs information in sexual assault cases could be an important tool for detecting the number of male donors to an evidence specially in male/female mixtures.

Otherwise, locus duplication along the Y-chromosome can provide greater strength to a match between two samples, however should not prevent the reliable interpretation of mixtures.

Special attention should be given to these cases in order to prevent wrong inclusions or exclusions from a profile.

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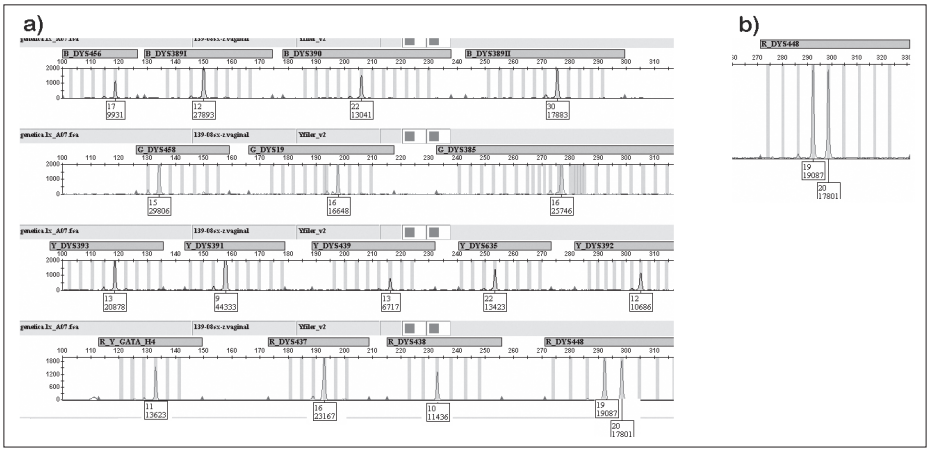

Figure 1 – vaginal swab Y-Filer profile a) and DYS448 loci allele duplication b)

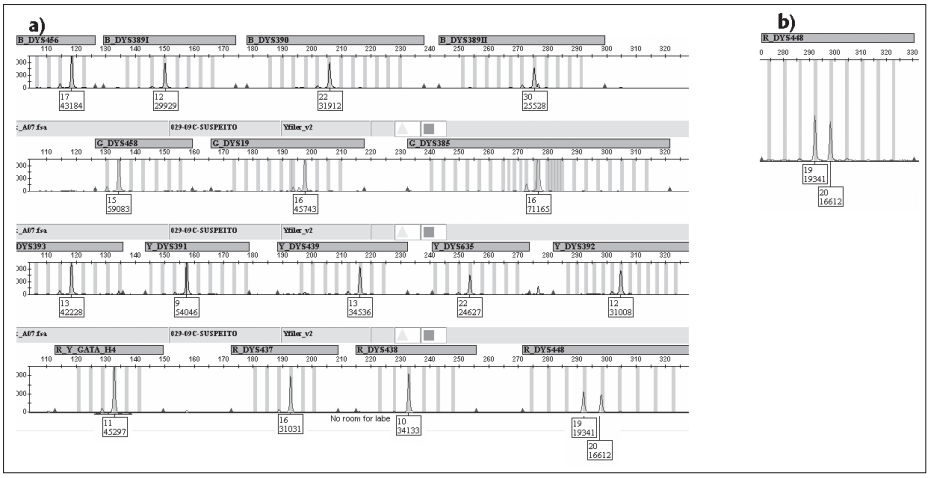

Figure 2 – Suspect Y-Filer profile a) and DYS448 loci allele duplication b)

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THE INTEREST OF MITOCHONDRIAL DNA CODING REGION SNPS

Abstract: As analysis of mtDNA hypervariable regions is a laborious technique, it is desirable to type mtSNPs before performing sequencing. This study presents a 10 SNP multiplex, selected from mitochondrial coding region *loci*. These markers can subdivide any population samples in H* and non-H* haplogroups, assigning non-H* haplogroups, which in same cases is useful for forensic casework. The SNP multiplex detected 13 different non-H* haplogroups encountered in the two most common populations in our casework – European and African ancestry populations. Haplogroups U, J, T and L1/L2 are the most frequent non-H* haplogroups in the Portuguese population samples. A probable new variant was assigned as a U/K haplogroup. This coding SNP assay is a simple method and can increase the discrimination of hypervariable region haploypes.

Keywords: MtSNPs; coding region; SNaPshot analysis; non-H* haplogroups.

Introduction

In recent years, the interest in autosomal, Y-chromosome and mitochondrial Single Nucleotide Polymorphisms (SNPs) has increased in the forensic area. Mitochondrial DNA (mtDNA) is useful for identity testing and, above all, for analysis of degraded material or few DNA containing samples, such as skeletal remains or hair shafts. Analysis of mtDNA HVI and HVII hypervariable regions is sometimes the only available method in forensic casework but provide limited power of discrimination besides being a laborious technique. Coding mtSNP multiplex reaction prior to sequencing analysis can allow for a rapid screening in forensic casework (1). In this study we have selected 10 SNP *loci*, performed in one multiplex assay, for mitochondrial DNA non-H* haplogroup typing of the two most common populations in our casework – European and African ancestry populations.

Material and Methods

DNA was extracted from blood stains by Chelex method in a total of 80 Portuguese population samples studied. HVI and HVII regions were previously sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). A preliminary

classification of sample sequences into haplogroups was done following phylogenetic criteria. Ten non-H* mtSNP *loci*, selected from previous panels from other authors (1-3), were studied with single base extension using SNaPshot® methodology (Applied Biosystems). SNP *loci* selected were the following – G1719A, C3594T, T4216C, G4580A, C7028T, G8251A, A10398G, C10400T, C12705T and A12308G. Amplification products were analyzed in a 3130 Genetic Analyzer with GeneMapper® ID Software v3.2 (Applied Biosystems).

Results

Using this 10 SNP target site multiplex assay, several haplogroups can be detected – U, J, T, I, K, X, W, M, N, V, HV, L3, L1/L2 and H* (Fig.1), some of them shown in Fig.2, which agreed, in the majority of the samples studied, with haplogroups previously obtained from mitochondrial haplotypes. H* haplogroup is caracterized by C7028 and non-H* haplogroups are defined by different SNP polymorphisms as shown in Table1. Haplogroups U, J, T and L1/L2 are the most frequent non-H* haplogroups in the Portuguese population sample, as previously emphasized by HVI and HVII hypervariable region studies. It was not possible to assign a defined haplogroup (U or K) in two samples, as the 10398 SNP *locus* was not detected.

Discussion and Conclusions

This 10 *loci* SNaPshot® multiplex provides a less expensive and simpler method for coding region mtSNP typing compared to control region mtDNA sequencing. With this set of coding mtSNPs it is possible to subdivide any population samples in H* and non-H* haplogroups, assigning non-H* haplogroups, which has been done previously for H* haplogroups (4) and in same cases is useful for forensic casework analysis. With these selected markers, 13 non-H* haplogroups can be detected including the most common European and African population non-H* haplogroups. However, in two samples, it was not possible to assign a defined haplogroup (U or K), as the 10398 SNP *locus* was not detected, probably due to a mutation near this *locus* in our population. The new variant was assigned as a U/K haplogroup. This panel increases phylogenetic haplogroup discrimination which can be very useful for forensic casework, as also emphasized by other authors (5-7).

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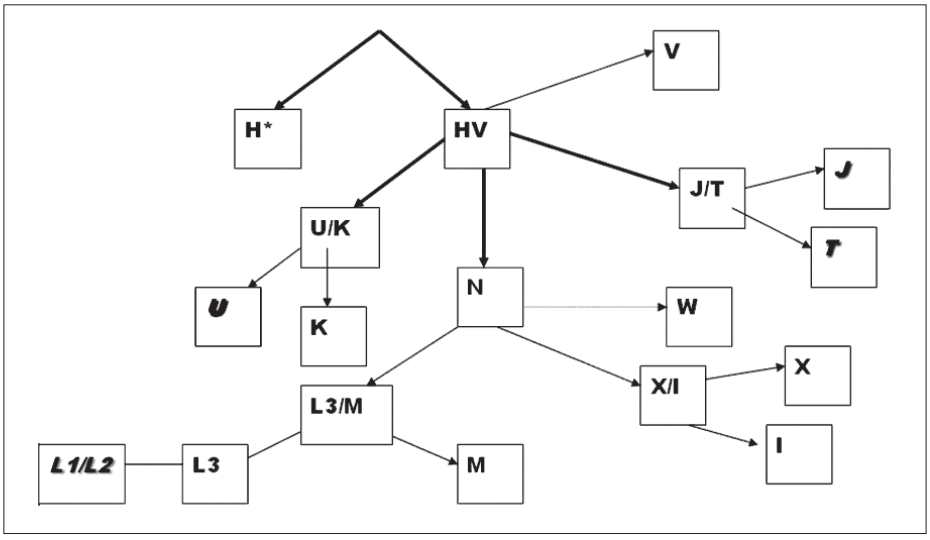

Figure 1 – Mitochondrial phylogenetic tree determined with 10 mtDNA coding region *loci,* emphasizing J, U, T and L1/L2 haplogroups, the most common non-H haplogroups in our population study

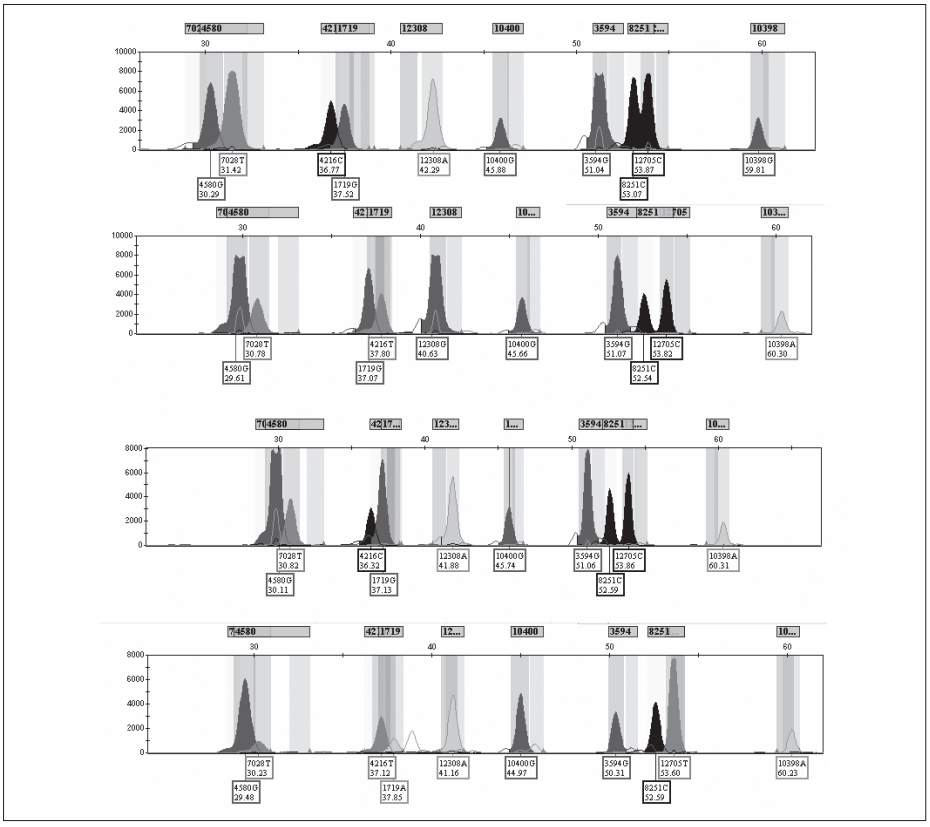

Figure 2 – Several mtDNA haplogroups can be obtained with 10 *loci* SNaPshot multiplex as shown, respectively, for J, U, T and X haplogroups.

G4580A	C7028T	G1719A	T4216C	A12308G	G10400A	G3594A	C8251T	C12705T	A10398G	Haplogroup
G	T	G	T	A	G	A	C	T	G	**L1/L2**
G	T	G	T	A	G	G	C	T	G	**L3***
G	C	G	T	A	G	G	C	C	A	**H***
G	T	G	T	G	G	G	C	C	**?**	**U*/K**
G	T	G	T	G	G	G	C	C	A	**U***
G	T	G	T	G	G	G	C	C	G	**K**
G	T	G	C	A	G	G	C	C	A	**T**
G	T	G	C	A	G	G	C	C	G	**J**
G	T	A	T	A	G	G	C	T	A	**X**
G	T	A	T	A	G	G	T	T	G	**I**
A	T	G	T	A	G	G	C	C	A	**V**
G	T	G	T	A	G	G	C	T	A	**N***
G	T	G	T	A	G	G	T	T	A	**W**
G	T	G	T	A	A	G	C	T	G	**M***
G	T	G	T	A	G	G	C	C	A	**HV***

Table 1 – SNP state for 15 different haplogroups – H* haplogroup is caracterized by C7028 and U*/K haplogroup by a possible mutation near the 10398 SNP *locus.*

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FORENSIC Y-STR STUDY IN Y-CHROMOSOME ABNORMALITIES

Abstract: Y-chromosome infertility is usually caused by deletions of genetic material in Azoospermia Factor (AZF) regions, localized in three nonoverlapping Yq regions – AZFa, AZFb and AZFc. However, some of the Y-STRs routinely studied in Forensic Genetics with multiplex kits are localized in these three regions giving rise to microdeletions observed in some *loci*. Rare alleles were also encountered in these samples with Y-chromosome structural changes. A complete Y-STR profile in a sample from a female individual was detected which may raise ethical implications. The aim of this work is to assess the validity in the use of Y-STR multiplex systems in structurally abnormal Y-chromosomes, which may occur in forensic casework analysis.
Keywords: AZF regions; Y-STRs; microdeletions.

Introduction

Y-chromosome infertility is usually caused by deletions of genetic material in AZF region, localized in three no overlapping Yq regions – AZFa, AZFb and AZFc (1-3). Microdeletions are rare in AZFa region, concerning 1-2% of total deletions in AZF region. The most frequent microdeletions are observed in AZFc region where we encountered 80% of total AZF region deletions (4,5). However, some of the Y-STRs routinely studied in Forensic Genetics are placed in these three regions (6). The aim of this work is to assess the validity in the use of Y-STRs in structurally abnormal Y-chromosomes, which may occur in forensic casework.

Material and Methods

Y-STRs were studied with AmpFlSTR® Yfiler® in 46 samples previously screened for AZF region deletions and with informed consent. DNA samples from these individuals were provided as blind samples, not being known how many individuals had AZF microdeletions. Samples of 50 individuals also with informed consent and with clinical indication of male infertility but with no Y chromosome deletions (control samples) were also studied. Control samples were also previously characterized at the molecular level, using Y chromosome specific STS (Single Tagged Site) concerning these

three AZF regions: AZFa – DFFRY3´, DBY; AZFb – sY1227, sY1224, sY134, sY119, sY134, RBMY1, sY143; AZFc – sY1192, sY254, RRM3, sY1291, sY283, sY1201.

DNA extraction was done by "Salting in salting out" method with Wizard Genomic DNA kit (Promega) and, more recently, automatic extraction with MagNa Pure LC DNA Isolation Kit (Roche). AmpFlSTR® Yfiler® PCR Amplification kit (Applied Biosystem) was used for studying the following 16 *loci* – DYS456, DYS389I, DYS390, DYS389II, DYS458, DYS19, DYS385, DYS393, DYS391, DYS439, DYS635, DYS392, GATAH4, DYS437, DYS438 and DYS448. Capillary electrophoresis was performed in a 3130 Genetic Analyser with 36 cm capillaries with POP^{TM}4 (Applied Biosystems), using specific software GeneMapper IDv3.2.

Results

Forty-six samples were previously screened for AZF region deletions by using Y chromosome specific STS (Single Tagged Site) (Table1). DNA samples from these individuals were provided as blind samples, not being known how many individuals had AZF microdeletions. Four cases with no amplification of DYS385, DYS392 and DYS488 *loci* were detected (Fig.1 and Table 2), suggesting microdeletions in the AZFb region (Table2). From the Y-chromosome map positions, DYS385, 392 and 448 are located in the AZFb region, while DYS434, 437, 435, 439, 389I/II, 388, 438 and 436 are located in the AZFa region. DYS 391, 393 and 19 are not assigned to these regions. Using Yfiler multiplex system, rare alleles were observed in DYS458 (allele17.2), DYS385 (allele12.1) and GATAH4 (allele14.1) loci. Concerning control samples, there was no suggestion of microdeletions although some rare alleles were also present in DYS458 (alleles16.2, 17.2 and 18.2) and DYS635 (allele17). A complete Y-STR profile was detected in a sample belonging to a female individual, although with a 46, XY karyotype (Fig.2).

Discussion and Conclusions

The existence of Y-chromosome microdeletions is a phenomenon with which Forensic Geneticists may have to deal. In case of such an occurrence, is of great importance to know how to interpret the nature of these results, given the structural changes of the Y-chromosome. In all control samples, a full Y-STR profile was obtained, confirming molecular information previously obtained with STS analysis. Four cases of non amplification of DYS385, DYS392 and DYS488 *loci* were detected, suggesting microdeletions in the AZFb region which was confirmed by single tagged site studies. Rare alleles not previously detected in Portuguese population studies were also encountered in several Y-STR *loci*.

A complete Y-STR profile was detected in a sample belonging to a female individual, although with a 46, XY karyotype. This is a well known clinical situation, even though of rare occurrence. Complete Y-STR profiles in samples from female gender individuals may raise ethical implications and, above all, increase the difficulty in the interpretation of physical evidence in criminal investigation casework.

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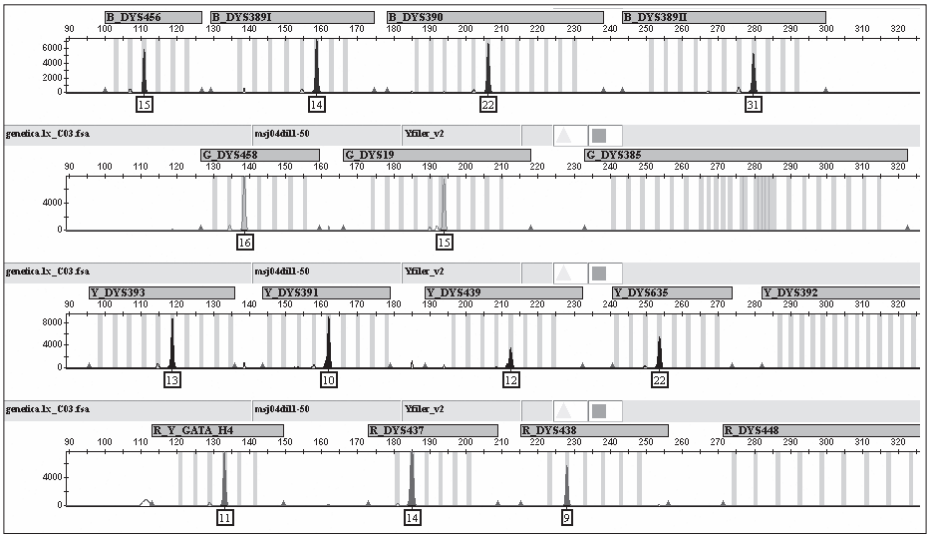

Figure 1 – Y-STR profile performed with Yfiler showing no amplification in DYS385, DYS392 and DYS448 *loci*

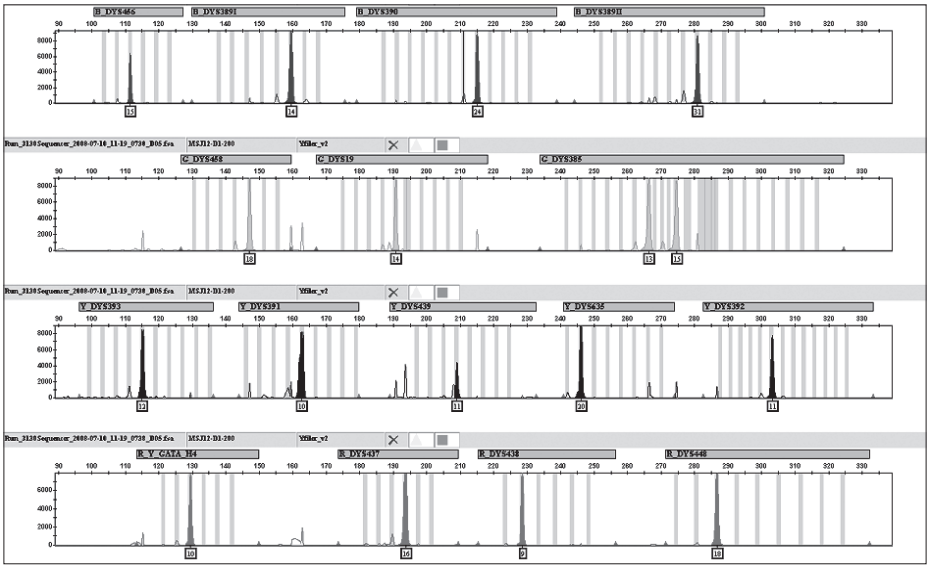

Figure 2 – A complete Y-STR profile was detected in a sample belonging to a female individual, although with a 46, XY karyotype.

AZF Region	STS's	Sample1	Sample2	Sample3	Sample4
AZFa	*DFFRY-3'*	Present	Present	Present	Present
	DBY	Present	Present	Present	Present
AZFb	sY1227	-	-	-	-
	sY1224	-	-	-	-
	sY119	-	-	-	-
	sY134	-	-	-	-
	RBMY1	-	-	-	-
	sY143	-	-	-	-
AZFc	sY1192	Present	-	-	-
	sY254	Present	-	-	-
	RRM3	Present	-	-	-
	sY1291	Present	-	-	-
	sY283	Present	-	Present	-
	sY1201	Present	-	Present	-

Table 1 – Four examples of microdeletion results obtained with single tagged sites concerning AZF regions.

Amostras	Sexo	DYS19	DYS389I	DYS389II	DYS456	DYS390	DYS458	DYS385	DYS393	DYS391	DYS439	DYS635	DYS392	GATAH4	DYS437	DYS438	DYS448
	M	14	13	29	15	23	18	11-14	13	11	12	23	13	12	15	12	19
	M	14	13	29	15	23	**17.2**	14-17	12	10	12	20	11	11	14	10	20
1	M	14	13	29	16	23	18	?	13	11	13	23	?	11	15	12	?
	M	14	13	29	16	24	17	11-13	13	11	12	24	13	**12**	**15**	**12**	**19**
	M	14	13	30	16	24	16	**11-12.1**	12	**11**	**12**	**23**	**13**	**12**	**15**	**12**	**19**
	M	14	14	31	16	25	17	11-15	13	11	10	23	13	12	15	12	19
	M	15	12	29	15	22	16	14	14	10	11	20	11	13	16	10	21
2	M	15	13	29	15	24	17	?	13	12	14	23	?	**14.1**	15	12	?
	M	15	13	30	15	24	17	11-14	**13**	**11**	**12**	**23**	**13**	**12**	**15**	**12**	**19**
3	M	15	13	31	15	24	15	?	12	10	11	21	?	10	14	10	?
	M	15	14	29	17	24	16	11-15	13	10	11	23	13	12	15	12	17
	M	15	14	30	15	24	17	11-14	13	11	11	23	13	12	15	12	19
4	M	15	14	31	15	22	16	?	13	10	12	22	?	11	14	9	?

Table 2 – Examples of Y-STR profiles showing 4 samples with no amplification in DYS385, DYS392 and DYS448 *loci* (samples in yellow).

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SPECIES IDENTIFICATION FROM GENETIC MATERIAL WITH CYTOCHROME b

Abstract: Cytochrome b gene has a genetic content preserved between animals and it is used especially for the vertebrate's identification. In the forensic context it is specially used to determine the origin of non human samples. The sequencing strategy that was implemented in our laboratory, BigDey/BetterBuffer/XTerminator, allowed to reduce time procedures, improve quality of data and reduce significantly the cost per reaction comparing to other described methodologies. Using the program BLAST the obtained sequence is aligned and compared with sequences of the cytochrome b gene registered in a database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The aim of this study is the identification of several vertebrate animals to allow the resolution of eventual forensic cases in a shorter time than traditional methodologies and with lower costs.

Introduction

The nucleotide sequence of the cytochrome b (cytb) region of the mitochondrial DNA (mtDNA) holds specific information about diverse animal species. The analysis of the sequence of the cytb has been used in the identification of species in phylogenetic and forensic genetics fields. There are cases in the forensic context (traffic accidents involving animals, illegal traffic or hunter of animals, human attack perpetrated by animals, among others) in which identification of species is necessary for the case to be solved (Branicki *et al.*, 2003).

The aim of this study is the identification of several vertebrate animals to allow the resolution of eventual forensic cases in a shorter time than traditional methodologies and with lower costs.

Materials and Methods

Blood samples were obtained from diverse mammals and birds. DNA was extracted using Chelex®100 method (Walsh *et al.*, 1991). PCR amplification was performed with primers L14816 and H15173 (Parson *et al.*, 2000) and chemistry of QIAGEN® Multiplex PCR Master Mix in a final volume of 25 µl. Thermocycling conditions

were performed in a GeneAmp® PCR system 2700 (Applied Biosystems), followed the Parson *et al.*, (2000) protocol. The cycle sequencing was performed using the ABI Prism® BigDye® Terminator v.1.1 Cycle Sequence Kit (Applied Biosystems); BetterBuffer (Microzone Limited, Sussek, UK) has been incorporated into the sequencing procedure. Before DNA analysis a simple bead purification method (XTerminator) was made, to remove the unincorporated BigDye terminators, unnecessary salts, and unused diluent buffer. The sequences were analysed in the sequencer 3130 – Genetic Analyser (ABI PRISM®) with the ABI DNA Sequencing Analysis Software v.5.2 and the SeqScape® Software v.2.5. Species were identified with BLAST 2.2.19+ (Smith *et al.*, 1996, Zhang *et al.*, 2000).

Results

Using the cytochrome b gene and Blast tool we were able to identify multiple species including *Homo sapiens, Canis familiaris*, *Felis catus*, *Ovis aries* and *Turdus viscivorus*. Figures 1, 2 and 3 show the steps that allow identifying samples species (in this case a bird sample).

Discussion

The sequencing strategy BigDye/BetterBuffer/XTerminator compared with others (for example dRhodamine/ ethanolic precipitation) allow to reduce time procedures, improve quality of data and reduce significantly the cost per reaction.

Conclusions

Results show that it is possible to identify multiple species with this low cost and faster methodology.

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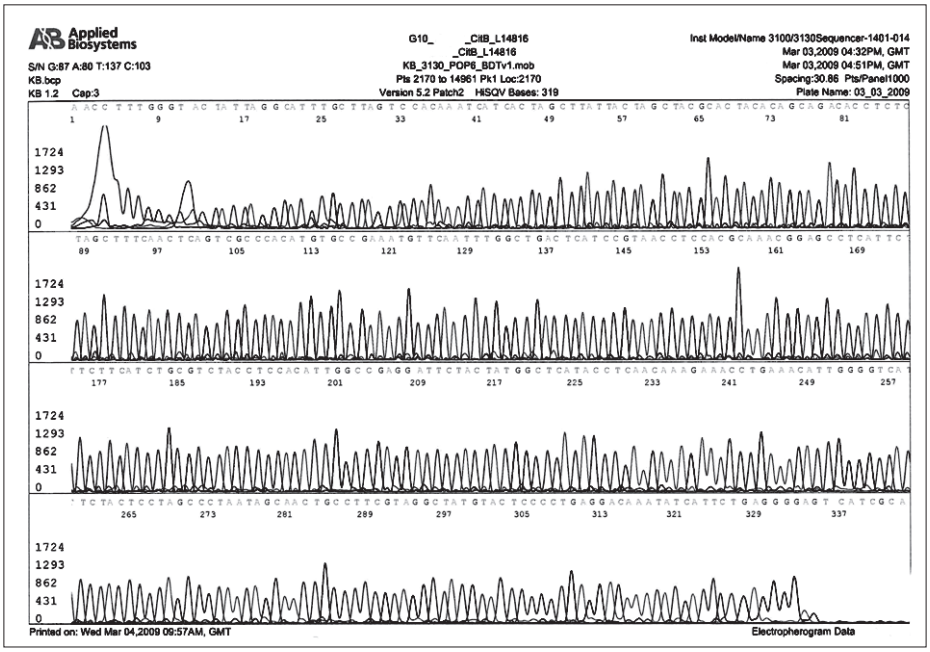

Figure 1 – Electropherogram sequence of *Turdus viscivorus* cytochrome b gene.

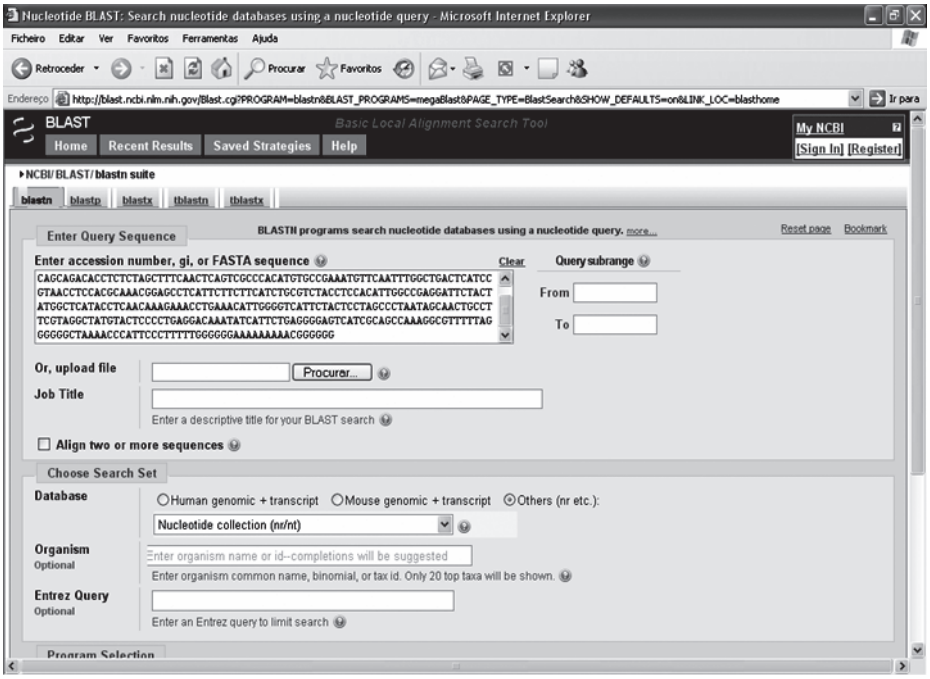

Figure 2 – Input on to Blast Web page of the nucleotide sequence as a query against all the public sequence databases [http://www.ncbi.nlm.nih.gov/BLAST/].

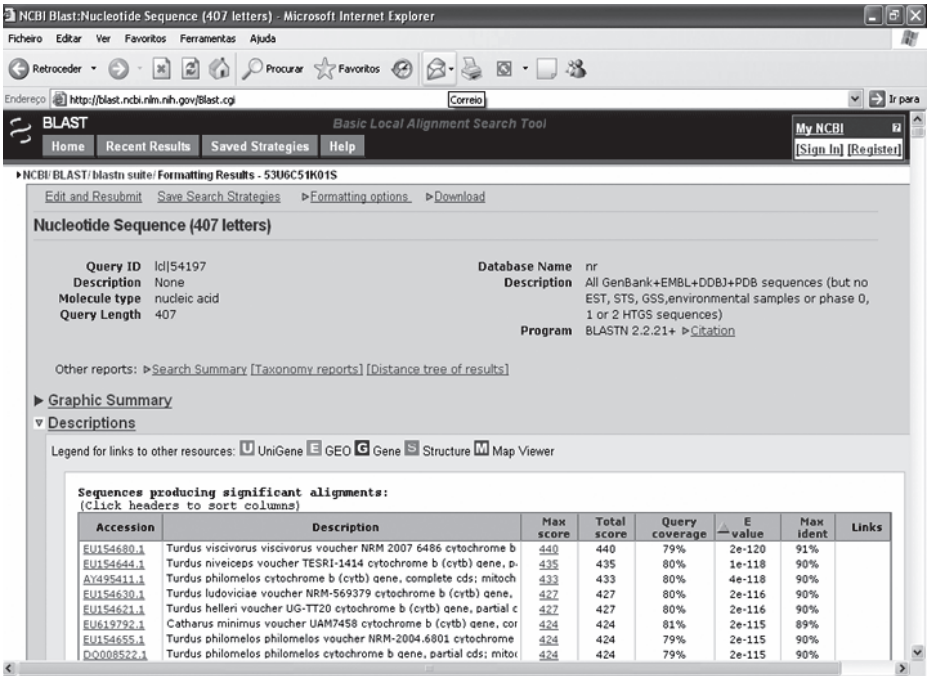

Figure 3 – Results of the search performed on the NCBI database and servers.

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AMPFLSTR® MINIFILER™ PCR AMPLIFICATION KIT: A POWERFUL TOOL FOR GENETIC IDENTIFICATION IN CRIMINAL CASES WITH LOW COPY NUMBER SAMPLES

Abstract: The AmpFlSTR® MiniFiler™ PCR Amplification kit (Applied Biosystems), a new available 8-mini-STR and the sex determining marker Amelogenin multiplex, includes the most common problematic *loci* (above 200 bp) of the AmpFlSTR® Identifiler™ PCR Amplification kit: FGA, D21S11, D18S51, D13S317, D7S820, D16S539, CSF1PO and D2S1338. In several casework samples, with different DNA contents, results allowed to complete partial Identifiler™ profiles and additional information was achieved in Low Copy Number (LCN) samples, revealing that this miniSTR kit can improve identification of compromised samples [1].

Introduction

Multiplex STR typing of forensic samples is a powerful tool for genetic identification that allows quickly to achieve a high combined discrimination power with low DNA consumption (AmpFlSTR® Identifiler™, Applied Biosystems, and PowerPlex® 16 System, Promega, being the most common used PCR Amplification kits in our lab). If full profiles are obtained at the majority of high quality DNA samples, in degraded samples the higher molecular weight markers fail partial or completely their amplification. In order to minimize this problem several miniSTRs (with redesigned primers that generate reduced amplicon fragments) have been developed. This work is about a criminal case of a woman homicide (strangulated) followed by the suicide (hanged by a rope) of the principal suspect of the crime.

Materials and Methods

DNA extraction of LCN samples was made with *Chelex* 100 method [2] (nails and hand scrapings from both victim and aggressor, ropes – Figure 1 – used by the aggressor when trying to commit suicide) or commercial kits, namely DNA IQ™ System, Promega (three hairs collected in the mouth and in the right side of the victim´s face). Samples were quantified with Quantifiler® Human DNA Quantification kit,

Applied Biosystems, using an ABI Prism® 7000, in accordance with the manufacturer´s instructions. Amplification was made with the AmpFlSTR® MiniFiler™ and the AmpFlSTR® Identifiler™ PCR Amplification kits, using an ABI Prism® 2700, in accordance with the manufacturer´s instructions. Samples were genotyped using an ABI Prism 3130 Analyser and the GeneMapper ID 3.2 software. In all laboratory procedures negative controls were tested, to despite contamination. To confirm results, all PCR amplifications were done twice.

Results

Just one hair had sufficient DNA quantity to be amplified with the AmpFlSTR® MiniFiler™Amplification kit, allowing to determine a female DNA profile, compatible with the victim's profile. In one of the analysed ropes, a male profile was obtained, using AmpFlSTR® MiniFiler™ Amplification kit, compatible with the aggressor's profile. In nails and hand scrapings from both victim and aggressor mixed profiles were obtained with the AmpFlSTR Identifiler™ PCR Amplification kit; sometimes these profiles were partial, with the need of being completed, through amplification with AmpFlSTR® MiniFiler™ Amplification kit. Negative controls were always clean. The minimum DNA quantity that allowed results with the MiniFiler™ was 15 pg/µl.

Discussion and Conclusions

MiniFiler™ is a commercial 9-plex miniature STR amplification kit, expected to revolutionize the way forensic scientists process casework samples by significantly increasing the ability to obtain information from DNA evidence, specially inhibited and/or degraded samples, that previously would have yielded limited or no genetic data. By combining innovative primer design, improved PCR amplification conditions and a properly mastermix, MiniFiler™ provides increased sensitivity, robust results in the presence of inhibitors and improved discrimination for casework samples. Working in conjunction with AmpFlSTR® kits, MiniFiler™ can recover more complete DNA from challenging samples enabling more crime and missing person cases to be resolved. MiniFiler™ PCR Amplification kit has been demonstrated to yield the greatest amount of information from samples that have previously produced partial profiles or no profile at all using other existing commercially available autosomic amplification kits. In the presence of PCR inhibitors, MiniFiler™ outperforms other kits with regard to genetic information recovery. The use of a dual-amplification strategy (MiniFiler™ and Identifiler™) is an adequate strategy to deal with compromised samples, allowing achieving information from the most common database autosomic markers, without the need of further population studies. In compromised samples, the largest molecular weight *loci* of Identifiler™, including FGA, D21S11, D18S51, D13S317, D7S820, D16S539, CSF1PO and D2S1338 most often fail to amplify. MiniFiler™ is a very useful kit in these situations (Figure 2), allowing to complete partial Identifiler™ profiles and to achieve additional information in LCN samples. Important advantages

of this new kit are also the possibility to verify the presence of false homozygotes and artefact peaks defined through Identifiler™. MiniFiler™ reduces stochastic effects produced in LCN samples amplifications.

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www.minifiler.appliedbiosystems.com.

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Figure 1 – One of analysed ropes.

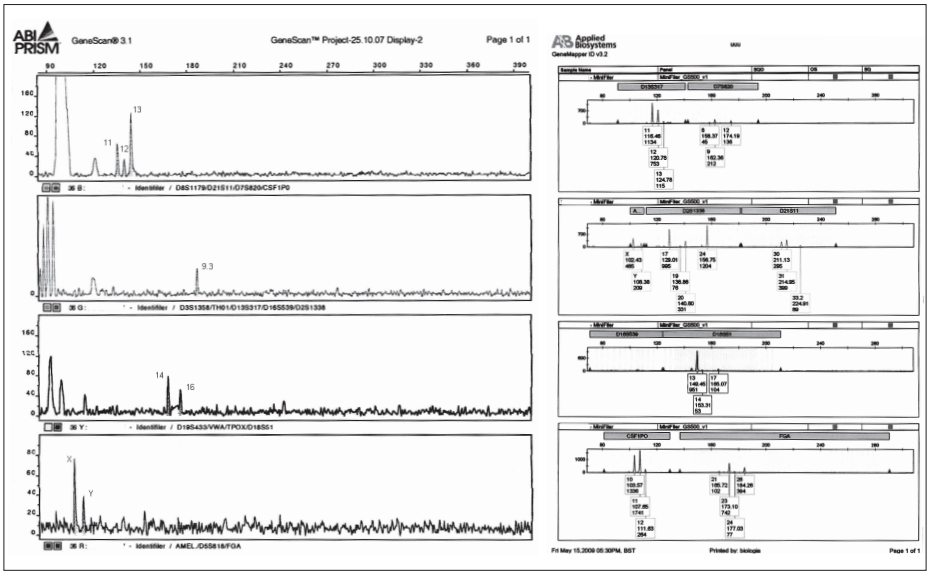

Figure 2 – Compromised sample (hand scrapings) analysed with Identifiler™ (a) and MiniFiler™ (b).

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GENETIC IDENTIFICATION OF ANIMAL SAMPLES (*CANIS FAMILIARIS* AND *FELIS CATUS*) IN FORENSIC CONTEXT

Abstract: Pets live with people and place biological samples everywhere, which may be useful in a forensic context linking suspects and victims, to an occurrence.
There were analyzed samples of 63 unrelated dogs to the STR´s markers PEZ1, FHC2054, FHC2010, PEZ5, PEZ20, PEZ12, PEZ3, PEZ6, PEZ8 and FHC2079, and 63 feline samples of unrelated animals for the FCA733, FCA723 and FCA731 markers.
Preliminary results show that it is possible to make genetic identification of individual animals of species under study, thus contributing to increase the potential of forensic samples of animal origin.
Keywords: Animal samples; STR´s; *Canis familiaris*; *Felis catus*.

Introduction

In a criminal investigation, the biological collected samples are mostly human, but they are not the only forensic evidence.

Pets such as cats (*Felis catus*) and dogs (*Canis familiaris*), live with people and place biological samples such as hair, saliva and blood everywhere, which may be useful in a forensic context linking suspects and victims, to an occurrence.

There are three different types of animal DNA evidence:

- the animal as a witness (*e.g.* struggles of animals);
- the animal as an aggressor (*e.g.* animals involved in attacks on people);
- the animal as a victim (*e.g.* the remains of an animal lost or stolen).

The aim of this study is to implement techniques for individual animal identification through the analysis of short tandem repeats (STRs) for each specie under study, with different samples, namely hair and blood.

Materials and Methods

Blood samples of 63 unrelated dogs were extracted by the Chelex100® method (Walsh *et al.*, 1991) and hair samples were extracted by the DNA IQTM (Promega)

commercial kit. Amplification of PEZ1, FHC2054, FHC2010, PEZ5, PEZ20, PEZ12, PEZ3, PEZ6, PEZ8 and FHC2079 STRs was performed with the multiplex Canine StockMarks (Applied Biosystems) with the markers, according to manufacturer's instructions. The amplified product was applied in an automatic capillary electrophoresis sequencer ABI PRISM ™ 310 Genetic Analyzer using the ROX 350 internal standard and analyzed with the GeneScan Analysis 3.1 software. The fragments sizes were compared with the described by Eichmann (2004).

For the feline samples blood of 63 unrelated animals was extracted by the Chelex100® method (Walsh *et al.*, 1991) and amplified for the FCA733, FCA723 and FCA731 markers in a multiplex reaction according Menotti-Raymond (2005). Detection of amplified product was performed as well as in dog samples using the Rox 500 internal standard

Results

The results presented in electropherograms (Figures 1 and 2) show that it can be possible to make genetic identification of *Canis familiaris* and *Felis catus* with the chosen markers.

Discussion

In a forensic context the limiting step can be the poor genetic material normally found in a crime scene.

The methods used in this study showed that it was possible to extract DNA from blood and hair leading to a good yield of DNA concentration (>3 ng/ul and <13ng/ul). All markers analysed appeared to be polymorphic, which is extremely important because it allows greater discrimination and thus a better individual identification.

Conclusion

The STRs markers used in this work showed that it is possible to make genetic identification of individual animals of both species under study, thus contributing to increase the potential of forensic samples of animal origin.

The studied markers proved to be highly informative and an important tool to assist in solving crime scene and casework related problems involving animal samples.

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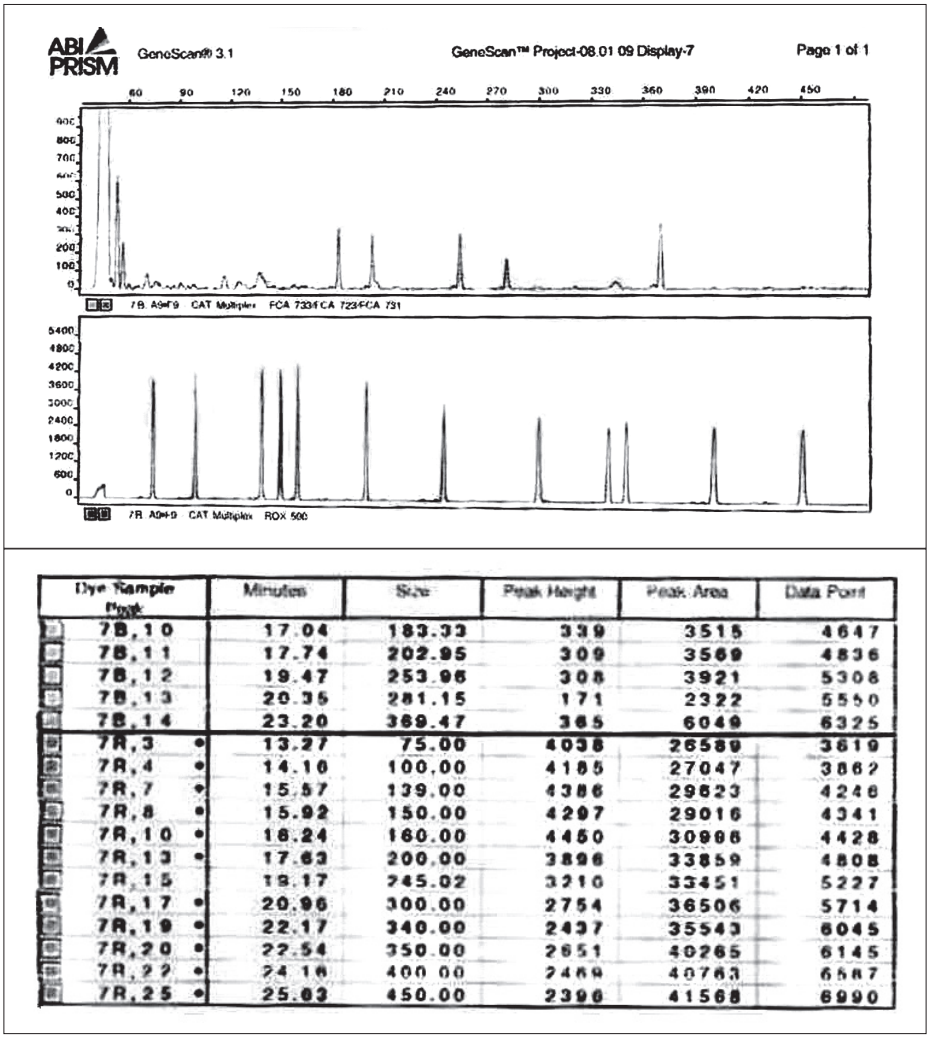
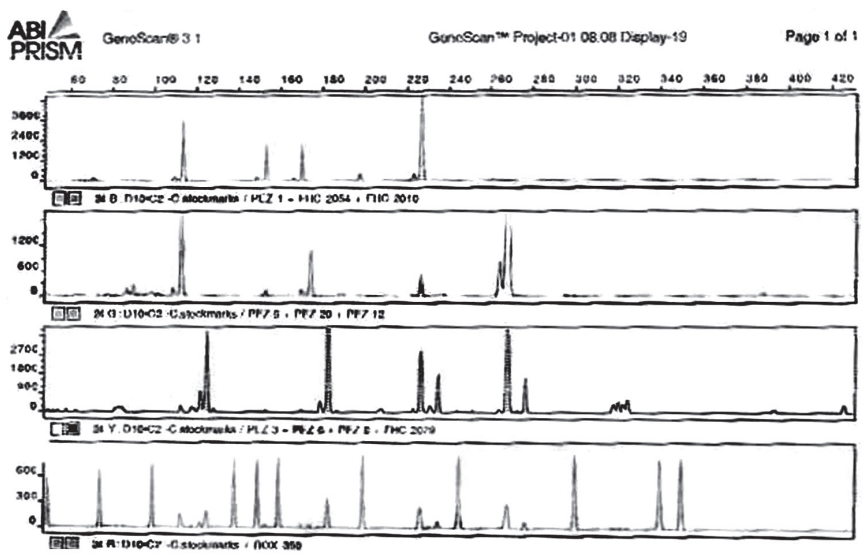
StockMarks® Horse, Cattle and Dog Genotyping Kits – Protocol, Applied Biosystems.



Dye Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
7B,10	17.04	183.33	339	3515	4647
7B,11	17.74	202.95	309	3569	4836
7B,12	19.47	253.96	308	3921	5308
7B,13	20.35	281.15	171	2322	5550
7B,14	23.20	369.47	365	6049	6325
7R,3 •	13.27	75.00	4038	26589	3619
7R,4 •	14.16	100.00	4185	27047	3862
7R,7 •	15.57	139.00	4386	29623	4248
7R,8 •	15.92	150.00	4297	29016	4341
7R,10 •	16.24	160.00	4450	30986	4428
7R,13 •	17.63	200.00	3896	33859	4808
7R,15	18.17	245.02	3210	33451	5227
7R,17 •	20.96	300.00	2754	36506	5714
7R,19 •	22.17	340.00	2437	35543	6045
7R,20 •	22.54	350.00	2651	40265	6145
7R,22 •	24.16	400.00	2469	40763	6587
7R,25 •	25.63	450.00	2396	41568	6990

Figure 1 (a,b) – Felid electropherogram for the STR´s FCA733, FCA723 and FCA731.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
24B,5	14.12	113.90	3605	25861	3850
24B,7	15.37	153.52	2258	14623	4192
24B,9	15.89	170.43	2184	14108	4333
24B,12	17.69	227.06	7183	66107	4823
24G,17	14.10	113.26	5250	34650	3844
24G,20	16.03	174.95	1114	9021	4372
24G,24	18.93	268.44	5311	77976	5163
24Y,17	14.52	125.78	3853	26153	3959
24Y,23	16.30	183.35	6432	45850	4445
24Y,29	17.70	227.42	2929	30603	4826
24Y,31	17.95	235.54	1793	12836	4894
24Y,35	18.93	268.44	3984	48415	5163
24Y,37	19.17	276.56	1603	12422	5228
24R,1 •	11.78	50.00	597	3674	3212
24R,2 •	12.80	75.00	696	4299	3489
24R,3 •	13.64	100.00	764	4607	3718
24R,6 •	14.95	139.00	831	4975	4076
24R,7 •	15.27	150.00	833	5065	4164
24R,8 •	15.57	160.00	831	5172	4244
24R,10 •	16.84	200.00	861	5615	4592
24R,13	18.25	245.45	843	6170	4976
24R,16 •	19.85	300.00	859	6836	5412
24R,17 •	20.94	340.00	828	7145	5710
24R,18 •	21.26	350.00	829	7338	5797

Figure 2 (a,b) – Canid electropherogram for the STR´s PEZ1, FHC2054, FHC2010, PEZ5, PEZ20, PEZ12, PEZ3, PEZ6, PEZ8 and FHC2079.

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THE INTEREST OF NAILS IN GENETIC IDENTIFICATION OF HUMAN DECOMPOSED CADAVERS

Abstract: The ability to recover DNA and STR data from bones and teeth exposed over time to a variety of environmental conditions, has become a valuable tool for individual identifications and/or kinship analysis. However, nails can be an advantageous alternative of these remain samples, since it is easier to perform the analysis process, without the need of powdering those mineralized elements. On the other hand, the high success rates for nuclear STR typing reported here, further confirmed that STRs could be considered a method of choice in casework involving skeletal remains. Nevertheless, when the DNA from the samples were degraded another approach to trying to recover information from them, is to reduce the size of the PCR products by moving primers as close as possible to the STR repeat region.

Introduction

A frequently encountered challenge in forensic casework is the analysis of highly degraded DNA that requires extraction from difficult material such as bones and teeth from long dead individuals (1). Forensic scientists are usually confronted with many problems, working with bones and teeth, such as insufficient quantity of DNA, high level of DNA degradation, and the presence of polymerase chain reaction (PCR) inhibitors. Therefore, careful optimization of all the stages of the procedures employed in the analysis of this type of samples is obligatory. The selection of appropriate procedure to promote the identification of human remains depends on the circumstances and the state of the examined material (2). Bone and teeth samples clearly protect DNA through their physical and/or chemical robustness to environmental degradation and/or biological attack. An elementary manifestation of this is that bone and teeth are often the only surviving material that can be tested (3). Nevertheless, shortly after death, blood and muscle can be easily collected from cadavers, as a DNA source for genetic identification. However, as previously stated, when the time elapsed between the death and the discovery of the body (*post mortem* interval) increases, the availability of these samples and the quality of the DNA decrease, which can hamper the establishment of a DNA profile. In such situations, DNA can be extracted from bones or teeth. Sampling is, in this case, invasive and DNA extraction from hard tissues will require

supplementary time consuming, for example, steps like powdering and decalcification of bones or fragmentation of teeth. Therefore, nails are very easy to collect and contain large amounts of good quality DNA that can be extracted within a few hours (4).

Short tandem repeat (STR) markers are the primary means used today for human identity and forensic DNA testing. However, with highly degraded DNA specimens a loss of signal is typically observed with larger sized STR products, either due to PCR inhibitors present in the forensic evidence or fragmented DNA molecules. Size reduction of STR markers, and thus improved success rates with degraded or inhibited DNA samples, may be accomplished by moving PCR primers in as close as possible to the STR repeat region (5).

The Forensic Genetic and Biology Service from the North Branch of the National Institute of Legal Medicine deals with different kinds of issues, such as kinship analysis, criminal cases and individual genetic identification, mainly human remains (Figure 1). A survey on genetic identification of human remains in the last four years (2005-2008), is referred to paternity testing (Table 1) or individual identification (Table 2) since the genetic analysis is required when the traditional methods failed. In the former situations, the human remains exhumation of the putative father (the more frequently absent in a trio) is required when close family members aren't available. Blood or muscle can be used as a DNA source for the genetic identification of recently deceased persons. However, if the *post-mortem* interval increases, bones and teeth are used, but in these cases, collection and DNA isolation is more difficult and time consuming. So, nails are an alternative genetic material source for the identification of decomposed cadavers, as referred before.

Material and Methods

In 26 human remains cases studied, 10 of them are related to paternity testing and 16 to genetic individual identification. The corpses, mainly those for individual identification, were found in different environments. In all the studied cases, bones (n= 25) are the most common sample sent to the laboratory. However, teeth and nails are also samples frequently collected. When nails (n= 17) were available, since it is easier to provide its DNA extraction without the need of previous treatment, they were cut with appropriate scissors. Before beginning the extraction procedure, all kinds of foreign substances were removed, and the nails were then washed with abundant quantity of sterile water at room temperature. Finally, to take away any exogenous or endogenous DNA, the nails were placed under a UV light during 30 minutes. DNA was extracted using a modified organic method followed by a Microcon® purification procedure. The DNA extraction from the reference samples was performed using the Chelex method. Autosomal STR profiles were obtained after amplification with the AmpFlSTR® Identifiler kit and the AmpFlSTR® MiniFiler™ kit. In the majority of cases we also used the AmpFlSTR® Y-filer™ kit to complement the information obtained from those STR loci, since the involved remains were from males (except three). The amplified products were detected and separated by capillary electrophoresis on an ABI PRISM® 3100 (Applied Biosystems). Fragment sizes were determined automatically using the Genescan® Analysis Software v 3.7 and allele designations using Genotyper® Software v. 3.7 (Applied Biosystems) typed by comparison with an allelic ladder.

Results

In twenty five of the total studied cases (n= 26) a full autosomal STR profile was obtained, in twenty four of them using the AmpFlSTR Identifiler® (Applied Biosystems). In one case, in which the sample was a femur from a deceased man buried 24 years ago (putative father), the DNA only performed results using the MiniFiler® (Applied Biosystems); in this situation the mitochondrial DNA analysis wasn't informative, because it was a paternity test in which a female descendant was involved. Only in a case where the analysis was made in two little bones found in a cemetery, sent to the laboratory without complementary information, including the *post-mortem* interval, no results were observed either with nuclear or mitochondrial analysis. The total of the results (n= 25) was obtained using bones, teeth and/or nails. Nails were the preferred sample used to make the study, although other type of sample was used, when available, to confirm the results (Table 2).

Discussion

In our routine casework we observed an increasing demand for paternity testing, when the putative father is deceased. Subsequently, the only option is the exhumation of the cadaver. We have also had deceased human bodies to establish the genetic identity, by comparing their genetic profile with personal items (direct comparison) or with relatives (indirect comparison).

When soft tissues were decomposed, bones and teeth were usually collected. However, DNA extraction from hard tissues requires supplementary time consuming, including steps like powdering. Previous studies showed that the most successful samples for STR testing were intact teeth and mid-shaft sections of femur. Bones that performed less well tend to be less dense and/or have a greater proportion of soft portions. For this reason, there was a laborious work to do before starting the DNA extraction process. Despite ease of collection and good resistance to decompose, nails are an advantageous sample used as a DNA source for cadaver's genetic identifications. In our case, when nails were available we used them, and the results obtained were similar to the ones found with other body sample, in accordance to this study.

STRs are highly polymorphic and capable of generating typing results from very little material through multiplex amplification, using the polymerase chain reaction (PCR). All the studied cases but one, were successfully concluded with the autosomal and Y STRs. However, with highly degraded DNA biological material, no results or allele dropout is typically observed with larger sized STR products, either due to PCR inhibitors present in the forensic evidence or fragmented DNA molecules. The AmpFlSTR® MiniFiler™ PCR Amplification Kit increases the ability to obtain DNA results from compromised samples that previously would have yielded limited or no genetic data. This situation occurred with a femur from a deceased man buried 24 years ago (putative father), that provided results with this kit when the analysis of conventional STRs failed.

Conclusion

Nails are very easy to collect and contain large amounts of good quality DNA that can be extracted within a few hours. So, they are an attractive genetic material source for identification of decomposed corpses, mainly because the starting process before the DNA extraction is easy and fast. Despite their utility in the identification of human remains, it is sometimes important to confirm the results with the study of other type of sample.

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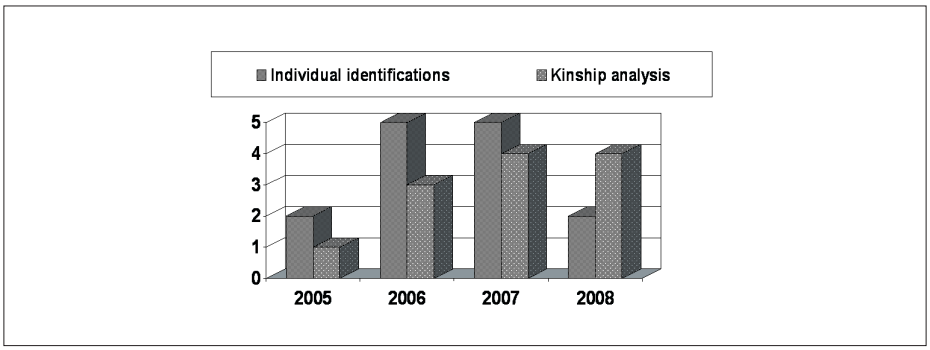

Figure 1 – Identification of human remains distribution

	Human Remains	Time of Burial	Collected Samples	Studied Samples
1	Putative Father	?	Teeth, bone, nails, muscle	Nails
2	Putative Father	+/- 4 years	Teeth, bone, nails, muscle	Nails
3	Daughter	+/- 6 months	Bone, nails	Nails
4	Putative Father	+/- 2 years	Teeth, bone, nails	Nails
5	Putative Father	+/- 25 years	Bone	Bone
6	Putative Father	+/- 5 years	Teeth, bone, nails	Nails
7	Putative Father	+/- 1 year	Teeth, bone, nails	Nails
8	Putative Father	+/- 3 years	Teeth, bone, nails	Teeth, bone, nails
9	Putative Father	+/- 1 year	Teeth, bone, nails	Nails
10	Putative Father	+/- 3 years	Teeth, bone, nails	Teeth, bone, nails

Table 1 – Paternity testing

	Place/ Reason of identification	Collected Samples	Studied Samples	Genetic Markers Results
1	Suicide (train)	bone, nails	Nails	Autosomal and Y STRs
2	Hill	Teeth, bone, nails	Nails	Autosomal and Y STRs
3	River	Bone, nails	Nails	Autosomal and Y STRs
4	Carbonized	Bone, teeth	Bone, teeth	Autosomal and Y STRs
5	River	Bone, teeth	Bone, teeth	Autosomal and Y STRs
6	Carbonized	Bone, teeth	Bone, teeth	Autosomal and Y STRs
7	?	Teeth, nails	Nails	Autosomal and Y STRs
8	House	Bone, teeth, nails	Nails	Autosomal STRs
9	House	Bone	Bone	Autosomal and Y STRs
10	Sea	Bone	Bone	Autosomal STRs
11	Possible exchange of identity	Bone, nails	Nails	Autosomal and Y STRs
12	House	Bone, teeth, nails	Teeth, bone, nails	Autosomal and Y STRs
13	Carbonized	Bone	Bone	Autosomal and Y STRs
14	Woods	Bone	Bone	Autosomal STRs
15	Cemetery	Bone	Bone	No Results
16	Hill	Nails	Nails	Autosomal and Y STRs

Table 2 – Individual genetic identification

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THE IMPORTANCE OF STR SPECIFIC DATABASES: A COMPARATIVE STUDY

Abstract: The STR allele frequencies have been used in studies of human populations to assess their genetic composition, variability, relationships and the evolutionary factors to which they are subjected. The Brazilian population is composed of individuals from different ethnical origins, varying according to the geographical region. Santa Catarina population is mainly originated from Portuguese (Azores archipelago). In this study, a Neighbor-Joining tree was constructed based on genetic distances of Nei, using the allele frequencies of the CODIS STR loci, from Santa Catarina population and other 23 different populations, including some of other ethnic groups. The results indicate the requirement of an extensive genetic study in order to implement population specific databases for forensic purposes.

Introduction

Short Tandem Repeat (STR) loci are widely used in individual identification, parentage testing and forensic casework, because of their higher discriminating information, sensitivity and reproducibility. Various multiplex PCR systems have been developed which allow the simultaneous amplification of several STR loci, they are rapid and powerful tools in forensic genetics. In this study we have analyzed the specific core of 13 STRs used to generate a nationwide USA DNA database, called the FBI (Federal Bureau of Investigation) Combined DNA Index System (CODIS).

The Brazilian population is composed of individuals from different ethnical origins, varying according to the geographical region. The Santa Catarina colonization began in the 16th century with the arrival of the Portuguese. The Azorean immigrants came later, in the 18th century, sent by the Portuguese king to complete the process. Despite the fact that a considerable amount of literature on the allele frequencies of these STRs is available, no data have been published about comparisons between different Brazilian states. Appropriate databases are imperative, because the variation in STR allele proportions between populations has been previously reported, reflecting different ancestral gene pools.

A comparative study, for the same markers, between Santa Catarina population and others was performed, being the main goal to demonstrate the need of the establishment of specific population databases for application in forensic and paternity investigations. A larger comparative study was not performed, because of the lack of additional Brazilian published population data.

Materials and Methods

Blood stains from 185 unrelated, autochthonous healthy donors from Santa Catarina were collected, with a major ethnic background considered to be Caucasian, 160 of them were used to perform the study previously published (Caine et al., 2003). All the methodology was made according to the afore mentioned study. Additionally, we used the PHYLIP version 3.6c software package (Felsenstein, 1986), to obtain a Neighbor-Joining tree (Nei and Saitou, 1987) from the genetic distances of Nei (Nei, 1972). The statistical robustness of their nodes was tested through a bootstrap approach (Efron, 1982; Felsenstein, 1985). Loci were resampled with replacement of 1000 iterations. The tree was visualized on TREE VIEW version 1.6.6 program (Page, 1996). For comparisons the following populations from the literature were used: Australia (Bagdonavicius et al., 2002), Azores (Velosa et al., 2002), Rio de Janeiro, Brazil (Góes et al., 2004), Mato Grosso, Brazil (Silva et al., 2004), Greece (Skitsa et al., 2003), Hong Kong (Law et al., 2002), Italy (Garofano et al., 1998), India (Sahoo and Kashyap, 2002), Japan (Hashiyada et al., 2003), Jamaica and Trinidad & Tobago (Budowle et al., 2001), Madeira (Fernandes et al., 2002), Morocco (Jauffrit et al., 2003), North Africa (Farfán et al., 2001), Portugal (Abrantes et al., 2004), Paraguay (Espin et al., 2002), Peru (Pérez et al., 2003), Poland (Pepinski et al., 2001), Scotland (Goodwin et al., 2001), Spain (Paredes et al., 2003), Swiss (Gehrig et al., 1999), Turkey (Akbasak et al., 2001) and Vietnam (Shimada et al., 2002).

Results

The observed allele frequencies (n=185), for the 13 core STR loci are similar to the frequencies obtained before (n=160), data not shown. The Neighbor-Joining tree based on Nei´s genetic distances is represented in figure 1.

Discussion

A comparison of Santa Catarina population with the others showed minor divergences with Caucasian populations. On the other hand, significant differences were found between Santa Catarina and other Brazilian (Rio Janeiro and Mato Grosso Sul), South American, Asiatic and Negroid populations. It can be seen that Caucasian populations, mostly European appear on the same cluster that contains Santa Catarina. A possible reason for this is the higher contribution of Europeans to the state formation. The closeness of Santa Catarina with Azores, Madeira and Portugal, may reflect their

Portuguese origin. The Brazilian Mato Grosso Sul population together with other South American populations (Peru and Paraguay) included in this analysis are grouped on a second cluster. A probable cause for this could be their geographic proximity.

The Asiatic populations can also be found in this group; however in a different branch. On the other hand, the Brazilian Rio Janeiro population is grouped with populations of Negroid origin. Assuming a high degree of admixture of this population (Portuguese, African descendants and native Brazilians), it was not surprising to find it in this group.

Conclusion

The genetic differences found between all compared populations, namely the Brazilian ones, grouped in different clusters, support the importance of developing specific local databases of the reference populations. In Brazil this necessity is essential because of the enormous population heterogeneity and the vast area occupied by this country.

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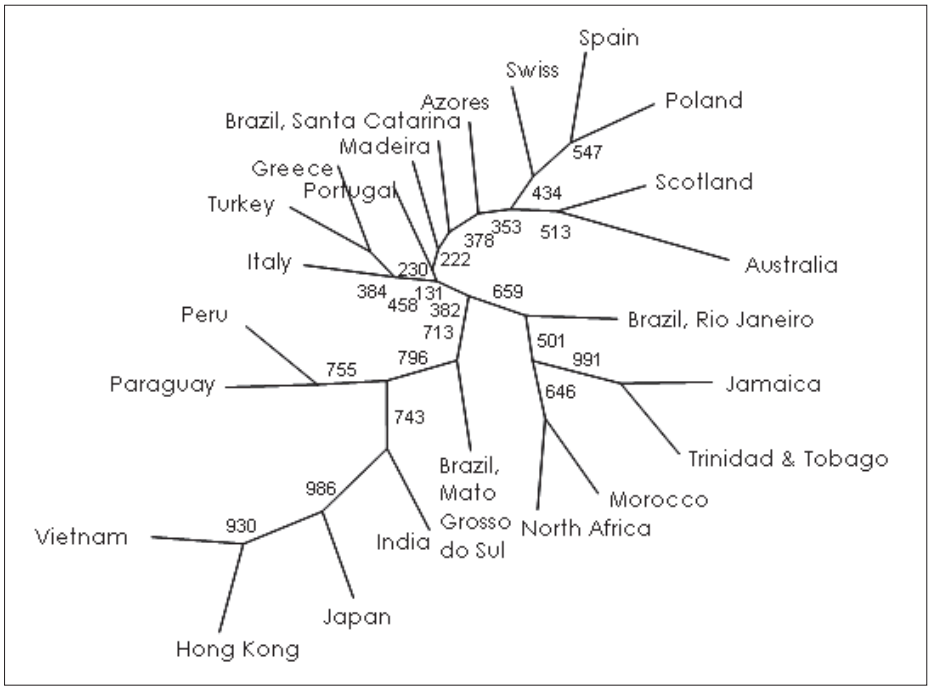

Figure 1 – A Neighbor-Joining tree based on Nei´s genetic distances for the 13 STRs loci in 24 populations. The numbers indicate the bootstrap value.

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PATERNAL AND MATERNAL LINEAGES IN CABO VERDE ARCHIPELAGO POPULATION

Abstract: The paternal and maternal lineages of Cabo Verde archipelago were characterized using 22 Y-Single Nucleotide Polymorphisms (SNPs) and Y-minimal haplotype (STRs) for paternal lineage and the two hypervariable segments (HVI and HVII) of the mtDNA control region for maternal lineage. A high variability of haplotype and haplogroup composition was found in the studied population. A total of 13 haplogroups was found with Y-SNPs and 24 haplogroups with HVI and HVII mtDNA. Using Y-STR minimal haplotype information, genetic distances were obtained between Cabo Verde and European/African populations. While almost all mitochondrial lineages were of sub-Saharan origin (95%), the Y-chromosome lineages reveal a high diverse composition, with more than 57% of Y lineages of European ancestry.

Introduction

The analysis of mitochondrial DNA (mtDNA) and Single Nucleotide Polymorphisms (SNPs) located on the Y chromosome specific region can be helpful in forensics, since they define haplogroups showing geographic specificity, providing information about the paternal or maternal ancestry of an individual or evidence under investigation. Moreover, the study of lineage markers can be extremely useful in order to evaluate population substructure in admixed populations, which is essential for definition of relevant forensic databases.

The aim of this work was to study the origin of paternal lineage (Y-SNPs) and maternal lineage (mtDNA) of Cabo Verde archipelago population by phylogeographic analysis of the observed haplogroups. This population was genetically characterized for Y-STR minimal haplotype (DYS19, DYS389 I, DYS389 II, DYS390, DYS391, DYS392, DYS393 and DYS385). The genetic distances obtained were compared with those reported to other populations from Europe and Africa, in order to evaluate the contribution of these populations to the genetic pool of Cabo Verde.

Materials and Methods

The population of Cabo Verde was characterized, in a sample of 42 unrelated males, for Y chromosome specific STR loci typing the minimal haplotype DYS19, DYS389 I,

DYS389 II, DYS390, DYS391, DYS392, DYS393 and DYS385. The DYS19, DYS389 I, DYS389 II, DYS390 and DYS393 were amplified as described by Gusmão *et al*., 1999 [1]. The DYS385 amplification conditions complied with the methodology described by Schneider *et al.*, 1998 [2], and multiplex amplification of DYS391, DYS392, DYS393 was carried out according to Kloosterman *et al.*, 1998 [3]. Alleles were designated according to the International Society for Forensic Genetics (ISFG) guidelines for forensic analysis using Y-STRs [4]. Pairwise Rst genetic distances were calculated using Arlequin v. 3.0 software Excoffier *et al.*, 2005 [5], and DYS385 was not considered.

To determine the frequency distribution of the male lineages, 22 Y-SNPs were typed in two PCR-SNaPshot multiplex reactions. The first multiplex included nine Y-SNPs (92R7, M70, M22, Tat, P25, SRY10831, M173, M213 and M9) and the second one included thirteen Y-SNPs (P2, M154, M293, M81, M85, M78, M35, M96, V6, M191, M33, M123 and M2). The first multiplex was performed according to Brión *et al.,* 2004 [6] and the second one by using a newly developed strategy.

The polymorphism of the two hypervariable segments (HVI and HVII) of the mtDNA control region was analyzed in 77 unrelated individuals from Cabo Verde, using the amplification method and *primers* referred by Wilson *et al.,* 1995 [7]. Sequences were obtained with ABI PRISM *Big Dye Terminator and dRhodamine Terminator Cycle Sequencing Ready Reaction Kits,* with *amplitaq DNA polymerase FS,* and were detected in an ABI 3100 Avant sequencer. Haplogroups were classified based on the different polymorphic positions of these two hypervariable regions using the software *mtDNA manager-forensic mtDNA database* [8].

Results and Discussion

The Y-chromosome haplotype and haplogroup and mtDNA haplogroup frequencies observed in Cabo Verde population were presented in Tables 1, 2 and 3.

The pairwise Rst genetic distances analysis obtained among male population sample from Cabo Verde and others from Iberia and Africa sub-Saharan, showed a similar significant differentiation from Iberian populations ($0.13352<RST<0.17027$, $p=0.00000$) [9, 10, 11] and from Africa sub-Saharan populations ($0.13401<RST<0.15268$, $p=0.00000$) [12, 13, 14].

The two most frequent Y-haplogroups were R1b1-P25 with a frequency of 26.19% and E1b1a(xE1b1a4,7)-M2 with 28.57%. The haplogroup R1b1-P25 reaches high frequencies in Weastern Europe, being the most frequent in Iberia [15]. The other haplogroups that are usually represented in Western European populations [6, 15] were also found in our sample, namely E1b1b1a-M78, E1b1b1b-M81, K2-M70, J2-M172, P(xR1)-92R7, I2a2-M26, I(xI2a2)-M170 and G-M201. Altogether, the proportion of the lineages that can be explained by European contributions reaches a frequency of 57% in our sample from Cabo Verde. Haplogroup E1b1a(xE1b1a4,7)-M2 is known to be of sub-Saharan origin, being the most frequent in all Bantu speaking populations [16], and was found to be the second most frequent in our sample.

For mtDNA, the majority of haplogroups (95%) were of sub-Saharan origin with exception of X and X2d that were of West Eurasian origin and D4k and N9b that were of East Asian origin [8].

Conclusion

Our results, like other recent studies [17, 18], confirm a strong male/female asymmetry concerning the European and African contributions to the genetic composition of the nowadays Cabo Verde population. While almost all mitochondrial lineages were of sub-Saharan origin (95%), the Y-chromosomes lineages reveal a high diverse composition, with more than 57% of Y lineages having an European ancestry.

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Frequency	Haplotype*	Haplogroup
3	14/10/26/24/11/13/13/11-14	R1b1-P25
2	13/12/29/24/10/11/13/17-18	E1b1b1a-M78
2	14/13/29/23/11/13/13/11-14	R1b1-P25
2	15/12/29/22/11/11/13/11-18	E1a-M33
2	15/13/30/21/10/11/14/15-16	E1b1a (xE1b1a4,7)-M2
1	13/13/29/24/9/11/13/10-14	E1b1b1b-M81
1	13/13/30/24/11/11/13/16-18	E1b1b1a-M78
1	13/13/31/22/10/11/13/14-16	E1b1b1a-M78
1	13/13/31/24/10/14/13/15-16	P(xR1)-92R7
1	14/12/28/24/10/12/13/11-17	B2b(xB2b3)-M112
1	14/12/30/23/10/11/12/13-17	J2-M172
1	14/13/29/24/11/13/13/10-14	R1b1-P25
1	14/13/29/24/11/14/13/12-14	R1b1-P25
1	14/13/29/24/12/13/14/11-13	R1b1-P25
1	14/13/30/24/11/13/13/11-14	R1b1-P25
1	14/13/31/23/10/15/11/15-18	K2-M70
1	14/14/30/24/11/13/13/11-14	R1b1-P25
1	14/14/31/23/9/11/13/10-14	E1b1b1b-M81
1	15/11/27/22/9/11/13/11-12	A3b2-M13
1	15/12/26/24/11/13/13/12-14	R1b1-P25
1	15/12/29/21/10/11/14/17-17	E1b1a (xE1b1a4,7)-M2
1	15/12/30/25/11/11/13/16-18	E1b1b1a-M78
1	15/13/30/21/10/11/14/14-16	E1b1a (xE1b1a4,7)-M2
1	15/13/30/21/10/11/14/15-15	E1b1a (xE1b1a4,7)-M2

1	15/13/30/21/10/11/14/16-17	E1b1a (xE1b1a4,7)-M2
1	15/13/30/22/10/11/14/13-13	G-M201
1	15/14/29/23/10/11/13/12-12	I2a2-M26
1	15/14/31/22/10/11/13/16-18	E1b1a (xE1b1a4,7)-M2
1	16/12/29/22/10/11/13/15-15	E1a-M33
1	16/12/30/22/11/11/13/15-16	E1a-M33
1	16/14/30/21/10/11/14/18-18	E1b1a (xE1b1a4,7)-M2
1	16/14/33/21/10/11/14/15-17	E1b1a (xE1b1a4,7)-M2
1	17/12/29/21/10/11/14/17-17	E1b1a (xE1b1a4,7)-M2
1	17/12/30/25/11/11/14/13-17	I(xI2a2)-M170
1	17/14/31/21/10/11/13/16-17	E1b1a (xE1b1a4,7)-M2
1	17/14/31/21/10/11/13/17-17	E1b1a (xE1b1a4,7)-M2

*The minimal haplotype: DYS19/DYS389I/II/DYS390/DYS391/DYS392/DYS393/DYS385

Table 1. Y-SNP Haplotype frequencies and haplogroup in Cabo Verde archipelago (N=42)

Haplogroup	Cabo Verde (N=42)
E1b1a (xE1b1a4,7)-M2	28.57
R1b1-P25	26.19
E1b1b1a-M78	11.90
E1a-M33	9.52
E1b1b1b-M81	4.76
K2-M70	2.38
J2-M172	2.38
P(xR1)-92R7	2.38
I2a2-M26	2.38
I(xI2a2)-M170	2.38
A3b2-M13	2.38
B2b(xB2b3)-M112	2.38
G-M201	2.38

Table 2. Y-SNP Haplogroup frequencies (%) in Cabo Verde archipelago

S	H	HVI and HVII Specific Control Region Sequences*	N
H1	*D4k*	73G 93G 146C 150T 152C 182T 195C 198T 204C 309.1C 325T 16192T 16223T 16278T 16390A	1
H2	*L0a1*	93G 152C 189G 200G 236C 247A 309.1C 16129A 16148T 16168T 16172C 16187T 16188G 16189C 16223T 16230G 16311C 16320T	1
H3	*L1b*	73G 152C 182T 185T 189G 195C 247A 16126C 16187T 16189C 16223T 16264T 16270T 16278T 16311C	1
H4	*L1b1*	73G 152C 182T 185T 195C 247A 357G 16114G 16126C 16187T 16188T 16189C 16223T 16264T 16270T 16278T 16293G 16311C	1

S	H	HVI and HVII Specific Control Region Sequences*	N
H5	*L1b1*	73G 152C 182T 185T 195C 247A 367G 16126C 16145A 16187T 16189C 16223T 16264T 16270T 16278T 16293G 16311C	1
H6	*L1b1*	73G 152C 182T 185T 195C 247A 16104T 16187T 16189C 16223T 16270T 16278T 16289G 16293G 16311C	1
H7	*L1b1*	73G 152C 182T 185T 195C 247A 16126C 16187T 16189C 16223T 16264T 16270T 16278T 16293G 16311C	1
H8	*L1b1*	73G 152C 182T 185T 195C 247A 16126C 16187T 16189C 16223T 16256T 16264T 16278T 16293G 16311C	1
H9	*L1b1*	73G 152C 182T 185T 195C 247A 16114G 16126C 16187T 16189C 16223T 16264T 16270T 16278T 16293G 16311C	1
H10	*L1c*	73G 151T 152C 182T 186A 189C 195C 247A 291T 297G 316A 16129A 16187T 16189C 16223T 16248T 16261T 16278T 16311C 16360T	1
H11	*L1c1*	73G 151T 152C 182T 186A 189C 195C 198T 247A 316A 16129A 16163G 16187T 16189C 16223T 16278T 16293G 16294T 16304C 16311C 16360T	1
H12	*L1c1*	73G 151T 152C 182T 186A 189C 247A 316A 16129A 16163G 16187T 16189C 16223T 16278T 16293G 16294T 16304C 16311C 16360T	1
H13	*L1c1*	73G 151T 152C 182T 186A 189C 195C 198T 247A 316A 16129A 16163G 16187T 16189C 16223T 16278T 16293G 16294T 16304C 16311C 16360T	1
H14	*L1c1*	73G 151T 152C 182T 186A 189C 195C 247A 316A 16129A 16163G 16187T 16189C 16223T 16278T 16293G 16294T 16304C 16311C 16360T	2
H15	*L2a*	73G 146C 152C 195C 16223T 16230G 16278T 16294T 16390A	1
H16	*L2a1*	73G 143A 146C 152C 195C 16111T 16223T 16278T 16294T 16309G 16390A	1
H17	*L2a1*	73G 143A 146C 152C 195C 309.1C 16183C 16189C 16223T 16274A 16278T 16294T 16309G 16390A	1
H18	*L2a1*	73G 143A 146C 152C 195C 198T 16086C 16223T 16278T 16294T 16309G 16390A	1
H19	*L2a1*	73G 143A 146C 152C 195C 309.2C 16093C 16189C 16223T 16264T 16278T 16294T 16309G 16390A	1
H20	*L2a1*	73G 143A 146C 152C 195C 264T 16183C 16189C 16192T 16223T 16278T 16294T 16309G 16390A	1
H21	*L2a1*	73G 143A 146C 152C 195C 16111T 16223T 16278T 16294T 16309G 16390A	2
H22	*L2b*	73G 146C 150T 152C 182T 195C 198T 207A 16093C 16114A 16129A 16213A 16223T 16271C 16278T 16390A	1
H23	*L2b1*	73G 150T 152C 182T 195C 198T 204C 249d 309.1C 16114A 16129A 16213A 16223T 16278T 16355T 16362C 16390A	1
H24	*L2b1*	73G 150T 152C 182T 195C 198T 204C 16114A 16129A 16213A 16223T 16278T 16355T 16362C 16390A	1
H25	*L2c*	73G 93G 146C 150T 152C 182T 195C 325T 16223T 16278T 16311C 16390A	1
H26	*L2c*	73G 89C 93G 146C 150T 152C 182T 195C 198T 325T 16192T 16223T 16278T 16390A	1
H27	*L2c*	73G 93G 146C 150T 152C 182T 195C 198T 325T 16178C 16223T 16278T 16380T 16390A	1
H28	*L2c*	73G 89C 93G 146C 150T 152C 182T 195C 198T 309.1C 325T 16192T 16223T 16261T 16278T 16390A	1
H29	*L2c*	73G 93G 146C 150T 152C 182T 195C 198T 325T 16051G 16223T 16278T 16390A	1
H30	*L2c*	73G 146C 150T 152C 182T 195C 297G 325T 16177G 16223T 16278T 16311C 16390A	1
H31	*L2c*	73G 93G 146C 150T 152C 182T 185C 189G 325T 16223T 16278T	1
H32	*L2c*	73G 93G 146C 150T 152C 182T 183G 195C 198T 199C 204C 325T 16223T 16278T 16390A	1
H33	*L2c*	73G 93G 146C 150T 152C 182T 195C 198T 309.1C 319C 325T 16051G 16223T 16278T 16390A	1

S	H	HVI and HVII Specific Control Region Sequences*	N
H34	*L2c*	73G 146C 150T 152C 182T 195C 198T 325T 16223T 16261T 16278T 16390A	2
H35	*L2c*	73G 93G 146C 150T 152C 182T 195C 198T 204C 325T 16192T 16223T 16278T 16390A	1
H36	*L2c*	73G 93G 146C 150T 152C 182T 195C 198T 309.1C 325T 16223T 16278T 16390A	1
H37	*L2c*	73G 146C 150T 152C 182T 195C 325T 16177G 16223T 16278T 16311C 16390A	2
H38	*L2c*	73G 93G 146C 150T 152C 182T 195C 198T 325T 16223T 16278T 16320T 16390A	1
H39	*L2c2*	73G 93G 146C 150T 152C 182T 195C 198T 325T 16084A 16093C 16220G 16223T 16264T 16278T 16311C 16390A	2
H40	*L2c2*	73G 93G 146C 150T 152C 182T 195C 325T 16093C 16126C 16223T 16264T 16274A 16278T 16390A	1
H41	*L2d1*	73G 146C 150T 152C 195C 310C 16129A 16182G 16183C 16193.1C 16278T 16300G 16354T 16390A	1
H42	*L2d1*	73G 146C 150T 195C 16093C 16129A 16189C 16259T 16278T 16300G 16354T 16390A	1
H43	*L2d2*	73G 146C 151T 152C 182T 185A 189G 16111A 16145A 16184T 16189C 16223T 16239T 16278T 16291T 16292T 16355T 16390A	1
H44	*L3b*	73G 309.1C 16124C 16223T 16234T 16278T 16362C	2
H45	*L3b*	73G 151T 152C 16124C 16223T 16234T 16278T 16362C	3
H46	*L3b*	73G 150T 16124C 16183C 16189C 16214T 16223T 16278T 16362C	1
H47	*L3b*	73G 309.1C 16124C 16188T 16223T 16278T 16362C	1
H48	*L3b*	73G 189G 16124C 16223T 16278T 16355T 16362C	1
H49	*L3d*	73G 152C 189G 195C 207A 16124C 16223T	2
H50	*L3d*	73G 150T 152C 16124C 16223T	2
H51	*L3d*	73G 146C 152C 16093C 16124C 16223T	1
H52	*L3d*	73G 152C 199C 309.1C 16111T 16124C 16223T	1
H53	*L3d1*	73G 146C 152C 195C 16124C 16223T 16319A	1
H54	*L3e2b*	73G 150T 195C 16172C 16183C 16189C 16223T 16259T 16320T	1
H55	*L3e4*	73G 150T 16051G 16223T 16264T 16299G	1
H56	*L3e4*	73G 150T 309.1C 16051G 16223T 16264T 16299G	1
H57	*L3e4*	73G 150T 16051G 16093C 16223T 16247G 16264T 16311C	1
H58	*L3e4*	73G 150T 309.1C 16051G 16223T 16264T	1
H59	*L3e4*	73G 150T 309.1C 16051G 16223T 16257T 16264T	1
H60	*L3e4*	73G 150T 16051G 16148T 16223T 16264T	1
H61	*L3e4*	73G 150T 257G 16051G 16223T 16264T	1
H62	*L3f1*	73G 189G 16153A 16209C 16223T 16230G 16260T 16292T	1
H63	*N9b*	73G 150T 195C 16172C 16182G 16183C 16189C 16223T	1
H64	*U6a*	73G 150T 309.1C 16172C 16183C 16189C 16219G 16278T	2
H65	*X*	73G 146C 152C 185A 189G 16111A 16145A 16183T 16189C 16223T 16239T 16278T 16292T 16355T 16390A	1
H66	*X2d*	73G 146C 182T 195A 207A 316A 16189C 16223T 16259T 16274A 16278T 16390A	1

* All sequences (S) carry 263/315.1C mutations/insertions.

Table 3. mtDNA haplogroup (H) frequencies in Cabo Verde archipelago (N=77).

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20 SNP-PLEX AS A COMPLEMENT METHOD IN PATERNITY TESTING

Abstract: This study intended to examine a set of 20 autosomal Single Nucleotide Polymorphisms (SNPs) selected from the 52-plex developed by the SNP*for*ID Consortium for human identification and to study its usefulness in investigation of paternity cases. We designed two 10-plexes and investigated 50 paternity cases, previously examined in this laboratory with standard STR methodologies. There was a total agreement between exclusion and not exclusion cases with the results obtained by STR analysis, except for one case where it was not possible to exclude the father with SNP analysis, probably due to the small number of SNPs studied. In paternity exclusions, between one and seven incompatibilities were detected for the SNP *loci* studied. This study demonstrates that analysis of a small number of SNP *loci*, as 20 polymorphisms, can be very useful in biological kinship investigation as a complement to standard STR methodologies, being an advantage to increase the number of *loci* to strengthen SNP study as a complement methodology.

Keywords: SNPs; paternity testing; SNaPshot.

Introduction

All over the world, Forensic Geneticists use Short Tandem Repeats (STRs) in the resolution of all kind of cases, being the most important tool in paternity investigation. However, there are cases where STRs usually used in routine analysis are not sufficient for the emission of a report. This is usually derived from the existence of genetic inconsistencies between alleged father and child, derived from meiotic mutation [1] or even from standard technologies used [2], resulting in low paternity indexes and paternity probabilities. In response to this problem, geneticists tend to raise the number, and sometimes the kind, of *loci* studied in order to raise the confidence of the results obtained, studying a larger number of autosomic STRs, besides X-STRs and Y-STRs whenever possible. Nevertheless, this resource is always subjected to the same problems that originated their use, that is, there could be some genetic inconsistencies between the alleged father and the child in the new *loci* studied, derived from the relatively high mutation rates of some STRs [3].

In the past years there has been a growing interest in the use of SNPs in several areas of biological sciences, not being exception the field of Forensic Genetics. This is mainly due to the characteristics of these polymorphisms: i) their short amplicon

sizes, ii) the available high throughput genotyping technologies, and, especially, iii) its very low mutation rate, 100 thousand times lower than the conventionally analyzed polymorphisms, STRs [4]. These characteristics makes SNPs very suitable for genetic identification studies and, therefore, for paternity testing. This study, in continuity of previous work [5], intended to examine a set of 20 autosomal SNPs, selected from the 52-plex developed by Sanchez *et. al* and the SNP*for*ID Consortium for human identification [6], and to study its usefulness as supplementary markers in investigation of paternity cases, as other authors demonstrated for the complete 52 SNP-Plex [7,8].

Material and Methods

With the use of SNP*for*ID browser [9], we designed two 10-plexes to analyze a total of 20 SNPs by SNaPshot® methodology (Applied Biosystem). The SNPs chosen from the 52 previously studied by the Consortium were the ones expected to have an allelic frequency closer to 0.5 in the Portuguese population, mainly South-Portugal resident population based on previously studies in the Spanish Galicia population. *Loci* studied were the following: rs1490413; rs1029047; rs763869; rs735155; rs2107612; rs1454361; rs2111980; rs1005533; rs8037429; rs891700; rs2046361; rs717302; rs1886510; rs729172; rs1024116; rs1463729; rs2076848; rs1355366; rs907100; and, rs737681.

To test the behavior of the selected loci, we investigated 50 paternity cases, with different ethnic-geographical background, previously examined in routine analysis with standard STR methodologies (Promega PowerPlex® 16 and Applied Biosystems AmpF*l*STR® Identifiler® using the manufacturer instructions). SNP *loci* were amplified in two 10-plexes using Sanchez et. al [6] conditions. Products of SNP amplification, as STR amplification, were analyzed in 3130/3130xl Genetic Analyzers with GeneMapper® ID Software v3.2 (Applied Biosystems).

Results

From the analysis of studied cases with SNPs, there was an agreement with the results obtained by STR analysis in exclusion and non-exclusion cases, as can be exemplified in figures 1 to 4. Figures 1 and 2 show the same paternity with two alleged fathers, where alleged father 2 is excluded from paternity in STR analysis (figure 1) as in SNP study (figure 2). Similarly, figures 3 and 4 illustrate another paternity case, also with two alleged fathers, where alleged father 1 is excluded from paternity in STR analysis but not alleged father 2 (figure 3), the SNP *loci* showing the same results (figure 4). However, there was one case where it was not possible to exclude the alleged father with SNP analysis. In paternity exclusion cases, between one and seven incompatibilities were detected for the SNPs studied. No mutations were found in this study.

Discussion and Conclusions

This study demonstrates that the analysis of as few as 20 SNP *loci*, with SNaPshot® methodology, can be very useful in biological kinship investigation as a complement to standard STR methodologies. Only in one paternity exclusion case, no genetic incompatibilities were found between the alleged father and the child. This was probably due to the small number of SNPs studied, although this set of SNP *loci* demonstrated to be very useful. This is true even for cases with different ethnic-geographical background, as is the case in our studied population. However it would be an advantage to increase the number of *loci* to strengthen SNP study as a complement methodology.

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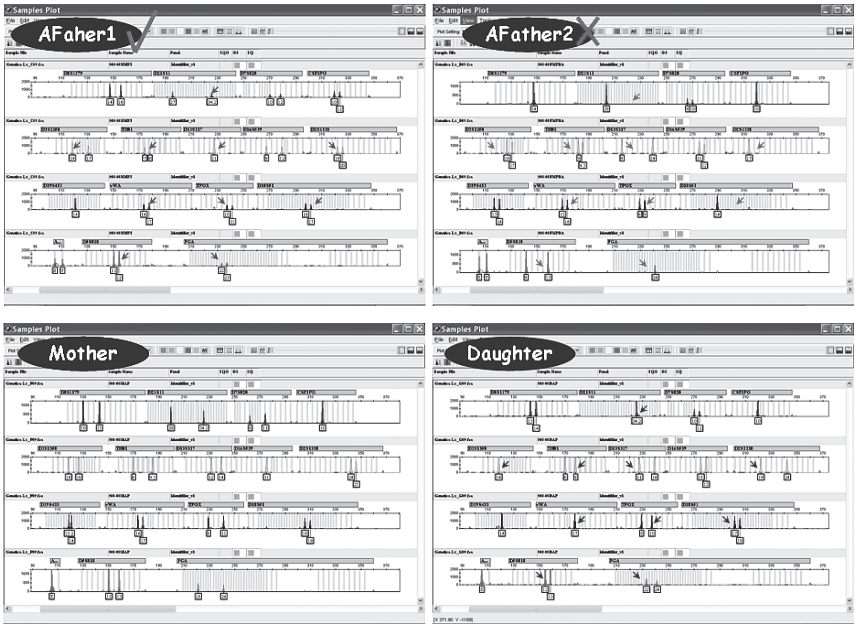

Figure 1 – Case 1 electropherograms (EPG), obtained using Identifiler. It is shown that Alleged Father 1 is not excluded from paternity and Alleged Father 2 is excluded with incompatibilities in 10 STRs.

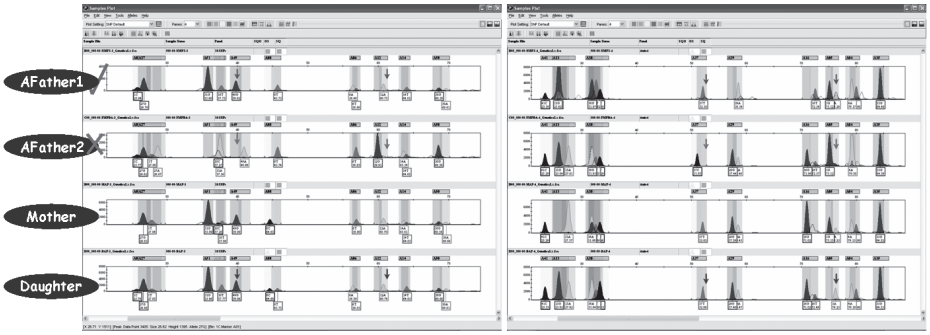

Figure 2 – EPGs obtained with the two 10 SNP-plexes for case 1. It can be seen that there are 4 incompatibilities with the alleged father 2, also excluded from paternity with STRs.

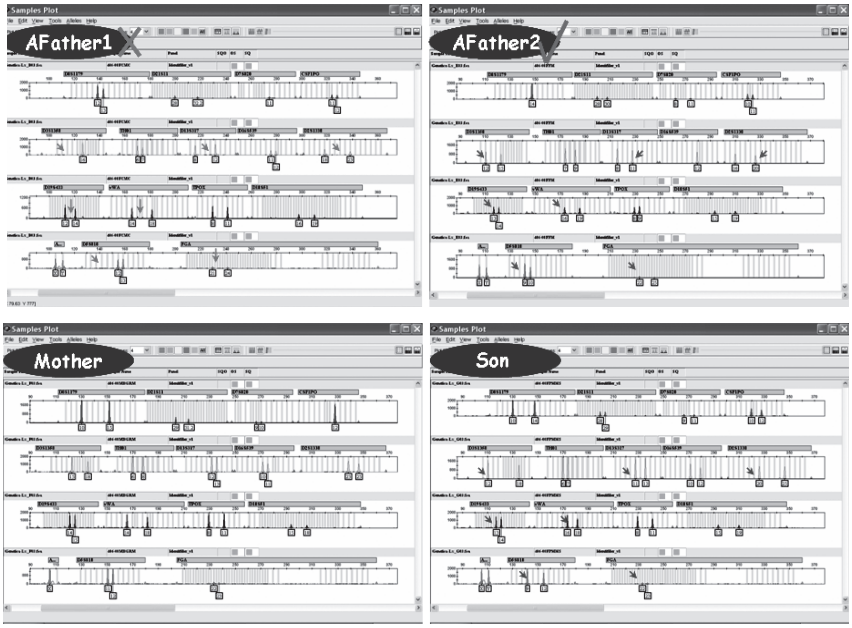

Figure 3 – Case 2 EPGs, obtained using Identifiler. It is shown that Alleged Father 2 is not excluded from paternity and Alleged Father 1 is excluded with incompatibilities in 8 STRs.

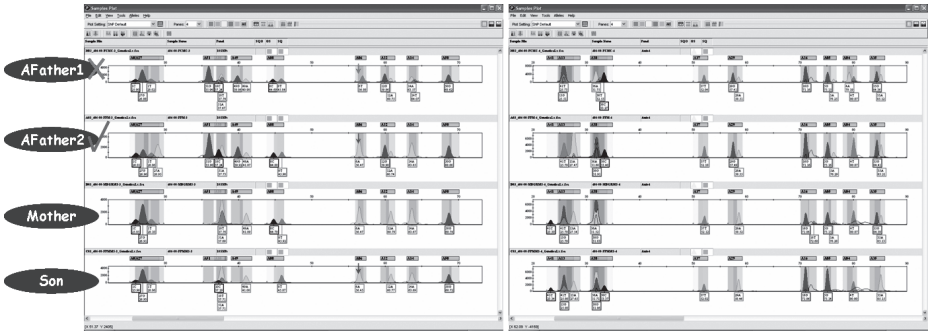

Figure 4 – EPGs obtained with the two 10 SNP-plexes for case 2. It is shown that there is only one incompatibility with Alleged Father 1, excluded from paternity with STRs.

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NATIONAL SCENERY OF THE USE OF GENETIC IDENTIFICATION TECHNIQUE IN THE OFFICIAL SERVICES OF IDENTIFICATION AND THE DENTIST PARTICIPATION

Abstract: DNA analysis can be considered a major technical advance in criminal investigation since the discovery of fingerprints. It is incorporated in forensic routine by police of first world countries and now it has been used in forensic reports in some states of Brazil. This paper aimed to know the Brazilian context regarding this technology. Questionnaires were applied in Institutes of Criminology and DNA forensic laboratories of 20 Brazilian states. The results of this study allowed us to verify the greatest influence of DNA technique in identification processes, the professional diversity of teams and the description of the procedures, which incorporated specific knowledge from dental professionals in the examples of teams with the presence of dentists.
Keywords: forensic DNA; forensic dentistry; human identification; dental DNA.

Introduction

Post-mortem human identification is an extensive study and research area in Forensic Dentistry, a science that has been evolving in a highly significant way. It used to be based on simple methods of observation and comparison and nowadays it employs sophisticated laboratory tests, including genetic exams (OLIVEIRA[8], 2008).

The analysis in molecular biology was introduced in forensic context and started to be used by forensic experts, dental professionals, and forensic doctors. They are associated with classical forensic techniques and result in more objective and reliable reports (SILVA et al.[10], 2007).

However, the introduction of new technologies in human identification services depends on financial resources available in each state for the acquisition of equipment and/or adaptation or construction of infrastructure. Moreover, there is the necessity of creating or negotiating the nursing staff and updating techniques in order to work with new methodologies (OLIVEIRA[8], 2008).

Thus, the mapping of the states that have already benefitted from the use of forensic DNA allows the understanding of the Brazilian context regarding that new technology, its implantation, structure, applied methodology, the categories of professionals, showing the differences in several units spread all over the country.

Objective

This study aims to verify the influence of the DNA technique in the identification processes in Brazilian identification services, checking the diversity of professionals involved in the analysis and the most common procedures.

Material and method

A questionnaire was used in data collection with the aim of establishing the centers that use DNA technique in forensic identification, types of biological samples, the existence of accreditation certificate in laboratories, number of procedures, category of professionals that belong to the team of forensic DNA, the number of dentists in the identification institutes and how many professionals work with forensic DNA.

The questionnaire was applied during the year 2008 with the of interviews with the experts who were responsible for the identification process by means of personal contact that happened during scientific events in the area, or in a complementary way after this first contact by means of telephone and/or e-mail.

Results

Contact was established with the Central of Legal Medicine Institutes in capitals of the 26 Brazilian states besides the Federal District. The questionnaire was answered by 20 Institutes and in 3 states the data were not incorporated in the discussion since the service in Rondônia was being implanted; joint venture was being renovated between Civil Police and the Federal University in Alagoas – the exams were temporarily being conducted in Bahia. The State of Pernambuco informed that it did not own a DNA laboratory and had the collaboration of the States of Paraíba and Bahia.

The States of Amapá, Amazonas, Pará, Roraima, Tocantins, Bahia, Ceará, Maranhão, Paraíba, Piauí, Rio Grande do Norte, Goiás, Minas Gerais, Rio de Janeiro, São Paulo, Paraná and Rio Grande do Sul have effectively contributed to the analysis.

The States of Acre, Sergipe, Mato Grosso, Mato Grosso do Sul, Espírito Santo and Santa Catarina; besides the Federal District did not answer or refuse to answer the questionnaire.

From the 20 States that were initially part of the sample, the dental professinal is present in 11 (Amapá, Bahia, Rio Grande do Norte, Pará, Goiás, Tocantins, Rio Grande do Sul, São Paulo, Paraíba, Minas Gerais and Paraná).There is also one professional linked to the DNA laboratory in Minas Gerais and Bahia and two others in Paraíba (Graphic 1).

The DNA forensic team from the 17 States that had already implanted the service by the late 2008 consisted of 83 professionals: 37 pharmacists (44%), 31 biologists (37%), 08 biomedical doctors (10%), 04 dentists (5%), 02 chemists (3%) and 01 doctor (1%) (Graphic 2).

The type of biological sample depends on several factors, such as the condition of conservation of the donor of this sample, the type of crime. Blood is the most employed biological sample (39%) (Graphic 3).

In most cases (60%), that biological sample comes from sexual crimes (Graphic 4).In spite of that fact, in 43% of the states, the collection of saliva in bite marks, when present, is part of the forensic examination (Graphic 5). This exam is usually performed by forensic doctors, and there are no dentists responsible for this procedure in the States involved.

Regarding accreditation in the 17 operating laboratories by the late 2008, 15 of them have answered the questionnaire, and 8 affirmed not performing any tests and 7 performed tests in association with the Brazilian Genetic Society, the Ibero-American Working Group in DNA analysis or the Spanish and Portuguese Group of the International Society of Forensic Genetics.

The number of DNA exams performed until December 2008 has varied in each State from ten to more than three thousands summing up 9.480 exams. The DNA analysis services have been implanted since 1998 in Minas Gerais; 1999 in Rio Grande do Sual and Goiás; 2000 in Paraná and Pará; 2001 in the State of São Paulo; 2004 in Paraíba; 2005 in the States of Rio de Janeiro, Bahia and Roraima; 2006 in Maranhão and Amapá; 2007 in Amazonas, Rio Grande do Norte and Ceará and 2008 in Tocantins and Piauí.

Discussion

The particularity of this research when interviewing forensic official services has brought some difficulties and, due to this fact, some States did not participate, since the hierarchic characteristic of those services does not provide autonomy for their professionals to release data without the approval of those in charge. Regarding the States that did not provide information, it is known that Mato Grosso, Espírito Santo and Santa Catarina, besides the Federal District, own laboratories of DNA forensic analysis.

The relation between the dentist and molecular biology and their presence in the official services of human identification can be traced back to the Law 5.081/66,from August 24th 1966 (BRASIL[3], 1966), in its article 6º, that defines the dentist's competencies in: I – practice all the acts regarding Dentistry, derived from acquired knowledge in under-graduate or graduate courses;

IV – perform dental forensic exams in civil, criminal, labor relations and in administrative office.

It is worth citing The Resolution CFO-63/2005(Conselho Federal de Odontologia[4], 2005) that ruled in its 64th article the areas of performance of the Forensic Dentistry professional, among them:

"Human identification; reports in correlated evidence, including spots or fluids originated or present in oral cavity."

The forensic techniques applied to human identification are methods that produce fully reliable results (SILVA et al.[10], 2007).

The dentist who is introduced in the forensic context can be really helpful in situations where the corpse is skeletonized, carbonized or in advanced state of decomposition (SILVA et al.[11,] 2008).In the presence of bite marks , its primary forensic approach is related to the analysis of dental characteristics presented in the victim's

injury or in the object found at the crime scene. However, when those characteristics do not produce satisfactory results, the DNA analysis obtained from the cells that are present in the oral cavity and collected from the bite mark consists an important phase to determine the individual's identity that has produced the evidence(ATSÜ et al.[1], 1998; MCKENNA et al.[6], 2000).

The multidisciplinary character of DNA forensic exams has its evidence in the presence of six different professional categories that compose the functional staff of those services and that, according to BILGE et al.[2] (2003), several techniques are used to identify a corpse in complex cases.

Despite the teeth are not the biological sample of election , they appear among other biological samples as important factors in the identification process and criminology due to the high probability of the dental characteristics that are never the same in two individuals, as well as relatively high level of physical and chemical resistance of the dental structure(OLIVEIRA[8], 2008).

In those situations, the teeth act as elective material to analysis and the extraction of the deoxyribonucleic acid (DNA) is obtained by the dental pulp, or by the tooth itself. This is due to the hardness of dental structures (enamel, cement, dentine and the alveolar bone around the tooth) that provide conditions to DNA preservation and integrity even in adverse environmental circumstances such as high temperatures (Tsuchimochi et al.[13], 2002).

The association of classical forensic techniques in genetic exams has allowed significant evolution in forensic reports. Case investigations of sexual violence that were once limited to semen analysis of the sample, serological tests, such as blood testing , are nowadays able to produce accurate results with the possibility of analyzing genetic material extracted from fluids, capillary bulb and fetal material(GOES et al.[5], 2002; SILVA et al.[9] , 2004).

The results reaffirm the fact that sexual violence is the one that mostly employs techniques of genetic investigation, however, human bite marks, evidence that is frequently found in such crimes, are discarded by a great number of institutes. DNA from saliva found in those bites is sometimes fundamental to find the aggressor (Sweet et al.[12], 1997).

In a study, Walsh et al.[14] (1992) proved the efficacy of human saliva as an adequate biological material to forensic analysis after evaluating the reliability of DNA extraction in different biological materials, including saliva and saliva spots.

If compared to blood puncture, saliva presents advantages since it is simple to collect biological samples and it presents less probability of contamination. It is a non-invasive, painless and non-traumatic method and in cases of paternity, children are able to collaborate with it (NICOLÁS; CANELA[7], 1999).

Regarding laboratory accreditation, one may observe low adherence in services possibly because such tests have not been mandatory yet in Brazil and there are not representative organizations to apply those tests. A second motive derives from the fact that official services are subjected to public trust. Anyway, proficiency from international organizations such as GEP-ISFG gives credibility to the tests and protects the Institution in case the results are contested.

Contribution of every State in the sum of exams performed varies and reflects the chronological differences in service implementation.

Conclusion

The techniques of genetic identification are important tools introduced in forensic practice to solve questions that were once considered unsolvable to Criminalistics, Medicine and Forensic Dentistry. The multidisciplinary character of the forensic practice and the experience of fewer States where the dental professional has already been practicing in the DNA forensic team suggest that the presence of a dental professional in the team is fundamental.

Acknowlegment

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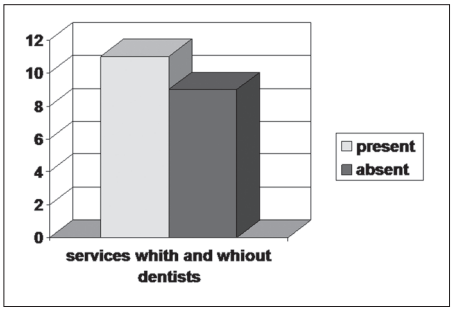
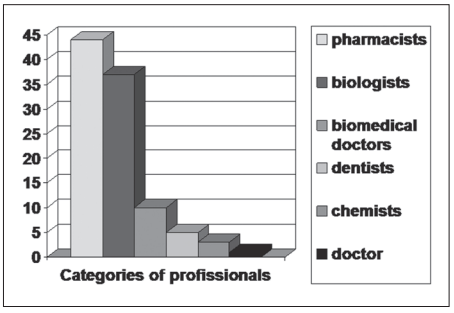
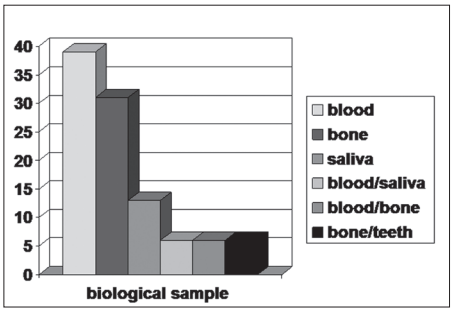


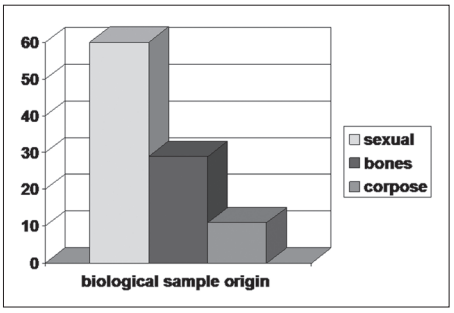
Graphic 1 – Presence of a dentist in Criminalistic Institutes



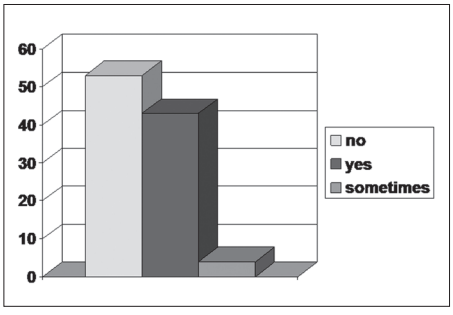
Graphic 2 – Categories of profissionals present in the DNA forensic team



Graphic 3 – Biological sample of election



Graphic 4 – Biological sample origin



Graphic 5 – Performance of suabe in bite marks

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HETEROPATERNAL TWINS INVESTIGATION WITH MISSING ALLEGED FATHER

Abstract: Twins paternity investigation (male and female) was performed as requested by the Court of Justice. As the alleged father was missing, the Court asked for DNA analysis comparison with a biological son of the missing man and their mother. Seventeen autosomal STRs and eleven Y-STR *loci* were analysed. The results showed that twins had different biological fathers.

Introduction

Superfecundation describes a situation where two ova can be fertilized giving rise to dizygotic twins. Sexual intercourse of a woman during the same polyovulatory period with two different partners may lead to superfecundation with the resulting twins having two different fathers, a phenomenon known as heteropaternal superfecundation**.** Although rarely among humans, this phenomenon has been described by other authors, particularly involving genetic disease studies and disputed paternities (1- 4). In this study we describe a heteropaternal twin investigation in a case of missing alleged father.

Materials and Methods

DNA was extracted by Chelex method from individual buccal swabs from the twins, the biological son of the missing alleged father and their mother. DNA analysis was performed using AmpF1STR® Identifiler (Applied Biosystem) and PowerPlex® 16 System(Promega) to study a total of 17 autosomal STR *loci*. Additionally, 11 Y-STRs contained in the PowerPlex®Y System (Promega) were investigated. Samples were analyzed in a 3130xl Genetic Analyser (Applied Biosystem) with Genemapper® ID v3.2. Statistical analysis was performed with "Familias" program (version 1.5).

Results

After studying 17 autossomic STR *loci* (Table1), "Familias" program was performed for statistical brotherhood analysis. A twin sisterhood probability of 99,99942% to the alleged brother and a twin brotherhood probability of 0,00841% was found. These results were confirmed by Y-STR analysis – six genetic inconsistencies between the male twins and the alleged brother were detected (Fig. 1). These results showed that twins had different fathers.

Discussion and Conclusions

Performing paternity testing investigations, we have detected several twin heteropaternal cases. The first report on a STR mutation in a double paternity case where both biological fathers were indisputable identified was performed in our laboratory (5). The frequency of twin cases with different biological fathers is probably underestimated, because this phenomenon is mainly detected when paternity investigation is performed. This situation depends not only on whether tests are done, but also on social behaviour (3). Thus, special attention should be taken in similar situations when twins' paternity is being investigated.

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Table1 – Results of the 17 STR loci from twins, their alleged brother and their mother.

Locus	Mother	Female Twin	Male Twin	Alleged brother
D3S1358	16-17	15-17	15-17	17
TH01	6-8	6-8	6-7	8-9
D21S11	28-29	28-29	28-29	28-31
D18S51	15-17	13-15	13-17	17
PENTA E	8-12	7-8	7-12	8-12
D5S818	11-12	11	11-12	11-12
D13S317	12	12	11-12	12-13
D7S820	10	8-10	8-10	10-11
D16S539	11	11	11	9-11
CSF1PO	7	7-12	7-12	7-12
PENTA D	12-14	5-12	5-12	12-14
vWA	15-19	17-19	14-19	15-20
D8S1179	12-14	13-14	12-14	12-14
TPOX	9-11	8-11	8-11	8-11
FIBRA	23	23-24	23	23-24
D2S1338	16-23	16-25	16-25	16-22
D19S433	13-14.2	13-14	14-14.2	12.2-14.2

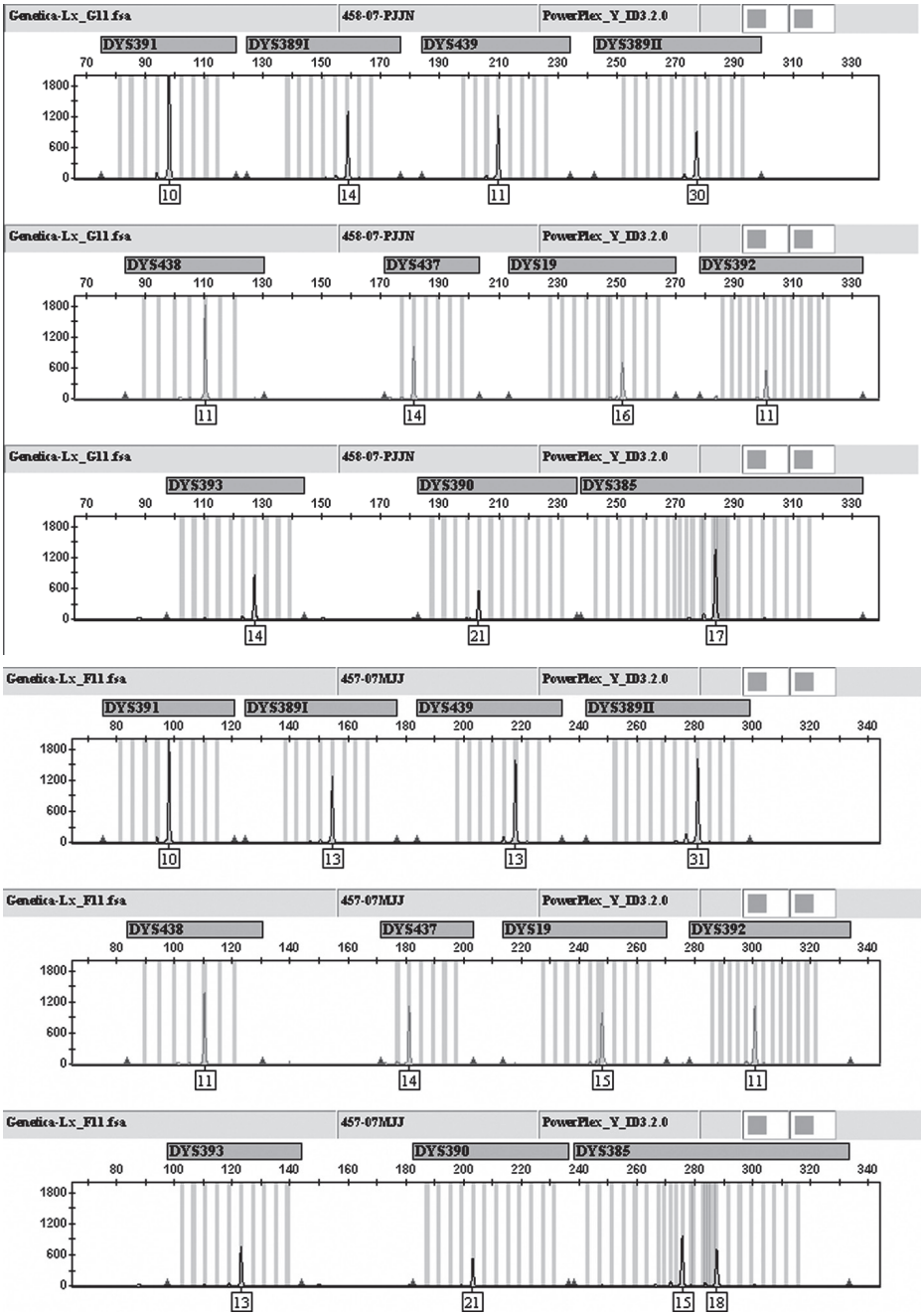

Figure 1 – Y-STR electropherograms of the alleged brother (A) and the twin male (B) showing six genetic inconsistencies in DYS391I, DYS439, DYS391II, DYS19, DYS393 and DYS385 *loci*.

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DEGRADATION OF BURIED DNA SAMPLES IN DIFFERENT TYPES OF SOIL

Abstract: Biological samples buried in different types of soil are often found in crime scenes. These samples are usually highly degraded which difficult its analysis. Several factors contribute to the degradation of biological material including temperature variation, humidity, UV light and especially the presence of microorganisms.

Blood was collected to three non related male donors and blood stains were made in fabrics such as jeans, cotton and lycra. Blood stains were dried at room temperature and buried in three different types of soil, to promote its degradation.

It was found that samples suffer a high degradation over time which difficult their analysis. The marshy soil proved to be the most aggressive, leading to rapid degradation of the different analyzed fabrics, probably because of its high percentage of moisture and microbial proliferation.

Introduction

Biological samples buried in different types of soil are often found in crime scenes. These samples are usually highly degraded which difficult its analysis. Several factors contribute to the degradation of biological material including temperature variation, humidity, UV light and especially the presence of microorganisms.

The cellular post-mortem degradation starts with the autolysis of the cell membrane. As a consequence the DNA is released to the environment and once in the soil it can 1) connect to minerals and humic substances such as humic acid (HA), 2) be degraded by bacterial DNases and used as nutrients for growth of plants and microorganisms or 3) be incorporated into the bacterial genome.

The preservation of DNA from buried samples is influenced by physical, chemical and biological properties of DNA and soil, such as pH, moisture percentage, concentration of humic substances, mineral content and cation concentration, and is dependent on its connection to certain minerals, humic substances and organomineral complexes.

The increase of humidity percentage leads to an increase in the number of microorganisms and consequently to a higher DNase activity. Another factor which affects the DNA degradation rate is temperature: whenever it rises, the half-life time of DNA decreases, as a consequence of increased activity of DNases [1, 2, 3].

Materials and Methods

18 ml of blood were collected from three non related male donors and 36 blood stains with approximately 7 cm of diameter were made in three different fabrics such as jeans, cotton and lycra, previously washed and decontaminated for 20 minutes with UV light. Blood stains were dried during 3 days at room temperature before being buried in three different types of soil (sand, marsh and clay).

Small pieces of each stain (12,5 cm^2) were collected after 15, 30 and 90 days. At this time, day, hour, place, presence of vegetation, temperature and humidity were registered. Photographic registration of the places and stains were also obtained, as well as, graphical registers from the closest meteorological stations, such as temperature, pressure, humidity and rainfall of the 31 days that precedes the collection. The different soils were also chemically characterized (Table 1).

Positive controls (blood stains of each individual) were made in all types of fabric. All fragments, as well as the control samples, were properly conditioned and frozen at – 80ºC until its analysis.

DNA extraction was performed using Chelex 100 method [4], QIAmp Investigator kit (Qiagen) and DNA IQ™ System kit (Promega). Samples were quantified with Human Quantifiler™ kit (Applied Biosystems), according to manufacturer's instructions using an ABI Prism® 7000 (real-time PCR).

Results

After ninety days jeans and cotton fabrics buried in marshy soil disappeared (Figures 1 and 3). Since only lycra remained in this type of soil for so long (Figure 2), it seems that this is a highly resistant fabric. Despite of its resistance, the DNA in lycra fabric undergoes a high degradation, not allowing its analysis.

In spite of the fabrics buried in the other types of soil didn't disappear after ninety days (Figures 4 to 9), quantification results after 15, 30 and 90 days, showed a high DNA degradation rate over time.

The greater quantity of DNA was obtained with samples buried during 15 and 30 days in sandy soil, extracted with QIAmp Investigator kit (Table 2).

Discussion

It was found that samples suffer a high degradation overtime which difficult their analysis. The marshy soil proved to be the most aggressive, leading to rapid degradation of the different analyzed fabrics, probably because of its high percentage of moisture and microbial proliferation, which are also responsible for the extensive DNA degradation verified after 15 days. The sandy soil with the highest pH showed the lowest degradation rate.

Conclusion

It is important to continue seeking for new methods of DNA extraction as well as improve the existing ones, to enable recover even the smallest amount of DNA present in degraded samples.

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Samples	SiO2 (%)	Al2O3 (%)	CaO (%)	MgO (%)	Na2O (%)	K2O (%)	Fe2O3 (%)	P2O5 (%)	TiO3 (%)	Mn (ppm)	Cu (ppm)	Zn (Ppm)	pH
Marshy soil	55,14	17,56	4,12	2,57	1,07	1,63	3,53	0,65	0,80	978	27,1	160	5,91
Sandy soil	68,32	8,66	7,51	1,05	0,89	1,12	1,24	0,09	1,23	234	12,6	71	6,84
Clay soil	54,63	20,31	2,13	1,54	0,84	1,72	4,96	0,56	0,78	563	32,1	97	5,80

Table 1 – Chemical characterization of the different soils

15 days - Extraction with QIAmp			
	Soil	Quantification	IPC
N. Denim	Sand	0.8705	31.26
N. Lycra	Sand	0.7747	32.04
N. Cotton	Sand	undetermined	31.44
A. Denim	Sand	undetermined	*
A. Lycra	Sand	2,625	30.01
A. Cotton	Sand	0.0053	*
P. Denim	Sand	0.0134	*
P. Lycra	Sand	2,282	31.04
P. Cotton	Sand	2,793	30.47
N. Denim	Clay	undetermined	39.32
N. Lycra	Clay	undetermined	*
N. Cotton	Clay	0.0101	*
A. Denim	Clay	undetermined	*
A. Lycra	Clay	0,0117	*
A. Cotton	Clay	undetermined	*
P. Denim	Clay	undetermined	*
P. Lycra	Clay	0.0065	*
P. Cotton	Clay	ind	30.89
N. Denim	Marsh	0,0112	*
N. Lycra	Marsh	undetermined	32,47
N. Cotton	Marsh	0,002	*
A. Denim	Marsh	undetermined	und
A. Lycra	Marsh	undetermined	30,47
A. Cotton	Marsh	undetermined	32,98
P. Denim	Marsh	0,0023	*
P. Lycra	Marsh	undetermined	*
P. Cotton	Marsh	0,0004	und

30 days - Extraction with QIAmp			
	Soil	Quantification	IPC
N. Denim	Sand	undetermined	und
N. Lycra	Sand	undetermined	und
N. Cotton	Sand	0.0026	*
A. Denim	Sand	0.004	*
A. Lycra	Sand	1,9384	30.35
A. Cotton	Sand	0.0038	35.02
P. Denim	Sand	0.0082	*
P. Lycra	Sand	0.0659	36.54
P. Cotton	Sand	undetermined	und
N. Denim	Clay	0.0055	*
N. Lycra	Clay	0.0129	*
N. Cotton	Clay	0.0083	*
A. Denim	Clay	0.0018	*
A. Lycra	Clay	0.0037	*
A. Cotton	Clay	undetermined	*
P. Denim	Clay	0.0113	*
P. Lycra	Clay	0.008	*
P. Cotton	Clay	0.0035	*
N. Denim	Marsh	undetermined	und
N. Lycra	Marsh	undetermined	und
N. Cotton	Marsh	undetermined	30.19
A. Denim	Marsh	undetermined	und
A. Lycra	Marsh	undetermined	38.76
A. Cotton	Marsh	undetermined	und
P. Denim	Marsh	undetermined	und
P. Lycra	Marsh	undetermined	und
P. Cotton	Marsh	undetermined	und

Table 2 – Quantification results of samples buried in the different soils for 15 and 30 days. N., A., and P. correspond to the blood donors. IPC values: und – undeterminated; * – normal (<30)

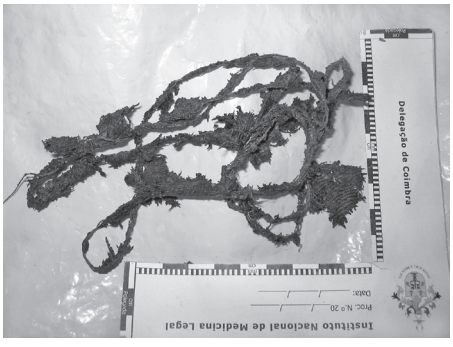

Figure 1 – Denim 90 days in marshy soil

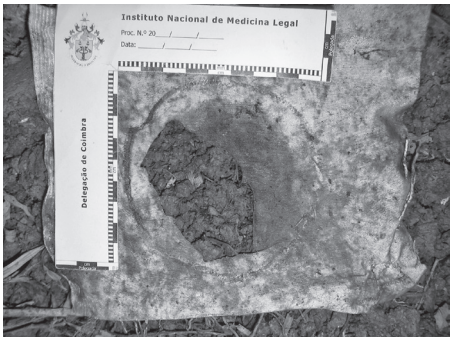

Figure 2 – Lycra 90 days in marshy soil

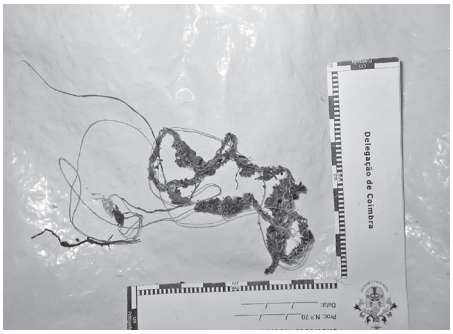

Figure 3 – Cotton 90 days in marshy soil

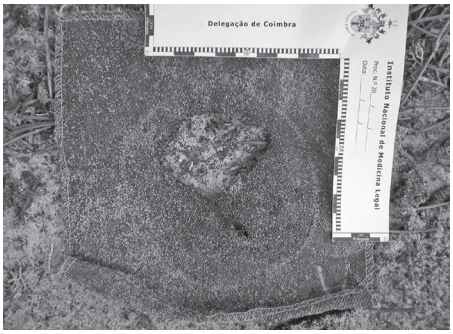

Figure 4 – Denim 90 days in sandy soil

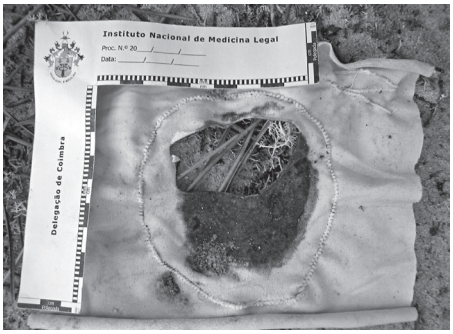

Figure 5 – Lycra 90 days in sandy soil

Figure 6 – Cotton 90 days in sandy soil

Figure 7 – Denim 90 days in clay soil

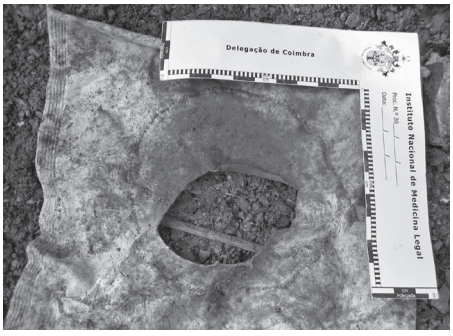

Figure 8 – Lycra 90 days in clay soil

Figure 9 – Cotton 90 days in clay soil

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GOOD PRACTICE IN COLLECTING SAMPLES FROM HUMAN FETUS WITH FEW GESTATION WEEKS

Abstract: Sometimes when young girls are victim of sexual assault the court determines the interruption of few weeks' gestation pregnancies and the posterior paternity testing to identify the crime perpetrator. In those cases is a good practice to collect samples (blood, skin or oral swabs) by forensic medical experts after the abortion procedure in order to avoid complex and not always successful technical procedures if further are received bones, muscle, paraffin-embedded blocks or, even worst, the whole fetus fixed in formalin.

Introduction

Our Genetic Department performs paternity testing according to court demand. Sometimes when young girls are victim of sexual assault the court determines the interruption of few weeks' gestation pregnancies and the posterior paternity testing to identify the crime perpetrator. If the fetus reference sample is not collected during surgical procedure later we will probably receive bones, muscle, paraffin-embedded blocks or, even worst, the whole fetus fixed in formalin. This will lead to complex and not always successful technical procedures in order to identify their genetic profile. In this context are reported two cases in which legal abortion was made at 15 and 16 gestation weeks. In both cases forensic medical experts were present to perform the collection of reference samples from the mother (blood and saliva) and fetus.

Methodology

It was performed a rigorous sterile preparation of material and work table. In the operating room, just after abortion procedure, the forensic medical experts made an esternotomy with scalpel. The procedure of incision should be made very carefully since heart must remain intact (figure 1). A 5cc blood cardiac sample was collected with needle and syringe in order to make a bloodstain (figure 2). In one of the cases were also collected skin tissue (preserved at –20ºC) and an oral swab.

DNA from blood samples, skin tissue and oral swab was extracted by Chelex method [1] and quantified in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). To identify the fetus genetic profile, the DNA extracted from the three types of samples was amplified by PCR with both commercial kits Identifiler [2] and PowerPlex16 [3]. The detection of PCR products was carried out with an ABI Prism™ 310 Genetic Analyzer using internal standards (LIZ-500 and I.L.S. 600) and allelic ladders from each kit.

Results

In all samples were identified complete genetic profiles (17 STRs) that allowed to establishing the fetus paternity. In the case that was also collected an oral swab and skin tissue, the genetic profiles were identical to the one identified in blood.

Conclusions

Blood, skin and oral swabs leads to very good results and are less time consuming and labour intensive with reduced costs than other kind of sample. In cases of interruption of pregnancy is a good practice to collect samples by forensic medical experts after abortion. The coordination between institutions is essential. Court should inform where and when abortion will occur.

References

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[3] PowerPlex® 16 System. Promega.

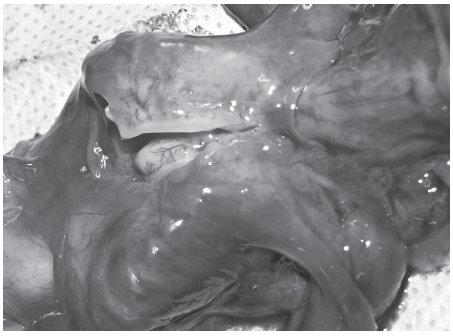

Figure 1 – Heart remains intact after esternotomy with scalpel.

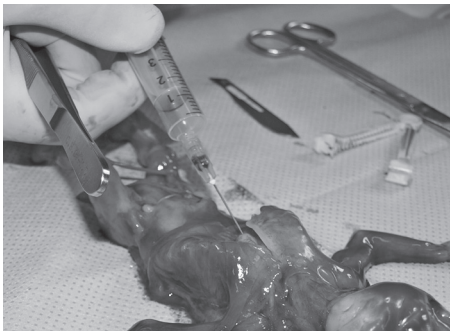

Figure 2 – Collecting a blood cardiac sample.

FORENSIC ODONTOLOGY

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DENTAL AGE ESTIMATION IN SPANISH AND VENEZUELAN CHILDREN. COMPARISON OF DEMIRJIAN´S AND CHAILLET´S SCORES

Abstract: Orthopantomographs taken from 308 Spanish-Caucasian and 200 Venezuelan-Amerindian children, aged between 2 and 18 years, were analysed following the Demirjian method. The applicability of this method was tested, and new predictive models for both populations were developed, using both, the original French-Canadian scores described by Demirjian (1976) and the new multi-ethnic dental scores proposed by Chaillet et al. (2005) when the ethnic origin is unknown. A high ethnic influence in dental development was found, with a clear delay in the Venezuelan-Amerindian population in relation to the Spanish-Caucasian one. New graphs were produced to convert the maturity scores to dental age for Spanish and Venezuelan children. With these graphs the Demirjian scores showed to be inadequate after the age of 12 in both populations, while Chaillet scores offered useful information until 14 years of age.

Introduction

Some of the more accurate methods of age estimation in the juvenile and young adult have been based on the assessment of the degree of dental development. One widely used method is that of Demirjian et al., first described in 1973 and based on a large number of French–Canadian children [1,2]. The method evaluates the development of seven mandibular teeth from a panoramic radiograph and calculates dental age. Two were the aims of this study: The first one was to test the applicability of the Demirjian method to two different etnic populations, a Spanish-Caucasian and a Venezuelan-Amerindian. The second objective was to develop age prediction models (polynomial or multiple regression models) for both populations using the original French-Canadian scores described by Demirjian [2] and the new multi-ethnic dental scores proposed by Chaillet et al. [3] when the ethnic origin is unknown, comparing the results for both scores and populations.

Material and Methods

Orthopantomographs taken from 308 Spanish-Caucasian children (157 girls, 151 boys) aged between 4 and 17 years, and 200 Venezuelan-Amerindian children (97 girls

and 103 boys) aged between 2 and 18 years, were analysed. The Demirjian's method using standard tables (separate for boys and girls) from Demirjian et al [2], and from Chaillet et al [3] was applied. Statistical analysis of data was performed using the SPSS package, version 15.0. First, the differences between dental age and chronological age for each patient were calculated. Finally, different regression models were explored in calculations of dental age as a function of maturity score for both samples.

Results

A high racial influence in dental development was found, with a clear delay in the Venezuelan-Amerindian population in relation to the Spanish-Caucasian one. Results showed that the Demirjian´s method overestimates the age in the Spanish Caucasian sample using both scores. In the Venezuelan Amerindian sample the opposite was found: the Demirjian´s method underestimates the age using both scores. Fig. 1 shows the differences between dental and chronological ages using the Demirjian method in both populations.

The Chaillet method allows the estimation of the dental age in a wider age range: Fig. 2 shows the relation between the mean maturity score, for both scales, and the mean age. As can be seen, using the Demirjian scores in the Spanish sample the 100% of maturity is achieved at the age of 12 in girls, and of 13 in boys, and in the Venezuelan sample, at the age of 14 in both, girls and boys. Using the Chaillet scores the mean 100% of maturity is achieved 2 years later.

Finally we calculated different polynomial functions between the chronological age (taken as the dependent variable) and the maturity score (taken as the independent variable), for the seven mandibular teeth. After trying the different options the statistical program offered, two models were selected: the compound and the cubic. Fig. 3 and 4 show a more stepped distribution applying the Chaillet maturity scores regarding the chronological age in both samples. So, once again Demirjian scores showed to be inadequate after the age of 12 in both populations, while Chaillet scores offered information until 14 years of age.

Discussion

The Demirjian's method to estimate dental age has been used in different populations and, in general, most authors agree that is a useful and easy to use method, but that overestimates the age of children (overestimation of dental age ranging from 0.02 to 3.04 years, depending on the population) [4-7]. Most of them also state that specific studies should be done to adapt the method to a specific population. Therefore, our results for the Spanish population are in accordance with previously published data for other Caucasian populations. Little information is known about dental development in Amerindian populations. Cameriere et al [8] found that Demirjian dental maturity in the Peruvian sample was advanced compared to that of the original study, which shows an overestimation of age in Peruvian-Amerindian children. In our sample of Venezuelan-Amerindian children the overestimation was only found in children under

8, whilst those older than 8 were delayed in development, in both sexes. Our results are in disagreement with the general tendency, but also with the specific Camerier´s results for the Peruvian population. This underestimation can be due to a small sample size, but in our opinion, in the case of the Venezuelan children, the ethnics could explain the differences with the original study because the French-Canadian population has a strong Amerindian genetic contribution but also European influence [3]. It is possible that the dental development in the French-Canadian population is in an intermediate point between the delayed Amerindian (Venezuelan) and the advanced Caucasian (Spanish).

Several authors have calculated new scores [9,10]. In 2005, Chaillet et al. [3] calculated an international weighed score in order to give new dental maturity curves for children when the ethnic origin is unknown, using the Demirjian's method. Since our sample was composed of two different ethnic population groups, we decided to test the Chaillet´s scores. Most of the previous studies elaborated the polynomial functions considering the "age" as the independent variable and the score as the dependent one [11-14]. Nevertheless, according to Muñoz et al. [15] when a perfect lineal fit is not possible between both variables, the unknown variable (age, in forensic cases) must be considered the dependent one. After calculating the polynomial functions for both populations, we found that the distribution of maturity scores related to chronological age was much better with the Chaillet scores, as can be seen graphically (Fig.3 a-b, Fig.3 and Fig.4).

Conclusion

We found *ethnic influence* in dental development, with a clear delay in the Venezuelan-Amerindian population in relation to the Spanish-Caucasian one. Even with specific polynomial functions, those calculated using the Demirjian´s scores showed to be inadequate after the age of 12 in both populations. On the contrary, those calculated using the Chaillet scores offered information until 14 years of age. Demirjian's method is simple, fast and easy to apply, but the *use of Chaillet International scores is more appropriate* than the curves originally proposed by Demirjian.

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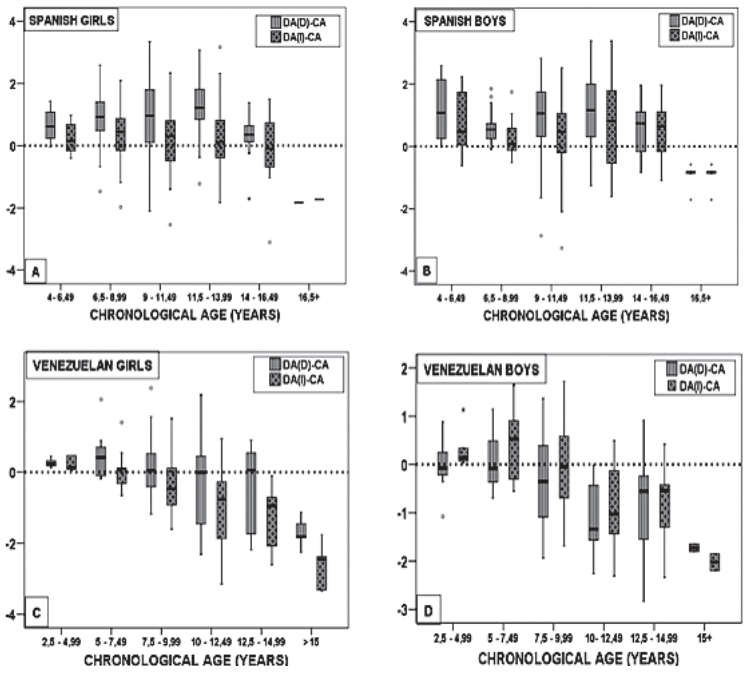

Figure 1 – Boxplots of differences between dental and chronological ages using the Demirjian method

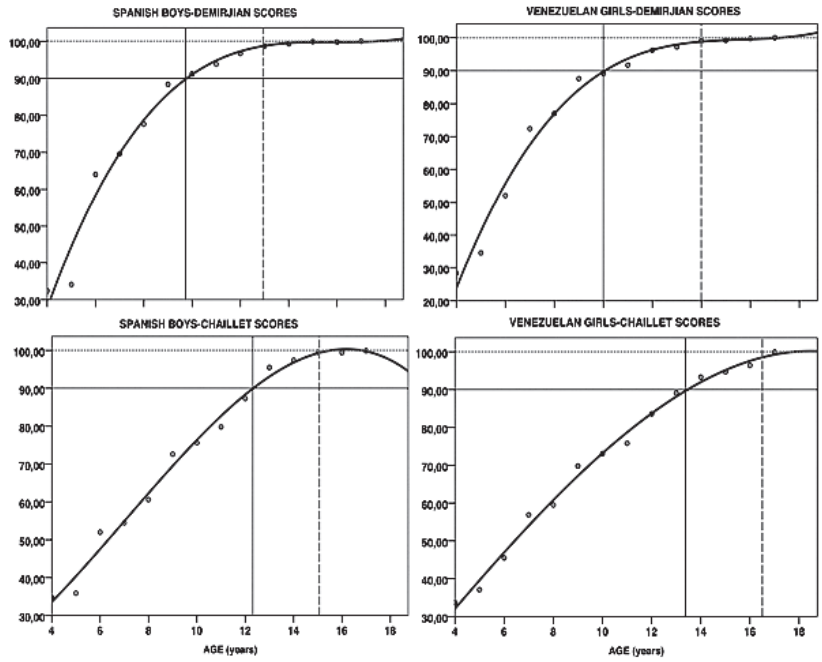

Figure 2 – Relation between the mean maturity score and the mean age, for both scores and populations

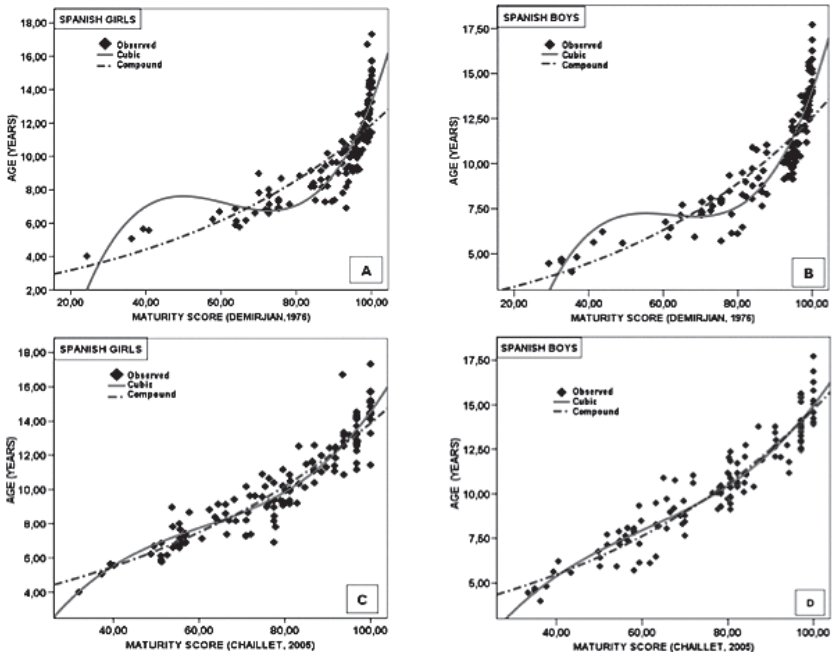

Figure 3 – Scatterplots of Maturity Score against Chronological Age in Spanish Children. Lines represent mean regression prediction in both function models

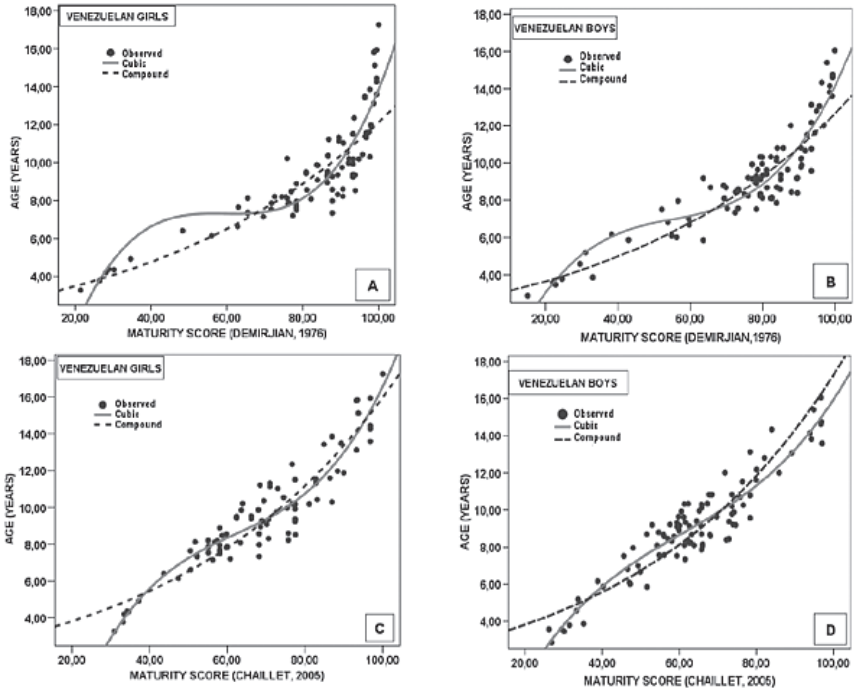

Figure 4 – Scatterplots of Maturity Score against Chronological Age in Venezuelan Children. Lines represent mean regression prediction in both function models.

MISCELLANEOUS

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PATTERNS OF SUICIDE IN CRETAN WOMEN

Abstract: In Greece so far little attention has been given to suicide among women and no systematic analysis has been reported. The goals of the current study were to estimate the proportion of female suicides on the island of Crete, Southern Greece; to describe the victims' sociodemographic profile and suicide-related variables; and to identify potential changes in rates and suicide methods during a predefined time period or between age groups. A retrospective study was undertaken, reviewing all female suicides between 1999 and 2007, and information was extracted into a computerized database. The female suicide incidence in the region was estimated to be 3.2 per 100,000, 1.7 times higher than the corresponding incidence reported for mainland Greece. These women were more likely to be over 55 years of age, to have lived in the western part of the island and to have committed suicide by hanging and self-poisoning. The most interesting finding was that self-immolation accounted for the 4.8% of the female suicide cases in the study area, while there is no mention of suicide by burning in Greece hitherto.

Introduction

Globally, one of the most consistent findings in suicide studies is a phenomenon called the gender paradox of suicidal behaviour [1-3]; women have higher rates of nonfatal suicidal behaviour but lower rates of suicide mortality than men. Nevertheless in several of the world's countries, including Greece, there is an apparent lack of national data on nonfatal suicidal behaviour [3], and furthermore the data on suicidal behaviour available through the WHO database or a number of comprehensive overviews of suicide patterns internationally [4,5] come mostly from industrialized countries.

With respect to Crete, preliminary data on suicidal acts during a 9-year period revealed a surprisingly higher suicide rate compared with the few previous suicide studies in Greece [6-8]; this was possibly a result of the observed differences in social, economic, traditional and life style features between various regions in the country [8,9]. Within this framework, the goals of the current study were to estimate the proportion of female suicides, to describe the victims' sociodemographic profile and suicide-related variables (i.e. seasonal variation and regional differences of suicides), and to identify potential changes in rates and suicide methods during a predefined time period or between age groups.

Materials & Methods

Catchment Area

Crete constitutes the largest island in Greece and one of the 13 administrative regions into which the country is divided. Geographically isolated, the island covers an area of 8,336km^2, and separates the Aegean from the Libyan Sea, marking the boundary between Europe and Africa. With a population of 601,131 in 2001, as recorded by the National Statistical Service (NSS), Crete is divided into four prefectures –Chania, Rethymno, Heraklion and Lassithi- and represents 5.5% of the total population of Greece. The urban and semi-urban population accounts for the 60% of the total, while the remaining 40% is rural. The 23.4% of the population is employed in the primary sector, the 16.1% in the secondary sector and the 55.6% in the tertiary sector, whilst the corresponding national percentages are 14.4%, 21.7% and 58.6% (2001) and the per capita GDP of the region of Crete represents the 95.9% of the national average [10].

Data Basis

A retrospective study was undertaken, reviewing all female suicides in the region from January 1, 1999 to December 31, 2007. Data on female suicides were collected from the Department of Forensic Sciences of the Faculty of Medicine of the University of Crete and the Department of Justice for the region, and then extracted into a computerized database. In an attempt to eliminate any possible discrepancies [11], the researchers cross-checked their data with official records kept in the police departments and the public prosecutor's files in each prefecture. Suicide deaths were defined by the International Statistical Classification of Diseases (ICD) 9th revision [12], codes E950-E959, and the 10th revision [13], codes X60-X84. Suicide cases were also processed according to the distribution of the prefecture and districts (urban, semi-urban or rural). Urban populations reside in cities and towns with more than 10,000 inhabitants, semi-urban in towns with between 2,000 and 10,000, and rural in villages with fewer than 2,000 inhabitants.

Statistical Analysis

Data are expressed as mean±standard deviation (S.D.) or median (in case of violation of normality) for continuous variables and as percentages for categorical data. Yearly and mean age-specific suicide rates were calculated per 100,000 population, in six-year age groups. The total suicide rates were age-standardized on the Greek population of census year 2001. The Chi-Square goodness-of-fit test compares the observed and expected frequencies in each category of variables to test if all categories contain the same proportion of values. All tests are two-sided, statistical significance was set at $p<0.05$. All analyses were carried out using the SPSS v16.0 [14].

Results

On the island of Crete from 1999 to 2007, 83 female suicides out of 374 suicide cases were recorded (22.2% of the total). Female suicides had a minimum value of 6 in the year 2001 and a maximum value of 16 in the year 2000; the average over the 9-year period was 9.2. The overall female suicide rate ranged between 4.3 per 100,000 population in 1999 and 2.3 per 100,000 in 2007, with mean incidence of 3.2 (Table 1).

The mean and the range age of the suicide victims were 54.4 ± 20.3 and 17-94, respectively (Table 2). All except seven females were of Greek origin and over half of the suicide cases (55.4%) were non married. The total incidence in the age groups was 1.7 per 100,000 population in 15-24 year-olds, 2.4 in 25-34 year-olds, 4.0 in 35-44 year-olds, 3.5 in 45-54 year-olds, 4.0 in 55-64 year-olds and 6.0 in females ≥65 (Table 3).

Table 4 demonstrates the mean annual suicide rate for each method. Hanging ranked first in terms of method preferred (41%), followed by self-poisoning and jumping from high places. Firearms were employed in only two cases of less than 34 years of age. When differences between age groups were analyzed, a significant increase of violent methods (hanging and self-immolation) in women ≥65 was observed (Table 5). In parallel, self-poisoning was the most frequent suicide method chosen (60%) in the 35-44 age group.

Table 6 displays the time-related characteristics and seasonal variation of suicide cases, but given the size of our sample no significant relationships emerged; there was no observable trend according to days, months or seasons rather than a random variation of suicide deaths. The correlation between prefecture and district is presented in Table 7 with no significant differences identified (x^2=2.51, p=0.474), with the exception of the prefecture breakdown of suicide cases, where it is striking that there is a higher incidence of female suicides in the western part (4.1 vs 2.5/100,000) of the island. Two thirds of the suicide cases occurred in semi-urban and rural areas.

Discussion

While the current study was a retrospective research and relied on a relatively small sample, so the results may not be generalized to the Greek population, the data reported herein warrant consideration since several key conclusions can be drawn from the data analysis. Female suicide incidence in the Cretan region was estimated to be 3.2 per 100,000 for the period under study (1999-2007), whereas the corresponding incidence for mainland Greece (period 1980-1995) or the region of Epirus (North-West Greece, period 1998-2002) was 1.89 and 1.29 per 100,000 respectively [7,8]. This discrepancy, though, should be interpreted with caution in the light of weighing the factors that may have contributed to the differential geographical impact on female suicide rates.

Regarding the age patterns of female suicides in this report, the ≥65 year age group has the highest age-specific rate followed by the 35-44 and 55-64 age groups. Furthermore, no appreciable alterations in the overall rates of female suicides or types of methods being used in the study area were recorded, but there has been an

increase in the use of more violent suicide methods with increasing age. Likely, the most interesting finding is that self-immolation accounts for the 4.8% of the female suicides recorded on the island of Crete, whereas in Greece until today no information is available concerning individuals who committed suicide by burning.

In addition, despite the fact that recent data from several European countries have noted that women are more likely to use drugs in fatal poisoning [5], pesticides were encountered as the most common agents involved in all age groups herein in accordance with findings from rural Latin American and Asian countries [4]. To date, most of the growing body of research into suicide methods has long recognized that the choice of a particular method for suicide depends on the availability, familiarity and accessibility of the mean in a given geographical location which consequently increases the risk of suicide, while cultural and social factors also have a strong influence on the predominant method of committing suicide [1,2,15].

Conclusions

In conclusion, new studies are needed in order to ascertain the underlying factors for the differential geographical impact on female suicide rates, and moreover, further empirical investigation on the particular psychological, social and cultural features that seem to influence the women's suicidal behaviour is considered necessary as a first step toward implementing effective prevention strategies.

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Year	Suicide cases	Female suicides	Percentage of suicide cases per year (%)	Percentage of female suicides per year (%)	Rate per 100,000 population	
					Total	Females
1999	43	12	11.5	14.5	7.6	4.3
2000	61	16	16.3	19.3	10.6	5.7
2001	36	6	9.6	7.2	6.2	2.1
2002	37	8	9.9	9.6	6.2	2.7
2003	42	9	11.2	10.8	7.1	3.1
2004	28	8	7.5	9.6	4.7	2.7
2005	47	8	12.6	9.6	7.8	2.7
2006	43	9	11.5	10.8	7.0	3.1
2007	37	7	9.9	8.4	6.0	2.3
Total	374	83	100	100	–	–
Average	41.6	9.2	11.1	11.1	7.0	3.2

Table 1 – Year-wise distribution of suicide cases and mean annual suicide rate

Variables	N	%
Number of individuals	83	100
Age (years)		
(mean – median – min – max)	(54.4 – 54.0 – 17 – 94)	
15-24	6	7.2
25-34	10	12.0
35-44	15	18.1
45-54	11	13.3
55-64	11	13.3
≥ 65	30	36.1
Nationality		
Greek	76	91.6
Other	7	8.4
Marital Status		
Single	20	24.1
Married	37	44.6
Divorced/Separated	6	7.2
Widowed	20	24.1

Table 2 – Sociodemographic characteristics

	Years / Age group	1999	2000	2001	2002	2003	2004	2005	2006	2007	Mean Rate
Females	**15-24**	0	2.4	0	2.5	0	5.3	5.4	0	0	1.7
	25-34	4.4	4.3	0	2.1	0	4.4	0	2.2	4.5	2.4
	35-44	2.6	12.7	2.5	2.4	4.7	2.3	4.5	2.2	2.2	4.0
	45-54	9.0	5.9	2.9	0	2.9	2.8	2.7	2.7	2.7	3.5
	55-64	0	13.2	0	6.6	3.3	0	3.3	9.6	0	4.0
	≥ 65	11.6	3.8	7.1	5.2	9.2	3.5	4.0	5.0	4.7	6.0

Table 3 – Age-specific suicide rates per 100,000 population

Method	Female suicides
E950 Poisoning by solid or liquid substances	1.1
E951 Poisoning by gases in domestic use	0
E952 Poisoning by other gases and vapours	0
E953 Hanging, strangulation and suffocation	1.3
E954 Submersion (drowning)	0.2
E955 Firearms and explosives	0.1
E956 Cutting and piercing instruments	0
E957 Jumping from high place	0.4
E958 Other means (self-immolation)	0.2

Table 4 – Mean annual suicide rates by different methods

Age group	E950 – E952		E953		E954 – E958	
	N	%	N	%	N	%
15-34	4	25	5	31.3	7	43.8
35-44	9	60	4	26.7	2	13.3
45-64	9	41	8	36.4	5	22.7
≥ 65	5	16.7	17	56.7	8	26.7

Table 5 – Percentage of female suicides per method in terms of age group

Variables	N	%	Significance
Time of death			
6 am – 12 pm	33	39.8	p=0.005
12 pm – 6 pm	19	22.9	
6 pm – 12 am	21	25.3	
12 am – 6 am	10	12.0	
Month of death			
January	5	6.0	p=0.284
February	8	9.6	
March	10	12.0	
April	5	6.0	
May	12	14.5	
June	9	10.8	
July	8	9.6	
August	8	9.6	
September	4	4.8	
October	8	9.6	
November	3	3.6	
December	3	3.6	
Season			
Autumn	15	18.1	p=0.143
Winter	16	19.3	
Spring	27	32.5	
Summer	25	30.1	
Day of death			
Sunday	17	20.5	p=0.203
Monday	4	4.8	
Tuesday	15	18.1	
Wednesday	11	13.3	
Thursday	13	15.7	
Friday	12	14.5	
Saturday	11	13.3	

Table 6 – Time-related characteristics and seasonal variation

		Urban	Semi-urban & Rural	Total	Female population per prefecture	Rate per 100,000 population
Chania	N	9	14	23	73132	3.5
	%	**39.1%**	**60.9%**	**100%**	**24.6%**	
Rethymno	N	5	13	18	40736	5.0
	%	**27.8%**	**72.2%**	**100%**	**13.7%**	
Heraklion	N	13	18	31	145167	2.4
	%	**41.9%**	**58.1%**	**100%**	**48.9%**	
Lassithi	N	2	9	11	37694	3.3
	%	**18.2%**	**81.8%**	**100%**	**12.7%**	

Table 7. Correlation between Prefecture and District

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STUDIES FOR THE ASBESTOS EXPOSURE IN JAPANESE URBAN POPULATION

Abstract: It has been well known that exposure of asbestos results in pulmonary diseases. Pulmonary concentration of asbestos bodies (Ab) is a good indicator of asbestos exposure. The aim of the present study is to determine the pulmonary concentration of Ab in the Japanese urban population. We observed age dependent increase of incidence of Ab. This may indicate that the very low level exposure of asbestos would be continued in a daily life.

Introduction

Asbestos had been widely used as an industrial material, because of its physical properties such as heat-resistant, high tensile strength and flexibility (1). It has been well known that exposure of asbestos results in pulmonary diseases such as asbestosis, lung cancer and mesothelioma (1). Pulmonary concentration of asbestos bodies (Ab) is a good indicator of its exposure, and used for the assessment of the occupational exposure. It is widely recommended to identify persons with a high probability of exposure to asbestos dust at work over 1000 Ab/g of dry lung tissue (Helsinki criteria) (2). Only a few study have been reported Ab concentration in Japanese general population (3-7). The aim of the present study is to determine the pulmonary concentration of Ab in the Japanese urban population.

Materials and Methods

The lung tissue samples (n=530) were collected from the autopsy cases (above 10 years old) between 1974 and 1987 at the Department of Legal Medicine, Hyogo College of Medicine, Japan. The pulmonary concentration of Ab is determined by light microscopy, according to the method of Kohyama (8). In brief procedures are as follows; after small pieces of lung tissue (approximately 1g of wet weights) was dried in air bath and weighted exactly, the samples were digested with laboratory breech (Clean99 K-200®, Clean chemical, Osaka, Japan) for few hours. The digested

solutions were washed with distilled water, and adjusted the volume. A part of solution were filtrated through a membrane filter (pore size; 0.45μm). The filter was fixed on a glass slide with acetone vapor and observed by light microscope. In the present study, we classified the pulmonary Ab concentrations, slightly modified the category of previous reports (4,7).

This study was approved by ethical committee of Hyogo College of Medicine.

Results and Discussion

Ab are asbestos fibers that have been coated with ferroprotein by macrophages in the lung tissue (9), which is a good indicator of asbestos exposure. In the present study, we have investigated 530 autopsy cases. The 530 subjects are composed of 376 males (range: 10-85 year-old, mean: 44.9 year-old) and 154 females (range: 10-86 year-old, mean: 47.8 year-old).

Table 1 shows the incidence and distribution of the Ab concentration in lung samples. In 108 cases (20.4%), we observed more than 100Ab/g (male: 87cases (23.1%), female: 21cases (13.6%)). According to the Helsinki criteria, some of the investigated cases would be speculated as the occupational exposure, but not confirmed because of no detailed information of past occupational history. Figure 1 shows the incidence and distribution of the Ab concentration in each range of ages. We observed relatively high incidence of more than 100Ab/g at the age of forties or above (forties; 26.8%, fifties; 31.4%, sixties; 32.4%, seventies; 64.2%) in males, and at the age of sixties or above (forties; 10.0%, fifties; 9.1%, sixties; 23.5%, seventies; 33.3%) in female. This data indicates that age dependent increase of incidence of the observation of more than 100 Ab/g lung tissue. This may also indicate that the very low level exposure of asbestos would be continued in a daily life. The observation of high incidence in the male is earlier and higher than that of female. This may be owing to the difference of the life style between male and female. In general, the work of the male in the open air is longer than that of female.

Figure 2 shows the distribution of the number of Ab in each year. The increase tendency of the exposure to asbestos may be observed year after year since late 1970. In Japan, asbestos had been widely used until the prohibition of its use in October, 2004. The vast majority of asbestos used in Japan was imported, and it was peaked at the middle of 1970's (10). As the asbestos-related diseases have a long latent period, these diseases will continue to increase next 10-20 years. Further investigation would be required to clarify its relationship.

Conclusion

Our data indicate an age dependent increase of incidence of the Ab concentration (more than 100 Ab/g lung tissue). This may suggest that the very low level exposure to asbestos would be continued in a daily life. The increase tendency of the exposure to asbestos may be observed year after year. Further investigation would be required to clarify its relationship.

Aknowledgement

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	Concentration of Ab (per g dry lung weight)				
	<100	101-350	351-500	501-1000	1000
Male (n=376)	288	54	12	17	4
Female (n=154)	136	10	6	4	1

Table 1 – The incidence and the concentration of Ab in this study.

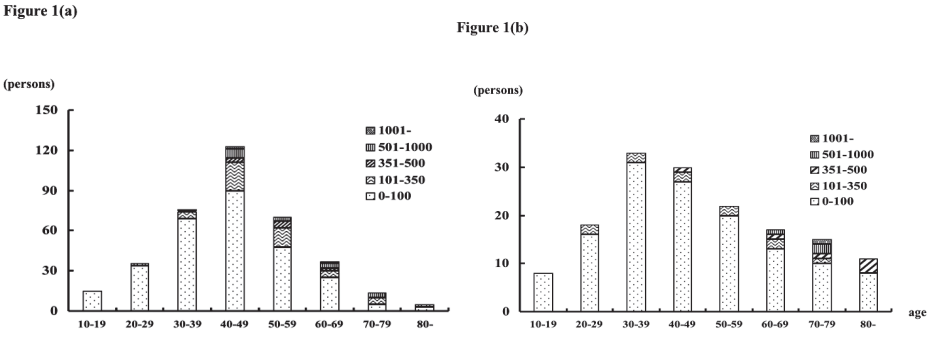

Figure 1 – The incidence and distribution of the Ab concentration (Ab/g lung tissue) in each range of ages of male (a) and female (b).

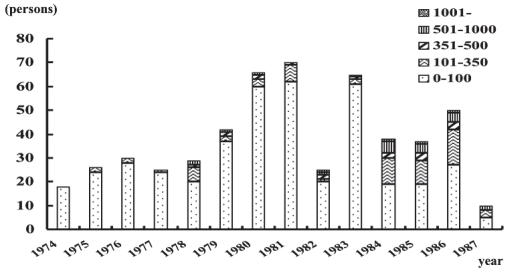

Figure 2 – The distribution of the number of Ab in each year.

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FORENSIC MEDICINE PROFESSIONAL SERVICES IN CANTON TICINO (SWITZERLAND):3 YEARS OF EXPERIENCE

Abstract: A report about the co-operation between the Justice Department of a Swiss Federal State (Canton Ticino) and the Forensic Medicine Institute of Insubria University (Italy) from October 2005 to September 2008 is presented.
The medico-legal activity performed consisted in death scene investigation, external examinations of corpses, autopsies and examinations on people in suspected sexual assault and criminal injuries.
Statistical elaborations of our activities and, in particular, of the causes of death are illustrated.

Introduction

Since October 2005 the Forensic Institute of Insubria University of Varese (Italy) cooperates with the Justice Department of Canton Ticino Swiss Federal State. Our Institute is located about ten kilometres from Swiss customs, so when our assistance is requested by Scientific Police (on Public Attorney indication), the forensic doctor on duty can arrive in only few minutes.

A report about 3 years of medico-legal activity performed, from October 2005 to September 2008, is here presented. Our medico-legal activity performed consists in death scene investigations, external examinations of corpses, autopsies and examinations on people in suspected sexual assaults and criminal personal injuries.

In the present diagram (Fig.1) our forensic activity resume on dead people, performed in three years is represented. Autopsies number is grown in the course of time, while external examinations and death scene investigations number is decreased. Statistical data at USTAT (Uffcio di Statistica del Canton Ticino) web-site reveal that in the year 2007 the resident population in Canton Ticino was about 324.851 people, and dead people was 2.762.

During this year we performed sixty-six autopsies and thirty-six external examinations. This means that the 3.7% of all deaths were of forensic interest.

With reference to autoptical activity we made a statistical elaboration about death causes.

In particular, in three years, we performed 195 autopsies, 133 of which (68%) concerned violent deaths and only 62 (32%) natural deaths (Fig.2). In acknowledgement of international data on death causes, the majority of natural death causes is consequence of vascular-heart diseases, such as heart attack and aneurysms (Fig.3).

As far as violent deaths, 95 of them (the majority) were caused by accidental events, 35 by suicides and only 4 by homicides (Fig.4). This last datum seems to be a very intersting information if we consider that on about 325.000 residents, in three years, there were only four events of this kind. In paritcular an homicide occured by gun-shot, two by assaults (in one of this cases the perpetretor and the victim were disturbed) and one by manual strangulation.

Very significant appear also data about suicidal deaths (Fig.5). There is a perceptible difference in regard of manners releted to the gender,following what known in criminological literature. Males prefer precipitation and gun-shot, followed by hanging and drugs and alcohol poisoning. Instead females prefer drugs poisoning followed by drowning and precipitation. A singular case is represented by a woman who chose to be assisted in her suicide (this practice is allowed by Swiss law). Likewise interesting is a case of a young boy, who committed suicide by poisoning with nitrogen.

With reference to accidental deaths (Fig.6) the first cause of death is represented by traffic accidents (mostly motorcycles and motor vehicles, only in few cases pedestrian are involved).

In Canton Ticino a lot number of death are caused by drugs overdose, mostly by heroine and for the most part males are involved. This datum is in contrast with what happens on Varese Prosecuting Attorney's Office, where deaths by drugs intoxication are very uncommon. Four of deaths caused by accidental precipitation are due to accidental falls during mountain trails (in relation with morphological area characteristics).

Our medico-legal activity regards also examinations in cases of sexual assaults and personal injuries (Fig.7). The sexual assault number seems to be constant in the three years considered, while the number of examinations in case of personal injuries is exponentially grown (tripled). This datum should be considered important from a social-criminalistic point of view.

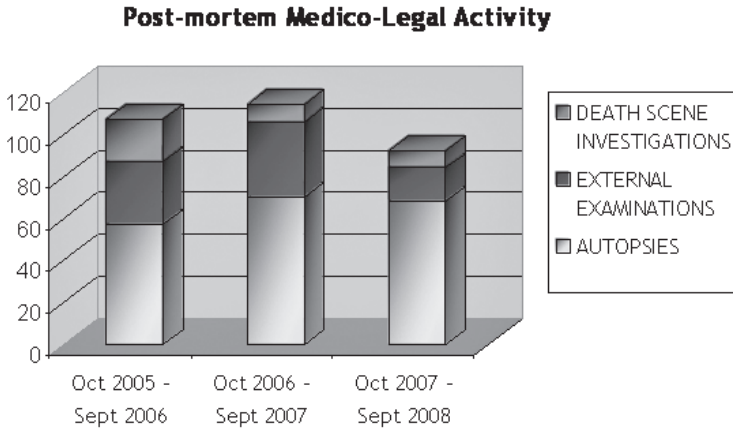

Figure 1 – 3-years post mortem medico legal activity, from October 2005 to September 2008.

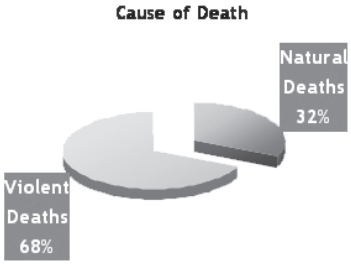

Figure 2 – Causes of death: autopsies performed revealed that the 68% of all deaths concerned violent causes and the 32% natural causes.

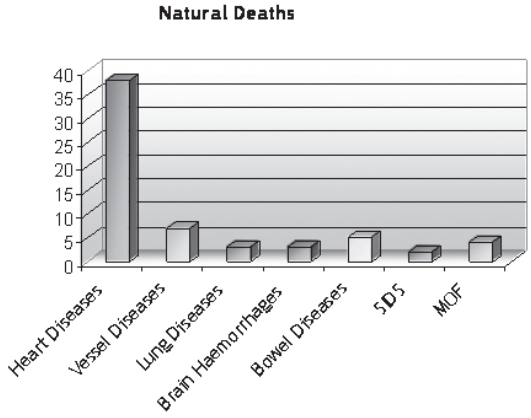

Figure 3 – Natural causes of death. The first natural cause of death is represented by heart disease.

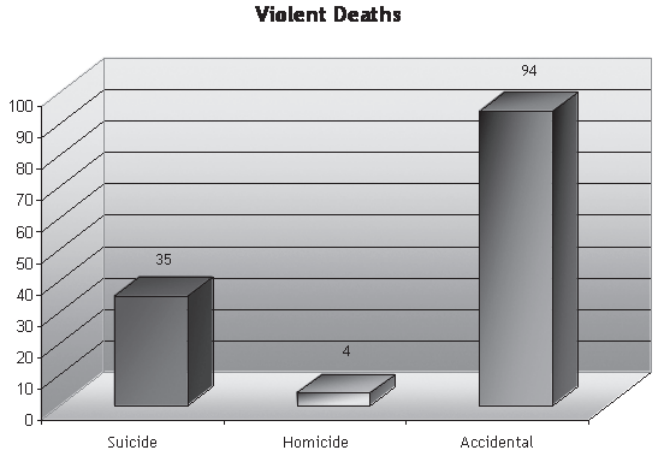

Figure 4 – Violent causes of death: 95 accidental events, 35 suicides and only 4 homicides.

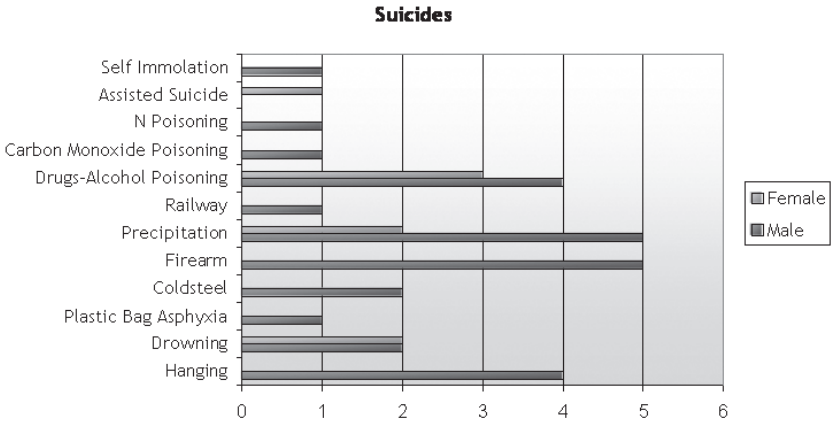

Figure 5 – Suicidal events and gender subdivision.

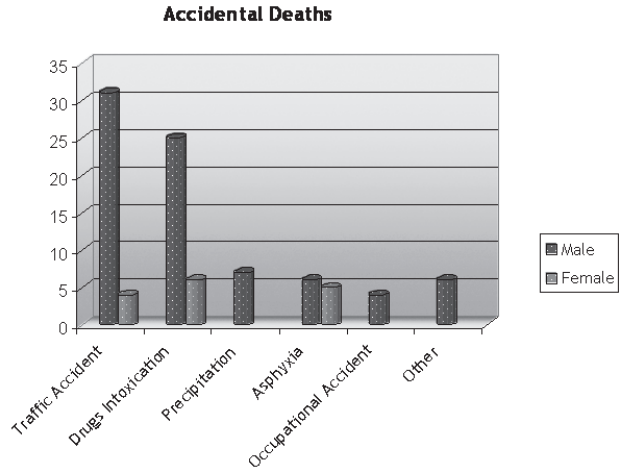

Figure 6 – Accidental deaths and gender subdivision.

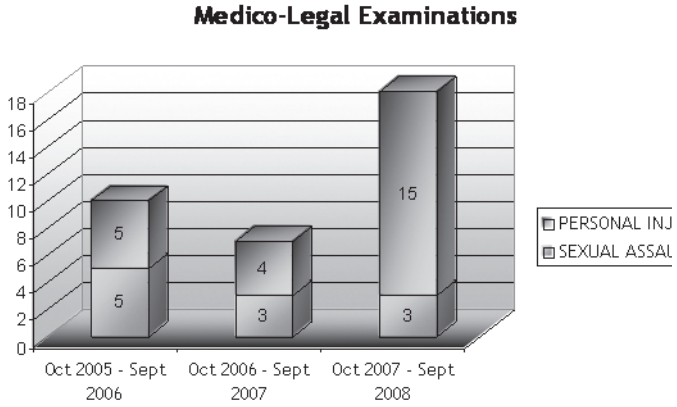

Figure 7 – Medico legal examination performed in case of sexual assaults and personal injuries.

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ALCOHOL AND CELL PHONE ASSOCIATED EFFECTS ON CAR DRIVERS TESTED IN AN EXPERIMENTAL ROADTEST

Abstract: The present study objectified the analysis of the physiological alterations in drivers who associate alcoholic beverages and mobile phone in the conduction of a vehicle. Practical tests in an experimental roadtest had been carried through, simulating common situations of transit, with four volunteers, who had lead a vehicle in different levels of alcoholemy and combined to the use of the mobile phone. After the accomplishment of the practical tests, it was perceived that with alcoholemy higher than 0,6g/L and the use of cell phone, 66% of the volunteers had not respected the landmark of the passage, 33% of them had wrongly understood the commands of the person who orientated and 100% of them had not correctly answered 80% of the tests of logical reasoning. It was concluded that there is not a parameter of safety of the usage of alcoholic beverages in the conduction of a vehicle, still observing the increase of the possibility of automobile accidents when associated to the use of cell phone.

Keywords: Driver under influence; alcohol; mobile phone; alcoholemy effects.

Introduction:

Traffic accidents kill more than a million of people per year globally and leave between 20 to 50 million wounded people[1]. In Brazil, in the year of 2005, around 500 thousand traffic accidents with victims occurred, causing the death of 35 thousand people, the majority of them in the age group that similarly corresponds to the interval between 18 and 59 years, affecting drivers, passengers and pedestrians[2,3,4]. In California / USA, 45% of the accidents with victims and 70% of deceased in traffic accidents presented significant alcoholemy[5]. With the purpose of reduction of the accidental events in result of the evidences of abuse of alcoholic beverages and traffic accidents, the new Brazilian Transit Code (2008) establishes that driving under the influence of any alcohol concentration per liter of blood subjects the conductor to the penalties foreseen for the law, being configured criminal penalty to drive under the influence of alcohol or any other psychoactive substance that determines dependence[3,5,6,7].

Another factor related to the increase of automobile accidents, even so considered medium infraction for the Brazilian Transit Code, is the use of mobile phone during driving[8,9,10]. British studies demonstrated that the reaction to an unexpected situation of someone who

is speaking on the mobile phone is 30% slower than of a person who had drunk a little above the limit allowed for the British law and 50% slower than of a driver in normal conditions[11,12,13]. The main damages caused for the association of the binomial cellular-driving constitute in the distraction, that occurs for the interaction between the conductor and his interlocutor, and in the commitment of a good technique of driving due to the withdrawal of one of the hands of the wheel for handling the device[5,14,15,16]. It is deduced that still more powered effects could be gotten when associating the triad: alcohol, cellular and driving with incalculable catastrophic results and losses [12,16,17,18]. Therefore, this paper is justified for the increase of automobile accidents involving drunk drivers and for the lack of studies in Brazil relating the use of alcoholic beverages and the use of cellular in the transit.

Materials and Methods

Practical tests had been carried through on September 14th of 2008, in an automotive vehicle in the city of Rio Acima (near the city of Belo Horizonte), State of Minas Gerais/Brazil. Common events of the transit in a track consisting in one tarred part and another one of land and gravel, duly interdicted had been simulated for competent authorities. The sample used in the experiments constituted from four volunteers, subdivided in two groups: Group A (two women) and Group B (two men).

VOLUNTEERS AGE AND WEIGHT.				
DATA	GROUP A		GROUP B	
	Volunteer I	Volunteer II	Volunteer III	Volunteer IV
Age (years).....	21	23	32	27
Weight (Kg).....	59	63	87	64

Table 1

As measures of security in the tests, a prepared car of driving school was used with auxiliary system of control of the vehicle, manned by a qualified instructor. An allowed maximum speed was established and an ambulance was available for medical attendance, in case it was necessary. One breathalyzer was used in order to indicate the breath sample to estimate the blood alcohol content – alcoholemy (Figure 1). The test was divided in two parts: non-chronometered (Figure 2) and chronometered (Figure 3). In the non-chronometered test, the time and the variations of speed of execution of the maneuvers were disregarded, being only analyzed the correct execution of the following maneuvers: reverse parking in garage, parallel parking (Figure 4) and clutch control on a hill (Figure 5). In the chronometered test, the possible variations of speed of the vehicle throughout the circuit had been evaluated, as well as the time of execution of the tests. The carried through maneuvers had been curves, eight-shapped curves (Figure 6), "slalons" (Figure 7), prevention accident test (Figure 8) and the braking test (Figure 9). The landmark of the circuit occurred with the use of continuous striped ribbons equidistant 3m and cones equidistant 5m. The adopted speed standard was from 15 to 20 Km/h throughout all the circuit, except in the test of prevention of accidents in which the speed standard corresponded to a principle of 40Km/h. Moreover, the number of knocked down cones were accounted.

The tests were carried through by the same driver in various situations, being that before the beginning of the first test each volunteer could make the recognition of

the circuit. In situation 1 (control), the driver made the passage without any alcohol concentration in the blood and without any factor that could deviate his attention. After that, the variables of the experiment that had consisted of increasing increase of the alcoholemy and questions of logical reasoning and/or answered mathematical tests to the cellular had been gradually added.

It is standed out that it had been maintenance of the hidden elements with the mathematical questions and of logical reasoning. Moreover, the orientations of conversion in the test of prevention of accidents and the lowering of the flag in the braking test were different for the same driver, however the same to all the volunteers. The situations (Figure 10) in which the driver was submitted were:

Situation 1: alcoholemy 0 g/l and without mobile phone use.
Situation 2: alcoholemy 0 g/l and with mobile phone use.
Situation 3: alcoholemy under 0,6g/l and without mobile phone use.
Situation 4: alcoholemy over 0,6g/l and without mobile phone use.
Situation 5: alcoholemy over 0,6g/l with mobile phone use.

For practical reasons, it was used as standard alcoholic beverage a distilled one, vodka, which alcoholic concentration is 40°GL. The dosage that each conductor received was proportional with his weight.

Results

In the first situation of the non-chronometered test all the volunteers were approved. However, when adding gradually the variable of the experiment, it was perceived the increase of errors of maneuvers in relation to the controlled situation; in the second situation it had an increase of 3 times the number of errors; in the third situation, the volunteers were wrong about 2 times more than in the first situation; while in the fifth situation it has been observed 5 times more errors than in the controlled situation. The maneuver most missed was the clutch control, followed of the parallel parking. In relation to the chronometered test, in higher alcoholemy over 0,6g/l, it had 2 times more errors and, when added by the cellular, it had about 3 times more errors than the controlled situation. The maneuvers most missed had been the sequence of curves in the asphalt and the test of prevention of accidents.

As for the test of logical reasoning, in alcoholemy 0g/l the average of rightness was 25,75% and in higher alcoholemy than 0,6g/l the average was only 9.15%. Moreover, evaluating reflexive capacity of the drivers for the braking test, it was observed that 100% of the volunteers had increased the distance followed when the alcoholemy was below 0,6g/L (Situation 3). When only the use of the cellular device was present (Situation 2), it was observed that 50% of the volunteers had increased the distance followed. Evaluating the time of accomplishment of the chronometered test, it had an increase of the time in 75% of the tested situations (TABLE 2).

CATEGORY OF EVALUATION	EVALUATED SITUATION				
	I	II	III	IV	V
Time of completion (min).....	02:28	02:45	02:17	02:35	03:00
Distance followed (m).....	10,36	11,90	13,91	18,98	08,40

Table 2. Time of the circuit and distance followed in the braking test, by evaluated situation

Discussion

It was verified that the increasing alcoholemy and the use of cellular telephone caused damages of attention, reduction of the cognitive and reflexive capacity of all the evaluated volunteers, proven for the biggest number of knocked down cones, low performance in the logical reasoning tests and errors in the accomplishment of maneuvers of the passage. When these two variables were associated it was observed that in 100% of the evaluated cases it had been alteration of the good technique of driving, evidencing that alcoholic beverage, use of cellular telephone and direction were incompatible. In Situation 5, most of the errors of the non-chronometered test had occurred in the clutch control and parallel parking. In the chronometered test of this same situation, the sequence of curves and test of accident prevention were the most missed ones; it also occurred an increase of the time for accomplishment of the test. These errors could be explained by the alteration of motor coordination caused by the alcohol and the use of only one of the hands to do the maneuvers.

Conclusion

This pilot study indicate that there is no safe parameter of usage of alcoholic beverage in the transit, therefore the psycho-motor reactions are individual and distinct in the various levels of tested alcoholemy. Moreover, the use of mobile phone is an important factor of distraction in the conduction of a vehicle, being dangerously potentialized when associated with alcoholic beverages. In 2009, a new study is being carried through with a higher number of volunteers and involved situations.

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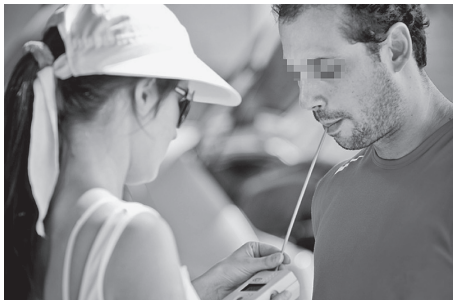

Figure – 1

Figure – 2

Figure – 3

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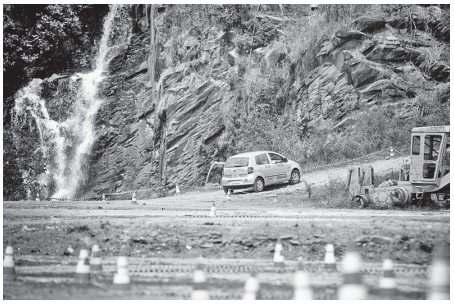

Figure – 5

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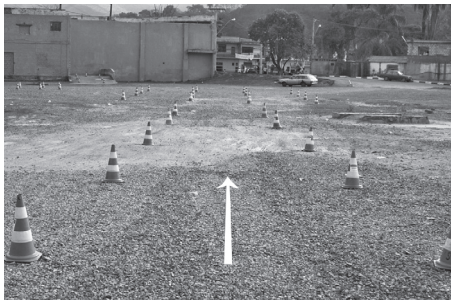

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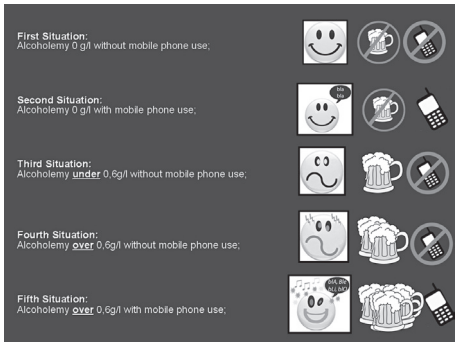

Figure – 10

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A BAYESIAN ASSESSMENT OF UNEXPLAINED FRACTURE AS A FORENSIC TEST OF CHILD ABUSE; QUANTIFICATION OF UNCERTAINTY USING THE ERROR ODDS APPROACH

Abstract: All forensic opinions can be characterized as probabilities, and all forensic methods upon which the probabilities are based can be described as tests of relevant evidence. Probability can also be described as a way to quantify uncertainty (as [1-probability] = uncertainty) and thus a means of assessing the validity of a forensic opinion. In the present paper a method of quantifying the uncertainty in forensic test results called the Error Odds is described. The Error Odds is a Bayesian metric that allows for calculation of the degree of uncertainty in a test result by using ordinary clinical terms and concepts. As an example of how the method can be easily applied to a common test result, the Error Odds calculation is used to quantify the uncertainty in the use of unexplained pediatric fracture as a test for child abuse. It is suggested that an Error Odds of 10:1 is the minimum threshold for the consideration of a single test result as evidence of guilt in a criminal proceeding.

Introduction

In February of 2009 the U.S. National Research Council of the National Academies released a report of a comprehensive analysis of deficiencies in the forensic sciences, including a list of 13 recommendations for the improvement of forensic science.1 The third recommendation addressed the lack of research pertaining to accuracy, reliability, and validity in forensic sciences, and recommended the development of quantifiable measures of the uncertainty in the conclusion of forensic analyses. Uncertainty is quantified by probability, as the one is the complement of the other; *i.e.* [1-probability] = uncertainty, and [1-uncertainty] = probability.2

In a forensic setting, probability is used to quantify one's belief in the truth of a conclusion regarding the interpretation of evidence. One approach to probability is that of the Frequentist, in which many prior observations are used to predict a future event or an event with an unknown outcome. An example of this approach is the 17% probability that the roll of a die will result in a 6, based on historical observations of many rolls of the die. In a forensic setting, such an approach would often be overly simplistic; for example, the prior observation that 81% of known offenders in the

UK are men does not allow for the conclusion that it is more probable than not that an individual crime was committed by a man, particularly when there is evidence that a woman committed the crime.3 In this example the probability that a crime was committed by a woman would be "conditioned" by the evidence, so that the probability more accurately reflects the known facts.

In the application of forensic medicine methods and tests to the investigation of a crime, where evidence may be present that modifies or conditions a probabilistic conclusion, a more accurate approach is that of the Bayesist, based upon the application of Bayes' Law. Simply stated, when applied in a forensic setting Bayes' Law tells us what we want to know given what we do know.4 Symbolically, Bayes' Law as applied to a positive test result can be depicted as $P(A|+\text{test})$; the probability that condition A is truly present given a positive test result for condition A. In a forensic setting, Bayes' Law is what allows for the identification and avoidance of the Conditional Probability Fallacy, in which the erroneous assumption is made that the terms are reversible; that $P(A|+\text{test}) = P(+\text{test}|A)$.5 An example of the Conditional Probability Fallacy would be the incorrect conclusion that if 90% of Spaniards speak Spanish then it is equally true that 90% of Spanish speakers are from Spain. As applied to a forensic test, the Conditional Probability Fallacy occurs when it is assumed that the probability that a test will be positive when a condition is present is the same as the probability that a positive test means the condition is present.

Error Odds

A simple application of Bayes' Law for assessing the uncertainty in a forensic opinion that relies upon a positive test result is the Error Odds assessment (also known as a post-test probability in Bayesian terminology). The result of the Error Odds assessment is the ratio of true positive to false positive tests (also known as the likelihood ratio) given the expected "base rate" or prevalence of the condition of interest (also known as the pre-test probability). The Error Odds test can be illustrated with a theoretical drug test that has a 90% false positive rate (the same as the "sensitivity" of the test to the presence of drugs) and a 10% false positive rate (the rate at which the test misidentifies subjects with no drugs as positives, [1-specificity]) that is applied to two populations; one of felons and the other of factory workers. If we estimate, from a hypothetical epidemiologic study, a base rate of drug use among the felons of 90% and 10% among the workers, the Error Odds test gives a very clear assessment of the degree of uncertainty in a positive test result for both populations. This is illustrated in Figures 1 and 2.

Note that the result of the Error Odds assessment (OE) for the felons indicated that the drug test had a very high ratio (81:1) whereas the OE assessment for the same test applied to the factory workers demonstrated an equal probability of true and false positive (1:1). This example illustrates how the base rate is the most potent value in the OE calculation; while the ratio of base rate for the felons to the workers was 9:1 (90% vs. 10%) the ratio of the OE result was the square of the base rate ratio; 81:1. Also note that the OE value is actually the odds *against* error; the rationale for this inversion is so that a positive test result with a low degree of uncertainty is always

a whole number. As an illustrative example, the 81:1 Error Odds calculated for the drug test among felons would be 0.012 if the terms were inverted.

The Error Odds can also be calculated using the following formula:

$$O_E = \frac{\text{true positive rate}}{\text{false positive rate}} \times \frac{\text{base rate}}{(1\text{-base rate})}$$

Using Bayesian terms this calculation would be described as the Likelihood Ratio multiplied times the Pre-test Odds, respectively. For the felons the calculation would be as follows:

$$O_{E\ felon} = \frac{0.9}{0.1} \times \frac{0.9}{0.1} = 81$$

and for the workers it would be:

$$O_{E\ worker} = \frac{0.9}{0.1} \times \frac{0.1}{0.9} = 1$$

Methods

In the present investigation we describe the application of the Error Odds assessment of test validity to the use of unexplained fracture as a proxy for child abuse among infants and toddlers. Skeletal fractures have been described in as many as 1/3 of cases of suspected abuse.6 Because the fractures are often occult, not correlated with a first-person account of injury, and occur in a particularly vulnerable population, the presence of unexplained fracture is sometimes interpreted as a reliable indication of intentional violence, despite the fact that most pediatric fractures are the result of unintentional trauma.7 Additionally, some children are more susceptible to fracture because of metabolic and other conditions that affect skeletal integrity.8

Intentional violence against infants and toddlers is far from rare, and cases of confirmed child abuse with fracture will most often have collateral evidence of abuse. In the case in which unexplained skeletal fracture is present in an infant or toddler and there is no supporting evidence that the cause of the fracture was abuse a quandary arises; is unexplained fracture a valid and reliable proxy for child abuse?

In order to perform an Error Odds assessment of fracture as a test for abuse the three unique elements of the calculation must be identified and estimated (true and false positive rate and base rate). When a test is used as a proxy for a specific condition, meaning, for these circumstances, that when an unexplained fracture is present that there is always abuse, the true positive rate for the test is 100%. Use of the test as a proxy also means that the test does not have the ability to correctly identify cases in which a fracture did not result from abuse, and thus the test has a 100% false positive rate as well.

The determination of a base rate to complete the Error Odds calculation can be derived from the literature. The most comprehensive publication on the base rate of abuse among children with fracture is the work of Kemp and colleagues.9 In their systematic comprehensive review of the medical literature these authors identified 32 out of 439 reviewed studies that allowed for the meta-analysis of abuse rates for fractures to various parts of the body. The authors found that the site of fracture with the highest probability of abuse was the ribs (71%, with a 95% confidence interval

of 42-91%), and next was the humerus (54%, CI 20-88%), the skull (30%, CI 19-46%), and then the femur (28%, CI 14-44%). Based upon their results Kemp et al. concluded that no single fracture should be used as a test for abuse, although they did not describe a threshold value that would make such a test acceptable as evidence of abuse.

Results

Using the values reported by Kemp et al. an Error Odds estimate of the validity of skeletal fracture as a proxy for child abuse can be calculated. Since the true positive rate and false positive rate are both 100% they cancel out in the OE calculation (making the likelihood ratio 1), and therefore the Error Odds is arrived at quite simply by dividing the base rate of abuse by [1- base rate] of abuse. The range of OE values for unexplained fracture as a proxy for abuse is presented in Table 1.

Discussion

It has been previously suggested that a minimal threshold value to consider the use of test results as evidence in a criminal matter is a post-test probability or Error Odds of 10:1, as this is approximately equal to a 90% confidence interval.10 By this standard only the extreme upper end of the confidence interval for rib fractures would meet the criteria (OE = 10.1), and the majority of the probable values are below the minimal value for consideration.

Prior authors have described a high rate of metaphyseal fracture amongst pediatric victims of homicidal abuse, and this finding has been misinterpreted by some as evidence that there is an equally high probability that abuse is the cause of such fractures when they are discovered.11 As described earlier in this paper, this misconstruction is a Condition Probability Fallacy, in which the mistaken conclusion is drawn that $P(Abuse \mid Fracture) = P(Fracture \mid Abuse)$. With no information on the base rate of abuse among children with metaphyseal fracture the presence of such fractures can only be considered as important but incomplete evidence of intentional violence.

Conclusions

In keeping with the NAS recommendations on the forensic sciences, we have described a method of assessing the uncertainty inherent in a forensic test for child abuse that relies solely upon the presence of an unexplained fracture. The Error Odds application of Bayes' Law is a simple method of uncertainty quantification that uses common clinical rather than Bayesian language to describe the terms in the calculation. This approach provides an easily understood metric for assessment of the relative weight that forensic specialists and fact finders can give to individual test results.

It is apparent from the results of the Error Odds assessment presented herein that the degree of uncertainty inherent in the use of unexplained fracture as test for child

abuse indicates that it is not reliable for this purpose when additional evidence of abuse is not present.

Acknowledgement

The author gratefully acknowledges the editorial input of Prof. Doug Mossman in the preparation of this manuscript.

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Fracture site	Confidence Interval	Base rate/ [1-base rate] range	Error Odds range
Rib	0.42-0.91	0.42/0.58, 0.91/0.09	0.72-10.1
Humerus	0.20-0.88	0.20/0.80, 0.88/0.12	0.25-7.3
Skull	0.19-0.46	0.19/0.81, 0.46/0.54	0.23-0.85
Femur	0.14-0.44	0.14/0.84, 0.44/0.56	0.17-0.79

Table 1

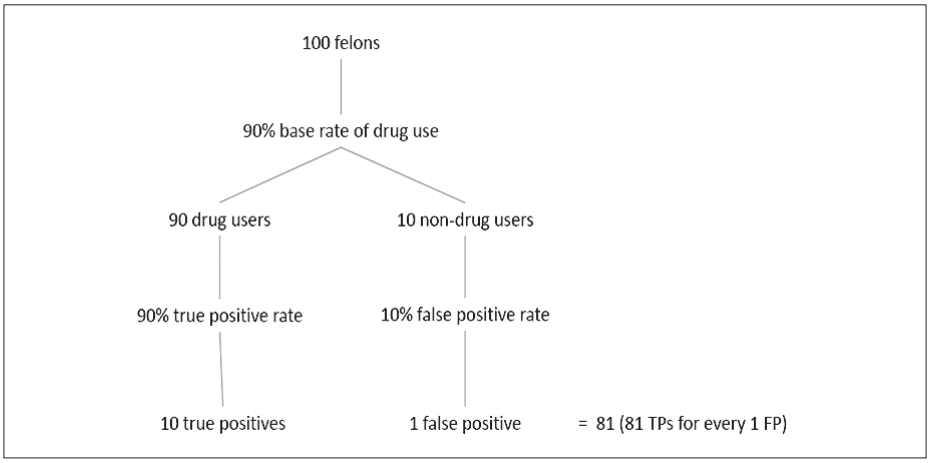

Figure 1 – Error Odds assessment of drug testing of felons

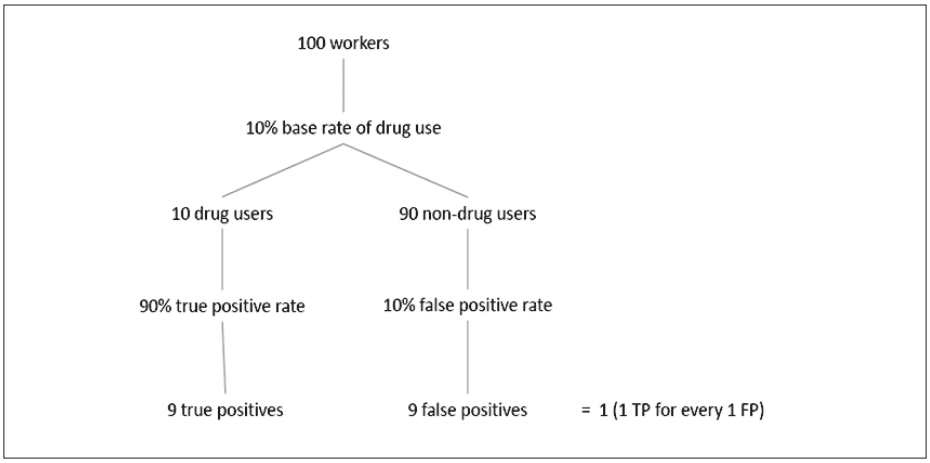

Figure 2 – Error Odds assessment of drug testing of workers

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PERCEPTION AND RISK OF EXPOSURE TO XYLENE BY PATHOLOGIC ANATOMY STUDENTS

Abstract: This study intends to assess the perceived and real risk of occupational exposure to xylene by students in bachelor degree of pathologic anatomy, cytology and thanatology. A questionnaire was produced and validated (Likert scale) applied to a sample of 217 students in higher education. Air samples were collected using activated charcoal cartridges. The quantification of the three xylene isomers (ortho, meta, and para) was performed by GC-FID.

With respect to perception and the classification of the risk of exposure to xylene for 35.9% of individuals corresponds to moderate risk (MR), 30.8% of high risk (HR), 28.2% of low risk (LR) and 5.1% of no risk (NR). The handling of this compound without gloves for 59.9% of students in MR, 21.5% for VR, 16.3% for LR and 2.3% of NR. For 56.7% of students there is danger of exposure to xylene. Xylene concentrations in laboratories environment were found at the range of 113.15-714.93 ppm and vary along of the week. The average concentration of total xylenes was 169.38 ± 76.15 ppm, which is below the Threshold Limit Values – Time Weighted Average (TLV-TWA). The highest occupational exposure occurred at slide mounting stage of the histological processing.

Introduction

Laboratory is inherently potentially dangerous environments and there will be always a level of risk associated with the work undertaken. In laboratories (where a variety of hazards exist) the workers must be closely supervised at all times. People who work in histology laboratory and related disciplines are at risk from exposure to risk agents. This risks can be traumatic for individuals, as well as extremely toxic (Buesa, 2007; Vecchio, Sasco and Cann, 2003).

The chemical hazards depending on the specific compound has the potential to poison (toxics, including carcinogens, teratogens and mutagens), burn (corrosive), irritate, produce allergenic reactions, explode, ignite or asphyxiate. They can affect us by inhalation, skin contact and ingestion. So may pose immediate consequences for the health or long-term (Buesa, 2007). The presence of technicians of pathologic anatomy, cytologic and thanatologic, it is essential to ensure a smooth functioning of a laboratory of pathology and cytological (Ferrand and Bernard, 1995). Inexperienced workers are persons at special

risk (including undergraduate students and school pupils on "work experience" schemes), deserves special attention. The adequate processing of tissues and body fluids require the use of chemical substances. A hazardous chemical by the Occupational Safety and Health Administration (OSHA), is a substance that may cause health effects in short- or long-term exposed employees, based on statistically significant evidence from at least one study conducted using established scientific principles (OSHA, 1994). It is certainly a broad definition that applies to all, or almost all of the chemicals typically used in laboratories. During academic training, the students need to handle chemicals, which can be classified according to Portuguese legislation, Portaria n.º 732-A/96 as extremely flammable, highly flammable, flammable, highly toxic, toxic, harmful, corrosive, sensitizing, irritant, toxic for reproduction, dangerous to the environment, explosive, incendiary, mutagenic and carcinogenic, putting in danger the man and the environment (Ministério da Economia, Ministério da Saúde and Ministério do Ambiente, 1996).

In pathology and histology laboratories, the chemicals more used are xylene, formaldehyde, the acids and ethanol, among other toxic substances that easily contaminate the air (Roy D. R., 1999), as well generate hazardous waste (xylene) (Environmental Protection Agency, 2000). The xylene is an important component of the routine, almost indispensable in laboratory and is often perceived by the technicians as a source of problems for the health(Agency for Toxic Substances and Disease Registry, 1999, 2005). The theme of the risk, as part of job security is recent and is not studied completely. The perception of risk interferes in behavior and with the preventive measures against the procedures that can cause injury and / or accidents (Sanders and McCormick, 1993). The main purpose of the psychological approach to Shrader-Frechette, focuses on responding to questions on the perception and acceptability of risks, after considering the views expressed by individuals, to which you are requested assessments of certain activities and / or dangerous technologies (Shrader-Frechette, 1985).

This study intends to assess the perceived and real risk of occupational exposure to xylene by students in bachelor degree of pathologic anatomy, cytologic and thanatologic.

Materials and Methods

Questionnaire

A descriptive survey was conducted during the period November of 2007 to May 2008 and information was collected on a sample of 217 students who attended randomly selected superior institute schools in Gandra (Portugal).

The questionnaire was extensive, but only a small portion of all the items will be used.

The question asked for a rating of global attitude to exposure occupational to xylene in Pathological Anatomy Laboratory, on a four-step category based in Likert scale. The categories on this group varied from "no risk" to "must risk". In questionnaire socio-demographic data (gender, age, and academic level) were collected, and consisted in sources of danger: classification of risk of exposure to xylene; classification of risk of manipulation of xylene without gloves;

Study Population

All students in the sample received an anonymous self-administered questionnaire and a letter explaining the purpose of the study, advising that they were under no obligation to complete the questionnaire, explaining that the information obtained would remain confidential, that the research team was available to provide further clarification of questions when necessary.

The students were divided into four academic groups:

– First Degree: no classes in histology laboratory
– Second Degree: 2 hours per week in histology laboratory
– Third Degree: 21 hours per week in histology laboratory
– Fourth Degree: 2 hours per week in histology laboratory

Statistical analysis

The internal consistency reliability coefficients using Cronbach's alpha or scale reliability coefficient from SPSS 16.0.1 for Windows software (SPSS Inc, Chicago, Illinois, USA) were examined. The intraclass correlation coefficient was evaluated by Pearson's test and Mann-Whitney test. For all statistical tests a p-value below 0.05 was considered significant.

Air of Pathological Anatomy Laboratory Sampling

Air sampling was performed for the 3rd October of 2008 to 15th of January of 2009. Air samples (129) were collected using activated charcoal cartridges, between 9:00 am and 5:00 pm, during the 21 hours a week of academic training, all days except Tuesday and week-end.

Personal air values were measured by means of passive air samplers (SKC solid adsorbent badges, inc. catalog number 530-11) equipped with of charcoal (cartridge (SKC coconut shell charcoal adsorbent sample tubes, inc. catalog number 226-01)). As reported in the SKC certificate of quality, these cartridges are calibrated at 25°C. Confounding factors of temperature and humidity in determination of chemicals were not relevant. The passive-spread samplers were positioned in proximity to the respiratory airways at the collar of each subject during the daily work day (between 9:00 am and 5:00 pm). The sampling times were of around 15 min. The samples were analyzed using a GC Chrompack CP-9000 Series equipped with a flame ionization detector (FID) and capillary column VF-5ms, 30m x 0.225 mm ID, film 0.25µm.

Results

Validation

The used questionnaire was first tested in a pilot scale for testing feedback by format to enhance clarity, reliability of responses and validation. No modifications were necessary to the final questionnaire.

Demographics

Two hundred and fourteen undergraduated students of pathologic anatomy, citologic and thanatologic (1st, 2nd, 3rd and 4th) respondents were enrolled. There were 32 (14.7%) men and 185 (85.3%) women, the students characteristics are presented in Table I.

The test Komolgorov-Smirnov (KS) revealed that all variables do not have a normal distribution (the value of D ranges between 0.156 and 0.452; Lilliefors α = 5%).

Risk perception

In classifying the risk of exposure to xylene in the laboratory of histology, 35.9% of the sample survey classifies it as being of moderate risk, 30.8% of very risk; little risk of 28.2% and 5.1% of no risk. Although, the test Mann, revealed that there are no significant differences between the 1st year and 3rd year ($p = 0.084$; $\alpha = 0.05$), and the 2nd and 4th grade ($p = 0.239$; $\alpha = 0.05$). However there are significant differences between students of 1st year and 2nd year ($p = 0.003$; $\alpha = 0.05$); 1st year and 3rd year ($p = 0.049$; $\alpha = 0.05$) 1st year and 4th year ($p = 0.002$; $\alpha = 0.05$); 2nd year and 3rd year ($p = 0.000$; $\alpha = 0.05$); 3rd year and 4th year ($p = 0.000$; $\alpha = 0.05$); (Table II).

The use of Personal Protective Equipment (PPE) is in itself an increase of demands for workers, often causing discomfort and other undesirable effects, especially when used for long periods of time. When handling chemicals, it is recommended that gloves with the right kind of material be used to protect the worker from accidental spills or contamination. The use of PPE's contributed to reducing the exposure of all or part of the risks, as the reduction of accidents at work. The classification of the risk of xylene manipulation without gloves: for 59.9% of students is much risk, 21.5% moderate risk, 16.3% of low risk and 2.3%. The Mann-Whitney test showed that there are no significant differences between the 1st and 4th grade ($p = 0.055$; $\alpha = 0.05$). Although there are significant differences between students of 1st year and 2nd year ($p = 0.000$; $\alpha = 0.05$); 1st year and 3rd year ($p = 0.006$; $\alpha = 0.05$); 1st year and 4th year ($p = 0.000$; $\alpha = 0.05$); 2nd year and 3rd year ($p = 0.000$; $\alpha = 0.05$); 2nd year and 4th year ($p = 0.001$; $\alpha = 0.05$); 3rd year and 4th year ($p = 0.000$; $\alpha = 0.05$) (Table III).

The personal monitor air measurements were compared to the values of atmospheric air at the three histological stages (histological staining, slide mounting and chemical waste disposal. Figure I shows the results obtained for the ambient air contaminants. Each area represents the mean of 129 measurements of total xylenes. The mean of each gas was determined separately, and subsequently, these values were summed to arrive at data shown in Figure I.

The concentrations obtained for the three xylene isomers was show to vary along of the week. Xylene concentrations were found at the range of 113.15-714.93 ppm. The average concentration of total xylenes was 169.38 ± 76.15 ppm (mean ± SD), which is below the Threshold Limit Values – Time Weighted Average (TLV-TWA). Significantly higher levels of xylene, were found during at histological staining and slide mounting. The highest occupational exposure occurred at slide mounting stage of the histological processing.

Discussion

The questionnaire showed an excellent Cronbach's alpha or scale reliability coefficient of 0.943 (lower bound of 95% CI of 0.935) indicating good internal consistency of the test instrument. This was probably due to a large number of items in the questionnaire.

Risks tend to be perceived differently for one's own person compared with others (Söjberg L. and Engelberg E., 2009). The degree of risk attributed to increases in the light of an increasing frequency academic year, except for students enrolled in 3rd grade. The majorities of students in 1st grade has no opinion or attach little risk, the 2nd grade low or moderate risk, the 3rd grade considered little risk or no risk and the degree of risk assigned by the students and 4th grade ranges from low risk to much risk. Authors in another studies search similar tendencies for personal and general risks and risk taking for oneself and others(Stone E. R., Yates A. J. and Caruthers A. S., 2002).

Regarding the perception of risk to xylene is largely aware of the risk to which they are subject however the perception of students is higher in the 2nd and 4th grade. Regardless of exposure time there is always risk.

A higher level of Pathological Anatomy Laboratory air contamination was found in the slide mounting area with greater concentrations of xylenes. The values founded were higher than 100 ppm (established in standard NP 1796:2007).

Conclusion

The results demonstrate that the manipulation of organic solvents, which includes xylenes, requires, correct and adequate information, to the students and lab workers in general, about the chemical and health risks, since, roughly 25% of students had no opinion on the risk of exposure to xylene, which may indicate lack in the consciousness of the danger. Students should have more information about dangerousness of those chemical substances. The individual perception of risk is a critical component of the behavior of students.

Implementation of measures to improve indoor air quality in order to reduce occupational exposure, as there are environmental concentrations exceeds the TLV-TWA, mainly in the stages of staining and slides mounting.

Concentrations above 100 ppm may endanger the student's health, mainly assigned to develop their curricular activity for 21 hour in this laboratory, i.e. 24% of the population; Strategies for prevention which includes environmental monitoring and biomonitoring should be periodically.

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Characteristic	N	%	Mean ±SD
Gender			
Male	32	14.7	
Female	185	85.3	
Academic Level			
1st	62	29%	
2nd	49	23%	
3rd	49	23%	
4th	54	25%	
Age			22 ± 2

Table I – Selected Characteristics of the Study Population

	no risk (%)	little risk (%)	moderate risk (%)	much risk (%)
1st Year	14.3	21.4	57.1	7.1
2nd Year	0	7.7	56.4	35.9
3rd Year	10.2	69.4	4.1	16.3
4th Year	1.9	7.4	44.4	46.3
Total	5.1	28.2	35.9	30.8

Table II – Classification of Risk of Exposure to Xylene

	no risk (%)	little risk (%)	moderate risk (%)	much risk (%)
1st Year	3.3	10.0	40.0	46.7
2nd Year	0	0	7.0	93.0
3rd Year	2.1	51.1	14.9	31.9
4th Year	3.8	1.9	28.8	65.4
Total	2.3	16.3	21.5	59.9

Table III – Classification of Risk of Manipulation of Xylene Without Gloves

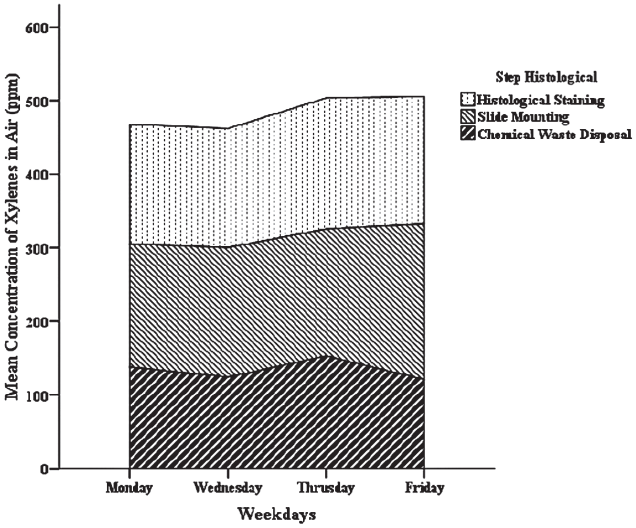

Figure I – Indoor Levels of Xylenes by Weekdays and Histological stage

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REVIEW OF INFORMED CONSENT IN GYNECOLOGICAL AND OBSTETRICAL INTERVENTIONS WITH CURETTAGE: A STUDY OF 20 SPANISH CENTERS

Abstract: Informed consent is a very important document in legal medicine, as well as in the doctor-patient relationship. The informed consent has been widely developed in Spain with Law 41/2002 that regulates Patient Autonomy and Health Documentation and Information related Right and Obligations. The Spanish Society of Obstetrics and Gynecology suggests that many claims in their speciality have their origin in this issue. So, we reviewed this document in 20 Spanish medical centers (public and private) in curettage interventions and various medical societies and public institutions. Protocols for administration and the content of the informed consents checked were acceptable in most of them, except the private centres for voluntary interruption of pregnancy. We conclude that curettage should have a more homogeneous protocol of informed consent between public and private centres; the information should be adapted for each patient; a signed copy of the document should be given to the patient; and informed consent should be provided at least 24 hours before the intervention.

Introduction

Informed consent is a very important document in legal medicine, as well as in the doctor-patient relationship. The informed consent has been widely developed in Spain with Law 41/2002 that regulates Patient Autonomy and Health Documentation and Information related Right and Obligations.

In gynecology and obstetrics, the defects around informed consent are present in more than half of the claims according to the Spanish Society of Gynecology and Obstetrics (SEGO) in their study of Accidents (from 1994 to 2004)[1].

So, it was proposed to review this document in Spanish hospitals (public and private) in curettage interventions because it is one of the most common surgical procedures in this speciality.

[1] Spanish Society of Gynecology and Obstetrics (SEGO). Professional Civil Responsability. Spain (1994-2004). Study developed by Uniteco Profesional.

The curettage can be divided into:

- Vacuum curettage: it could be scheduled or urgent. It is the intervention when there is a delayed abortion or to remove a tumor.
- Diagnostic curettage: it is used in cases where it is necessary to obtain a sample of the endometrium or myometrium.
- Curettage for a voluntary interruption of pregnancy (VIP) or induced abortion: these are performed mostly in specialized private centres. It is also called dilatation and sharp curettage (D&C). This curettage is often accompanied by vacuum aspiration of embryonic material and placenta tissues. In Spain, this is the procedure that it is used in more that 85% of the VIPs performed, usually in the first trimester of pregnancy.

Methodology

We have reviewed the documents of informed consent from 20 medical centres, public and private, throughout Spain. In the study they are included the different types of curettage, the moment when the informed consent is given and, finally, we have studied if the documents follows what is required by Law 41/2002 and SEGO´s recommendations.

The informed consents that we looked through belonged to the following medical centres: Hospital Universitario Gregorio Marañón, Madrid Hospitals Group (three centres), Getafe´s Hospital, Móstoles´ Hospital, Alcorcón´s Hospital, Moncloa Hospital – ASISA, Vistahermosa Clinic – ASISA, San Jose Clinic, Ginetec Clinic, Ginemedex Clinic, EMECE Clinics (two centers), Isadora Clinic, Hospital Universitario Central de Asturias, Cabueñes´ Hospital (Asturias), San Agustin´s Hospital (Asturias), Talavera de la Reina´s Hospital (Toledo) and Hospital Clínico de Granada. Furthermore, we studied the models recommended by the Council of Health of Valencia. Totally there were 30 models from 20 centres.

The items selected to be verified in the informed consents were:

1. Criteria set out in Law 41/2002:

1.1. **Article 2** says that "the consent for any medical procedure must be obtained after the patient receives adequate information." Also, the law establish that the information must be "understandable" and "adequate", and it indicates in which cases the informed consent shall be in writing.

So, we introduced an item that would analyse if the explanation of the procedure was appropriate in manner and form.

1.2. **Article 10** of Law 41/2002 concerning the conditions of informed consent in writing:

"The doctor should provide information to the patients before obtaining their written consent. The information given must include at least:

a) The relevant consequences that for sure the procedure will have.

b) The risks associated with personal or professional circumstances of the patient.

c) The risks of the technique in normal conditions and based on experience and the state of the science or directly related to the type of intervention.
d) Contraindications of the patient to this procedure."

We evaluated if all this criteria were included in the selected consents.

1.3. **Article 8** of the Law: "The patients may freely revoke their consent in writing at any time."

In this case, we analysed if it was possible to revoke the consent or not.

2. Minimum criteria for the SEGO:

From SEGO´s model we took out three items for comparison among the other documents:

It is possible or not to identify the doctor/s who give the information and who make the procedure?

Does the document mention the possibility from which transfusion of blood might be needed?

Does the informed consent include that material obtained from curettage will be sent for histopathologic exam?

Results

The results are summarized in Table 1.

The information provided and the moment when the informed consent was administrated was acceptable in most of the documents studied. On the other hand, we identified some shortcomings in content and form, especially in the explanation of the contraindications, alternatives, possible need for transfusion and information on the histopathological studies. There were three public centres (Hospital Universitario Gregorio Marañón, Móstoles´ and Alcorcón´s hospitals) which included a reference to the authorization for the treatment of personal data according to Spanish Law (Organic Law 15/1999).

1. Criteria established in Law 41/2002:

Related to items from Law 41/2002 and taking as standard the document provided by the SEGO, five centres give further information or more complete. However, most of the documents include in the explanation of the procedure incomprehensible concepts like "ovular remains", or like Isadora Clinic that includes in their list of probable risks the word "etc.", which is not suitable in these cases because they are too general.

None of the models include any reference to sure consequences of the procedure. It should be included in the VIP centers, as the sure consequence of the intervention is the induced abortion. Also, we observed that the four private centres that do the interruptions for pregnancy have lots of shortcomings in the document.

None of the informed consent models include the compulsory section of the contraindications.

2. Items recommended by SEGO:

The model of informed consent proposed by the SEGO does not include two important items identified in Law 41/2002: the contraindications of the curettage and the alternatives to the procedure, including the posibility of not to perform the intervention. These posibility should be present in the case that the curettage be elective.

Moreover, the model proposed by the SEGO is basically followed by all the centres, except the inclusion of the possible need for transfusion, that it is not included in two public hospitals.

Nevertheless, informed consents of the VIP centres do not meet the criteria of the model SEGO in almost any case and they are much more incomplete than this one.

3. Other comparative studies:

3a. Comparison between public and private centres: if we do not include the five VIP centers, we can conclude that all the centres, four private and nine public, provide in their documents an information quite similar in quality and quantity. Anyway, the content of the informed consent from ASISA hospitals was the most complete from all, with the exception of the item of the contraindications, that it is neither included.

3b. Related to the type of curettage: there were no differences between the form and content of documents used in the informed consent of the curettage used to evacuate and the one used in the diagnosis.

3c. We have found great differences in form and content among the four VIP centres and the rest, being the former the most defective.

3d. Moment of application of informed consent: most of consulted centres reported that they provide the document at least 24 hours before the procedure, so that it could be read and studied by the patient. In some centers, the document is explained and given weeks before the intervention. However this information, we were not able to confirm it personally at any centre, nor in public, neither in private centres.

Discussion:

We have studied 30 different models of informed consent at ginecological curettage, 20 from medical centers, public and private, as well as the model propose by the Council of Health of Valencia. We have verified in each model the criteria established by Basic Regulatory Law 41/2002 on Patient Autonomy and Patient Rights and Duties Regarding Clinical Information and Documentation.

It is relevant that curettage, a procedure that, initially, we may think that is seemingly simple has shown enough differences among the documents of informed consent among the centers studied.

On the other side, it has been seen that centres that develop VIP have the documents with more shortcomings, specially in the explanation of the procedure and risks. And, in general, these clinics are not respecting Law 41/2002 and SEGO´s criteria. However, we can not generalize by saying that all informed consent from clinics of VIP have poor documentation because our sample includes only four documents used

in five centres and, in Spain, there are more than 140 public and private institutions accredited for VIP.

Information must be provided verbally and in writing to the patient. It is also important for a good comprehension that the information is adapted to the cultural level of the patient.

From what is referred in each centre, we have also deduced that, with certain exceptions not verified, information is provided in good time. By the way, the Spanish Society of Aesthetic Plastic Surgery and Repair (SECPRE)[2] have developed some recommendation on informed consent. The SECPRE have long experience in claims because of defaults in informed consent and their indications are: "Informed consent documents must be delivered to patients with the greatest possible number of days preceding the procedure. The minimum is 24 hours. Actually, it has been demonstrated that the patient retains less than half 50 percent of which is discussed in the consultation." This is why we consider specially irregular to provide the informed consent in the same day of the intervention. But, as we have already reported, this information could not be confirmed personally at each centre.

The SECPRE also makes the following recommendation: "the consent with patient and doctor´s sign must be filed with the clinical history but, also a signed copy of the informed consent document should be delivered to the patient." As in the previous issue, we were unable to verify this point at our sample but it would be very interesting to check this item on upcoming studies on the subject.

Conclusions:

Curettage procedures should have a more uniform protocol among public and private hospitals and clinics performing VIP. Mainly, all centres must follow the indications on the Law 41/2002, as well as recommendations from the model of the SEGO. As main recommendations we suggest the following:

- Before performing a curettage, the physician must provide the information both verbally and in writing, and ensure a proper understanding of the terms by the patient.
- It is mandatory to give informed consent adapted to the circumstances of each patient before the curettage.
- Informed consent must be provided with the maximum possible time before to the intervention if there is no medical emergency, and, at least, 24 hours before the the curettage.
- The physician should give to the woman a signed copy of the document of the informed consent at the time of signature.
- In the written form, the informed consent should make a reference to data protection, unless the patient had already signed at the centre a document of consent to the processing of personal data.

[2] Spanish Society of Aesthetic Plastic Surgery and Repair. Informed consent. Available online: http://www.secpre.org/documentos%20consentimiento.html. Checked in June 2009.

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Table 1. Results of the comparison of the different items studied in the 30 models of written informed consent from 20 different centers, including SEGO and the Council of Health of Valencia.

	Law 41/2002						SEGO Model		
	Explanation of the proceedings	Patient related risks	Probable risks	Contraindications	Alternatives (including not to perform the surgery)	Revocation	Name of the doctor	Transfusions	Histopathological studies
SEGO Model (V & D)	**Yes**	**Yes**	**Yes**	**No**	**No**	**Yes**	**Yes**	**Yes**	**Yes**
San José Clinic (V & D)	SEGO								
Asturias Hospital Universitario Central (V & D)	SEGO								
Cabueñes` Hospital (Asturias) (V & D)	SEGO								
San Agustín`s Hospital (Asturias) (V & D)	SEGO								
Talavera de la Reina`s Hospital (Toledo) (V & D)	SEGO								
Hospital Clínico de Granada (V & D)	SEGO								
Hospital Universitario Gregorio Marañón (V)	+	+	+	**No**	**Yes**	**Yes**	**Yes**	**No**	**Yes**
Alcorcón`s Hospital (V & D)	+	**No**	=	**No**	**No**	**Yes**	**Yes**	**No**	**Yes**
Getafe`s Hospital (V & D)	+	=	=	**No**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Móstoles`s Hospital (V)	=	+	+	**No**	**No**	**Yes**	**Yes**	**Yes**	**Yes**
Madrid Hospitals Group (3 centers) (V & D)	=	=	=	**No**	**No**	**Yes**	**Yes**	**Yes**	**Yes**
Moncloa Clinic – ASISA (V)	+	+	+	**No**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Vistahermosa Clinic – ASISA (V)	+	+	+	**No**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Ginetec, Ginemédex and EMECE (2 centers) Clinics (VIP)	-	=	=	**No**	**No**	**No**	**Yes**	**No**	**No**
Isadora Clinic (VIP)	**No**	**No**	-	**No**	**No**	**No**	**No**	**Yes**	**No**
Council of Health of Valencia (V & D)	=	=	=	**No**	**Yes**	**Yes**	**Yes**	**No**	**No**

Abbreviations: SEGO: Spanish Society of Gynecology and Obstetrics; V: vacuum curettage / aspiration; D: diagnostic curettage; VIP: voluntary interruption of pregnancy; (+): more information; (-): less information; = similar quantity of information.

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FATAL ELECTROCUTION
A 10-YEAR RETROSPECTIVE STUDY IN THE LISBON AREA[1]

Abstract: Electric current can determinate a fatal outcome – electrocution – which is a relatively unique death. The aim of our 10-year retrospective study is to characterize the trend of deaths by electrocution occurred within the Lisbon Area. Database of the Forensic Pathology Department, between 1999 and 2008, was scanned for fatal electrocution, and several medico-legal variables were analysed. Of the 25 victims, only one was female, 60% were between 18 and 34 years-old and 64% had professions related to construction and electricity industries. Death occurred on place in 76%, the passage of current was direct in 76%, related to low voltage in 52%, and in 68% was the result of a labour accident. Electrical burns were found in 84% and thermal burns in about 50%. Signs of passage of electric current were identified in about 50%. Ethanol and cocaine were present in 2 separated cases. Death was caused directly by the passage of electric current in 84%, of which 24% were associated with blunt force trauma or thermal burns. Results are quite similar to those of other studies, and underscore the importance of a better understanding of the phenomenon in order to prevent this kind of fatal incidents.

Keywords: Fatal electrocution; electric burns; labour accidents; forensic pathology.

Introduction

Electric current is a physical agent that acting within the body can determine a fatal outcome. Nevertheless, despite the common use of electricity, electrocution also referred to as electrical injury, is relatively rare [1]. Most cases result from accidents, suicides are less usual and homicides extremely rare. Fatalities caused by electrocution depend on many factors such as individual's characteristics, environmental factors and electric current features, especially the source of electricity [2]. In most fatal electrical accidents, death is caused by the passage of the electric current itself and should be suspected whenever an individual falls while near a charged source [1]. Not uncommonly the electrotrauma becomes associated with mechanical injury. Usually

[1] Preliminary results presented at the XXI Congress of the International Academy of Legal Medicine, May 2009, Lisbon – Portugal.

these type of deaths often lack characteristic morphologic findings and specific results are absent in autopsies, which can cause considerable problems to the correct diagnosis of electrocution [1, 3-5].

Therefore the objectives of our retrospective study is to characterize the victims of fatal electrocution, investigate the presence of any trend of deaths, to better understand this phenomenon and to ascertain characteristics features aiming to a better identification and prevention of these situations.

Materials and methods

Autopsy cases of fatal electrocution were identified after scanning the database of the Forensic Pathology Department of the South Branch of the National Institute of Legal Medicine of Portugal (NILMP) concerning the Lisbon Area during a 10-year period, between January 1, 1999, and December 31, 2008.

In order to understand this type of fatalities, deaths due to or related to electrocution were carefully analysed regarding several variables, such as social-demographic ones, circumstances of death, available information and results of medico-legal autopsies.

In this study, regarding alternating current, low voltage current is considered between 50-1000 V, and high voltage is >1000 V, while for direct current, low voltage current is between 120-1500 V, and high voltage is >1500 V.

Results

During the 10-year period considered, a total of 14.663 autopsies were performed at the Forensic Pathology Department of the South Branch of the NILMP of which 25 cases were related to fatal electrocution.

Of the 25 victims only one was female. Concerning age, victims had a medium age of 29 years-old (range 8 months to 56 years), with 15 of the cases (60%) between 18 and 34 years-old (Figure 1). Two cases were children: 8 months and 2 years-old. According to occupation, 16 victims (64%) had professions related to construction and electricity industries (Figure 2).

Concerning the place of death, 19 cases (76%) deaths occurred on place after contact with electric current: 15 at the work place, 3 at home and 1 on train tracks. One case was related to a baby still found in contact with the electric source. In 2 cases (8%), death occurred during the transport to the hospital, and in 4 cases (16%) it occurred during the hospital admittance or after a hospitalization period of more than 13 days. In relation to time of death, 60% of fatal electrocution was registered during daytime, mainly between noon and 6 pm, at the work place.

The passage of current was direct in 19 cases (76%) (Figure 3). Low voltage was present in 13 cases (52%), high voltage in 10 cases (40%), and in the remaining 2 cases (8%) there was no information about the type of electric current (Table 1).

External examination of the body revealed electrical burns in 21 cases (84%), being the hands the anatomical region most affected. Thermal burns were found in 13 cases (52%), mainly due to Joule effect or to the ignition of garments, most of

them 2nd and 3rd degree burns (Figure 4). Seven of all cases with thermal burns were related to high voltage, with a more heterogeneous distribution of 1st, 2nd, 3rd and 4th degree thermal burns than in the cases related with low voltage (Figure 5). External blunt force trauma was found in 13 cases (53%), of which 6 had concurrent internal blunt force trauma, and in 3 cases blunt force trauma was considered to be the direct cause of death.

In the internal examination of the body, excluding non specific findings, only 12 cases (48%) had signs of electric current flow, mainly focal diaphragmatic haemorrhages (Figure 6). In 20 cases (80%), petechial haemorrhages were found. Organ congestion was present in 22 of the cases (88%), including 16 cases of generalized congestion (Figure 7). Organ oedema was present in 17 cases (68%), including 11 cases of oedema of the lungs (Figure 8). Most of the cases with signs of internal blunt force trauma had traumatic lesions on the head.

Toxicology exams were positive in two cases: 1,97g/l of ethanol in a labour accident, and 2650 ng/ml of cocaine metabolites in an otherwise not specified accident – a youngster painting graffiti at a subway station.

In 21 deaths (84%), the direct cause of death was due to the passage of electric current, of which 6 (24%) were associated to other causes of death: 3 (4%) to blunt force trauma; 2 (8%) to thermal burns or 1 (4%) to acute cocaine intoxication. In 4 cases (16%) the death resulted from infectious complications consecutive to electrocution.

According to the manner of death, 17 cases (68%) were the result of a labour accident. The remainder 8 cases (32%) were related to 4 household accidents, 3 accidents not otherwise specified, and one case of undetermined manner of death (Figure 9). The household accidents included: two children, a woman in the bathtub, and a man at home.

Discussion and conclusions

Overall, the findings of this study are in accordance with other similar studies. In fact the review of the literature shows a male predominance (81-91%), mostly due to accidents (near 70%), including 30% of labour accidents. Most deaths are due to low voltage current (65-83%). Almost 81-83% present characteristic electrical burns, mostly located at the fingers and feet. Petechial haemorrhages are present in 12-74% [3,5].

In the present study, considering all the 25 victims of fatal electrocution, 68% of deaths were due to labour accidents, all males, mostly with ages between 18 to 34 years-old and mainly related to building construction and electricity industries professions. 16% were household accidents including: two children, a woman in the bathtub and a man at home. Lack of any information, one case was considered as undetermined manner of death, probably a labour accident since there was cement on the body's surface. In 76%, death occurred in place, the passage of current was direct in 76% and related to low voltage in 52 %. External examination of the body revealed electrical burns in 84%, and in about 50% of the cases, there were thermal burns. Internal examination revealed signs of the passage of electric current in 48% of the cases, the majority of which were focal diaphragmatic haemorrhages. Toxicology findings revealed the presence of ethanol and cocaine in 2 different cases. Death was

caused directly by the passage of electric current in 84%, of which 24% were associated with blunt force trauma or thermal burns. In the other 16%, deaths were due to post electrocution infectious complications.

The current results point out the importance of a more extensive research concerning this cause of death, namely the identification of the circumstances of electrical injuries and underlying factors, in order to take proper preventive measures, especially in labour-accidents field.

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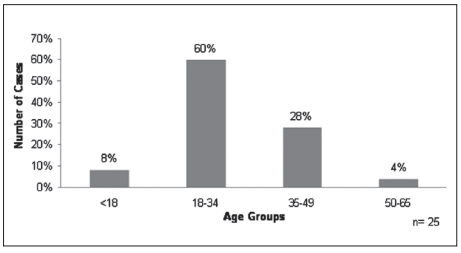

Figure 1 – Distribution by age groups

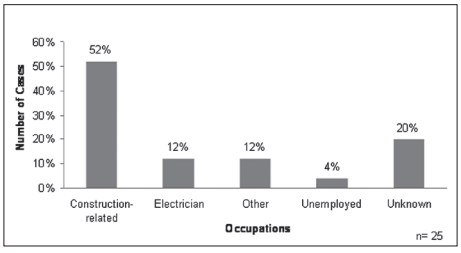

Figure 2 – Distribution by occupation

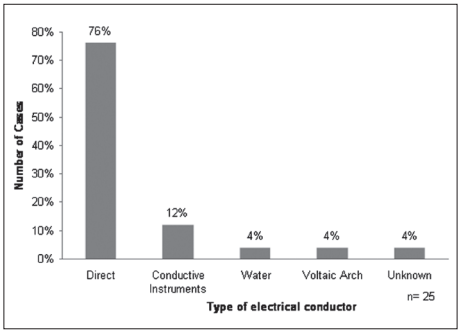

Figure 3 – Distribution by electrical conductor

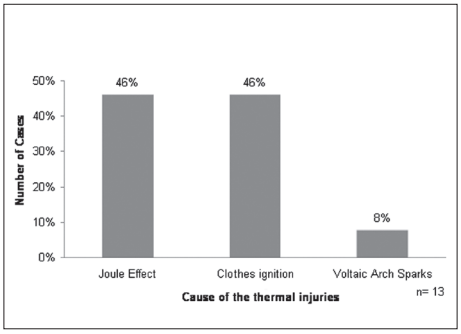

Figure 4 – Distribution by the cause of the thermal burns

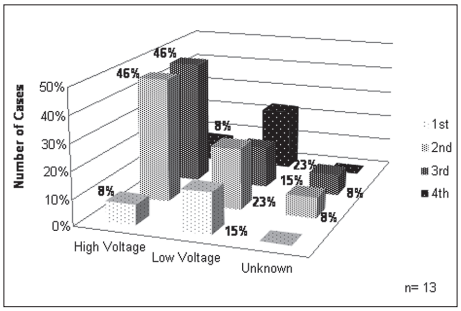

Figure 5 – Distribution of the thermal burns by the voltage of the electrical current

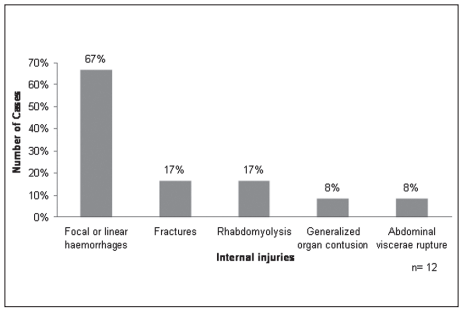

Figure 6 – Distribution of internal signs of current passage

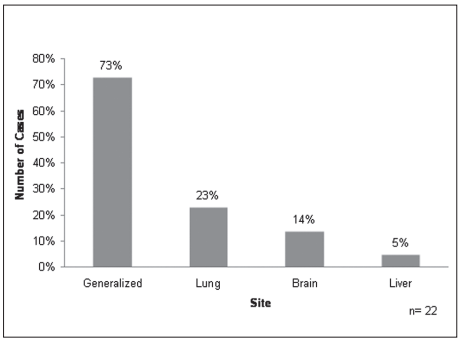

Figure 7 – Frequency of congestion

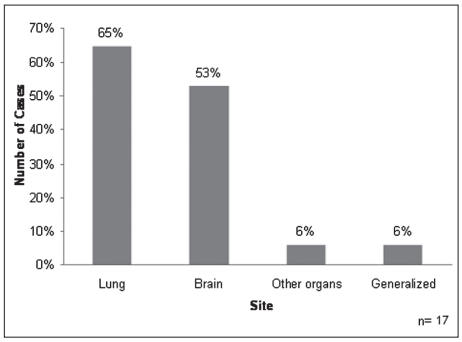

Figure 8 – Frequency of oedema

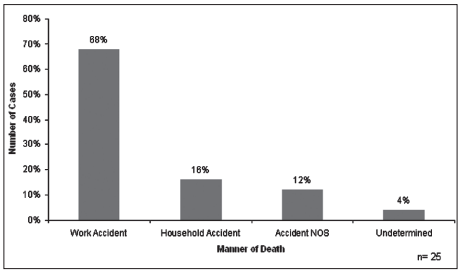

Figure 9 – Distribution by manner of death

Manner of death		Voltage		
		Low	High	Unknown
Accidents (total)		13	10	1
	Work accident	7	9	1
	Household accident	4	4	-
	Accident NOS	2	1	-
Undetermined		-	-	1
Total		13	10	2

Table 1 – Relation between manner of death and voltage of electric current

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DIAGNOSING DEATH BY DROWNING THROUGH THE ANALYSIS OF BLOOD MARKERS BY INDUCTIVELY COUPLED PLASMA – MASS SPECTROMETRY

Abstract: Investigation of bodies recovered out of water is a complex but frequent medico-legal task. The key problem is if the victim died due to drowning or by the means of other cause and placed in water. At the moment, the diagnosis of drowning is based on some unspecific findings during autopsy (lung distension and the presence of froth in upper airways and lungs) and the results of laboratory tests. Our project aimed to evaluate the usefulness of the determination by ICP-MS of trace elements (TE) in blood of the cardiac cavities of corpses found in aquatic environment as a tool to increase the certainty of the diagnostic of death by drowning. Blood samples were collected from 18 cadavers found in water, from 2006-2008, in Oporto area. The advantage of ICP-MS compared to other instrumental analytical techniques is clear and it proved to be useful. It allowed us to perform a multielemental analysis of blood, and the results highlight the importance of this kind of approach, compared to previous studies where we are dependent on the results of a single TE.
Keywords: Drowning; ICP-MS; Forensic pathology.

Introduction and objectives

Post-mortem diagnosis of drowning is one of the most difficult in forensic pathology. The majority of the diagnoses are based on some unspecific findings during autopsy (lung distension and the presence of froth in upper airways and lungs) and the results of laboratory tests [1]. Despite the many diagnostic methods used, the ideal diagnostic test as definitive proof for drowning still needs to be established, and more research is necessary. One of the diagnostic methods used is the quantification of drowning markers, such as the trace elements (TE) iron and strontium in blood [2-4]. The use of these "markers" is based on the different blood concentration between left and right heart cavities according to the type of water medium [4]. If the drowning takes place in a hypotonic medium compared to human blood (freshwater drowning) animal experimentation shows that TE blood concentration decreases in the left heart cavities as a consequence of hemodilution. On the other hand, TE such as Sr, due to its water concentration (and practically inexistence in human blood) will be higher in left ventricle (compared to the right one). Even though, the reviewed bibliographic

data showed that strontium diagnostic value is well established in seawater drowning, but not in freshwater drowning because of the lower Sr concentration in this kind of medium [5]. The authors wish to introduce an instrumental method with recent forensic application in detection and quantification of gunshot residues [6]: Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), which easily allow us to perform a multielemental analysis. So, the aims of this study are to investigate the application of other TE and their usefulness in drowning diagnosis and also evaluate Sr diagnostic value in freshwater drowning.

Materials and Methods

We collected blood samples from 18 cadavers found in water, selected from medico-legal autopsies performed in the North Branch of the National Institute of Legal Medicine (Oporto, Portugal) over the period 2006-2008. The criteria used for inclusion was based on the following aspects: the bodies had to be found in water, the absence of mortal trauma and the presence of findings compatible with drowning during the autopsy. Some cases where excluded from the study population such as those who were subjected to resuscitation maneuvers or found in advanced state of putrefaction.

The samples were collected during the autopsy, from ventricular heart cavities (after opening the pericardial sac by means of standard techniques) using disposable needle and syringe for each cavity.

The samples were then placed in propylene tubes (previously washed and decontaminated with nitric acid and deionized water) and stored at -70º. Subsequently they were processed for ICP-MS analysis. About 1 g of blood was digested with 1 ml of H2O2 30% and 2.5 ml of HNO3 65% in a microwave oven (Milestone MLS 1200 Mega). The digestion solution was diluted to 25 ml with ultra-pure water (Milli-Q system, Millipore) and analyzed using a quadrupole ICP mass spectrometer (VG Elemental PlasmaQuad 3. The quantification of TE Li, Be, Al, V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Ag, Cd, Sb, Cs, Ba and Pb (Figure 1) was performed (Si, Ti, Br and I were also measured in the "semi-quantitative" mode). Results correspond to the content in whole blood and were expressed in µg/kg.

The mean difference of the TE concentration between both heart cavities was statistically compared. The T test for paired samples was used to evaluate the difference between the two groups. A probability level of $P \leq 0.05$ was considered statistically significant.

Results and discussion

From the tested TE, some were not systematically higher (or lower) in left cavities blood versus right cavities blood, except for Sr, Li, Cu, Mn and Cd (Figure 2 & Table 1). Accordingly, Sr, which is typically present in water but not in blood, was found in concentrations higher in left cavities than in right cavities in 16/18 cases. So, even in the case of drowning in freshwater, where Sr is present at a lower concentration compared to seawater [5], our study points to some value of this TE, which can be complemented by another TE that behaved very similarly: Li in 15/18 cases.

Other TE behaved the opposite way and presented a decreased blood concentration in the left cavities (compared to the right ones) as a result of the hemodilution due to the hypotonic freshwater entrance. One of the TE in these circumstances was Cu that presented a blood concentration in left cavities much lower than in right cavities in all the cases, with a mean difference of 411,22±434,34 (P=0,01). Additionally we were able to find other two TE that behaved like Cu: Mn in 15/18 cases and Cd in 17/18 cases. In the case of Mn the mean difference between both heart cavities was 37,35±38,42 (P=0,01).

Conclusions

The advantage of ICP-MS compared to other instrumental analytical techniques is clear and it proved to be useful [7]. It allowed us to perform a multielemental analysis of blood, and the results highlight the importance of this kind of approach, compared to previous studies where we are dependent on the results of a single TE [2-3]. On these cases, whenever it isn't possible to validate the results, the conclusions also get compromised, in opposition to multielemental research where we can present reliable conclusions, even when one TE result is not significant. We were able to find "new" TE which showed a typical behavior during drowning, with Cu given the most promising results. The information obtained from these additional TE can complement the data obtained from classical ones (Sr). In addition we obtained consistent info regarding Sr potential in freshwater drowning. Even in this kind of medium, with the use of a highly sensitive technique such as ICP-MS [7] a higher concentration in left cavities could be observed in most cases, compared to previous studies whose sensibility didn't allowed to obtain valid results [5].

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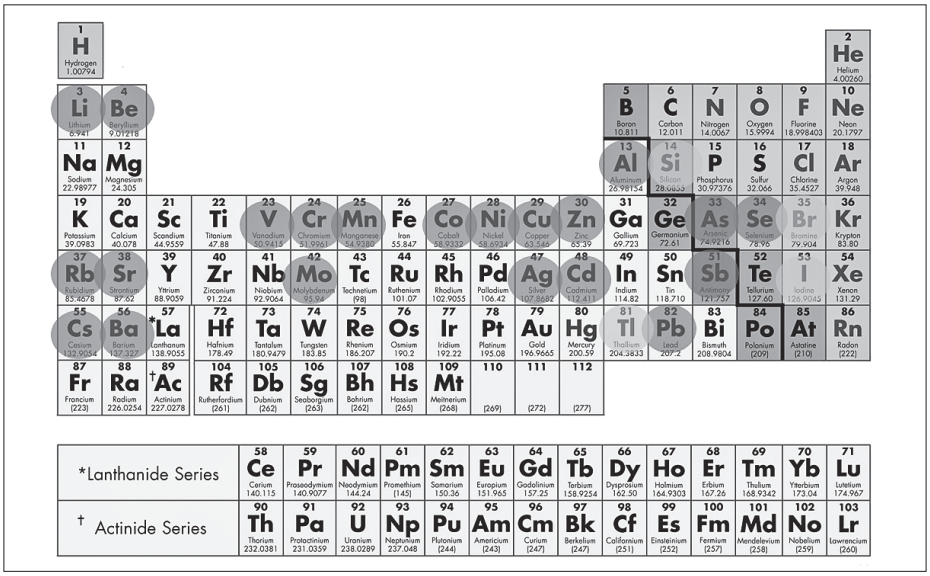

Figure 1 – Periodic table showing quantified TE

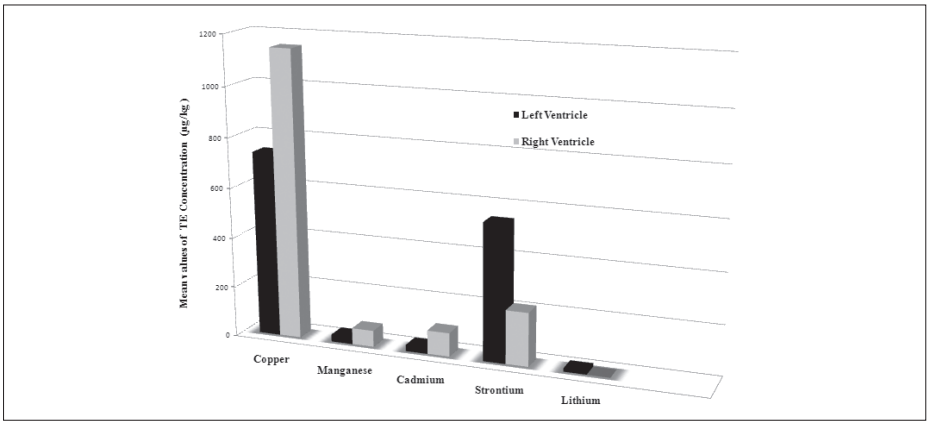

Figure 2 – Graphic of mean TE concentrations in left ventricle vs. right ventricle

TE	LV mean	RV mean
Cu	737,33 ± 225,15	1148,56 ± 448,01
Cd	25,53 ± 33,87	95,05 ± 134,08
Mn	28,61 ± 11,12	65,95 ± 38,03
Sr	545,38 ± 942,17	213,63 ± 290,02
Li	13,12 ± 21,08	5,5 ± 7,3

Table 1 – Mean TE concentrations values in left ventricle vs. right ventricle

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OCCUPATIONAL FATALITIES CHARACTERIZATION (2006-2008)

Introduction

Fatal work accidents, defined as sudden and unpredictable events, suffered by workers on the job site during working hours and which pertaining death occurs up to one year after the incident, are presently primary concerns of every occupational safety promotion policy. This issue involves the daily activities of the Government, the companies themselves, the workers and social partners. Being that the Portuguese legislation predicts a forensic autopsy in case of an immediate death caused by a work accident (Law n. 45/2004, 19th August) and despite having noticed a significant decrease in the rate of such accidents during the last few years (ACT – "Autoridade para as Condições de Trabalho" – Statistics of 31/07/2009), it is still fundamental to promote the awareness of workers, employers and such others, on the importance of occupational safety.

Material and Methods

The authors conducted a retrospective study of 979 autopsies performed, from January 2006 until December 2008, at the Forensic Pathology Department of the Centre Branch of the National Institute of Legal Medicine, considering all cases of occupational fatalities' victims. Data were analysed according to sex, age, nationality, professional group, circumstances, cause of death, post-traumatic survival time and the results of toxicological ancillary investigation. The incidence rate during this period was compared with a similar study conducted in this same Department in the years 2001-2005.

Results

In the aforementioned period, 45 cases of occupational fatalities were found (4,6% of the total autopsies), 44 (98%) of which concerned male individuals and 1 case (2%) a female worker. There was a peak of incidence in the range of 50 to 60

years old (33%) and the average age was determined as 43,2 years (DP+-14,6). The greater number of victims was Portuguese (90%). As for the professional group, the most accident-prone were those of manufacturing industry (28%) and construction industry (20%) sectors. The most common event was fall from a structure (35%) and the majority of deaths was due to traumatic injuries (75%). Toxicological ancillary analyses disclosed ethylic alcohol in blood samples from 8 cases (18%), 6 of which were above 0,5mg/dl (13%); such analysis were not carried through in 40% of the cases in which the victims remained hospitalized more than one day. In 17 cases (38%) the workers died immediately after the event.

Discussion and Conclusions

Considering the achieved results and having compared them to the national statistics of the ACT, it appears that the measures lately adopted by the current and former government entities as a means of preventing occupational hazards and risks – making of the "White Book of Companies Prevention Services" (1999), approval of the Statutes of IGT – "Inspecção Geral do Trabalho" (Law n. 102/2000, 2nd June), control of the implementation of labor regulations, opposing undeclared work, strengthening of the linkage and performance of different inspection services, promotion of systematic campaigns of specialized training, aiming to minimize and avoid work accidents – are having the anticipated results.

The awareness of employers and workers, resulting from knowledge, concern and the advantages of occupational safety, may have contributed to this decrease.

It is hoped that the recent revision of the Labor Code (Law n. 105/2009, 14th September) with the publishing of the new procedure regime, which is to be applied to labor-related offences (Law n. 107/2009, 14th September), will lead to a new decrease in deaths related to work accidents within the next few years, so as to bring the national rates closer to the European standards – according to the "National Strategy for Occupational Health and Safety – ACT, April 2008".

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LARGE-SCALE MUTATION SCREENING IN SUDDEN CARDIAC DEATH (SCD)

Abstract: One of the most common causes of death in developed countries is sudden cardiac death (SCD). Structural and arrhythmogenic diseases are the main abnormalities found in SCD cases, especially in the young, and both are due to genetic heart disorders such as hypertrophic cardiomyopathy, Long QT syndrome or Brugada Syndrome. In recent years, significant advances have been made in understanding the genetic basis of SCD. We have developed a high-throughput strategy using a semi-automated MALDI-TOF mass spectrometry system for detecting the most frequent mutations in different syndromes that can result in SCD.

Introduction

Most of the forensic pathologist workload deals with natural deaths and more than 50% of cases are sudden cardiac deaths (SCD). In about 1 of every 20 cases of SCD, the classical autopsy cannot establish the cause of death, even after the heart has been examined by an expert cardiac pathologist. This is then called Sudden Arrhythmic Death Syndrome, and most have a genetic component. The conditions responsible for SCD can be classified in two groups: a) structural anomalies, being the most frequent hypertrophic cardiomyopathy (HCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC), and b) non structural anomalies or channelopaties, such as long QT syndrome (LQTS) and Brugada syndrome (1,2).

In people over 35, the cause of death is usually due to coronary heart disease. When it comes to younger people and children, cardiomyopathies, congenital heart disease and conduction disorders take the lead (3).

Up to now many genes have been associated with the different syndromes responsible for SCD, making difficult the genetic diagnosis by classical technologies such as sequencing. Based on this knowledge, we have developed a high-throughput strategy using a semi-automated MALDI-TOF mass spectrometry for detecting the most frequent mutations in different syndromes. More than 600 mutations of HCM (4) genes and almost 400 mutations of LQTS genes have been analysed in sudden cardiac death cases and relatives. HCM is defined as a clinically heterogeneous but relatively common autosomal dominant genetic disease with a prevalence of 1:500

in a general population of healthy young adults (5). Congenital LQTS comprises a distinct group of cardiac channelopathies characterized by delayed repolarization of the myocardium, QT interval prolongation, an specific form of polymorphic ventricular tachycardia called Torsade des Pointes can also be found and the risk for syncope, seizures, and sudden cardiac death is higher in the setting of a structurally normal heart (6). It is a genetically heterogeneous disease affecting 1 in 5000 persons, with a natural history ranging from sudden death in infancy to asymptomatic longevity (7).

Efficiency and accuracy of the system have been evaluated and preliminary results are presented.

Materials and methods

Samples:

DNA was extracted from embedded paraffin tissues or from peripheral blood after autopsy in sudden cardiac death cases in individuals of 1-40 years old.

Sample inclusion criteria was:

A) Samples obtained after autopsy:
   - Negative autopsies
   - HCM evidences found after autopsy.
   - Autopsy cases with family medical history of cardiac events such as sudden cardiac death in relatives.

B) Patients after sudden cardiac event recuperation
   - The study includes two large families with family medical history of sudden cardiac death.

Mutation databases:

Familiar Hypertrophic Cardiomyopathy Mutation Database http://www.angis.org.au/Databases/Heart/heartbreak.html

Cardiogenomics Mutation Database http://cardiogenomics.med.harvard.edu/home

The Human Gene Mutation Database (Institute of Medical Genetics, Cardiff) http://www.hgmd.org/

Mutation detection method:

We have developed a rapid and efficient mutation detection system based on semi-automated MALDI-TOF mass spectrometry using the Sequenom MassArray system. This typing assay uses iPLEX GOLD assay reactions that ends after a Single Base Extension (SBE). Extension primer hybridise one base before the mutagenic locus. Sample genotyping is performed because of the addition of one ddNTP to the extension primer which is complementary to the locus we are studying. The extension primer is now one base longer. The mass difference after the iPLEX reaction is detected by means of matrix assisted laser desorption ionization time-of-flight mass spectrometry. Samples are automatically genotyped from each mass spectrum produced. Multiple sites can be typed simultaneously by multiplexing PCR and the extension reaction (8).

Two different strategies were designed to analyze on one hand 688 HCM genetic variants in 102 samples and 432 LQTS genetic variants in 75 samples.

HCM Strategy: HCM strategy include the detection of mutations described in literature not only in sarcomeric genes but also in other genes previously described as implicated in HCM. Number of genetic variants in each gene is shown in table 1.

Genetic variants added in our HCM mutation detection chip, including causal mutations and some polymorphic variants are distributed into 44 plexes as shown in table 2.

LQTS Strategy: Our LQTS screening strategy include mutations in the three most prevalent genes implicated in the disease: KCNQ1 (implicated in LQT1 syndrome), KCNH2 (implicated in LQT2 syndrome) and SCN5A (implicated not only in LQT3 syndrome but also in Brugada syndrome). Genetic variants analyzed in each gene also include causal mutations and some polymorphisms. Distribution of the mutation into 38 plexes is shown in tables 3, 4 and 5.

Results

CM Strategy results: Genotyping success rate was 84% in DNA extracted from peripheral blood and 74% in DNA extracted from paraffin embedded tissues. A total of 19 mutated samples were found out of 102 total analysed samples. Most of the variants were found in myosin binding protein C (MYBPC3) where a total of 9 cases with 4 different genetic variants were found. A total of 9 more cases were mutated in Troponin T (TNNT2),1 more case was found mutated for myosin heavy chain gene (MYH7) and 1 sample was found as positive mutation carrier Troponin I gene (TNNI3).

Results can be found in table 6. All the genetic variants detected in the platform were confirmed by direct sequencing. Variation R326Q detected in MYBPC3 gene as an example of a mutation found in Sequenom MassArrayä system is shown in figure 1 and the confirmation of this mutation by direct sequencing can be seen in figure 2.

LQTS Strategy results: Genotyping success rate was 84% in DNA extracted from peripheral blood. In this case, a total of 75 samples were analysed, 46 of them analysed for KCNQ1, KCNH2 and SCN5A and 29 samples specifically genotyped for SCN5A study.

2 samples up to 46 were found as positive mutation carriers with 2 different genetic variants in KCNQ1 gene and 1 case up to 29 was a mutation carrier in SCN5A gene. The results can be seen in table 7. All the genetic variants detected in the platform were confirmed by direct sequencing.

Discussion

Sequenom MassArray system efficiency for our mutation screening strategy is demonstrated since the genotyping success rate value is up to 80% in both strategies: MCH and LQTS. This value is lower when we analyze DNA extracted from paraffin embedded after formol fixation in which the quality of the DNA is worst. In spite of

the low DNA quality in these cases we were able to get good results in this degraded samples as the length of the analysed sequences in Sequenom MassArray system is short.

The accuracy is also proved as all the mutation detected was confirmed by means of direct sequencing in all cases.

This fast mutation screening may help to clarify the cause of the death after a non conclusive autopsy. Relatives of a deceased person can benefit from the screening by identifying mutation carriers in the family so our mutation detection screening has a useful application not only in the forensic sphere but also in clinical field.

Conclusions

Sequenom MassArray system allows us to get really fast analysis results since the genotyping analysis can be developed in 48 hours. The flexibility of the platform make possible to add new mutations as they are being described in the literature.

Low quality DNA samples can be easily genotyped even if they have been preserved for a long time or if they have been paraffin embedded after formol fixation

In addition, Sequenom MassArray genotyping is more cost effective than performing the analysis by means of traditional sequencing and the reliability of the platform is absolute since all genetic variants found were confirmed by direct sequencing.

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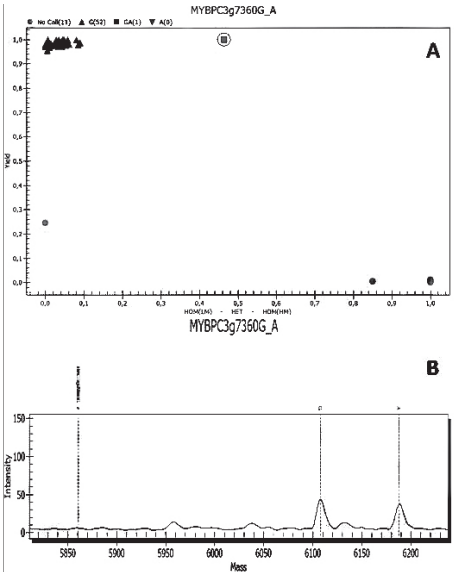

Figure – 1
R326Q mutation detected in MYBPC3 gene by means of Sequenom MassArray System: A) Cluster plot aspect B)spectrum aspect of the heterozygous sample

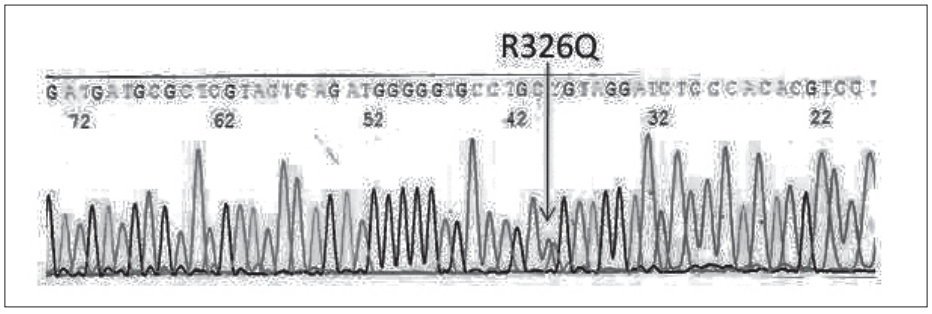

Figure 2 – Confirmation of R326Q detected in MYBPC3gene by direct sequencing.

Sarcomeric genes	Genetic variants	Other genes	Genetic variants
MYH7	280	PRKAG2	4
MYBPC3	249	TCAP	3
MYL2	14	GLA	1
MYL3	11	MYO6	1
TNNT2	45	MYLK2	1
TNNI3	35		
TNNC1	4		
TPM1	14		
ACTC	9		
TTN	10		
MYH6	6		

Table 1 – Number of genetic variants analyzed in each gene in HCM strategy.

Number of plexes	Number of genetic variants
1 PLEX	23
2 PLEXES	22
3 PLEXES	21
4 PLEXES	20
3 PLEXES	19
4 PLEXES	18
4 PLEXES	17
7 PLEXES	16
4 PLEXES	15
4 PLEX	13
1 PLEXES	12
2 PLEX	11
1 PLEXES	8
1 PLEX	7
1 PLEX	5
1 PLEX	4
1 PLEX	3

Table 2 – Distribution of the genetic variants into plexes in HCM strategy.

Number of plexes	Number of genetic variants
1 PLEXES	19
1 PLEX	17
1 PLEX	10
2 PLEX	8
2 PLEX	7
5 PLEX	6
1 PLEX	4

Table 3 – Distribution of the genetic variants into plexes in KCNQ1 strategy

Number of plexes	Number of genetic variants
1 PLEXES	23
1 PLEX	22
1 PLEX	19
1 PLEX	16
4 PLEX	5
7 PLEX	4

Table 4 – Distribution of the genetic variants into plexes in KCNH2 strategy

Number of plexes	Number of genetic variants
1 PLEXES	19
2 PLEX	18
2 PLEX	17
1 PLEX	15
1 PLEX	11
1 PLEX	7
1 PLEX	6

Table 5 – Distribution of the genetic variants into plexes in SCN5A strategy

Gene	Genetic variant	rs (dbSNP)	Number of cases
MYBPC3	R326Q	rs34580776	2
MYBPC3	K814del		1
MYBPC3	A216T		1
MYBPC3	Q689H		5
TNNT2	K247R		9
MYH7	M982T		1

Table 6. Results found in HCM strategy

Gene	Genetic variant	Number of cases
KCNQ1	G325R	1
KCNQ1	R366W	1
SCN5A	K1512W	1

Table 7 – Results found in LQTS strategy

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CLINICAL FORENSIC MEDICINE

AND DOMESTIC VIOLENCE

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INCEST: MEDICAL-LEGAL PERSPECTIVES

Abstract: Incest is a well-known phenomenon, defined as a sexual relationship between close blood relatives, usually between father-daughter, uncle-nephew and also brother-sister, or, in its broader sense, between a child and a stepparent or stepsibling.
Incest is a taboo in many countries; however incest and sexual aggression toward minors are not classified as a criminal behaviour in all part of the world.
Our study has the purpose to examine the legislator's attitude towards this social phenomenon in expansion. Clinical Forensic approach to this phenomenon in literature is an essential contributory to explain the physical and psychological consequences; but it is yet certainly possible to ameliorate legal-medical procedures of investigation. We report the laws in force in different countries so to appraise the exact connotation of this crime in our country.
The aim of this research is to take widest consciousness of the diffusion of this phenomenon in the health professionals and the difference of cultural approach in legal perspectives.

Introduction

Our study starts from the consideration that often the victim of incest becomes sexual abuser. The turn of the potential victim in executioner was not sufficient to urge the scientific community to the analysis and to the definition of treatment in favor of abusers. The therapeutic intervention on abusers is not practiced, perhaps, because of the social disapproval that this crime creates in each of us and which ultimately also affect the look of science. Our research, similarly to the Swiss experience, is going to organize efficient multi-professional strategies for early intervention that would build a model of effective treatment to stop the "cycle of abuse". Our project wants to provide, over time, an active contribution to the many professionals who deal with these subjects.

To achieve this aim we should also evaluate the different design of this crime in different countries of the world, in order to structure a protocol of care that is acceptable abroad. In fact, incest is a taboo in many countries; however incest and sexual aggression toward minors are not classified as a criminal behaviour in all part of the world.

Materials and Methods

The adoption of standardized procedures, shared by the various health professionals involved, can simplify the management of incest victim, pointing to further interdisciplinary integrated approach to the victims. It can also improve the public debate inherent how politicians and leaders of multidisciplinary public centres should prioritise these endeavours.

But we must also consider that with the more and more consistent melting pot of populations, people with different cultures are in contact; this fact can lead to the realization of incest, according to the conception of the host Country, that are not so intended by people who committes them. Seems clear that in implementing the project we realized we must take into account the different environmental, cultural and psychological substrates as the criminal event was realized.

Results

From investigations carried out in Italy we have seen that in 90% of cases the abuse takes place in the family and the rapist is a certain frequency with the same father or stepfather.

In Italy there are almost 3000 cases of incest the year, or 6 per 100,000 people.

Incest usually occurs within families with low socio-economic status.

Studies showed a clear dominance of the father-daughter relationship, or the increasing involvement of the man who within the family plays in fact a fatherly role, although not linked to the victim by a relationship of consanguinity (paraincest); a few were, however, cases of incest between siblings or between parent and child.

The authors of incest usually have the following characteristics:

- age between 40 and 50 years;
- violent personality and behavior, often with previous criminal convictions or being alcohol abusers or with psychiatric disorders;
- extremely low level of education;
- lack of stable employment.

The victims of incest, instead, present in most cases the following profile:

- age between 11 and 15 years;
- 1/5 of the sample is represented by individuals with serious psychological and intellectual problems;
- for children under 10 years old, the acts of lust outweigh; for teenagers, instead, rape is more frequent (study conducted by the Maternal Child Services at the Health Authority of the City of Genoa).

Various studies conducted, showed also a tendency to cyclicality of the crime of incest in successive generations of one family: the abused becomes in turn abuser ("cycle of abuse").

The incestuous relationship may cause to minors severe mental disorders and especially mental development, alteration in the development of character and serious sensibility disorders.

The most frequently disorders described in literature are:

- psychosomatic disorders and neurotic reactions;
- depression and attempted suicides;
- attempts to escape, and wandering;
- psycho-sexual disorders such as frigidity, homosexuality and promiscuity;
- feelings of guilt.

Discussion

Our study has, firstly, the purpose to examine the legislator's attitude towards this social phenomenon that is in expansion or, sometimes, becomes a spectacular media phenomenon, as recent judicial events in Austria. Clinical Forensic approach to this phenomenon in literature is an essential contributory to explain the physical and psychological consequences; but it is yet certainly possible to ameliorate legal-medical procedures of investigation. We report now the rules in different countries so to appraise the exact connotation of this crime in our country.

The incest, by definition, is not the simple performance of sexual acts of any kind, but it is consumed only with the completion of sexual intercourse. However, there is also who considers sufficient acts of sexual nature, even different than physical connection from the persons specified, so that resulting public scandal.

It is complex the predictability of competition between sexual violence (art. 609 bis) and incest (art. 564 cp). To examine whether sexual violence contributes with incest it is necessary to reintroduce the distinction between conjunction and carnal sexual acts other than this, in spite of the "unification" between the figures for rape and acts of violent lust provided for the new law. This is perhaps a lack of legislative attention to issues of sexual violence or an inability to coordinate between the rules of the Criminal Code; the competition between the crimes in question can not, in fact, matter in court by a differentiation which is considered culturally and legally separate.

The psychological element of the offense is the "general intent", then there must be both aware of the existence of the bond between the authors of the fact (it's just a bond of filiation illegitimate as long known to the authors) and the knowledge and desire to have sexual intercourse with a person referred to specifically in art. 564 cp.

The Italian Penal Code establishes (ex art. 564) the imprisonment for one to five years against everyone who commits incest with a descendant or an ancestor, or with a close relative, or with a brother or sister, so that it arouses a public scandal. Just the simple notion of "public scandal" is the objective condition of punishment. Incest instead, as a form of sexual abuse, as physical and psychological constriction to do, or to undergo, actions against one's own will, is a penal crime according to Law n. 66/1996 ("Norme contro la violenza sessuale" – "Rules against sexual violence"). Every "imposed" incestuous relationship is not a simple "incest" but the worst hypothesis of "sexual violence". In these cases incest has to be considered "aggravation" of the crime of sexual violence.

Unlike in Italy, in France as in Belgium, the laws that condemn incest were abolished by Napoleon. In Spain this offense has been decriminalized, but the marriage between blood relatives is not yet possible, and Sweden, however, is the only country in Europe which allows marriage between siblings who share only one parent. In New Jersey there is no penalty for the majority, while in Massachusetts it is expected to imprisonment up to twenty years for sexual activity with a closer relative of the cousin of First Instance. In Japan, incest is legal (the law that condemned him was abolished in 1881), although generally regarded as immoral, and, however, consanguineous can not marry. In Israel, incest between consenting persons who have both completed the age of majority is not a crime while it is punished the incestuous relationship with a minor. In Brazil, finally, the law allows incest only in certain cases, that is uncle and nephew can have a relationship, but on condition that they firstly are subjected to genetic control.

From what emerged from our study we can see that incest does not exist as a criminal case in all Countries; in some of these, in fact, you can marry even if blood relatives: the prerequisite is the consent between the parties. It becomes clear that the term incest is better and more specifically referred to cases where there is a violent relationship, in which one party is forced to suffer abuse or is too weak, even from a psychological point of view, to oppose a good resistance. This does not occur in Germany, where the union between close relatives has always prevented, even when aware and consenting adults, which in light of the current legislation, that is part of the old policy of "racial hygiene" promoted by the Nazis and still in force.

Conclusions

The aim of our contribution is to take widest awareness of the diffusion of this phenomenon among health-professionals perspective and differences of cultural approach in legal views.

When physical, sexual and psychological abuses on children take on the contours of incest, offense is even more serious and even the risk of the phenomenon of the cycle of abuse is more concrete. The early identification of a victim is inescapable obligation of all the professions of experts, really competent to provide support only if properly trained for this purpose. Improving the professional interdisciplinary abilities in this field of person abuse can help victimes.

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A MULTIDISCIPLINARY NETWORK: PERSPECTIVE ON THE DOMAIN OF PSYCHOLOGICAL VIOLENCE IN CONJUGALITY – PART 1

Abstract: Domestic Violence generates hard conditions for the abused ones. Damages can be either physical or psychological. For Judiciary purposes it is fundamental to establish cause-connection and to evaluate the risk rate for every case. Thus, the role of Legal Medicine becomes crucial for Judiciary work as well as the Psychiatric and Psychological health care services in terms of risk evaluation and curing approaches. The multidisciplinary approach is needed. A successful experienced at Coimbra is reported. Aiming to solve hard situations and caring for the condition of the abused ones, a network involving 11 institutions (3 judiciary, 4 health and 4 social) was set up under the name of "Grupo Violência".

Introduction

Domestic violence is a serious problem around the world. It violates the fundamental human rights of the victims and often results in severe injury and death.

All over the world the prevalence of cases of violence against women by their partner is overwhelming. It is estimated that one, in every five European women, experienced violence and that at least seven hundred women in Europe die every year as result of violence perpetrated by an intimate partner *(*Athena, 2006*)*.

Most women that die as a consequence of a homicide are murdered at home by their partners. On the contrary, men that die as a consequence of a homicide are killed outside by non relatives.

Studies show that domestic violence is the greatest motive of death and invalidity among women.

In general, the victims of domestic violence are women. However, there are also cases where the women are perpetrators of violence against men, mainly of psychological nature. Children and older people are also, often, victims of domestic violence.

It is a phenomenon that occurs within all social, economic, religious and cultural groups, and it is a reality, both in developed countries and in underdeveloped ones.

UN Declaration on Elimination of Violence against Women recommends that state members promote research, collect data and compile statistics concerning domestic violence, with the goal of understanding the problem in order to find solutions for them.

Therefore, countries must develop policies to face the problem, such as passing adequate legislation, creating mechanisms do protect the victims from the aggressors, promoting the recuperation of the offender through special programs for aggressors.

Domestic violence involves enormous costs to treat all the symptoms and diseases caused by this kind of violence. Sometimes, those symptoms are not immediately evident and perceptible.

A criminal offence

It is a criminal offence. Neither the society, nor the judicial system, should ever regard the violence inflicted on someone by an intimate partner, less serious than the violence inflicted by a stranger. However, in spite of that, according to Amnesty International, in seventy nine countries of the world there is no legislation dealing with the matter of domestic violence.

Portugal: a public crime

In Portugal domestic violence is a public crime only since the year two thousands. It means that victims do not have the responsibility for initiating criminal charges because the State assumes this as its own duty.

All public institutions, like police, hospitals and care centres, must communicate to the Department of Justice all the cases that they identify as domestic violence. This change of the law was crucial, since it allowed that an enormous number of cases came out from the shadow and, consequently, it also allowed the corresponding criminal investigation.

Elza Pais, the President of the Portuguese Commission for Citizenship and Gender Equality, made a study about spousal murder in Portugal and she settled that, in every six homicides, one was a spousal homicide.

In 2006, two hundred and twelve men were in prison for the murder of their wives, partners or girlfriends. In 2008, forty nine women were killed in Portugal. Almost all of them were victims of domestic violence. In most cases all those women, before the murder, had a history of being battered by their partner who put an end to their life (CIG, 2007)

If one looks at the preceding story, whenever a murder occurs in a partnership relation, it will be noted that, despite the signs, nobody did anything to prevent the unhappy end.

When someone is a victim of domestic violence, it is important to make an adequate and swift intervention, regarding the aggressor, the victim and even the family, in order to prevent that more serious attempt against victims occurs, such as homicide.

Men that kill their wives, partners or girlfriends, act not moved by an impulse that occurs on that particular moment. On contrary, they kill after premeditating about it for a long period; they had thought carefully how to do things; they had threatened; they had warned the victim and yet nobody, even the victim, recognised the warning signs. But those signs where there and it was imperious that someone had to look for them.

As Mullender (2000) said "The domestic violence cases of today are often the murders of tomorrow".

The court

A lot of complaints from victims of domestic violence are filled in the court. This is a very special crime because it occurs among people that had shared their lives for a long time, that had dreams and goals together, that had children they care about. It is very difficult for them, in one way, to recognize that their project of life has failed, as well as to face a new life, alone, with the uncertainty of the future.

Aiming to give to each crime of domestic violence an adequate response, it is fundamental to have an interdisciplinary approach, involving all the institutions that work with the phenomenon – those that investigate the crime, those that made the assessment of the victims and offenders and those that protect the victims.

While the police and the court take measures against the aggressor, the victims must be immediately supported, protected and assisted, by health and social care workers.

The net work

In order to achieve this, in Coimbra, a network has been set up which involves a lot of institutions from all areas – health, social and justice – named "Violence Group – Information, Investigation and Intervention".

This Group is trying to find and to develop strategies to prevent domestic violence throughout an articulated work. It is also committed to bring to the surface the shadow sides of violence and victimization.

The public hospital is the entrance door of a lot of cases of domestic violence, which are camouflaged as downfall and accidents, what means that an inappropriate cause has been mentioned.

Nurses and doctors in the emergency departments are on the front line of interpersonal violence and are in a vital and unique position to initiate the process that may stop the cycle of violence (*Healey, 1998).*

Because of this, at the emergency rooms of the Central Hospital in Coimbra it is now being developed a program to implement the screening of victims of domestic violence, to collect all physical and psychological evidence and to give to the victims a special treatment in order to protect and support them.

Legal Medicine

Legal Medicine is an interface between the health care system and the legal system.

The National Institute of Legal Medicine is a national reference and it is the official entity that cooperates with the courts and the judicial system, making all the forensic assessments, which are essential to the investigation.

So, in order to allow a complete and precise assessment of every case, all the information's of public hospitals are useful when Legal Medicine has to elaborate the reports for the court.

Therefore, it is important that health care officials are properly trained in the area of domestic violence. Doctors must also screen for domestic violence, during physical examinations. Likewise, police and social workers must be trained to work with cases of domestic violence in order to detect, earlier, the warning signs and to give an adequate protection and support to the victims.

The fear of failing once more, the fear of not being able to begin again a new project, the fear of not being able to take care of their children, the fear of becoming homeless and the lack of money, are enough motives for the victim to maintain the situation and to continue being battered. So, they are not able to decide for the court intervention and this ambivalence of the victims brings about difficulties to the criminal investigation. *(Roberts & White, 2007).*

Very often, when the first aggression occurs, the victim doesn't believe that it is true. In most of the cases, she does nothing. She suffers in silence and says nothing to anybody. And, sometimes, she is being abused for years.

Psychologists and psychiatrists said that, when someone is beaten by an intimate partner, a frontier is passed. And when that frontier is passed, victims must react very strongly. Since that once created the fear of further attacks, it requires nothing more from the aggressor than a verbal threat to maintain the atmosphere of constant fear and anguish.

The feeling of impunity of the aggressor becomes propitious for more and more aggressions.

Everybody knows that, within the psychological damage brought on by abuse, is common to find symptoms of stress, depression and anxiety, which aggravates the situation and makes it more difficult to escape from the abuse.

We can say that self-esteem is the mental immunity system of man and when a victim looses her self-esteem; she also looses the capacity to complain and to take care of herself.

The cause connection

Aiming to establish the cause-connection it is crucial for the court to know which psychological damages are presented by a battered person and whether those damages were caused by the aggressive behaviour of the defendant.

Verbal abuse can be more damaging to a victim's psychological well-being, than physical abuse.

A forensic assessment must be considered, whenever the public prosecutor has the perception that a victim of violence is experiencing intense feelings of fear, shame, panic and anxiety. It also must be considered whenever he needs to understand the offender behaviour. An increased knowledge, which allowed the prosecutors and the judges to sentence properly, is the goal of these assessments.

So, the importance of the Legal Medicine for the investigation and for the make up of this crime is unavoidable.

The reports of Legal Medicine will inform the public prosecutors and the judges whether that victim presents symptoms of having been abused, what kind of symptoms she presents and if they could be caused by the aggressor's behaviour.

It is crucial to establish the cause connection because, without that, all the charges of psychological damages will fail.

Note that the specificities of this crime do not allow achieving a correct diagnosis of cause-effect in only one strike.

It is important to study the case, to know the aggressor and the victim, to understand how they interact, in order to find the causes of the aggressiveness, in such a way, that the court is able to understand what is going on.

So, the report can suggest, for instance, an intervention over the defendant, over the victim or over the family.

In Coimbra, a new approach for the assessment of victims of psychological domestic violence had been executed with the guarantee of Legal Medicine.

The assessment of victims and offenders

The Delegation of Coimbra of the National Institute of Legal Medicine make the assessment of physical damages and delegates on the Family Violence Department of the Central Psychiatric Hospital of Coimbra the task of making the psychological and psychiatric assessment of victims of domestic violence and defendants sent by the Department of Investigation and Penal Action (DIAP) of Coimbra.

The Family Violence Department, besides making the reports of assessment, pronounces about the need and success of an intervention for the aggressors and for the victims and suggests to the court a program that the defendant, although in a coercive situation, must follow up.

The Portuguese legislation offers pre-trial mechanisms to give the offender the opportunity to review his behaviour, in order to maintain the relationship without further aggressions and injuries.

Before the charge and before the trial, the public prosecutor can put the defendant on probation. The aggressor is expected to adapt a new conduct and is aware of the coercive context. If he agrees with the conditions, and if he accomplishes those conditions, he will not be charged.

Considering the forensic report, these conditions can include, for the aggressors, the mandatory attendance of sessions of the Intervention Program in the Family Violence Department,.

Final considerations

The control system response must achieve different results: must rehabilitate the aggressor, protect and support the victim, and dependents, and make victims more comfortable and compensated after the law intervention.

We think that an efficient response to this kind of criminality requires an articulated and interdisciplinary intervention.

Besides judicial system, that has the coercion power; it must call for the cooperation of health and social systems.

The objective of bringing this to you, in a way, is to appeal for the decisive importance of Legal Medicine in the criminal investigation.

On the other hand, to call attention to the importance of the psychological and psychiatric assessment of aggressors and victims, and how this assessment can be decisive in sentencing someone, who commits a domestic violence crime.

I also believe that this subject requires a network response, adjoining all the actions of different profiles and areas. So far, it has been my fulfilling experience, at Coimbra.

Finally, I feel the need to state, that the duty of the judicial system, will only be effectively accomplished towards the victim, and to the society, if, after all, the victim experiences a change, for better, in her life.

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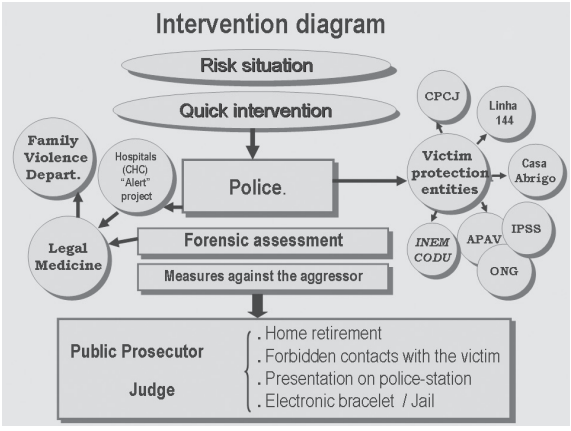

Figure – 1

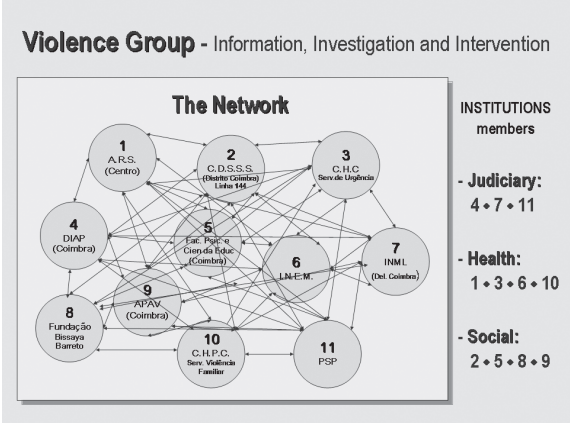

Figure – 2

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DOMESTIC VIOLENCE AND FORENSIC ASSESSMENT: A MULTIDISCIPLINARY NETWORK PERSPECTIVE ON THE DOMAIN OF PSYCHOLOGICAL VIOLENCE IN CONJUGALITY – PART 2

Abstract: This paper discusses the strategy adopted by the Family Violence Department (FVD) of the Coimbra Psychiatric Hospital Center, towards the forensic assessment requested by the Investigation and Prosecution Department of Coimbra (DIAP) due to intimate partner violence (IPV) associated with psychological violence. In accordance with the National Institute of Legal Medicine (Coimbra Delegation), we are often requested to "produce a report in order to establish the psychological status of the victim and the contribution of the accused, by establishing a causal link, if possible". With the purpose of trying to provide answers to the questions made by the Justice Department to the Health Department, five integrated levels of knowledge are proposed in this paper. It is in the crossroad of the forthcoming narratives of these *theories* that some of the questions by DIAP can be answered.

In the past, researchers considered psychological abuse, in a domestic violence context, to be a consequence of other forms of abuse, particularly physical or sexual abuse. Now, however, psychological abuse is understood as a separate and distinct form of abuse and researchers have confirmed that it represents a common and significant form of interpersonal violence in terms of its frequency, and its short and long-term effects (*Psychological Abuse: A Discussion Paper*. Ottawa: Public Health Agency of Canada, 2008)

The changes caused by trauma can occur on a conscious or unconscious level. They may cause behavioural modifications, or impact on the emotional, cognitive or relational fields; so that by its quality and/or amount of events, they are experienced as a traumatic consequence that exceeds the victim's tolerance, producing a breakdown of their homeostasis. The slightest change in their adaptive defense system will be sufficient to produce health prejudice.

In order to help in forensic assessment in the area of psychological violence in conjugality, five integrated levels of knowledge are proposed.

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1. A theory about intimate partner violence

Violence in general. The types of violence proposed by World Health Organization divides violence into three broad categories according to characteristics of those committing the violent act. **(SLIDE 1)**. Family and Intimate Partner Violence[2], includes forms of violence such as child abuse, intimate partner violence and abuse of the elderly.

For the purposes of this paper the term Intimate Partner Violence or Domestic Violence will be used when referring to the abuse that occurs between two people in a close relationship.

Intimate Partner Violence (IPV). One of the most common forms of violence against women is that performed by a husband or an intimate male partner. This is in stark contrast to the situation for men, who in general are much more likely to be attacked by a stranger or acquaintance than by someone within their close circle of relationships. Research suggests that physical violence in intimate relationships is often accompanied by psychological abuse, and in one-third to over one-half of cases by sexual abuse (WHO, 2002).

Although international studies have focused on physical violence because it is more easily conceptualized and measured, qualitative studies suggest that some women find the psychological abuse and degradation even more intolerable than the physical violence. As most survivors of partner abuse report, the physical violence is the least damaging abuse they suffer: it is the relentless psychological abuse that cripples and isolates the women. Accordingly Hegarty, et al (2006) from a health perspective, IPV, can be better understood as a chronic syndrome that is characterized not by the episodes of physical violence that punctuate the problem but by the emotional and psychological abuse that the perpetrator uses to maintain control over the partner.

Risk factors for IPV. We need to understand the multiciplity of risk factors that contribute to man's violence against women, so that we can appropriately target interventional programs for men and couples. Violence is the result of the complex interplay of individual, relationship, social, cultural and environmental factors. Understanding how these factors are related to violence is one of the important steps for public health approach to prevent violence. Ecological model **(SLIDE 2)**, first introduced in the late 1970s, may aid us to understand the multifaceted nature of violence. The model explores the relationship between individual and contextual factors and considers violence as the product of multiple levels of influence on behaviour. Studies to advance the understanding of violence are needed on a variety of levels. **(SLIDE 3)**

The consequences of intimate partner violence. A growing body of research evidence is revealing that sharing her life with an abusive partner can have a profound impact on a woman's health **(SLIDE 4).** Although violence can have direct health consequences, such as injury, being a victim of violence also increases a woman's risk of future ill health. Given the long-term impact of violence on women's health, women who have suffered abuse are more likely to be long-term users of health services, thereby increasing health care costs and it does appear to influence a woman's earnings and her ability to keep a job (WHO 2002).

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[2] Violence largely between family members and intimate partners, usually, though not exclusively, taking place in the home (WHO 2002).

2. A *theory* about trauma

When trauma hits, the patterns and structures of self-organization that were there, whether in childhood or as an adult, become frozen in time. Personal assumptions of safety and connection are shaken at their roots. Strong defenses form against such terror and pain. Survival modes of living become locked into "trauma bubbles" in the face of life-threatening experiences and continue to be practiced unconsciously many years after trauma ends. Brains affected by trauma are unable to put meaning to unprocessed experiences. These fragmented memories are stored in the right brain and organized around affect, not words. In the past years, research in neurobiology shows that flashbacks and body memories, common post-traumatic stress disorder symptoms, activate the emotional but not cognitive parts of the brain. Is also showing physical, measurable, biochemical changes in the brain of people who have experienced trauma. "Body remembers what the mind forgets". (Hudgins, 2002)

3. A *theory* about strategies and instruments associated with the information collected

The life history of the victims and aggressors – including their violence history, personal and developmental history, family history, socio-professional history and their clinical history **(SLIDE 5)** – is essential for a more precise knowledge of the histories of the associated problems and needs. Accordingly, we are developing in the Family Violence Department **(SLIDE 6)** a protocol to collect information to allow a more precise elaboration of the diagnosis of the situation (e.g. risk assessment, potential for changing, clinical diagnosis) and to help define an interventional strategy – more appropriate to the realistic needs of each case – as well as a prognosis. In relation to the aggressor personality assessment, we sometimes complement the evaluation carried out in the interviews with the application of the Minnesota Multiphasic Personality Inventory.

Taking into account that violence should be understood as a network phenomenon – where each violence history reflects the crossroads of life stories of the "actors" involved in the problem of violence and the many subsystems that interact with them during their life cycle – elements of primary networks (relatives and friends) and secondary network (professionals of institutions involved) are also called on to participate in the elaboration of that history. The participation of these supportive networks also represents an important contribution to the attempt of evaluating a "before" and an "after" to the event of illness in the personnel's victim's history and its eventual relation with the history of violence.

Preceding the interview and evaluation with these networks, we carry out with both, together or separately (depending on the relationship quality level and the couple's risk of retaliation), the Genogram and the Personal Social Network Map **(SLIDE 7)**.

4. A *theory* about network support in relation to the emergence of narratives about families and individual histories, also of violence, and the development of a multidisciplinary and multisectorial intervention.

The personal social network includes all those with whom the individual interacts and that distinguish him from the anonymous mass of the society. The network's

narratives is the field of histories that are common to a family or to a social network, where the focus of attention is no longer the individual, or the family, or the network as such, but histories embedded in the virtual space of the conversation between people, that is, the narrative.

Working with networks of victims and perpetrators, allows us the access to several "actors" and narratives.

In evaluating, for example, how the primary network builds its narrative around IPV, we can observe the group's structure and dynamic relationship, how this group is structured in a moment of stress, its adaptation to the life cycle, loyalties, alliances, recurrence patterns and how they deal with family secrets and myths. These provide support for the construction of narratives, where inevitably the existence of violence will emerge. Extending the assessment to the services' network gives access to the various stories that define the relationship between victims and perpetrators with those services. This approach will increase even more the probabilities of us knowing other narratives about IPV.

Adopting more holistic frameworks that integrate different approaches enables us to address abuse at the individual and societal level and to better account the diverse and complex factors associated with psychological abuse. The existing connections between the FVD and the *Violence: Information, Investigation and Intervention* group **(SLIDE 8)** – a multidisciplinary and multisectorial care network in the domestic violence area, in Coimbra – has given a strong contribution in order to offer to those who use these services more quicker and efficient answers, improved accessibility, continuity and personalization of care, along with revictimization prevention. All those aspects constitute globally important strategies in order to help diminish tensions and gain the confidence of the users of this care system.

5. A *theory* about forensic assessment and the importance of a concomitant support/therapeutic intervention

To evaluate the situation with victims and aggressors, in conjunction with forensic evaluation, in order to define a concomitant support/therapeutic intervention at the FVD, seems a strategy with noticeable benefits to both parties. It allows, on the first stage, aside with risk assessment, revictimizing prevention and evaluation of the clinical-social situation, to soften the tension, also associated with the social visibility of the problem and the services intervention on "family matters".

After the first 3 to 4 sessions, the subsequent system extension to other relatives and friends (most of the times, with the victim's acceptance, and in several cases with the aggressors resistance), will contribute to other perceptions and perspectives of the problem as well as to enlarge its "visibility".

In several cases, the aggressor and the victim continue on the FVD, following the treatment that had been previously defined, after the judicial measure enforcement or the process suspension. It is relevant to underline that some of the aggressors, which abandon the Violence Family Department earlier, suffer from personality disorders and end up separated or divorced from the victim.

On a global perspective, such a joint intervention is assuring and protective of the victim and it increases a more effective "visibility" of the violence (since the evaluation occurs on a most relaxed environment). In this change, the support networks also plays an important role, which usually accepts positively the idea of both, the victim and the aggressor, being followed up by a specific service in the family violence field.

Final considerations

The richness and complexity of the relationship between traumatic experiences, the many variables that influence the response to these experiences and the symptoms and syndromes that are associated represent a serious obstacle to its "visibility".

In the trauma investigation field, it is essential to have a knowledge of the related theories. It is important to understand and to know how to assess the answers to traumatic experiences, and to have the appropriate methods to validate the impact of the trauma, along with an effective and efficient clinical practice in treating traumatized people. The non-revictimization of those who suffer is a central aspect of this area of investigation. It is imperative to maximize the accuracy of assessments and minimize the effects on the victims.

In this framework, and with the aim of a most effective evaluation of psychological damage, it is essential, along with the previously mentioned, to continue to pursue the development of a matrix, both in the theoretical and conceptual framework of the issues in analysis, along with the integration and standardization of appropriate tools and strategies for correct analyzing and understanding of the problem (e.g. specific evaluation tools, protocols).

It is also important to enhance the importance of invisibility of the psychological damage in the legal and health speeches, taking into account the severity of this problem's impact in the quality of life of those who suffer.

Given the many problems related to domestic violence it is fundamental to create a care structure or organization – focus on multidisciplinary, multisectoral and network's intervention – along with the investment, in partnership with the Legal Medicine, on a research project in order to improve the gathering of evidence within the psychological violence in marriage.

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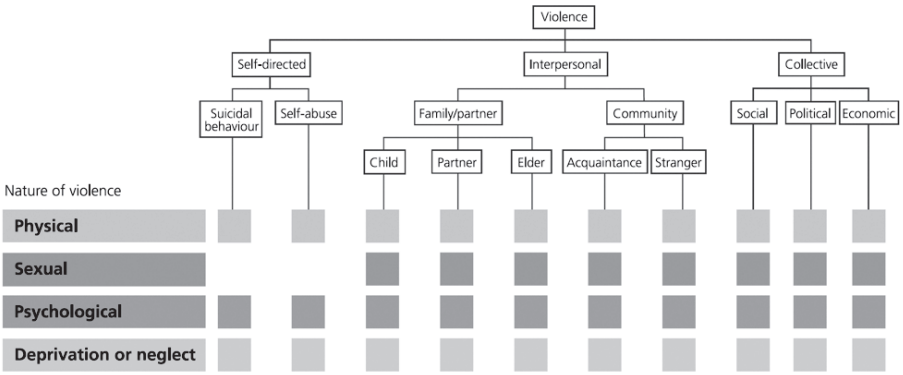
Violence typology

The World Health Organization defines violence as "The Intentional use of physical force or power, threatened or actual, against oneself, another person, or against a group or community, that either results in or has a high likelihood of resulting in injury, death, psychological harm, maldevelopment or deprivation" *(WHO, 2002)*

WHO, *"World Report on Violence and Health"* (2002)

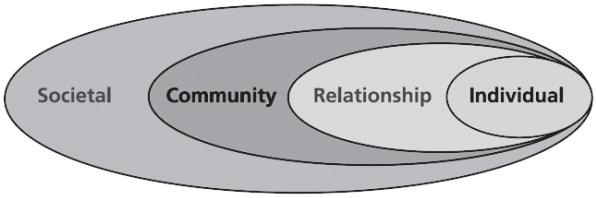


Intimate partner violence refers to any behaviour within an intimate relationship that causes physical, psychological or sexual harm to those in the relationship. Occurs in all countries, irrespective of social, economic, religious or cultural group.
Initially viewed largely as a human rights issue, partner violence is increasingly seen as an important public health problem

Slide – 1

Ecological Model

(...) The model explores the relationship between individual and contextual factors and considers violence as the product of multiple levels of influence on behaviour.



Factors associated with man´s risk for abusing his partner

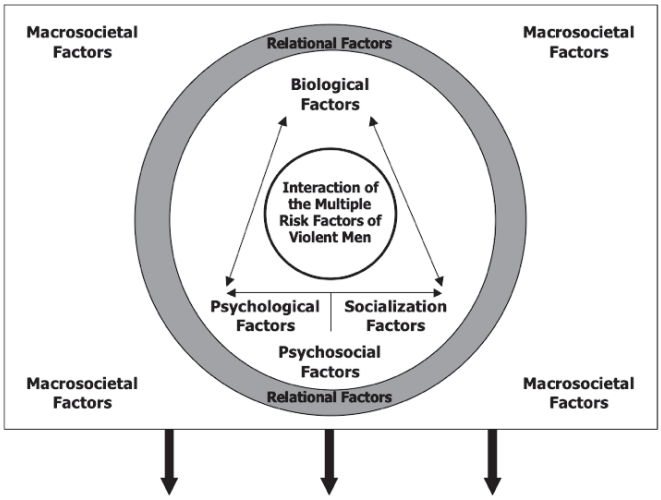
Individual factors	Relationship factors	Community factors	Societal factors
• Young age • Heavy drinking • Depression • Personality disorders • Low academic achievement • Low income • Witnessing or experiencing violence as a child	• Marital conflict • Marital instability • Male dominance in the family • Economic stress • Poor family functioning	• Weak community sanctions against domestic violence • Poverty • Low social capital	• Traditional gender norms • Social norms supportive of violence

WHO, *"World Report on Violence and Health"* (2002)

Slide – 2

A Revised Multivariate Model Explaining Men´s Risk For Violence Against Women (Harway & O'Neil, 1999)

Being at risk for suffering relationship violence is more likely the result of an interaction of factors. Seven factors are shown in the figure (macrosocietal, relational, biological, psychological, socialization, psychosocial and interacting risk). Only through research can we verify whether these relationships are valid



The National Academy of Science and the American Psychological Association have convened task forces that recommend the study of multiple factors that cause relationship violence (APA, 1996a, b; Crowell & Burgess, 1996; Koss et al., 1994).

Slide – 3

Health Consequences of Intimate Partner Violence

"In general, the following are conclusions emerging from current research about the health consequences of abuse:
- The influence of abuse can persist long after the abuse itself has stopped
- The more severe the abuse, the greater its impact on a woman's physical and mental health
- The impact over time of different types of abuse and of multiple episodes of abuse appears to be cumulative" (WHO, 2002)

Fatal health consequences
- AIDS-related mortality
- Maternal mortality
- Homicide
- Suicide

Sexual and reproductive
- Gynaecological disorders
- Infertility
- Pelvic inflammatory disease
- Pregnancy complications/miscarriage
- Sexual dysfunction
- Sexually transmitted diseases, including HIV/AIDS
- Unsafe abortion
- Unwanted pregnancy

Psychological and behavioural
- Alcohol and drug abuse
- Depression and anxiety
- Eating and sleep disorders
- Feelings of shame and guilt
- Phobias and panic disorder
- Physical inactivity
- Poor self-esteem
- Post-traumatic stress disorder
- Psychosomatic disorders
- Smoking
- Suicidal behaviour and self-harm
- Unsafe sexual behaviour

Physical
- Abdominal/thoracic injuries
- Bruises and welts
- Chronic pain syndromes
- Disability
- Fibromyalgia
- Fractures
- Gastrointestinal disorders
- Irritable bowel syndrome
- Lacerations and abrasions
- Ocular damage
- Reduced physical functioning

WHO, *"World Report on Violence and Health"* (2002)

Slide – 4

Possible Indicators of Psychological Abuse

The effects of psychological abuse can be manifested in many different forms and may be difficult to detect. The abuse does not leave physical markings, but it does have substantial, and often long-lasting, impacts on the victim that may escalate or transform over the life-span

** Indicators of PTSD include haunting memories, nightmares, social withdrawal, anxiety, depression, sleep disturbances, fatigue, difficulty concentrating, memory loss, and feelings of helplessness, fear and anger (Meyers 2004).*

(Canada. National Clearinghouse on Family Violence. *Psychological Abuse: A Discussion Paper*. Ottawa: Public Health Agency of Canada, 2008)

Children (Infancy to age 12)	Adolescents (Ages 13-19)	Adults (Ages 20-64)	Older Adults (Age 65 and up)
PTSD* (older children)	PTSD (both male and female victims)	PTSD likely for both men and women	Signs of PTSD
Non-organic failure-to-thrive (infants)[10]	Psychological abuse in dating relationships (both male and female victims).[17]	Fear for self, children and/or pets (female victims)	Discomfort or fear around caregiver
Elevated levels of cortisol (a stress hormone) that may cause damage to areas of the brain important for memory formation and emotional regulation (infants /preschoolers)[11]	Poor school performance	Shame	Difficulty with normal life transitions (e.g., retirement)
Risk of being bullied	Involvement in bullying as either victim or perpetrator (both male and female victims)[18]	Physical problems that have no medical basis (both men and women)	Extreme passivity and learned helplessness[23]
Significant delays in language development (infants)[12]	Depression,[19] social withdrawal, poor identity development, eating disorders and self-mutilation (more likely for female victims)[20]	Depression, withdrawal and abuse of alcohol (gender differences same as teens).[22]	Exhibit behaviours (e.g., rocking, sucking, biting) commonly associated with dementia (and therefore may be misdiagnosed as dementia patients)
Anxiety and depression	Delinquent acts, abuse of alcohol/drugs and abusive dating behaviour (more likely for male victims)[21]	Low self-esteem	Signs of general psychological distress:
Social withdrawal and limited peer interaction[13]	Suicide attempts or discussion (both male and female victims)	Risk-taking behaviour common (gender differences, e.g., women may risk unintended pregnancy; men might drive too fast)	- depression - fear - anxiety - low self-esteem - shame - anger - self-harming
Severe cognitive and academic difficulties[14]		Suicide attempts or discussion	Difficulty sleeping
Overt aggression (e.g., fighting, making threats, bullying) common as short-term outcome (male and female school-aged victims)[15]			Sudden loss of appetite unrelated to physical disease or aging
Indirect aggression (e.g., gossiping, telling other's secrets) common as long-term outcome (female school-aged victims)[16]			Substance abuse (in particular, of alcohol)

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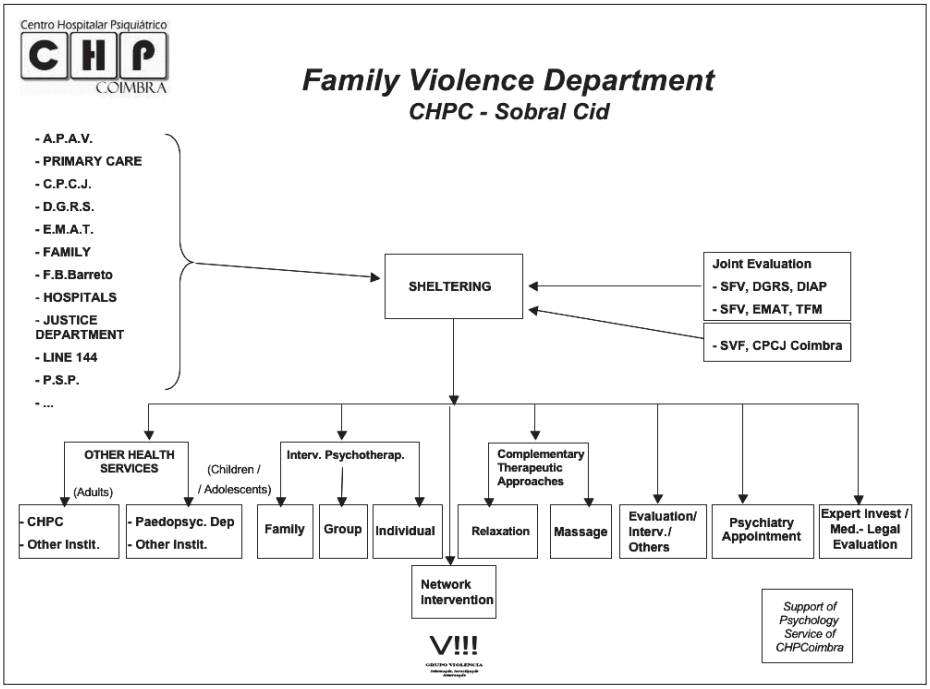
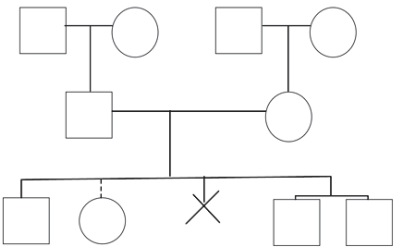


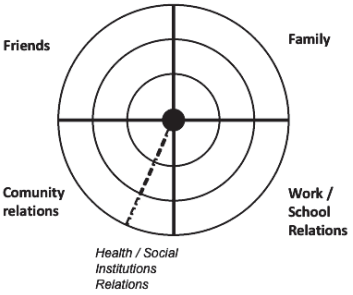
Slide – 6

Genogram

The genogram is an analytical tool which enables one to visualize the family structure and to allow assessment of individual, interactional, and intergenerational patterns within family systems. Includes at least three generations of family members



Personal Social Network Map



Our personal social network — that rather stable but continually evolving interpersonal fabric constituted by close and distant family members, friends, work and study connections, and relationships that result from informal and formal participation in community organizations (religious, social, political, health-related, etc.) — constitutes a key depository of our identity, our history and our well being (Sluzki, 1996)

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Regional Health Administration - Centre (ARSC)
Dr. Fernando Gomes (Family Doctor); Dr. Mª José Hespanha (Family Doctor)

Regional Institute of Solidarity and Social Security – District of Coimbra
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Coimbra Hospital Centre (CHC)
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- Emergency Department of the General Hospital
Dr. Maria João Frade (Neurosurgeon)

Investigation and Penal Action Department - Coimbra (DIAP)
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Faculty of Psychology and Education – University of Coimbra
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National Institute for Medical Emergencies (INEM)
Dr. Sara Rosado (Psychologist)

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In a **NETWORK** it is important
that everyone knows everything
that everyone is equally responsible
ensure transparency

Slide – 8

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INTIMATE PARTNER HOMICIDE-SUICIDE A 5-YEAR CASE REVIEW (2004-08)[1]

Abstract: Homicide followed by suicide is an extremely rare tragic incident, specially in the context of intimate partner violence. The aim of this 5-year retrospective study, from 2004 to 2008, in the Lisbon area, is to provide a better understanding, in a medico-legal perspective, of intimate/partner homicide-suicide. Database of the Department of Forensic Pathology was analysed concerning all the incidents involving at least a female homicide victim and a related suicide or suicide attempt. According to our study, 54% of all intimate partner homicides were followed by suicide (71%) or suicide attempts (29%); 71% with one single victim and 29% were double homicide with involvement of children/young adults; in most cases, the male homicide perpetrator was older and married to the victim; the majority of incidents occurred at the victim's home; firearms were used in most cases, to both homicide and suicide, with the same single anatomic location injuries in the head area. The results gathered are in accordance with other similar studies, and point towards the importance of prevention programs and the enforcement of existing domestic violence laws.
Keywords: Intimate partner homicide-suicide; partner violence; forensic pathology.

Introduction

Homicide followed by suicide is an extremely rare tragic incident committed by an individual who subsequently commits suicide within one week of the homicide [1]. Despite the low rate of ocurrence, homicide-suicide incidents are of great concern, specially in the context of intimate partner violence. Intimate/partner homicide-suicide is considered the most extreme form and consequence of intimate partner violence [2], because they often result in the death of family members, young children, and cause an additional morbidity and family disruption [3].

Previous studies of homicide-suicide incidents shows that usually these include one female victim and one male perpetrator, with a current or former intimate relationship between them [4], being the most typical, a man shooting a family member during

[1] Preliminary results presented at the XXI Congress of the International Academy of Legal Medicine, May 2009, Lisbon – Portugal.

a separation process [5]. A substancial proportion includes the homicide of a child, and a small proportion can be attributed to "mercy" killings [4]. People who commit homicide have a high death risk themselves and are especially prone to suicide [5]. Between 18% and 40% of perpetrators of intimate femicide commit suicide afterwards [2]. Most suicides occurr at the same location as at least one of the homicides [1], with homicides taking place in the victim´s home [4]. Firearms, specially handguns, are more likely to be used for both homicides and suicides and only in a minority of cases the offender uses a different weapon for homicide and suicide [3,5]. Amongst suicides, the head, face or neck are the most frequent locations of single wounds, while in homicides, the head is also the most common location with more multiple wounds locations than in suicides [3]. Alcohol or drug use is a common risk factor in homicides, independent of the suicide of the perpetrator [6].

The aim of this 5-year retrospective study, from 2004 through 2008, in the Lisbon Area, therefore, is to provide contextual information and a better understanding of intimate/partner homicide-suicide incidents concerning Portuguese population. Thus, the author's purpose was to analyse sociodemographic, clinical and medico-legal aspects of both victims and perpetrators involved in these kind of deaths.

Materials and methods

Intimate partner homicide-suicide were identified, after scanning the South branch database of the National Institute of Legal Medicine of Portugal (NILMP), related to Forensic Pathology Department and Lisbon area, over a 5-year period, between January 1, 2004 and December 31, 2008.

All the incidents involving at least a female intimate partner victim of homicide, with 18 years-old or more, and one male perpetrator that committed suicide or suicide attempt, within one week after the homicide(s) were identified. The expression "intimate partners" includes: spouses by marriage, common-law spouses, girlfriends, ex-intimate partners and extramarital consort. The incidents in which the perpetrator was not an intimate partner of the victim were excluded. Cases involving male intimate partner victims were not searched. Cases were reviewed and data collected from the medico-legal autopsy records, and other information (police, newspaper, etc.) when available.

In order to compare perpetrators and intimate partner victims characteristics, the following variables were sistematically analysed: sociodemographics aspects (gender, age and race/ethnic group); nature of the relationship; circumstances of death, precipitating factors and motivations if available; location of incident; autopsy results (location and type of fatal injuries; type of weapon) and toxicological information.

Results

During the 5-years examined, a total of 6.696 autopsies were performed at the Forensic Pathology Department of the NILMP South Branch. Of almost 57 female homicide victims, 26 cases (46%) were considered intimate partner homicides. Out of these 26 cases, 14 (54%) were identified as homicide-suicide incidents: 10 homicides

(71%) followed by suicides and 4 (29%) by suicide attempts of the perpetrator. Of all 14 homicide-suicide incidents, the offender killed one victim in 10 cases (including all the cases of suicide attempts), and two victims in 4 cases, accounting a total of 28 deaths. Table 1 provides a comparison of all homicide victim(s) and perpetrators characteristics found.

Perpetrators

All the 14 perpetrators were male with a medium age of 55 years-old (range 33 to 79). Of the 10 males who committed suicide, all but one were white. Suicide was completed in 10 cases, the majority by firearm injuries (5 cases with handguns and 4 cases with shotguns) and the other suicide was due to a fall from height. Of the 4 cases of suicide attempts, a sharp instrument was used in 2 cases and a handgun in other 2. In 3 incidents, the perpetrator used a different weapon for homicide and subsequent suicide (shotgun and handgun/shotgun, manual strangulation/fall from the height, manual strangulation/sharp instrument for suicide attempt). Considering only firearm injuries in completed suicides or suicide attempts (11 cases), all of them were located in the head including 9 cases with single anatomic location. In other hand, 3 cases were quite different in homicide versus suicide, with multiple injuries in female victims and single ones in male perpetrators. All but one suicides occurred in the same place of the homicides. Two males tested positive for benzodiazepines and only one had a blood alcohol level of 0,63 g/l. Out of the 4 cases of attempted suicide, only one offender had a history of previous attempts.

Female Victims

Concerning female intimate partners, the victims had a medium age of 48 years-old (range 21 to 80) and all but one were white people. All but one females were younger than their intimate partner perpetrator.

Females were current or former intimate partners of the perpetrator: 9 spouses by marriage or common-law spouses; 3 ex-intimate partners (including spouses and girlfriends) and 2 extramarital consort. Except 3 cases (including those within extramarital consort), the majority were cohabitating with the perpetrator at the time of the incident. In 11 incidents (79%), the perpetrators used a firearm for homicide, in 2 manual strangulation, and in 1 a sharp instrument. Among firearms, handguns were more likely to be used (7 cases) than shotguns (3 cases), with 1 case where both type of guns were used. Most female victims (64%) died as result of firearm injuries on the head, 57% single located. Only one female had passive and active defense wounds. 72% of the female homicides took place in the victim's home while the others occurred outside home. In 4 cases benzodiazepines were detected. One of the cases of suicide attempt, the female victim had physical health problems (submitted to cancer surgery, Alzheimer´s disease and bedridden) that could be attributed to "mercy" killing.

Other Victims

Four homicide victims of the total homicide-suicide incidents comprised males: 2 children and 2 young adults, with a medium age of 20 years-old (range 8 to 34), all white, killed in the same event as their mothers. All but one were perpetrators offspring, the exception being an ex-step-son. In all 4 cases, firearms were used, mostly handguns (3 cases) and injuries were located in the head. In all cases, the victims were living at the same house of the perpetrators and the homicide took place at home.

Discussion and conclusions

In substance, the findings of this study are in accordance with other similar studies. In fact, the review of the literature show that over than 30% of all male perpetrators/ offenders of intimate partner homicide ultimately ended their own lives [4]. 85% of the offenders were males [7,8]. Most homicide victims are female [4]. Approximately one quarter of involved persons are over the age of 55. The medium age of offenders was 51 years in males [7,8]. More often (over half of the victims, 58%), the perpetrator was a former or current husband or other intimate partner [4]. Most suicides, near 80%, following a homicide/suicide incident occurred in the same place of at least one of the homicides [4]. Homicides occurred more often at the victim's home [4]. Most incidents occur in a residence [4,8]. Alcohol or drug use is a common risk factor in homicides. In homicide/suicide incidents, 34% of the perpetrators had detectible blood alcohol content and other substances were identifiable in 18% of the same group [3]. Male perpetrators are also more likely than victims to be under the influence of alcohol at the time of the incident [4]. Almost 30% of perpetrators tested positive for drugs or alcohol [4]. Firearms were used in a majority of incidents for both homicides and suicides (nearly 80%). The next most common weapon used were sharp instruments 6% [4]. In 80%, the offenders used a gun for both the homicide and suicide. In 16%, the offender used a different weapon for homicide and suicide [7,8]. Among firearms, handguns were more likely to be used than shotguns or rifles [8]. Among suicides, the head, face or neck were the most frequent location of the wounds with only one wound location. While the head area was also the most common location among homicide victims (72%), a significative percentage of these had wounds in multiple locations (22%) [4].

In the present study, considering all female victims of homicide, 54% of all intimate partner homicides were followed by suicide (71%) or suicide attempts (29%), the majority (71%) with one single victim. In most cases, the male homicide perpetrator was older than the intimate partner and married to the victim. Firearms were used in most cases, to both homicide and suicide, with the same single anatomic location injuries in the head. Most homicides and suicides occurred at the victim's home.

The current results undescore the importance of: (1) prevention programs and policies for victims of intimate partner violence, (2) enforcement of existing domestic violence laws, and (3) restricting access to guns.

Additional research is needed to study and identify risk factors and precipitating events in this special type of homicide-suicide incidents, as legal, job or financial

problems, physical health problems, mental illness, substance abuse disorders, relationship rupture, suicidal behavior and violence, in order to understand and prevent them. Another interesting study would be the correlation of homicide, suicide and homicide-suicide incidents.

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	No.		Gender	Age	Race	Relationship victim/ /perpetrator	Living at the same home of perpetrator	Weapon used	Location and type of injury	Place of the incident
Homicide-suicides	1	Perpetrator	Male	39	Black	-	-	Firearm (shotgun)	Head - single	Outside home
		Victim	Female	36	Black	Spouse by marriage	Yes	Firearm (shotgun)	Head - single	Outside home
	2	Perpetrator	Male	72	White	-	-	Firearm (shotgun)	Head - single	Home
		Victim	Female	45	White	Spouse by marriage	Yes	Firearm (shotgun and handgun)	Head - multiple	Home
	3	Perpetrator	Male	49	White	-	-	Firearm (handgun)	Head - single	Outside home
		Victim	Female	25	White	Extramarital consort	No	Firearm (handgun)	Head,torax,abdomen – multiple	Outside home
	4	Perpetrator	Male	60	White	-	-	Firearm (handgun)	Head - single	Outside home
		Victim	Female	51	White	Extramarital consort	No	Firearm (handgun)	Head - single	Outside home
	5	Perpetrator	Male	74	White	-	-	Firearm (shotgun)	Head - single	Home
		Victim	Female	80	White	Spouse by marriage	Yes	Firearm (shotgun)	Head - single	Home
	6	Perpetrator	Male	61	White	-	-	Fall from height	Head,torax,abdomen,limbs - multiple	Home
		Victim	Female	56	White	Spouse by marriage	Yes	Manual strangulation	Neck - multiple	Home
	7	Perpetrator	Male	55	White	-	-	Firearm (handgun)	Head - multiple	Home
		Victim	Female	50	White	Spouse by marriage	Yes	Firearm (handgun)	Head,abdomen - multiple	Home
		Victim	Male	26	White	Son	Yes	Firearm (handgun)	Torax,abdomen - multiple	Home
	8	Perpetrator	Male	40	White	-	-	Firearm (shotgun)	Head - single	Home
		Victim	Female	34	White	Common-law spouse	Yes	Firearm (shotgun)	Torax - single	Home
		Victim	Male	8	White	Son	Yes	Firearm (shotgun)	Head - single	Home
	9	Perpetrator	Male	56	White	-	-	Firearm (handgun)	Head - single	Home
		Victim	Female	49	White	Ex-common-law spouse	Yes	Firearm (handgun)	Head - multiple	Home
		Victim	Male	11	White	Ex-step son	Yes	Firearm (handgun)	Head - single	Home
Homicide-suicide attempts	10	Perpetrator	Male	65	White	-	-	Firearm (handgun)	Head - single	Home
		Victim	Female	69	White	Spouse by marriage	Yes	Firearm (handgun)	Head - single	Home
		Victim	Male	34	White	Son	Yes	Firearm (handgun)	Head - single	Home
	11	Perpetrator	Male	47	*	-	-	Firearm (handgun)	Head - single	Home
		Victim	Female	44	White	Spouse by marriage	Yes	Firearm (handgun)	Head - multiple	Home
	12	Perpetrator	Male	33	*	-	-	Firearm (handgun)	Head - *	*
		Victim	Female	21	White	Ex-girlfriend	No	Firearm (handgun)	Neck - single	Outside home
	13	Perpetrator	Male	41	*	-	-	Sharp instrument	Limbs - multiple	Home
		Victim	Female	38	White	Ex-spouse by marriage	Yes	Sharp instrument	Torax - multiple	Home
	14	Perpetrator	Male	79	*	-	-	Sharp instrument	Neck,abdomen - multiple	Home
		Victim	Female	76	White	Spouse by marriage	Yes	Manual strangulation	Neck - multiple	Home

* Unknown information.

Table 1 – Comparison of perpetrator and victim(s) characteristics of homicide-suicide incidents

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IMAGING IN EMERGENCY RADIOLOGY: CLINICAL AND FORENSIC ASPECTS

Abstract: Radiologists are frequently faced with different clinical situations that may have medico-legal implications. In these cases, Radiology can help document all findings and is, therefore, of great value when medical-legal issues are raised, both in the clinical field and in post-mortem examination. The main objectives of forensic medicine are to document, analyze, and elucidate scientific medical findings in both living and deceased persons in a comprehensible way for courtroom presentation. This work presents several cases in which importance of radiological examination is self-evident. These include: foreign material into the body (body packer, intraabdominal metal splinter, aspiration of a foreign body), professional malpractice (inadequate placement of a catheter, intraabdominal migration of an intrauterine device) or intentional injuries (cranial fracture). Emphasis has been placed on the contribution of the new imaging techniques (Computed tomography, Magnetic resonance) which constitute the basis of the so-called virtopsy.

Introduction

The radiological examination can be a very useful ancillary method for the expert in both clinical forensic medicine and forensic pathology. In the clinical forensic setting, imaging can be the only or even the best evidence of the problem. Of equal importance is the anticipation of what injuries to look for in the autopsy, or to obtain an objective clue that could be related to the cause of death when the patient has undergone medical o surgical treatment prior to death. The new imaging techniques (CT and MRI) increase considerably the efficacy of radiological applications in forensic medicine [1,2].

This work presents several clinical cases in which the radiological examination performed (CT) could be fundamental for the courtroom presentation of the evidence in both fields.

Cases Report

1. Foreign material inside the body

A. Body packer: A 21-year-old man, known to have swallowed a number of packages of cocaine in the previous 48 hours, presented to the Emergency Department of the

Hospital complaining of abdominal pain and vomiting. A plain abdominal radiograph revealed a number of packages within the abdomen (4x1 cm), and evidence of bowel obstruction, that were confirmed by CT (Figure 1). An emergency laparotomy was performed to remove the cocaine packets. He was discharged on the tenth day without complications.

Unenhanced MDCT thus not only essentially contributes to the clinical emergency management of cocaine dealers, but also helps resolving legal questions by furnishing the immediate proof of ingested drugs, if any. The plain abdominal radiography is imperfect as a screening method.

B. Foreign objects accidentally introduced in the body:

B.1. Intra-abdominal metal splinter: A 45-year-old British man was admitted to the Emergency Department with abdominal contracture and strong pain on palpation. He refers that an unknown object entered his abdomen while working with a lawnmower. Plain radiology and CT confirm a foreign metallic object in the abdominal cavity (Figure 2). Perforation of the small intestine is confirmed by laparotomy and intestinal resection and reconstruction with T-T anastomoses was performed. The evolution was good and the patient discharged eight days later.

B.2. Aspiration of a foreign body: A 59-year-old woman visits the Emergency Department with chronic cough and, in the last 24 h, expectoration and fever. Chest radiography and thoracic CT showed an opaque segment corresponding to the left superior pulmonary lobe. Also, a hyperdense (~bone) linear image (± 2.5 cm) nearby the left bronchus was incidentally seen (Figure 3). A diagnosis of atelectasis of the left superior pulmonary lobe with stenosis of the corresponding bronchus was made.

C. Foreign objects in the context of professional malpractice:

C.1. Intra-abdominal migration of an intrauterine device (IUD): A 28-year-old Brazilian woman visits the emergency department with headache, fever, chills, nausea and vomiting. She had an IUD inserted seven years ago, with no further revision. She refers abundant vaginal bleeding that resulted in anemia. An abdominal CT shows a large irregular fluid-density lesion within the right lobe of the liver which was confirmed to be a pyogenic hepatic abscess. Also, an IUD was seen outside the uterine cavity (Figure 4). Patient underwent abscess drainage and antibiotic therapy.

C.2. Inadequate placement of a catheter: A 52 year-old man with a history of an epidermoid carcinoma of larynx was admitted to hospital with acute respiratory problems (shortness of breath) caused by an obstructive mass on the epiglottis. After the patient was stabilized, a total laryngectomy was carried out. Because of complications after surgery, medication and nutrition through a nasogastric tube were indicated. In the follow up a nasogastric tube in vena cava was revealed in radiology as an incidental finding (Figure 5).

C.3. Gauze left in abdomen: A 38 year old woman was admitted to hospital with persistent abdominal/pelvic pain after previous surgery. An abdominal/pelvic CT shows the image of a foreign body on the left side of pelvic cavity (Figure 6).

As we can see, CT has the advantage of exactly locating the foreign object and demonstrating their topographic neighbourhood, thus facilitating its extraction. Also, postmortem CT can help document the correct/incorrect position of tubes/catheters etc, prior to any autopsy and is therefore, of great value when medical-legal issues are raised [3].

2. Intentional injuries:

Cranial fractures: A 90-year-old woman was admitted in the Emergency Department with an open traumatic brain injury. The patient presented multiples incised wounds in the parieto-temporal part of skull and a depressed fracture with loss of brain parenchyma. Cranial CT showed pneumoencephalus, skull fracture (Figure 7) and a parieto-temporal brain contusion. After seven days in the intensive care unit the patient died.

Fracture distribution can be easily visualized and examined in a non destructive fashion. Also, it becomes possible to answer questions regarding the dynamics of patterned injuries and to evaluate their linkability to suspected injury-causing instruments even after the body has been buried. These methods can be used for forensic purposes in both living and deceased persons [3].

Conclusions

The application of imaging methods for non-invasive documentation and analysis of relevant forensic findings in living and dead persons has lagged behind the enormous technical development of imaging methods [4]. Imaging techniques are nowadays excellent tools for forensic medicine. They allow permanent preservation of a document of proof, whether the victim is dead and undergoing post-mortem decay or surviving and losing evidence due to healing. This method of documenting forensic findings is investigator independent, objective and non-invasive and will lead to qualitative improvements in forensic clinical and pathology investigations.

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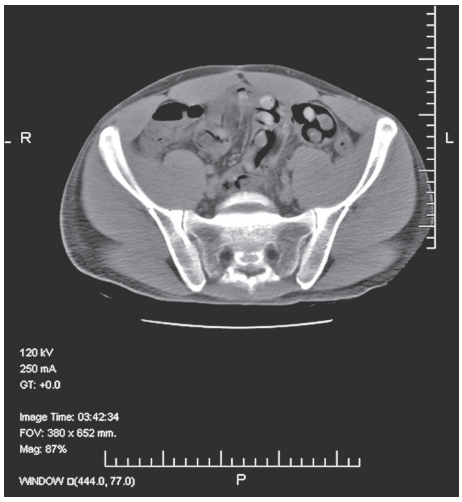

Figure 1 – CT image of the abdomen demonstrates multiple drug packages in the colon.

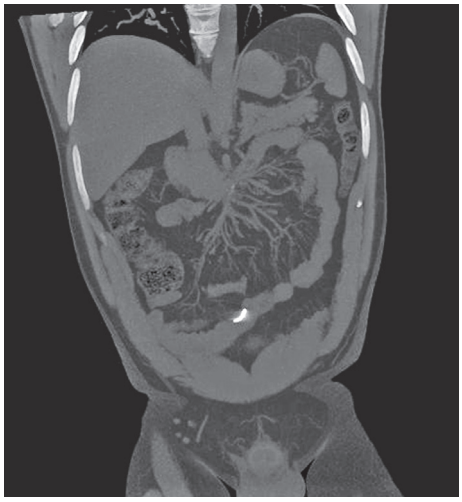

Figure 2 – Coronal MPVR-MIP confirms an intraluminal foreign body.

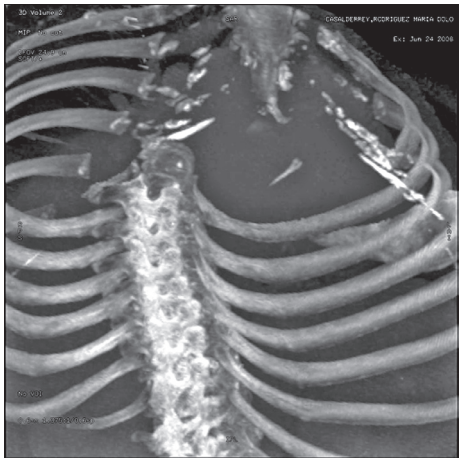

Figure 3 – MPVR identifies a high density lineal image compatible with a chicken bone.

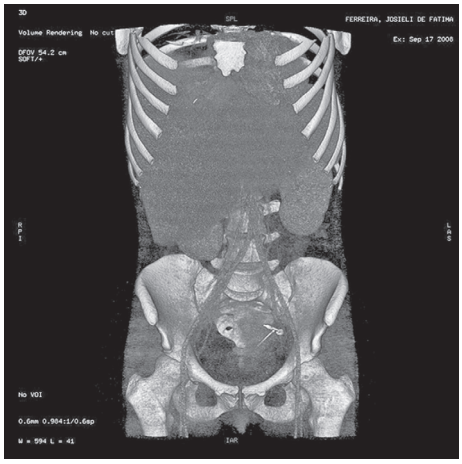

Figure 4 – MPVR clearly identifies the IUD outside the uterine cavity.

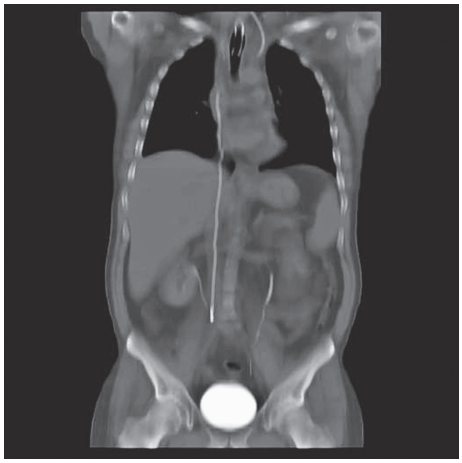

Figure 5 – MPVR shows the nasogastric tube following the path of vena cava. Incidentally, urethers can be visualized because of renal elimination of contrast.

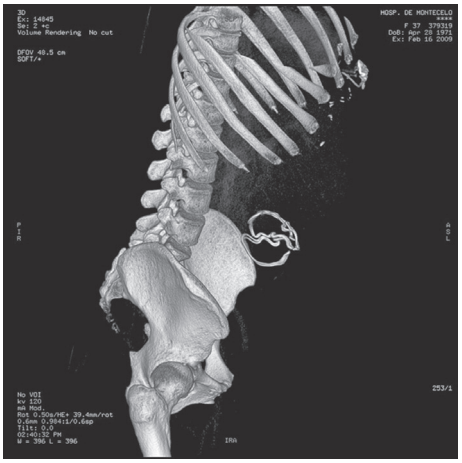

Figure 6 – MPVR showing the image of the retained gauze in abdominal cavity.

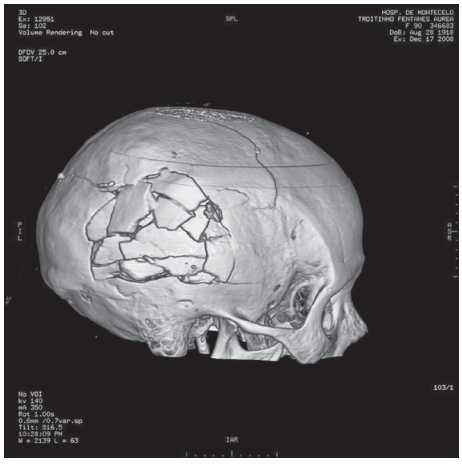

Figure 7 – Injury caused by blows to the head. Oblique lateral 3D VR CT image shows a typical local impression and ring fracture of the parietal skull due to blows with a blunt object.

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FORENSIC TOXICOLOGY

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DEATHS FROM ACUTE REACTION TO DRUGS AND PSICOACTIVE SUBSTANCES IN GALICIA IN 2005

Abstract: Drug abuse causes a large number of deaths in young people. Despite the fact that the number has decreased in recent years, it is still a major concern for our community. Drug related mortality is used as an indicator of abuse, and in Galicia these statistics are kept by the *Observatorio de Galicia sobre Drogas*. Analytical confirmation is done in the Forensic Toxicological Service of the Legal Medicine Institute of the University of Santiago de Compostela. The present study reflects the results for 2005, when 39 cases of death were suspected of drug abuse, of which 37 were analytically confirmed. Of the latter, 90% of them were males, and the median age was 35. Cocaine (56%) and opiates (54%) were the most frequently identified drugs, and poly-consumption was confirmed in 82% of the cases. Blood concentrations of drugs were very variable, and a bad correlation between opiates in blood/urine and hair was found.

Introduction

Recreative abuse of psicoactive substances is a major concern in developed societies. To know the intensity of this abuse some indicators have been developed, one of which is the Mortality Indicator (MI). In Spain the MI is elaborate based upon data obtained from the investigation of all the suspected drug-related deaths [1]. For this purpose biological samples are collected from the corpses and analyzed in different Forensic Toxicology Laboratories. In Galicia, northwestern Spain, the MI is elaborated by the *Observatorio de Galicia sobre Drogas* (OGD), founded in 1995. The toxicological analyses are performed in the Forensic Toxicological Service of the Legal Medicine Institute of the University of Santiago de Compostela.

In the ten years since the founding of the OGD, important changes have occurred related to the abuse of drugs, drug user profile, analytical techniques, and the types of samples collected. The objective of this work was to analyze the results of the cases investigated during 2005 in our Service in relation to the MI (the indicator includes all deaths related with adverse reaction to psicoactive substances) in order to establish the profile of the victim and the drugs more frequently involved in this kind of deaths

Material and Methods

Corpses from people between 10 and 64 years (range of age used to elaborate the MI), dead in Galicia in 2005 and whose cause of death was an adverse reaction to

psicoactive substances, were autopsied. Biological samples were collected and analysed in the Forensic Toxicology Service of the LMI – USC. Toxicological analyses comprised screening techniques (immunoassays and gas chromatography – mass spectrometry), and confirmation-quantitation techniques (radioimmunoassay, LC-MS or HPLC depending on the substance). The information of each case, provided by the forensic pathologist and the toxicologist, was filled in a form, which was finally sent to the ODG, to elaborate the MI. The form included information about socio- demographic, clinical, pathological or analytical variables. This information was used for this study. Analysis of data was done with the statistics package SPSS.14.0.

Results

In 2005 a total of 39 cases were investigated as suspected of death related with adverse reaction to psicoactive substances, but only 37 were confirmed by analytical methods, being 2 cases negative for any substance. Of them 90% were male, and the median age of the group was 35 years (range 19-64) (*Fig.1*). The corpses were found mainly at home (54%), followed by the street (23%). The detected drugs were, in order of frequency cocaine (56%), opiates (54%), benzodiazepines (51%), methadone (38%) and cannabis (21%). In relation with the number of different drugs detected, in most o the cases policonsume was detected, with 2 (26%), 3 (46%) and even 4 (10%) drugs identified. In the 18% of the cases only one drug was detected, and this drug was an opiate, cocaine or alcohol. The concentrations of the drugs were very variable (*Fig. 2, 3 and 4)* and, in general, they were inversely related with number of drugs (*Fig.5*), especially for the cocaine. The opiates were more frequently associated with cocaine (55%) followed by methadone (25%), while in the case of cocaine the main association was with opiates (52%) followed by benzodiazepines (43%). Finally, the most frequent association of methadone was with benzodiazepines (78%). Relation between the presence of drug blood and/or urine (indicating recent consume) and hair (indicating chronic consume) was also studied: the 46% of positive cases to opiates in blood/urine were negative in hair (*Fig.6*); in the case of positives to cocaine, only the 8% of positives in blood/urine were negative in hair.

Discussion

The number of cases was similar to the cases registered the year before in our laboratory, but lower than the figures for the previous decade, when the mean was 60 cases per year [2,3]. The distribution by sexes did not change, but the mean age of the group increased seven years (from 28 in the 90 decade until 35 in 2005). The type of drug detected changed also, from the opiates to the cocaine. This is in concordance with the pattern of abuse in the Spanish society, were the cocaine was (and is) the second illegal abused drug, after cannabis [4,5]. The policonsumer pattern is also in concordance with data from these studies. In relation with the concordance blood/hair, our results are similar to those of Druid et al [6], who reported the absence of opiates in the most recent hair segment in 18 out of 28 cases of drug-related deaths, suggesting that these individuals had a reduced tolerance to opiates. So careful segmental hair analysis can reveal recent opiate abstinence, very important in the interpretation of the tolerance. Tagliaro et al [7],

also found that the mean morphine content in the hair of opiate addicts who had died (heroin-related deaths) was lower than that of the alive addicts (active heroin addicts), (1,15 ng/mg vs 6,07 ng/mg). Under their opinion these findings support the theory of high susceptibility to opioid overdose after periods of intentional or unintentional abstinence, due to loss of tolerance. Finally, Drake et al[8] also found that fatal overdose cases were using considerably less heroin and other opiates in the period prior to death than active street users. Fatal overdose cases appeared to have been at risk from a lower tolerance to opiates and a higher level of alcohol consumption.

Conclusions

1. The number of drug related deaths in our community has stabilized in the past years. The dead profile is that of a 35 years old man, policonsumer of two, tree and even four drugs. The main drugs related with death are cocaine and opiates, but in association between them or with benzodiazepines, methadone, alcohol and THC.
2. The concentrations of the drugs were very variable, and inversely related with the number of positive drugs in the biological samples. So, potentiation of effects can be involved in some of the cases of death with low concentrations of drugs in blood.
3. Hair analysis is a useful tool in the interpretation of blood concentrations, because informs of the tolerance of the patient. Some cases of death with a low concentration of opiates in blood, and low or negative concentration of opiates in hair can be explained because of loss of tolerance. So hair analysis should be done, at least, in the cases were low blood concentrations´ of drug are found.

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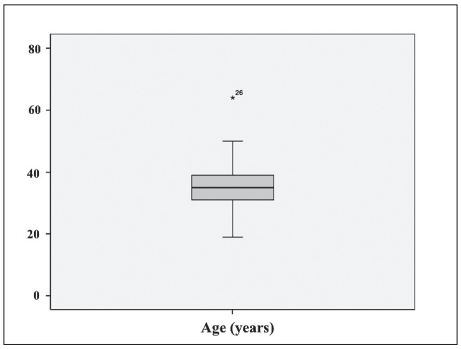

Figure 1 – Box Plot of data relative to age distribution.

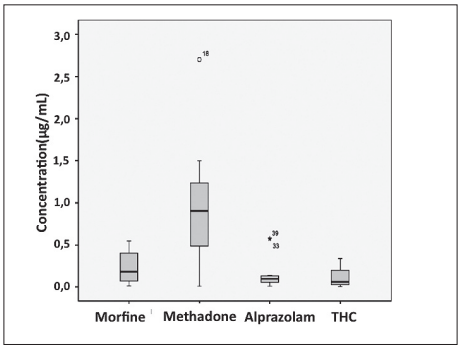

Figure 2 – Box Plot of the concentrations of some drugs in blood (µg/mL).

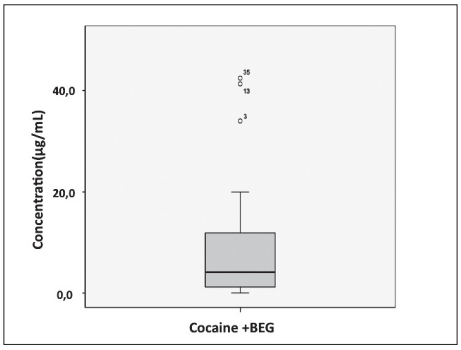

Figure 3 – Blox Plot of the blood Cocaine-BEG concentrations (µg/mL).

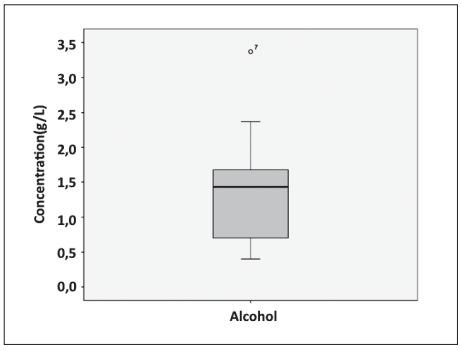

Figure 4 – Blox Plot of the blood Alcohol concentrations (g/L).

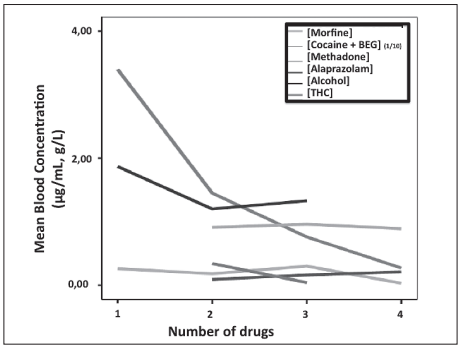

Figure 5 – Median concentration of some drug – Number of positive substances in blood ([cocaine-BEG] x (10^{-1}))

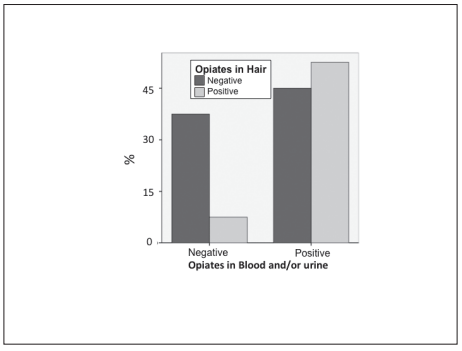

Figure 6 – Correlation between opiates in blood-urine and and opiates in hair.

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THE TOOTH AS AN ALTERNATIVE MATRIX IN FORENSIC TOXICOLOGY. APPLICATION TO THE DETERMINATION OF BENZODIAZEPINES

Abstract: Toxicological investigation of drugs in dentin can help in reconstructive identification of corpses with extensive soft tissues destruction. In this work the analysis of benzodiazepines in dental tissues was performed by LC/MS/MS. The method allows the identification and quantitation of 16 benzodiazepines, after a solid phase extraction procedure. The method was applied to 16 teeth real cases from patients under chronic treatment with these drugs. Twelve out of these cases tested positive to any benzodiazepine, while 4 were negative. This means that a positive result confirms the use of the drug, but a negative one does not exclude it.

Introduction

When studying unidentified, putrefied or skeletonised human remains, it may be difficult to obtain information on drug habits which may prove important for the construction of a biological profile [1], in the context of a reconstructive identification process. Dentin is a hard dental tissue that in vital teeth is in contact with the blood that irrigates the dental pulp and, therefore, the chemicals presents in blood can reach the dentin. But it is also a tissue with a slow metabolic turnover, meaning that the incorporation of chemicals into the dentin probably takes place at a slow rate. Therefore teeth should be evaluated as an alternative biological matrix in the investigation of chronic exposure to chemicals. Although hair is usually the matrix of choice for this purpose [2], in some forensic cases it may have disappeared (extensive destruction of soft tissue in burned corpses and, in general, in the cases where identification of the human remains is done by forensic dentistry techniques). In these circumstances, and due to the extraordinary hardness of dental tissues, teeth can be the only available biological matrix and an invaluable source of data from a toxicological point of view.

Material and Methods

Apparatus. The HPLC system was a Waters Alliance 2795 Separation Module with a Waters Alliance series column heater/cooler (Waters, Mildford, MA, USA). For the

chromatographic separation, an Atlantis® T3 3µm (2,1x100mm) (Waters, Mildford, USA) was employed, using 2 mM ammonium formate buffer pH 3 and acetonitrile as mobile phase at a flow rate of 0.2 mL/min (gradient mode). The column temperature was kept at 26ºC. The total run time was 17 min.

For the detection, a tandem mass spectrometer Quattro Micro™ API ESCI (Waters, Mildford, USA) with a triple quadrupole was employed. The instrument was operated in electrospray in the positive ionization mode (ESI +). Nitrogen was used as nebulization and desolvation gas at a flow rate of 500 L/h, heated to 450ºC, and as cone gas at a flow of 50 L/h. Capillary voltage and source block temperature were 3 kV and 140ºC, respectively.

Chemicals, reagents and standard solutions. Alprazolam, Oxazepam, Lorazepam, Tetrazepam, Clorazepam, Bromazepam, Zolpidem, Midazolam, Diazepam, αOH-Alprazolam, Triazolam, Lormetazepam, Nordiazepam, Flunitrazepam (FNZ), Zopiclone and 7-amino-FNZ, Zolpidem-d6, FNZ-d7, Alprazolam-d6, Oxazepam-d6, αOH-Alprazolam-d5, 7-amino-FNZ-d7 y Diazepam-d5 were obtained from Cerilliant (*LGC Standards* Barcelona, Spain). All reagents of analytical grade were obtained from Merck (Darmstadt, Germany). A stock standard solution for each compound was prepared at 10 µg/mL in methanol. Working solutions were prepared by appropriate dilution of these stock standards in methanol.

Teeth samples. Drug-free teeth samples were obtained from healthy donors from a public hospital and a private dental clinic. Teeth from patients under benzodiazepine treatment were obtained from the Special Patients Unit of the Dentistry Faculty (Santiago de Compostela, Spain).

Sample preparation and extraction. Teeth were first shredded in a crushing device consisted of two aluminum plates and a hydraulic press. Teeth fragments were then pulverized in a ball mill (Precellys 24), and intact enamel fragments were discarded. 300 mg of the pulverized dentin were transferred into a vial and 5 mL of borate buffer pH 9 added. After overnight incubation, the buffer was filtered and submitted to solid phase extraction (OASIS HLB 3cc, Waters). The eluate was then evaporated to dryness and reconstituted with 60 µL of mobile phase. Finally, 40 µL were injected into the HPLC system.

Results and discussion

The method was validated for 16 benzodiazepines, achieving limits of detection from 0,1 to 5 ng/g. Although full validation was not performed, selectivity, linearity, precision and accuracy (interday and intraday) were under the acceptable analytical criteria [3,4]. The analysis of blank teeth samples showed the absence of any trace of interfering peaks from endogenous compounds at the corresponding retention times of each analyte. Representative chromatogram of a real sample is shown in Figure 1.

The method was applied to 16 real samples, obtained from patients under chronic treatment (lasting from 1,5 to 15 years) with benzodiazepines. Results are shown in Table 1. In four cases any BZD was detected, and in the remaining 12 cases concentrations were highly variable (0.168-352 ng/g). In 11 out of the 16 cases, the identified benzodiazepine was the one prescribed to the patient. Alprazolam was the

most prevalent substance, detected in 7 out of 16 cases, which could mean this analyte has a higher affinity for the dental tissues. In case #1, besides the parent drug, some metabolites were detected. In eight samples, the active substance identified was different to the one referred in the anamnesis, maybe due to the failure of the anamnesis in identifying all the benzodiazepines taken by the patient.

In four cases the analysis was negative, even after 8, 10 or 15 years of treatment. This could be due to the lack of vitality of the tooth when the benzodiazepine treatment was prescribed. In these cases, blood supply to the dental pulp is eliminated, so any drug can reach the dental tissues. Vital conditions of the teeth were not known when the treatment was established. Under these results, dental tissues are suitable to identify chronic exposure to drugs. Nevertheless, although a positive result confirm the use of the substance, a negative result does not exclude it.

Other authors also identified drugs in dental tissues, such as opiates and cocaine [1,5], and found similar results. Under their opinion, the presence of drugs in dental tissues is difficult to interpret, but can help in the identification process of human remains. Pascual et al [6] also used dental tissues to quantify the environmental exposure of children to tobacco smoke, being able to identify nicotine and cotinine in deciduos teeth.

Conclusions

Dental tissues can be used in toxicological analysis when other common matrices are not available. The identification of benzodiazepines in teeth confirms the treatment with these drugs. This can help in a reconstructive identification process in those cases where forensic odontology techniques have to be used.

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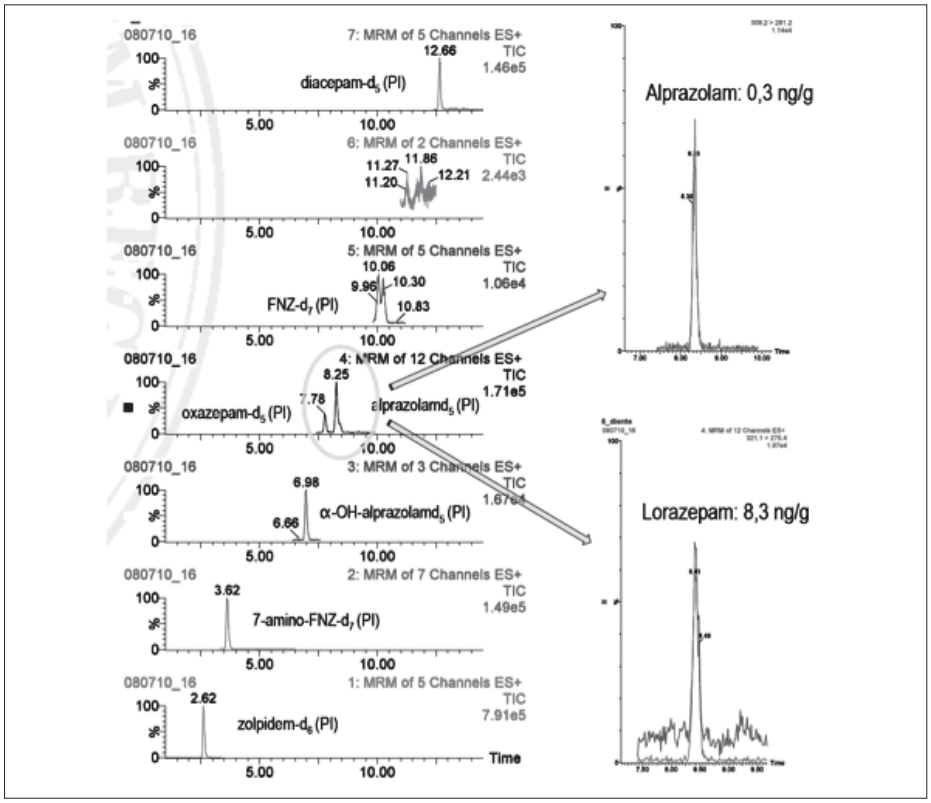

Figure 1 – Chromatogram of a real case showing alprazolam and lorazepam in dental tissues.

Anamnesis Information			Toxicological Results	
CASE Nº	PRESCRIBED BZD	TREATMENT PERIOD	IDENTIFIED BDZ	CONCENTRATION ng/g
1	Clorazepate Dipotassium	3 years	Oxacepam Nordiazepam Diazepam	5.14 352.34 Traces
2	Clorazepate Dipotassium	5 years	Diazepam	Traces
3	Ketazolam	Not Known	Oxacepam Lorazepam Nordiazepam	14.81 171.12 4.62
4	Bromaz/Lormetazepam	1.5 years	Lormetazepam Diazepam	1.46 0.96
5	Lorazepam	3 years	Midazolam Lorazepam	1.26 4.14
6	Diazepam	10 years	N/D	N/D
7	Bromazepam	15 years	N/D	N/D
8	Alprazolam	3 years	Alprazolam Lorazepam	0.36 8.35
9	Alprazolam	3 years	Alprazolam	1.05
10	Alprazolam	5 years	Alprazolam	2.80
11	Alprazolam	7 years	Alprazolam	5.64
12	Alprazolam	8 years	Alprazolam Nordiazepam	0.46 77.88
13	Alprazolam	8 years	Alprazolam	0.16
14	Alprazolam	8 years	Alprazolam Nordiazepam	5.30 19.53
15	Bromazepam	15 years	N/D	N/D
16	Alprazolam	8 years	N/D	N/D

(N/D = Not Detected)

Table 1 – Results of the analysis of the real cases.

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SEVERE COMPLICATIONS FOLLOWING LOW-DOSE APPLICATION OF METHOTREXATE

Abstract: Methotrexate is a folate antagonist used to treat malignant tumors, mostly combined with other cytostatic drugs and/or radiotherapy. However, it can also be applied for therapy of chronic polyarthritis and other joint-destroying rheumatic diseases. Low-dose methotrexate (15–25 mg/week) is indicated as the basic therapeutic agent for these diseases. Even this low-dose therapy may damage bone marrow and thus cause potentially lethal infections. In the context of basic antirheumatic drug therapy, the use of methotrexate requires strict indications and frequent laboratory follow-ups. Interactions with other pharmaceutical agents must also be considered.

Introduction

Methotrexate (MTX) is a folate antagonist used in combined chemotherapy or chemoradiotherapy regimens for treatment of malignant tumors, especially leukemias with central nervous system manifestations (1). Other indications are rheumatoid arthritis and other chronic-rheumatic illnesses, but doses are lower than those used for neoplastic diseases (2,3). Even this therapeutic dosage (low-dose therapy) can cause substantial side effects largely involving the hematopoietic system. Hepatotoxic effects, interstitial ("atypical") pneumonia and mucositis may also occur (4,5,6). These unwanted side effects appear mainly in the presence of factors that increase the accumulation and thus potentiate MTX toxicity. These factors include pre-existing infections and simultaneous therapy with more than five drugs as well as renal insufficiency, since MTX is renally excreted (7). The minimum lethal dose is 10 mg/week for patients with renal insufficiency. Even slightly reduced renal function can prolong the radioactive half-life of MTX (8) and thus lead to intoxication.

Material and Methods

We evaluated five cases of death from the Department of Forensic Medicine, University Medical Centre Hamburg-Eppendorf, Germany, where MTX application in low-dosages was performed.

Clinical appearance

Chronic rheumatoid arthritis is often an illness of the elderly (9), thus mainly affecting multimorbid patients with various pre-existing internal diseases such as diabetes mellitus, arterial hypertension, (compensated) cardiac and/or renal insufficiency, coronary heart disease and arrhythmias. These pre-existing diseases may complicate the clinical diagnosis of MTX intoxication under low-dose therapy, since symptoms of generalized immunodeficiency may also be due to advanced age or associated geriatric diseases and conditions, e.g., hematologic conditions. A characteristic finding is the rapidly deteriorating general condition of the patient. Rapidly decreasing thrombocyte and leukocyte levels lead to pancytopenia and agranulocytosis with toxic bone marrow depression, mainly accompanied by atypical pneumonia and nonspecific neurological symptoms such as confusion. Patients may also develop hemorrhagic inflammation of the gastrointestinal tract such as duodenitis, glossitis and stomatitis, followed by dysphagia and petechial bleedings in the oral cavity. Death is usually due to fulminant sepsis.

Autopsy

Common autopsy findings are septic multiple organ failure with shock lungs, hemorrhagic pneumonia, brain oedema, fibrinous pericarditis and ubiquitous petechial bleedings (due to disseminated intravasal coagulation). Noteworthy is the lack of immunoreactions.

Histology of bone marrow

Histological examination typically shows high-grade insufficiency with hypocellular bone marrow, reduced thrombopoiesis and erythropoiesis and severely depressed granulopoiesis with only scattered immature stages indicative of toxic marrow damage (Fig.1). Chloroacetate esterase reaction reveal almost no mature cells (Fig.2), a marked left shift of granulocytopoiesis with a preponderance of immature leukocytes is also noticeable (Fig. 3).

Histology of the lungs

Histological lung sections show fibrinous plaques in the alveoli and disseminated hemorrhagic and fibrinous pneumonic infiltrates with a distinctly noncellular appearance. There is no cellular inflammatory reaction (Fig 4).

Conclusions

Following current guidelines (10), a detailed history and a hemogram must be obtained before initiating therapy with MTX. This applies particularly to liver parameters as well as creatinine, alkaline phosphatase and thrombocyte levels. Under current MTX therapy, hemograms must be taken at intervals of 4-8 weeks to adapt MTX dosages. Caution is urged to avoid accidental MTX overdosing, which can occur easily, since MTX is mostly given to patients who have long been receiving nonsteroidal antirheumatics (NSAR). Simultaneous medication with MTX and NSAR may lead to an excessively

high concentration of MTX in blood. This may be explained by the renal excretion of MTX (7) and the analgesic nephropathy attributed to long-term NSAR intake (11). Furthermore, chronic rheumatic diseases are treated with corticoid drugs, which are also regarded as potentially nephrotoxic; chronic renal insufficiency is a relative contraindication for their application (12). The pancytopenia and toxic agranulocytosis resulting from renal decompensation might be clinically detected, but subsequent inflammations will often remain uncontrollable and resistant to antibiotics at this stage. MTX is known to interact with different antibiotics (13). According to the literature, aminoglycoside antibiotics are especially likely to potentiate the toxic effects of MTX and are associated with ototoxicity and nephrotoxicity triggered by electrolyte shifts (14,15). Thus renal insufficiency may also be induced or worsened. MTX overdoses should therefore be treated causally by folate administration and forced diuresis. Severe courses require haemodialysis, hemoperfusion, plasma exchange and treatment with Granulokine. Despite the above complications, antibiotic therapy should be initiated. Frequent monitoring of relevant parameters is essential to promptly control the side effects of MTX therapy. Furthermore, low-dose MTX therapy should be restricted to specialists – some deaths are at least partially attributable to iatrogenic complications. MTX therapy requires regular monitoring before and during its application as well as necessary dose adjustments.

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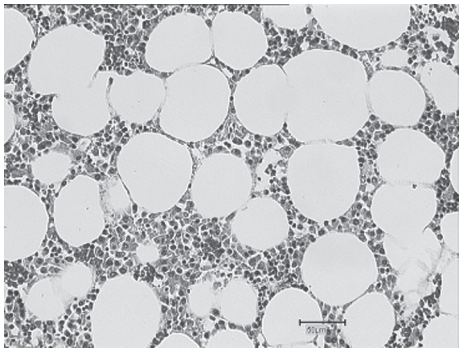

Figure 1 – Defective erythropoiesis and reduced granulocytopoiesis. No metamyelocytes, stab cells or mature granulocytes. Only scattered megakaryoblasts and histiocytes are detectable (HE x 50).

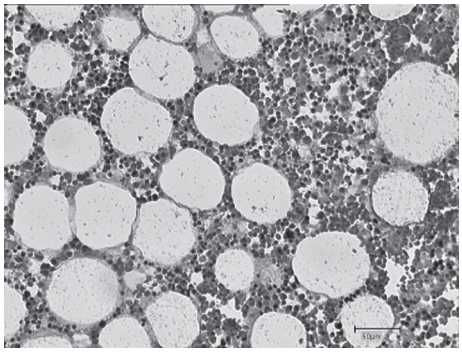

Figure 2 – Marked reduction of granulopoiesis and thrombopoiesis; lack of stab cells and polymorphs. Petechial bleedings on the right side of the screen (HE x 50).

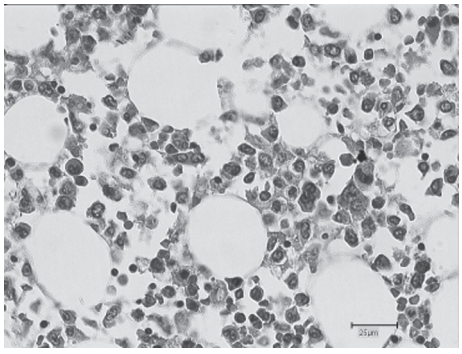

Figure 3 – Marked left shift of erythro-, granulo- and thrombopoiesis with defects mainly in granulopoiesis (HE x 100).

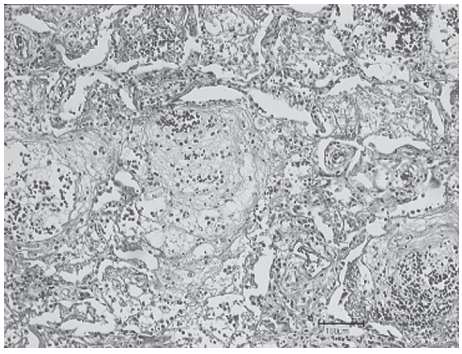

Figure 4 – chronic florid, partly organized pneumonia with loose, fibrinous mesenchymal structures in the alveoli. Few inflammatory cells with no mature granulocytes in the exudate (HE x 25).

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ERYTHROPOIETIN DOPING AS CAUSE OF SUDDEN DEATH IN ATHLETES – AN EXPERIMENTAL STUDY

Abstract: *Aims:* To evaluate the cardiovascular (CV) effects of rhEPO treatment in rats under chronic aerobic exercise and to assess the probable cause of sudden death in one rat.
Protocol: Male Wistar rats: control – sedentary; rhEPO – 50 IU/Kg/3xwk; swimming (EX) – 1hr, 3x/wk; EX+EPO. Haematology, catecholamines and serotonin, redox status and inflammation, were assessed. One rat of EX+EPO group suffered a sudden death episode.
Results: rhEPO treatment in trained rats promoted several markers of increased CV risk. The sudden death rat tissues presented: lungs without signs of drowning; brain with vascular congestion; LV hypertrpphy and deregulation of cardiac fibers, together with a "cardiac liver", suggesting the hypothesis of heart failure as cause of death.
Conclusion: The sudden death of a EX+EPO rat, due to a cardiac episode, together with the increased CV risk profile, strongly suggest a high life risk associated to the continuous rhEPO doping. The anatomo-pathological studies were determinant to establish the cause of death.
Keywords: rhEPO; doping; chronic aerobic exercise; sudden death.

Introduction

Erythropoietin (EPO) is a glycoprotein hormone synthesized predominantly in the kidneys which stimulates proliferation and maturation of erythroid cells in the bone marrow (1). The increase in circulating RBC may be used to increase O2 delivery to muscles, improving performance in sport (2). The availability of recombinant human EPO (rhEPO) allowed its use in doping. Sports authorities prohibited the use of rhEPO in 1988. The idea was, first, to limit both the degree of health risk and, second, the degree of performance enhancement. The abusive use of rhEPO promotes an increment of BP, which, together with the increase of Hct and blood viscosity, strengthens the probability of cardio/cerebrovascular events (3).

In the early 1990s, there was a considerable speculation about the involvement of rhEPO doping in the death of professional cyclists (4,5). The artificial increase in RBC and Hct, further enhanced by dehydratation during prolonged exercise, predisposes to thromboembolic complications, which might be connected to sudden death in sport practice (6). However, the cellular/molecular mechanisms underlying the sudden death episodes are poorly clarified, as well as whether rhEPO use was linked to this outrageous phenomenon.

This study intended to evaluate the CV effects of rhEPO treatment on rats under chronic aerobic exercise; we also studied the probable cause of sudden death occurred in one rat.

Material and methods

Animals and protocol

Male Wistar rats (Charles River Lab., Spain), 220-250g, were maintained in appropriate conditioned: 22-24°C; 60% humidity; 12-h dark-light cycles; standard rat chow (AO4, Panlab, Letica, Spain) and water *ad libitum*.

After a period of adaptation of 2 wks, 4 groups (n=8) were tested for 10 wks-treatment: control – sedentary (SED); rhEPO – 50 IU/Kg/3x/wk beta-EPO Recormon®, Roche Pharm. (EPO); Exercised (EX) – swimming (1 hr, 3x/wk); EX+EPO. The swimming rats were submitted to a 1 wk period of adaptation for minimizing the water stress (bath set at 35±1°C). Sessions started with 15 min, increased 5 min/day until a 60 min continuous period was achieved. Excepting 1 animal of the EX+EPO group, which suffered a sudden death episode during an exercise session (wk 8), all the animals have completed the 10-week protocol. Body weight (BW) was monitored, and blood pressure (BP) and heart rate (HR) measured.

Sample collection and preparation

Serum samples were obtained from blood collected with i.p. ketamine anesthesia at the end of treatments. The heart weights (HW) were measured in order to be used as trophy index (HW/BW). The following tissues were removed from the sudden death rat: lungs, kidneys, brain, heart/left ventricle (LV) and liver and analyzed for histomorphology (H&E staining).

Haematological data and renal function

Red blood cell (RBC) count, Hct and haemoglobin (Hb) were assessed by using an automatic Coulter Counter® (Beckman Coulter Inc., USA). Serum creatinine, ureia and uric acid concentrations were assessed through an automatic Hitachi 717 analyser.

Catecholamine and serotonin

Plasma noradrenaline (NA), adrenaline (A), 5-hydroxy-tryptamine (5-HT) and 5-hydroxy-indoleacetic acid (5-HIAA) concentrations were evaluated by HPLC-ED (7).

Redox status

The thiobarbituric acid reactive-species (TBARs) assay was used to assess serum products of lipid peroxidation, via malondialdehyde (MDA), according to previously described (7). Serum 3-nitrotyrosine (3-NT), which is an index of peroxynitrite formation, was measured through an enzymatic immunoassay (HyCult-biotec., Netherlands). Serum total antioxidant status (TAS) was assessed via the ferric reducing antioxidant potential (FRAP) assay (7).

Inflammatory profile

Serum levels of interleukin 2 (IL-2), IL-1β, transforming growth factor β1 (TGF-β1), tumour necrosis factor α (TNF-α) and C-reactive protein (CRP) were measured by Elisa kits (R&D Systems, USA).

Data analysis

Results are means ± s.e.m. Comparisons between groups were performed using one-way ANOVA and Fisher's test. Significance was accepted at p less than 0.05.

Results

Haematological data and renal function

EX+EPO rats presented a significant (8.23±0.14, $p<0.05$) increase in RBC count vs the EX group (7.59±0.15). Haematocrit and Hb showed a trend to identical variation. Exercised rats presented significantly lower values of serum urea (17.35±0.26, $p<0.05$) and uric acid (0.40±0.06 mg/dL, $p<0.01$). This reduction was prevented in the EX+EPO rats (18.60±0.63 and 0.50±0.03, respectively).

Blood pressure, HR and heart trophy

BPs were higher in the EPO group vs control. The same pattern was found for the EX group (SBP: 123.92±1.38, DBP: 108.33±1.34 and MBP: 113.25±0.99 mmHg; $p<0.05$ and HR: 394.58±8.66 beats/min). The EX+EPO rats presented a further increased in BPs (136.67±1.08, 123.22±2.04 and 127.33±1.62, respectively; $p<0.001$) and HR (418.44±6.57, $p<0.05$). HW and HW/BW were significantly higher in the EPO group vs control. The EX+EPO rats presented a further increment in HW (1.40±0.03 g, $p<0.01$) and HW/BW (3.06±0.16 g/kg, $p<0.05$) vs EX group (1.23±0.03 and 2.65±0.10).

Catecholamine and serotoninergic measures

In the EX+EPO rats, the plasma NA (9.32±1.43 ng/ml, $p<0.05$) and AD (1.96±0.18, $p<0.05$) were significantly higher when compared with EX (5.10±0.96 and 1.04±0.09). Concerning the serotoninergic plasma measures, the EX+EPO rats presented plasma 5-HT (30.07±4.45 ng/ml, $p>0.001$) and 5-HIAA (25.07±2.38, $p<0.05$) substantially higher than those found in EX (11.08±0.65 and 18.00±2.94).

Redox status and inflammatory profile

The EX+EPO rats presented a pro-oxidant effect, with a trend to increased values of serum MDA (0.34±0.01 μmol/L), MDA/TAS (1.53±0.05) and 3-NT (42.26±6.90 nmol/L) vs EX (0.30±0.02, 1.27±0.09 and 37.96±7.31, respectively).

Concerning the serum inflammatory markers, in the EX+EPO group there was significantly higher values of TGF-β1 (375.7±23.5 pg/mL, $p<0.05$) and a trend to higher values of IL-2 (59.08±3.76 pg/mL) vs EX (317.8±15.1 and 51.48±4.11, respectively).

Histomorphological analysis of tissues from the sudden death rat (Fig. 1 and Fig. 2)

The rat HW was 1.82 g and the HW/BW was 4.04, significantly hypertrophic vs the EX (1.23±0.03 and 2.65±0.10, respectively), demonstrating the tremendous effort of the heart to maintain its functions. The histomorphological studies provided the following results: the kidneys (1B1: glomerular and 1B2: tubular) from the suddenly death rat showed eosinophilia and congestion, when compared with control kidneys (1A1 and A2); the lungs showed signs of blood congestion, alveolar hemorrhage and anoxia, without markers of drowning (1B3), vs control (1A3); the brain presented vascular congestion (2B1) vs control (2A1); the liver showed centre-lobular congestion and signals of "cardiac-liver", probably due to the heart failure (2B2) vs the normal pattern of control (2A2); there was LVH and desregulation of cardiac fibers (2A3 vs 2B3), suggesting the hypothesis of heart failure as cause of death.

Discussion

Since rhEPO became available as an erthropoiesis-stimulating drug, its abusing use by athletes of endurance aerobic sports has been speculated and studied (2,8-10). In endurance sports, such as long-distance running, cycling and skiing, performance relies on an adequate O2-supply to the heart and skeletal muscle. Hence, the rate of maximal O2-uptake is an important determinant of aerobic physical power. However, athletes who abuse rhEPO seem to consider only the benefit to performance and ignore the short and long-term side-effects. There is a suspicion that rhEPO-induced erythrocytosis caused the death of about 20 world-class cyclists, although this was never proven (4,5), probably due to the lack of methodological capacity to distinguish between the endogenous and the recombinant EPO as well as due the lack of knowledge concerning the mechanisms underlying the side-effects. When Lasne and de Ceaurriz (11) were able to distinguish the endogenous and the rhEPO in human urine, the scandal of rhEPO use in sports was revealed, and the scientific/medical community was able to alert for the high health risks for the athletes.

The main risks of erythrocytosis (Hct>0.55 l/l) include hypertension (HT), heart failure, myocardial infarction and thromboembolic events. Endurance athletes are at increased risk during the competition, if their blood viscosity increases further due to the great loss of fluid associated with sweating (4,5,9,12). Interestingly, some deaths allegedly caused by rhEPO have not occurred during exercise but during periods of physical inactivity, suggesting that the deleterious effects are prolonged.

In our study, the rats under chronic exercise practice and rhEPO treatment showed several markers of increased CV/thromboembolic risk. The increased RBC count, Hct and Hb vs EX was confirmed, as expected. This was accompanied by development of HT and tachycardia. Increased BP is a common feature in patients and athletes under rhEPO treatment (8,9,13), and might result both from hyperviscosity and loss of hypoxia-induced vasodilatation. rhEPO treatment was also able to promote heart hypertrophy, which might be due to the blood hyperviscosity and could be viewed as a need to ensure proper blood circulation to peripheral tissues. Increased tachycardia might be explained by the increment in sympathetic activity, revealed by the increment in plasma NA and AD. This effect of rhEPO was previously documented, namely on hemodialyzed patients under rhEPO therapy (14). Furthermore, there was an increment in plasma 5-HT, which might result from platelet overactivation, thus releasing the granule contents. The increased platelet reactivity was reported by others (3), and is in favour of an increased BP and thromboembolic complications.

rhEPO has been successfully used in anaemic patients to correct their anaemia. However, its effects on non-hematopoietic cells and tissues, such as the brain and the heart, suggested new important insights to its use in other pathological conditions, such as the ischemia-reperfusion, heart failure and neurodegenerative diseases (15). The rationale for its potential use in those disorders is based on its antioxidant, anti-apoptotic and anti-inflammatory properties, already known as "pleiotropic actions" (16). In our study, both the rhEPO treatment (*per se*) and the exercise practice have demonstrated a beneficial effect on the redox status markers. However, rhEPO use in rats under exercise favoured oxidative stress, given by the higher MDA/TAS index and 3-NT content, which, considering the deleterious effect of ROS, represent an increased risk. This pattern was accompanied by a trend to higher values of IL-2 and CRP, and a significant increment in TGF-β1. While the increase in the proliferation marker TGF-β1 might eventually explain the heart hypertrophy, the increase in IL-2 and CRP suggest an inflammatory effect, further strengthening the deleterious actions of rhEPO treatment in situations of regular exercise.

All the changes reported for the EX+rhEPO rats seem to be in agreement with the sudden death episode occurred in one rat of the group, after 8 wks of protocol. Actually, the rhEPO treatment in trained rats promoted an increase in RBC count (contributing to hyperviscosity), HT, heart hypertrophy, sympathetic and serotonergic overactivation, as well as oxidative stress and inflammation. The anatomo-pathological tissue evaluation of a suddenly death rat, demonstrated that there were no drowning signs in the lungs, despite some congestion marks. The kidneys showed some eosinophilia and the brain revealed vascular congestion. Furthermore, and even more relevant, there was some LVH and deregulation of cardiac fibers, together with a "cardiac liver", suggesting the hypothesis of heart failure as the cause of death, which is in agreement with the increased risk of cardio/cerebrovascular and thromboembolic events that the functional studies in the EX+EPO also indicate.

Conclusion

The sudden death of a rat belonging to the Ex+EPO group, due to a cardiac episode, together with the increased CV risk profile, strongly suggest a high life risk associated to the continuous rhEPO doping. The anatomo-pathological studies were determinant to establish the cause of death.

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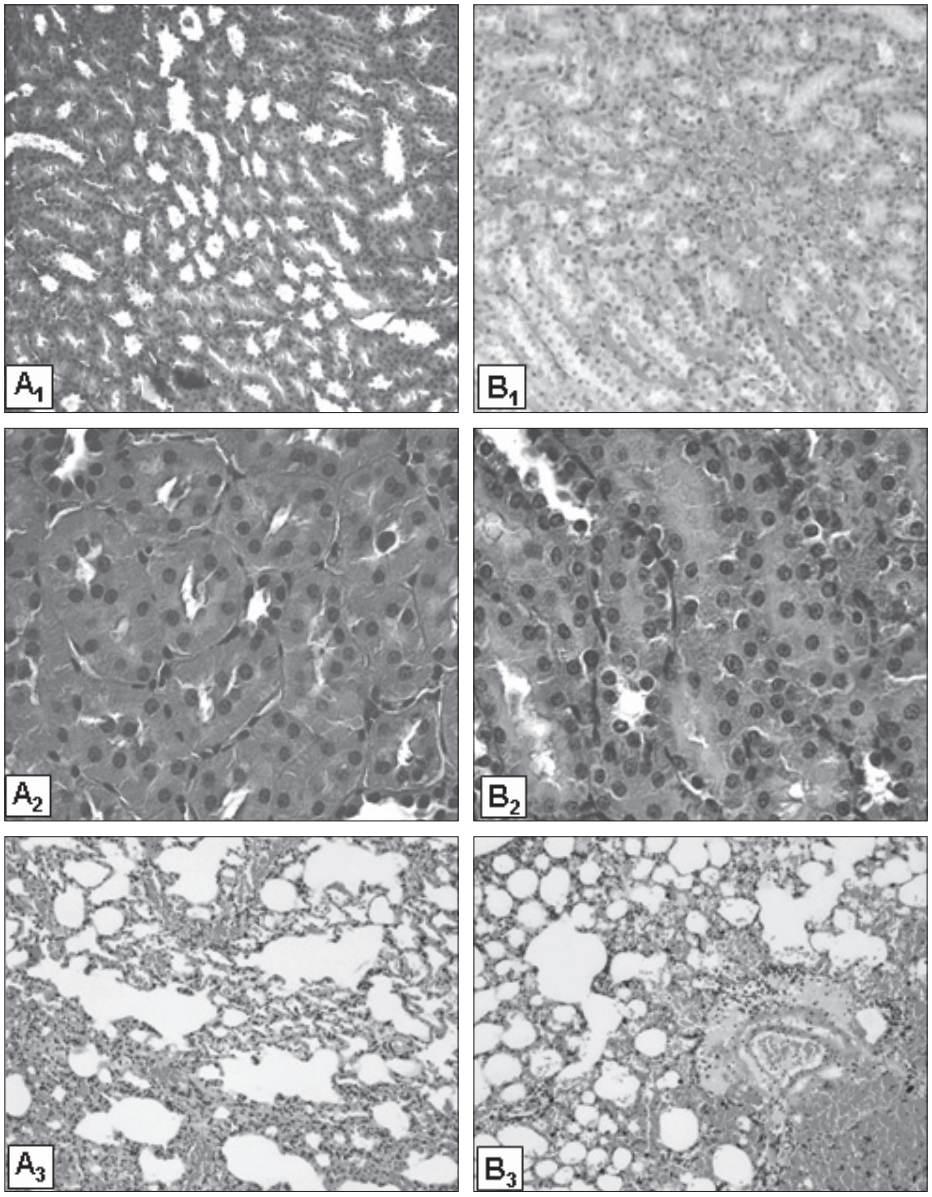

Figure 1 – Histomorphological H&E staining pictures from the kidney glomerular (1) and tubular (2) regions and from the liver (3) from the control rats (A) when compared with those of the sudden death rat of the EX+EPO group (B).

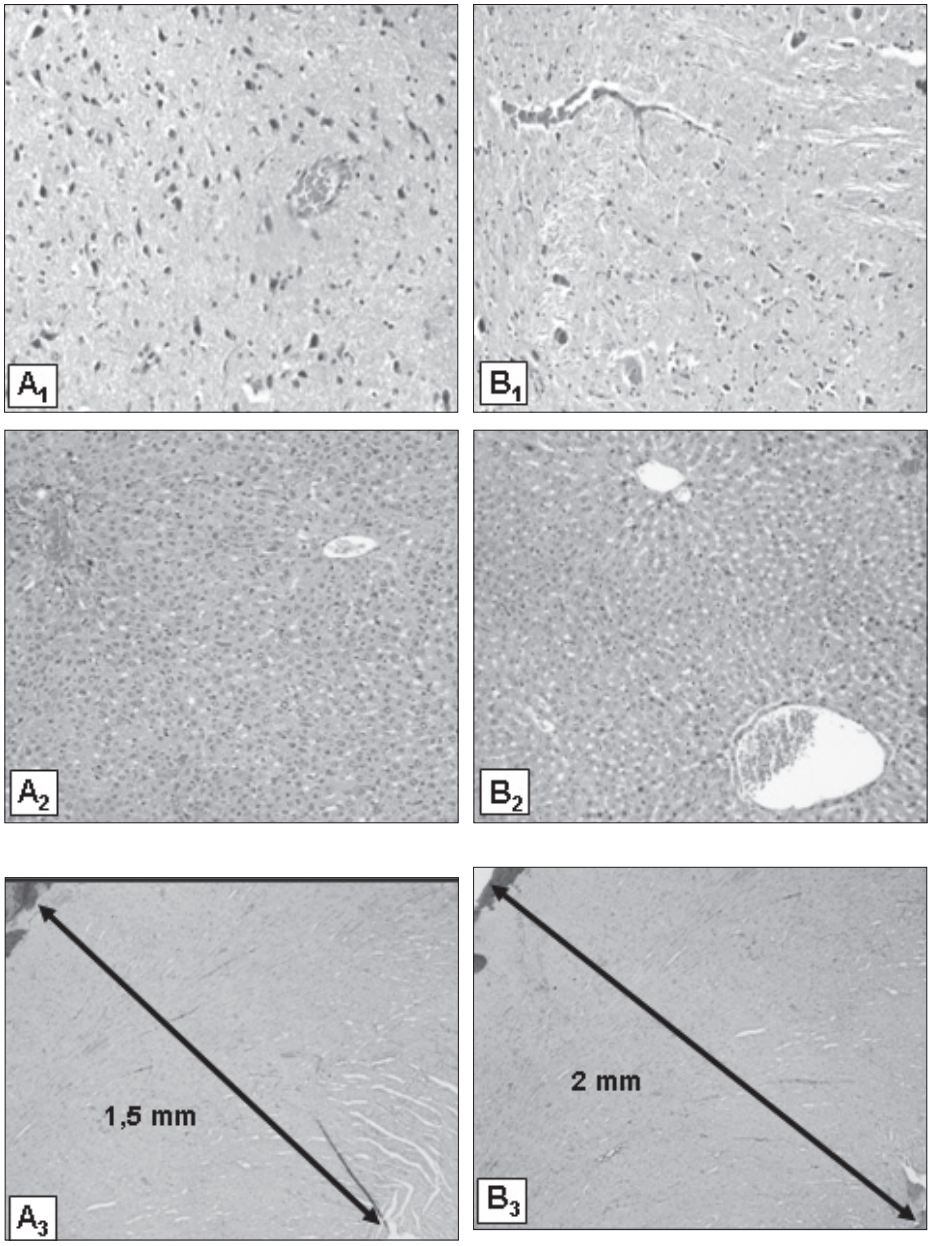

Figure 2 – Histomorphological H&E staining pictures from the brain (1), the liver (2) and the left ventricle (3) from the control rats (A) when compared with those of the sudden death rat of the EX+EPO group (B).

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TOXICOLOGICAL EFFECTS OF RECOMBINANT HUMAN ERYTHROPOIETIN DOPING – CHRONIC VERSUS ACUTE AEROBIC EXERCISE

Abstract: *Aims:* Compare the toxicological effects of rhEPO on rats under chronic versus acute exercise.

Protocol: Male Wistar rat groups for the chronic exercise (swimming) – 10 wks of treatment: – control – sedentary (SED); – rhEPO – 50 IU/Kg/wk; swimming (EX) – 1 hr, 3 times/wk; EX+EPO. For the extenuating exercise (rhEPO given for 3 wks prior to exercise): – swimming (Swi); – Swi+EPO (50 IU/Kg/wk); – running (Run); – Run+EPO. Blood and tissue samples were assessed for: haematology, catecholamine and serotoninergic measures and redox status.

Results: The chronic EX+EPO rats showed higher values of RBC, Htc and Hb vs EX and vs Swi+EPO of the acute sessions. Both chronic and acute swimming showed a remarkable sympathetic and serotonergic activation. rhEPO treatment in chronic training has promoted oxidative stress, in contrast with the antioxidant effect on Swi and Run of acute exercises.

Conclusions: rhEPO doping is more deleterious in rats mimicking high-performance athletes (chronic training) than in occasional consumers (acute sessions), due to increased CV risk.

Keywords: rhEPO; doping; toxicological effects; chronic *vs* acute aerobic exercise; haemogram; sympathetic and serotoninergic activation; oxidative stress.

Introduction

Erythropoietin (EPO) is a circulating glycosylated protein hormone, synthesized mainly in the kidneys, that is the primary regulator of RBC formation (1). The production of recombinant human erythropoietin (rhEPO), which has been widely used for correction of anaemia, allowed many patients to resume their normal daily activities due to increased energy (2).

The rationale for the use of rhEPO in sport, as doping, is based on the increased O2 capacity it provides, due to augmented erythropoietic stimulation (3). As soon as the anti-doping authorities were able to distinguish between the endogenous and the rhEPO (4), the scandal of its use in sport was revealed, with particular emphasis to cycling and cross-country skiing, between other sport modalities (5,6).

Athletes who abuse rhEPO consider only the benefit to performance and usually ignore the potential short and long-term liabilities. Elevated Hct and dehydratation associated with intense exercise may reveal undetected CV risk in some athletes (7,8). Illegal and abusive utilization of this hormone has been found in both endurance and short-duration sports, which require distinct energetic sources, but the potential deleterious effects and mechanism underlying, remain to be fully elucidated.

This study intended to compare the toxicological effects of rhEPO treatment on rats under chronic vs acute exercise, as well as to assess the differences between two distinct modalities of extenuating exercise.

Material and methods

Animals and experimental protocol

Male Wistar rats (Charles River Lab., Spain), 220-250g, were maintained in appropriate conditioned: 22-24ºC; 60% humidity; 12-h dark-light cycles; standard rat chow (AO4, Panlab, Letica, Spain) and water *ad libitum*.

For the chronic exercise (swimming), after a period of adaptation of 2 week, 4 groups (n=8) were evaluated for 10 wks-treatment: control – sedentary (SED); rhEPO – 50 IU/Kg/wk beta-EPO Recormon®, Roche Pharm. (EPO); Exercised (EX) – swimming (1 hr, 3 times/wk); EX+EPO. The swimming rats were submitted to a 1 wk period of adaptation for minimizing the water stress (bath set at 35±1ºC). Sessions started with 15 min, increased 5 min each day until a 60 min continuous period was achieved.

For the acute exercise, the following groups were tested: swimming (Swi); Swi+EPO; running (Run) and Run+EPO. rhEPO (50 IU/Kg/wk) was given for 3 wks prior to the extenuating sessions. Running was performed in a treadmill (Letica LE 8706, Spain) at the velocity of 54 cm/s and an inclination of 15%. Both exercises were made without previous adaptation and until extenuation. Duration and/or distance were monitored.

Sample collection and preparation

Serum, plasma and platelets were obtained from the rat blood collected i.p. ketamine anesthesia at the end of treatments. Some tissues were excised and stored for further analysis: adrenals, left ventricle (LV) and brain in HClO4 and gastrocnenius muscle in liquid nitrogen.

Haematological data

Red blood cell (RBC) count, haematocrit (Hct), haemoglobin (Hb), platelets count, mean platelet volume (MPV), platelet distribution width (PDW) and plaquetocrit (PCT) were assessed by using an automatic Coulter Counter® (Beckman Coulter Inc., USA).

Catecholamine and serotonin assay

Noradrenaline (NA) and adrenaline (A) concentrations in plasma, platelet, adrenals, left ventricle and brain, as well as plasma, platelet and brain 5-hydroxy-tryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) contents, were evaluated by HPLC-ED, according to previously described (9). Concentrations were expressed in ng/ml for plasma and platelets and in µg/g for adrenals, left ventricle and brain.

Redox status

The thiobarbituric acid reactive-species (TBARs) assay was used to assess serum and muscle products of lipid peroxidation (via malondialdehyde: MDA), according to previously described (9). MDA concentration was expressed as µmol/l in serum and as µmol/g tissue in muscle. Ferric reducing antioxidant potential (FRAP) assay was used to estimate serum and muscle total antioxidant status (TAS) (9).

Data Analysis

Results are presented as means ± s.e.m. Comparisons between groups were performed using one-way ANOVA and Fisher's test. Significance was accepted at p less than 0.05.

Results

Acute exercise performance

The animals from the EX group have swim for 50.67 ± 2.19 min, while the Swi+EPO rats have performed a longer swimming period (56.33±5.24 min). Similar rhEPO influence was found for the Run (38.67±2.19 min) and Run+EPO groups (43.00±6.11 min). In this assay, the distance of run was also higher in the rats under rhEPO treatment (678.33±75.08 m), when compared with those without rhEPO (662.67±30.69 m).

Haematological data (Table 1)

The most important findings were that rhEPO in the chronic exercise was able to increase RBC count (p<0.05), with a trend to increased Hct and Hb, while in the acute there was a significant reduction (p<0.01) of those parameters in the Swi protocol, with a trend to reduce in the Run. Platelet count showed a trend to increased values in the chronic Swi+EPO rats vs EX. Similar pattern, but significant (p<0.05), was found in the Swi+EPO of the acute exercise, contrasting with the trend to lower values in the Run extenuating sessions with rhEPO.

Catecholamine and serotonin measures (Table 2)

In the chronic Swi+EPO animals, there was an increment in plasma NA (p<0.05) and AD (p<0.01) contents, accompanied by a trend to NA reduction in adrenals and platelets and a significant decrease in the LV (p<0.001), together with a trend to AD increment in adrenals and a significant reduction (p<0.001) in platelets. An identical

plasma NA and AD (p<0.001) pattern of changes was found for the acute Swi, being the levels higher vs Run+rhEPO. The NA and AD increase in the acute Swi+EPO rats was accompanied by a reduction in platelet NA and by an increment in brain AD. In the acute Run+EPO, the main changes were a trend to higher values of NA in plasma and adrenals and reduction in platelets, together with a trend to lower values of AD in plasma and higher in platelets and adrenals.

While in the chronic training the changes were non-significant for 5-HT and 5-HIAA in the plasma, platelets and brain, in the Swi+EPO of the acute sessions there was an increment in plasma measures and a reduction in platelets. Similar pattern was found for Run+EPO vs Run.

Serum and muscle redox status (Table 3)

In serum samples, the redox status, evaluated by the MDA/TAS levels, increased (p<0.05) in the Swi+EPO chronic exercise, while there was a trend to a reduction in the Swi+EPO extenuating exercise. Similar pattern was found for the muscle assays, showing that rhEPO was pro-oxidant when given in chronic training conditions but antioxidant in acute. Concerning the extenuating exercises, the running is notoriously more oxidative than the swimming, but the rhEPO treatment demonstrated similar antioxidant profile, both in serum and muscle samples.

Discussion

The therapeutic use of rhEPO, particularly for the treatment of anaemia, allowed a significant reduction in the associated adverse effects and improved patient's quality of life (2). Unfortunately, some athletes and their coaches were eager to abuse rhEPO because it increases the O2 supply to muscles and boots performance in endurance sports, such as skiing, running and cycling (3). This led to a view among some athletes that to compete successfully doping with rhEPO was required, forgetting the increased health risk. The increased Hct above certain levels causes important side-effects, which includes hypertension (HT), heart failure, myocardial infarction, peripheral thromboembolic events and pulmonary embolism, as well as shorten lifespan (10,11).

Athletes are at increased risk during the competition, due to the blood hyperviscosity, further aggravated by the great loss of fluid associated with sweating (6-8). In the early 1990s, there was considerable speculation that doping with rhEPO was involved in the death of professional cyclists (8), some of them occurred during periods of physical inactivity, indicating that the deleterious effects remain after the competition. Abusive use of rhEPO might be viewed in both endurance and short-duration sports, which require distinct energetic sources, but the potential deleterious effects and mechanisms underlying remain to be fully elucidated. Our data confirmed that rhEPO promoted an augmented sports performance.

The consequences of physical exercise on the EPO concentrations have been poorly investigated. In a study with marathon athletes under rhEPO treatment, serum EPO levels increased after both 3 and 31 hrs after exercise, but were unchanged immediately after the end of running (12). In our study, rhEPO treatment was able to increase the

RBC, the Hct and Hb in chronic exercise, without significant changes in acute, or even with a decrease, particularly in swimming extenuating. Thus, prolonged rhEPO treatment (10 wks in chronic) seems to be able to promote important changes on erythropoiesis, while in short-term (3 wks prior acute) do not produce identical stimulation. This should have distinct implications in CV risk, with an expected hyperviscosity in chronic exercise with rhEPO use.

HT is one of the main deleterious effects of rhEPO therapy (13), but, apart from the blood hyperviscosity, the mechanisms underlying remain to be fully explained. Sympathetic nervous system (SNS) overactivity have been suggested as a possible explanation (14). In our study, swimming promoted more pronounced effects on catecholamines levels than running, confirming previous data from us (9). Both the chronic and the acute swimming exercise showed a remarkable sympathetic and serotonergic activation, which might be due to the cardio-respiratory involvement, favouring the CV risk.

Distinct types and intensities of exercise have been associated with different effects on oxidative stress. Regular training is able to promote antioxidant actions (15), while high intensity exercise or in non-adapted individuals might produce harmful effects, in a dual effect known as "exercise paradox". The final effect seems to depend, thus, on the intensity as well as on the type of protocol. rhEPO treatment have been associated with beneficial therapeutic effects on non-anaemic conditions, due to its cardio and neuroprotective actions, attributed to a pleiotropic action, such as its antioxidant ability (16). In our study, rhEPO was notoriously more deleterious when used in chronic conditions, demonstrating a pro-oxidative action, contrasting to its putative antioxidant effect when used in acute extenuating exercises. This effect might be due to the lower duration of rhEPO treatment prior to extenuating exercise session (3 wks), when contrasting with the 10 wks for the chronic exercise, as well as with particular characteristics of acute exercise. Therefore, extenuating exercise leads to important autonomic and haemodynamic adaptations that influence the CV system in order to maintain homeostasis in response to the increase of metabolic needs. This includes augment of cardiorespiratory responses to promote increase of O2 supply to peripheral tissues; SNS activation, which increases HR and cardiac output and, then, BP fluxes to peripheral tissues, particularly to the muscles that needs more energy to produce work (17). Under those conditions, rhEPO seem to be needed, playing thus an antioxidant effetc, contrasting with its deleterious pro-oxidant profile when used in prolonged and regular training condition, mimicking chronic rhEPO doping.

Conclusions

The effects of rhEPO doping in rats under exercise is notoriously more deleterious in circumstances that mimic high-performance athletes (chronic training) than in occasional consumers (acute sessions), particular due to increased cardiovascular risk.

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Parameters	CHRONIC EXERCISE (training)			
	Sedentary		Swimming	
	No rhEPO	With rhEPO	No rhEPO	With rhEPO
RBCs				
RBC count (x10^{12}/L)	7.31 ± 0.16	7.67 ± 0.08*	7.59 ± 0.15	8.23 ± 0.14*
Hb (g/dL)	14.45 ± 0.65	14.11 ± 0.15	14.86 ± 0.25	15.45 ± 0.45
Hct (%)	41.45 ± 1.65	39.59 ± 0.45	41.40 ± 0.82	44.05 ± 1.45
Platelet				
Platelet count (x10^{9}/L)	904.0 ± 9.0	986.3 ± 41.5	1008.4 ± 35.9	1021.0 ± 57.0
PCT (%)	0.55 ± 0.02	0.56 ± 0.02	0.57 ± 0.02	0.60 ± 0.07
MPV (fL)	6.15 ± 0.25	5.69 ± 0.06*	5.63 ± 0.14	5.85 ± 0.35
PDW (%)	16.85 ± 0.15	16.73 ± 0.18	16.37 ± 0.26	17.00 ± 0.10
Parameters	ACUTE EXERCISE (extenuating session)			
	Sedentary		Swimming	
	No rhEPO	With rhEPO	No rhEPO	With rhEPO
RBCs				
RBC count (x10^{12}/L)	8.11 ± 0.23	6.65 ± 0.21**	7.97 ± 0.20	7.96 ± 0.21
Hb (g/dL)	14.70 ± 0.12	13.05 ± 0.55**	15.17 ± 0.20	14.83 ± 0.03
Hct (%)	40.73 ± 0.42	35.80 ± 1.70**	42.67 ± 0.64	41.87 ± 0.62
Platelet				
Platelet count (x10^{9}/L)	993.3 ± 40.7	1223.5 ± 144.5*	929.0 ± 69.0	774.0 ± 18.2
PCT (%)	0.55 ± 0.01	0.64 ± 0.08	0.44 ± 0.06	0.46 ± 0.01
MPV (fL)	5.50 ± 0.17	5.25 ± 0.05	5.87 ± 0.47	5.93 ± 0.23
PDW (%)	16.17 ± 0.43	15.70 ± 0.00	16.47 ± 0.58	16.63 ± 0.46

Results are means ± s.e.m. *p<0.05, **p<0.01 and ***p<0.001 *vs* the No rhEPO group.

Table 1 – Effects of rhEPO on haematological data in chronic and acute exercise protocols

Parameters	CHRONIC EXERCISE (training)			
	Sedentary		Swimming	
	No rhEPO	With rhEPO	No rhEPO	With rhEPO
Catecholamines measures				
Plasma NA (ng/ml)	3.71 ± 0.60	4.81 ± 0.37	5.10 ± 0.96	9.32 ± 1.43
AD (ng/ml)	1.48 ± 0.21	1.52 ± 0.06	1.04 ± 0.09	1.96 ± 0.18
Platelet NA (ng/ml)	4.54 ± 0.61	0.60 ± 0.08***	8.02 ± 0.68	7.01 ± 0.47
AD (ng/ml)	0.69 ± 0.04	0.36 ± 0.08**	9.15 ± 2.26	0.50 ± 0.09
Adrenals NA (µg/g)	164.1 ± 8.0	130.4 ± 9.6*	149.8 ± 15.8	133.8 ± 7.9
AD (µg/g)	626.0 ± 47.6	602.0 ± 66.7	433.1 ± 24.6	579.4 ± 40.6
L.Ventricl. NA (µg/g)	0.14 ± 0.02	0.71 ± 0.05***	0.92 ± 0.04***	0.12 ± 0.02***
AD (µg/g)	0.02 ± 0.01	0.05 ± 0.01	0.15 ± 0.02***	0.13 ± 0.02***
Brain NA (µg/g)	0.20 ± 0.004	0.18 ± 0.007	0.21 ± 0.008	0.19 ± 0.008
AD (µg/g)	2.03 ± 0.09	2.38 ± 0.18	1.79 ± 0.25	2.57 ± 0.15*
Serotoninergic measures				
Plasma 5-HT (ng/ml)	18.56 ± 1.46	5.82 ± 0.60***	11.08 ± 0.65	30.07 ± 4.45***
5-HIAA (ng/ml)	11.53 ± 0.93	17.56 ± 1.20**	18.00 ± 2.94	25.07 ± 2.38*
Platelet 5-HT (ng/ml)	556.7 ± 40.9	830.0 ± 27.2***	1610.8 ± 55.1	1640.4 ± 39.6
5-HIAA (ng/ml)	3.92 ± 0.24	2.74 ± 0.18**	2.99 ± 0.22	3.68 ± 0.30
Brain 5-HT (ng/g)	0.25 ± 0.01	0.30 ± 0.01*	0.24 ± 0.01	0.22 ± 0.01
5-HIAA (ng/g)	0.13 ± 0.004	0.12 ± 0.007	0.13 ± 0.005	0.13 ± 0.006

Parameters	ACUTE EXERCISE (extenuating session)			
	Sedentary		Swimming	
	No rhEPO	With rhEPO	No rhEPO	With rhEPO
Catecholamines measures				
Plasma NA (ng/ml)	3.91 ± 0.85	5.29 ± 1.95	1.52 ± 0.33	2.29 ± 0.55
AD (ng/ml)	1.95 ± 0.33	4.60 ± 0.41**	1.76 ± 0.02	1.15 ± 0.29
Platelet NA (ng/ml)	4.41 ± 0.79	2.13 ± 0.37	2.68 ± 1.46	1.50 ± 0.36
AD (ng/ml)	1.09 ± 0.01	1.59 ± 0.30	2.01 ± 0.86	2.72 ± 0.46
Adrenals NA (µg/g)	188.3 ± 11.0	188.1 ± 96.7	96.9 ± 24.1	158.4 ± 16.1
AD (µg/g)	865.0 ± 107.6	778.5 ± 423.6	411.5 ± 117.4	645.0 ± 40.4
L.Ventricl. NA (µg/g)	0.48 ± 0.11	0.43 ± 0.07	0.48 ± 0.01	0.45 ± 0.05
AD (µg/g)	0.10 ± 0.03	0.11 ± 0.02	0.24 ± 0.03gg	0.16 ± 0.01i
Brain NA (µg/g)	0.16 ± 0.01	0.18 ± 0.03	0.15 ± 0.01	0.22 ± 0.02
AD (µg/g)	3.95 ± 0.06	10.35 ± 2.25*	7.57 ± 1.40	5.90 ± 1.57
Serotoninergic measures				
Plasma 5-HT (ng/ml)	85.97 ± 20.70	533.4 ± 60.7***	75.83 ± 39.12	27.31 ± 6.43
5-HIAA (ng/ml)	17.10 ± 0.71	21.72 ± 0.62	18.60 ± 2.75	21.62 ± 1.51
Platelet 5-HT (ng/ml)	1122.2 ± 135.9	727.6 ± 45.2	622.8 ± 266.6	528.8 ± 104.9
5-HIAA (ng/ml)	4.34 ± 0.74	0.65 ± 0.14*	4.75 ± 0.91	2.82 ± 0.54
Brain 5-HT (ng/g)	0.14 ± 0.04	0.21 ± 0.03	0.16 ± 0.01	0.18 ± 0.02
5-HIAA (ng/g)	0.25 ± 0.02	0.24 ± 0.03	0.27 ± 0.03	0.30 ± 0.06

Results are means ± s.e.m. *p<0.05, **p<0.01 and ***p<0.001 *vs* the No rhEPO group.

Table 2 – Effects of rhEPO on peripheral and central catecholamine and serotoninergic measures in chronic and acute exercise protocols

Parameters	CHRONIC EXERCISE (training)			
	Sedentary		Swimming	
	No rhEPO	With rhEPO	No rhEPO	With rhEPO
Serum redox status				
MDA (µmol/L)	0.40 ± 0.02	0.38 ± 0.04	0.30 ± 0.02*	0.34 ± 0.01
TAS (µmol/L)	0.24 ± 0.01	0.36 ± 0.03***	0.25 ± 0.01	0.22 ± 0.01
MDA/TAS	1.76 ± 0.16	1.13 ± 0.23*	1.27 ± 0.09*	1.53 ± 0.05
Muscle redox status				
MDA (µmol/L)	0.29 ± 0.03	0.36 ± 0.02	0.38 ± 0.04	0.47 ± 0.04*
TAS (µmol/L)	0.14 ± 0.003	0.12 ± 0.001	0.12 ± 0.003	0.12 ± 0.009
MDA/TAS	0.41 ± 0.04	0.63 ± 0.04**	0.58 ± 0.03*	0.73 ± 0.06*
Parameters	ACUTE EXERCISE (extenuating session)			
	Sedentary		Swimming	
	No rhEPO	With rhEPO	No rhEPO	With rhEPO
Serum redox status				
MDA (µmol/L)	0.29 ± 0.08	0.22 ± 0.13	0.29 ± 0.10	0.36 ± 0.06
TAS (µmol/L)	0.45 ± 0.14	0.65 ± 0.20	0.56 ± 0.13	0.71 ± 0.04
MDA/TAS	0.37 ± 0.02	0.28 ± 0.14	0.67 ± 0.33	0.53 ± 0.11
Muscle redox status				
MDA (µmol/L)	1.04 ± 0.31	0.73 ± 0.09	1.82 ± 0.61	1.47 ± 0.34
TAS (µmol/L)	1.36 ± 0.22	1.57 ± 0.34	1.04 ± 0.32	1.31 ± 0.20
MDA/TAS	0.84 ± 0.35	0.48 ± 0.10	1.89 ± 0.58	1.19 ± 0.33

Results are means ± s.e.m. *p<0.05, **p<0.01 and ***p<0.001 *vs* the No rhEPO group.

Table 3 – Effects of rhEPO on serum and muscle redox status markers in chronic and acute exercise protocols

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DRUGS OF ABUSE ANALYSIS IN FATAL VICTIMS OF ROAD TRAFFIC AND LABOUR ACCIDENTS IN THE CENTRE OF PORTUGAL BETWEEN 1990 AND 2007

Abstract: Driving under the influence of drugs is an issue of growing concern in the industrialized countries as a risk and a cause for road and labour accidents. The aim of this study was to assess the presence of drugs of abuse among drivers and workers involved in fatal accidents between January 1990 and December 2007, by presenting the requests and toxicological results of corresponding autopsies, from the Pathology Service of the Centre Branch of the National Institute of Legal Medicine and from a Legal Medicine Office. In the Forensic Pathology Service, between 1990 and 2007, a total of 3095 autopsies on road traffic accidents victims and 261 on labour accidents victims were performed and 336 28, respectively, in the office. In both cases, few requests and analyses of drugs of abuse were performed, emphasising the fact that the major percentage of the accidents involved individuals aged between 21 and 30 years-old, ages where the consumption is more often. Nevertheless, from the 261 victims of labour accidents autopsied, only 7 were subject of drug analysis, all with negative results.

Keywords: Drugs of abuse; road traffic and labour accidents.

Introduction

During the last years, driving under the influence of drugs other than alcohol has gained considerable attention as a problem to road traffic and labour accidents [1]. Increasing occurrence of driving under influence of non-alcohol drugs has been reported from several countries [2-4].

This paper presents a retrospective analysis of the toxicological investigation on biological samples from fatally injured drivers, between January 1990 and December 2007, to determine the role, if any, of ethanol amongst fatal road traffic accidents.

Material and Methods

This study has been carried out by the National Institute of Legal Medicine of Portugal. The target population consisted of drivers killed in road and labour accidents. The data available in each case was: year, sex, age and drugs concentrations.

The authors present the requests and consequent drug results of the road traffic accidents autopsies and on fatal victims of labour accidents performed from 1990 and 2007 in the Pathology Service of the Centre Branch of the National Institute of Legal Medicine (NILM) and performed from April 2001 (open date of the office) and 2007 in the Legal Medicine Office of Figueira da Foz. All drug analyses were performed in the Forensic Toxicology Laboratory of the Centre Branch of the NILM.

Several variables were studied and all the pertinent data was registered, separated and statistically treated with the SPSS program (Statistical Package will be Social Sciences).

Results and Discussion

Driving is a very complex task, during which the drivers continually receives information, analyzes it and react. The different steps involved in the practice of driving involve functions that are interrelated in a very narrow. The whole process is, likewise, closely related to the knowledge of the driver and his attitude quickly reflected in their behaviour while driving.

In the Forensic Pathology Service a total of 9409 autopsies were performed between 1990 and 2007, 3095 on road traffic accidents victims and 261 on labour accidents victims, 78% and 97% in male victims, respectively (Fig.1).

We found that the most troubling months in which there was a higher number of autopsies in road traffic accidents was the month of August for the Branch, but considerably different for the LMO, which had the highest number of cases in October since this could be justified by the increased movement on Portuguese roads due to holidays and tourism, family visiting emigrants abroad, or even due to sleep disturbance, especially during the afternoon, usually after lunch.

For the LMO, we can only assume that accidents occur mainly in October, probably due to unusual weather conditions, the early days of rain and consequent association of roads with oil and water, resulting in imminent risk of accident.

Drugs of abuse analysis were requested in only 4% of the road traffic accident cases and in 3% of the labour accidents (Figs. 2, 3).

In the Legal Medicine Office, from April 2001 to 2007, 1219 autopsies were performed, 336 to car accident victims, 82% men, and 28 to labour victims, with only one female victim (Fig.4).

In the car accident fatal deaths, 17% included drugs of abuse analysis, as well as 32% of the labour accidents (Figs. 5, 6).

As expected, the increase of the consumption of illicit substances in our country led to a growing concern, not only to their effect on health, but to all risk behaviours and deviant behaviour of the consumer [5]. Therefore, and as mentioned before, with the introduction of specific legislation in Portugal, concerning driving under the influence of alcohol and psychotropic substances, there is a greater awareness by all the experts with regard to the obligation to request drugs of abuse in cases of fatal car accidents. It is important to emphasise the fact that the introduction of a new law in 2007 also increased the requests, since before that only drivers involved in road accidents (seriously injured or death) would be subject to the screening of drugs of abuse. Currently, drivers can be monitored by the mere fact that law enforcement agents suspect the consumption of such substances. This was indeed a very important measure and valid introduced in our country.

Note, however, that despite the positive result of only about 9.5%, it is not possible to conclude that there is a low consumption of such substances when combined with driving. However, as mentioned before, this research was performed in only 4.4% (Branch) and 17.3% (LMO) of autopsied cases of traffic accident, leaving open all other drivers where such research was not even considered.

Important to add to this the fact that the greatest percentage of cases where no request was made for drugs focused on individuals aged from 21 to 30 years-old, corresponding to ages where the consumption of such substances have a greater effect.

From the road accident cases analyzed, 9 drugs were positive mainly in men (84%) aged between 21 and 30 years-old, being the opiates (47.1%) and canabinoids (50%) the most encountered groups.

Portuguese law concerning labour monitoring significantly addresses the problem of alcohol and psychotropic substances, because workers can, by law, be subject to a random check by the employer at any time of their activity. However, its review or determination in postmortem cases is not fully informed or legislated, in relation to how to act specifically in labour accident autopsies. Accordingly, the discrepancy of results concerns the practice carried out by experts who, more and more, are being aware and scientifically instructed to further research and evaluation of substance influence in labour accidents and consequent legal aspects implicated, such as life insurance, for example.

As already mentioned before, once again, for labour accidents, few requests of drugs of abuse were performed and only 9 of the 28 labour accidents victims were subject of drug analysis, all with negative results. From the total 58 requests (17%) for drugs in the road traffic victims, 5 were positive, mainly for cannabinoids and cocaine.

It is, however, extremely important to emphasize the fact that 96% of the cases autopsied in the Pathology Service did not include the drug analysis request, 21% with individuals aged between 21 and 30 years-old, ages where the consumption is more often. Nevertheless, from the 261 victims of labour accidents autopsied, only 7 were subject of drug analysis, all with negative results (Fig. 7).

Once again, in the Legal Medicine Office, 83% of the cases did not include the drug analysis request, 41.7% with individuals aged between 21 and 30 years-old, ages where the consumption is more often (Fig. 8).

The requests for drugs of abuse analysis in labour accident deaths were substantially reduced, taking into account the potential that these substances can have through the cognitive and psychomotor skills of an individual, which negatively affects their job performance and leading inevitably to accidents [6]. Note that, from a total of 261 accidents recorded in the Branch, only 7 included the requests of drugs of abuse.

Also in the LMO, only 9 requests for drugs were performed on the 28 victims of labour accidents. It can be perfectly understood since they were cases of individuals aged over 55 years. It should be noted, however, that, as mentioned above, both the Branch and the LMO, had high rates of accidents in people aged between 21 and 40 years.

We observed that none of the 16 cases examined were positive for drugs of abuse. It is important to state that in 18 years analyzed for the delegation, there were only 261 autopsies on labour accident victims and only 28 victims in the 7 years analyzed in LMO. However, only these 289 cases included 16 drug analyses and, given the shortage of requests, that information is clearly not statistically significant, impossible to predict the consumption of drugs of abuse in labour accidents.

Conclusion

Drugs of abuse can be defined as all chemical substances, psychotropic, which illicit consumption occurs more or less compulsively. Driving under the influence of drugs is an issue of growing concern in the industrialized countries as a risk and a cause for road and labour accidents.

The most common drugs (other than alcohol) found in fatally cases have been cannabis, benzodiazepines, amphetamine-like stimulants and opiates [7].

For road accidents, there were almost no requests of drugs of abuse, corresponding to only 4.4% of the cases in the Centre Branch and 17.3% in the LMO.

The requests for drugs of abuse analysis in labour accident deaths were substantially reduced: from a total of 261 accidents in the Branch, only 7 included this request. The same was observed for the LMO, where only 9 requests for drugs were performed on 28 victims of labour accidents autopsies.

It is easy to conclude that there are few requests for drugs of abuse in both car and labour fatal accidents. Traffic and workplace safety is a major concern for society. Aside from alcohol, there are other sources of impairment, like the illicit drugs, that may be related to accident risk. However, whereas the evidence of impairment from alcohol and fatigue has been sufficiently demonstrated to provide a basis for some policies, the evidence that certain classes of drugs may impair and, thereby, increase accident risk, is inconclusive. In contrast to alcohol, reliable epidemiologic data are not available and must be done, for e.g., by the increasing of drugs analysis requests in cases of road traffic and labour accidents.

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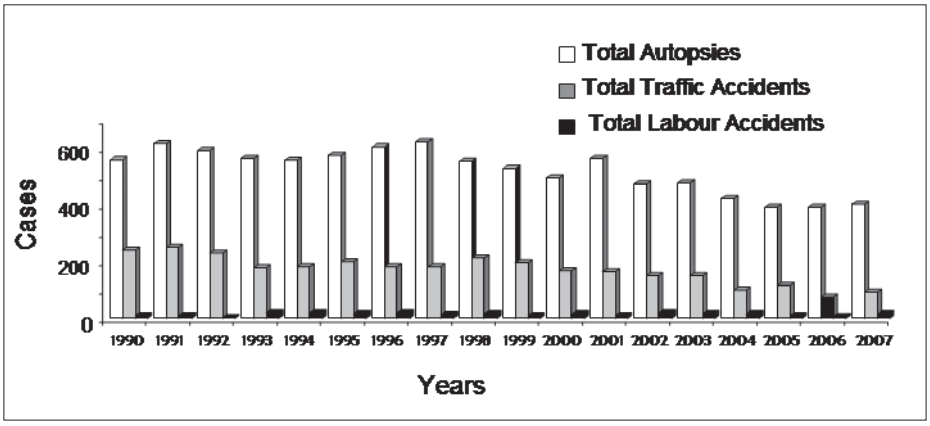

Figure 1 – Number of Traffic & Labour Accidents in the autopsies performed in the forensic pathology service between 1990 and 2007.

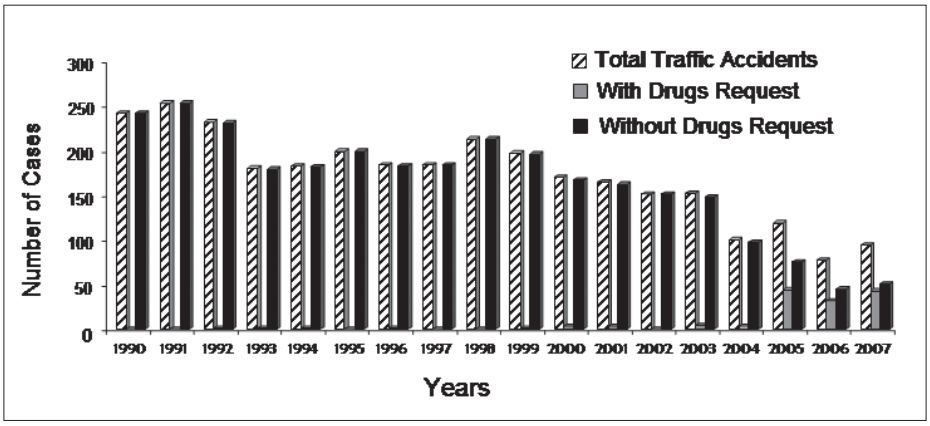

Figure 2 – Number of Traffic Accidents with and without Drugs request (in the forensic pathology service).

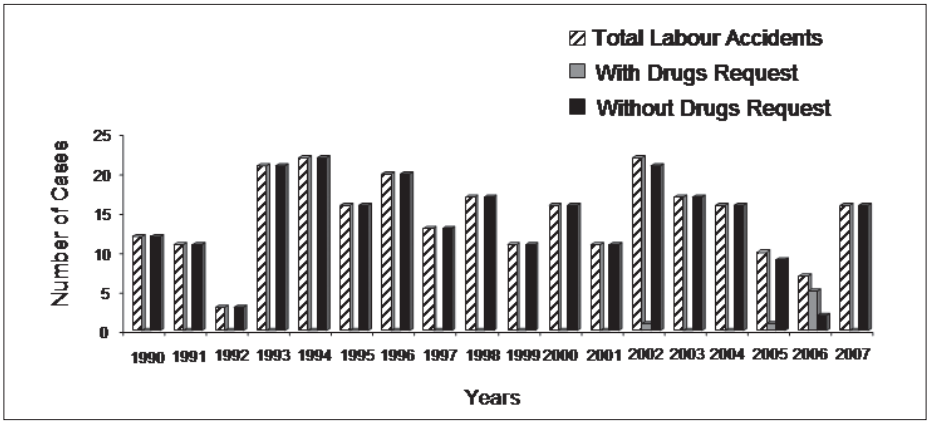

Figure 3 – Number of Labour Accidents with and without Drugs request (in the forensic pathology service).

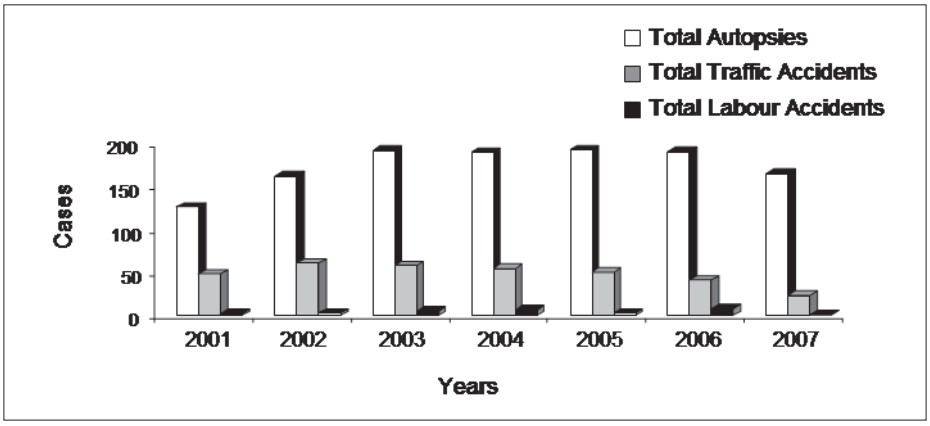

Figure 4 – Number of Traffic & Labour Accidents in the autopsies performed in the Legal Medicine office between 2001 and 2007.

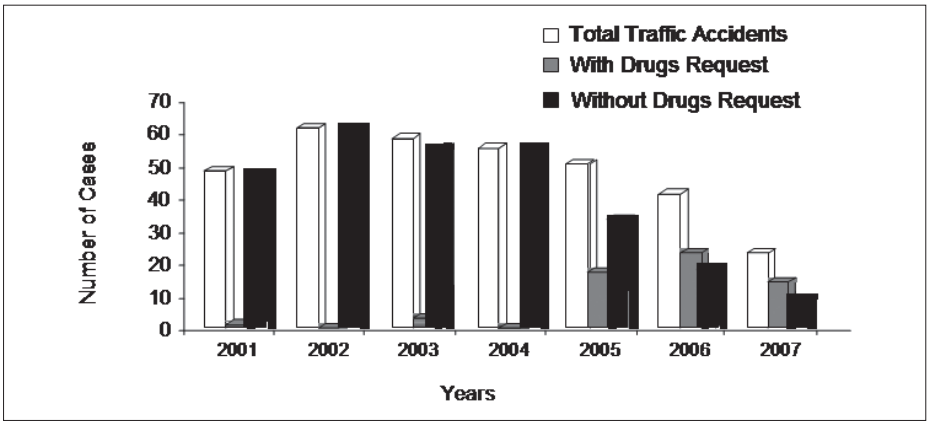

Figure 5 – Number of Traffic Accidents with and without Drugs request (in the Legal Medicine Office of Figueira da Foz).

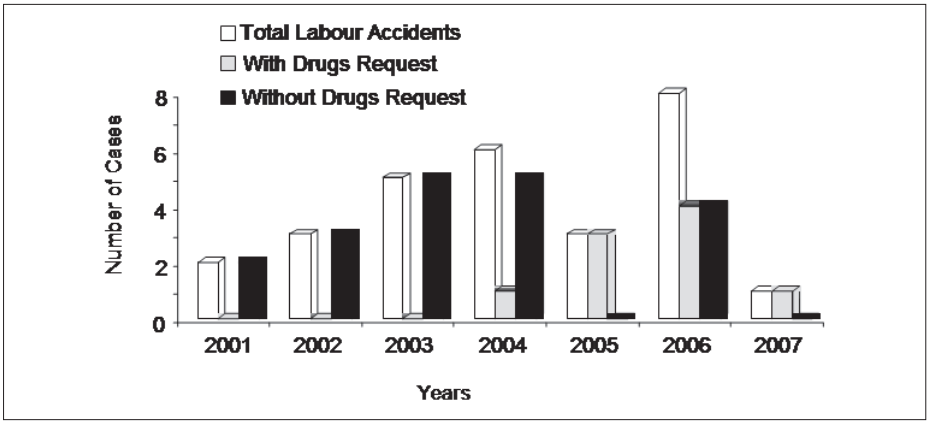

Figure 6 – Number of Labour Accidents with and without Drugs request (in the Legal Medicine Office of Figueira da Foz).

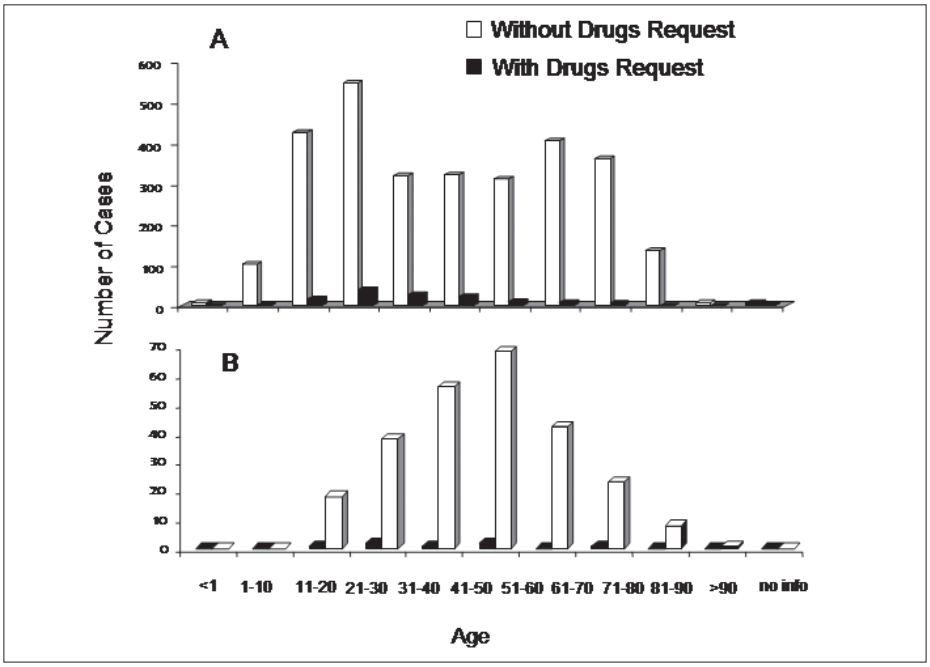

Figure 7 – Number of Traffic Accident (A) and Labour Accident (B) cases with/without drugs request per age (in the forensic pathology service).

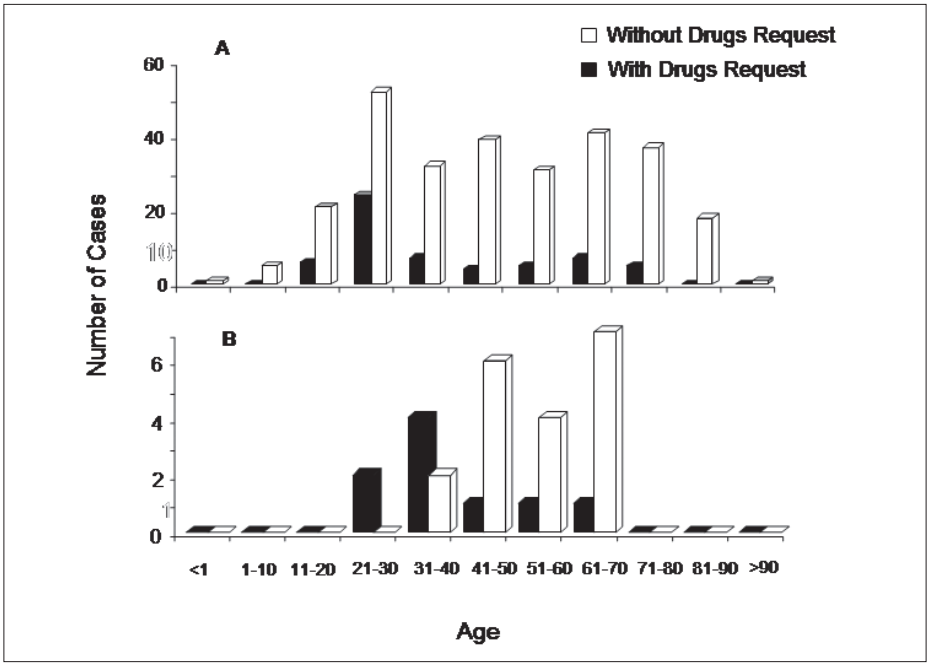

Figure 8 – Number of Traffic Accident (A) and Labour Accident (B) cases with/without drugs request per age (in the Legal Medicine Office of Figueira da Foz).

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ETHANOL CONSUMPTION & ROAD TRAFFIC ACCIDENTS: AN OVERVIEW BETWEEN 1990 AND 2007 IN THE CENTRE OF PORTUGAL

Abstract: Ethanol is the most frequently detected drug in deaths from all causes, particularly traffic and labour accidents. The aim of this study was to assess the presence of alcohol among drivers involved in fatal road traffic accidents between January 1990 and December 2007, by presenting the requests and ethanol results of the road traffic accidents autopsies, from the Pathology Service of the Centre Branch of the National Institute of Legal Medicine and from a Legal Medicine Office. In the Forensic Pathology Service and in the Office a total of 9409 and 1219 autopsies were performed, respectively, 3095 and 336 on road traffic accidents victims. Ethanol analysis was requested only in 54.5% and 75% of the road traffic accident cases, with 31% and 47% positive results, respectively. The major percentage involved concentrations over the maximum limit legally allowed (1.2g/L), being this undoubtedly present in males with ages between 21 and 30 years-old. Research has identified alcohol as a significant factor related to traffic accidents and this has led to specific legislation and methods of enforcement to prohibit this form of impaired driving.

Keywords: Ethanol; road traffic accidents.

Introduction

It has been estimated that more than 2 million people are killed each year in road traffic accident worldwide. Within the European Union, traffic accidents result yearly in 50 000 fatalities and 1.5 million injuries. Ethanol plays an increasing role in accidents, as accident severity increases [1]. Research has shown that alcohol impairs driving skills and increases crash risk. In Portugal, the legislation related to road traffic and safety is laid down in the Road Traffic Code and specific additional legislation. It states that it is forbidden to drive a vehicle under the influence of a substance of which the driver ought to know that it affects the driving proficiency. For alcohol, there are three legal limits, 0.5g/L, 0.8g/L and 1.2g/L, with different penalties.

This paper presents a retrospective analysis of the toxicological investigation on biological samples from fatally injured drivers, between January 1990 and December 2007, to determine the role, if any, of ethanol amongst fatal road traffic accidents.

Material and Methods

This study has been carried out by the National Institute of Legal Medicine of Portugal. The target population consisted of drivers killed in road accidents. The data available in each case was: year, sex, age and alcohol concentrations.

The authors present the requests and consequent ethanol results of the road traffic accidents autopsies performed from 1990 and 2007 in the Pathology Service of the Centre Branch of the National Institute of Legal Medicine (NILM) and performed from April 2001 (open date of the office) and 2007 in the Legal Medicine Office of Figueira da Foz (LMO). All ethanol analyses were performed in the Forensic Toxicology Laboratory of the Centre Branch of the NILM.

Several variables were studied and all the pertinent data was registered, separated and statistically treated with the SPSS program (Statistical Package will be Social Sciences).

Results and Discussion

Driver impairment is a significant factor that has been associated with accident risk. Research has identified alcohol as a significant factor related to traffic accidents and this has led to specific legislation and methods of enforcement to prohibit this form of impaired driving.

As mentioned above, the main objective of our work was the analysis of all road traffic accident cases from autopsies performed in the Forensic Pathology Service of the Centre Branch of the National Institute of Legal Medicine (NILM) between 1990 and 2007 and performed from April 2001 and 2007 in the Legal Medicine Office of Figueira da Foz (LMO), in order to know the prevalence of alcohol in this cause of death.

It is important to be aware that no related study has been published in Portugal and thus, no comparison with the results now achieved is possible. Consequently, the results will be discussed taking into account our legal medicine system and reality.

Throughout the years, we observed, in the Delegation and in the LMO, a decrease in the road traffic fatal accidents. In fact, in the Delegation, the year 2006 registered a less number of cases, with only 77 cases (2.5%), followed by 3%, in 2007. In the LMO, this less percentage was achieved in 2007, with only 23 cases, corresponding to 6.8%.

Indeed, it is important to remember that the road traffic safety European plan has as main objective the reduction on the number of accident victims in the European Union, in 2010, being "driving under the influence of alcohol and drugs" a phenomenon described as one of the most disturbing accident risk factor.

Thus, it has been recommended to all the UE countries some guidelines to fight against this problem, such as the application of a harmonized procedure to detect illicit drugs in all drivers involved in fatal accidents, the use of onsite screening devices, an adequate training of the police, etc. All these initiatives, added to the fact that, during the years, new hospitals have been build with better and increased assistance, lead to a major surviving percentage and the consequent decrease in deaths due to road traffic accidents.

In the Forensic Pathology Service a total of 9409 autopsies were performed between 1990 and 2007, 3095 on road traffic accidents victims, 78% in male victims (Fig.1). In the Legal Medicine Office, from April 2001 to 2007, 1219 autopsies were performed, 336 to car accident victims, 82% men (Fig.6).

Although Portuguese culture have suffered deep changing during the years, observing a huge number of women with active and professional activities and with driving licence, in fact, there will always be a major number of male drivers, as also stated by other authors [2, 3].

Moreover, despite the jargon assume that a female driving will result in constant danger, we believe that the data obtained can now be justified by a driving behaviour sometimes more aggressive and competitive by men [4], especially in individuals between 21 and 30 years (higher range detected, about 25% in road accidents studied), as described by Williams and Shabanova (2003), resulting in a less cautious behavior and consequent increased number of fatal accidents [4,5].

In the Forensic Pathology Service, ethanol analysis was requested in only 54.5% of the road traffic accident cases, with 31% positive results (Fig. 2) and in the Legal Medicine Office from all the car accident files analyzed, 75% included ethanol analysis request, with 47% positive results (Fig. 7).

In fact, with regard to the years in which this determination was not required, there was a clear predominance from 1990 to 1992, 2001 and 2004 (in the Centre Branch). We can possibly speculate that this lack of alcohol requests on drivers victims of road accidents in 1990 and 1992 was mainly due to a lack of specific legislation, obliging this determination, existent in our country only since 1998, not only through the highway code, but by separate legislation, Law 24/98, 30th of October, now repealed by the Law 18/2007, 17th of May.

The other cases with no alcohol analysis correspond to drivers or pedestrians with hospitalization and subsequent survival over 24 hours and, according to the rules implemented in the Forensic Pathology Services of the NILM, based on scientific evidence, is not required for blood alcohol determination.

On the other hand, it can also include all the cases where there were certain conditions, including thoraco-abdominal trauma or other injuries that led to the absence of biological samples, including blood, turning out impossible any determination. Finally, all the cases suspected of a possible postmortem synthesis of ethanol, such as signs of putrefaction were entirely excluded on the assessment of alcohol, since the results could be unreliable, especially in road accidents cases where the establishment of a state of influence or determination of a legal penalty is the main objective.

Of the positive cases analyzed, we observed that the majority of cases were above 1.2 g/L, with 51.4% (283 cases) for the Centre Branch (Fig.3), and 60.2% (73 cases) for the LMO (Fig. 8). Note that this concentration corresponds to the maximum legal limit permitted by law, while an offense punishable by law. Analyzing the cases in this BAC (BAC≥1.2 g / L), we concluded that there has been a regular consumption through each year, with a predominance of the month of August, in the Branch, and the month of September, in the LMO, possible months conducive to consumption, influenced by natural factors such as temperature, or social factors such as celebrations, holiday periods, highlighting again the sex male with about 95% of the cases, from 21 to 30 years-old, with about 25% of the cases (Figs. 4, 5, 9, 10).

Moreover, we performed the same analysis regarding the distribution of the positive cases by driver, passengers and pedestrians, and concluded that drivers were those with a higher number of positive cases for ethanol, with 51.5% of the cases in the Branch and 56.9% of the cases in the LMO.

In the absence of a total or absolute justification for these high values, we can only conclude that these extraordinarily high concentrations have been increasing over the years. It has been discussed in our country, not only the high alcohol consumption as a result of the culture as well as due to the fact that we are strong producers of alcoholic beverages, but the scenario effectively existent of a chronic use of this toxicological substance and consequent high concentrations achieved among drivers. In fact, the NILM is involved in an working group organized by the Institute for Drugs and Drug Addiction which aims to establish standards and strategies to reduce risks associated with alcohol, this plan set for 2009 to 2012.

Conclusion

The higher percentage of the analysed cases were violent deaths, with 76.8% of the cases in the Centre Branch and 66.7% in the LMO, being the road traffic accidents present in 35,8% and 31,8% of these deaths, in the Centre Branch and LMO, respectively.

However, there has been a decrease in the number of deaths from road accidents over the years due to the strong strategy to reduce road accidents and development of health systems.

The vast majority of fatal road accidents occur in males, with about 80% of the cases, drivers aged from 20 to 40 years, and the car is the vehicle of choice, followed by the motorcycle, with approximately 45% and 40%, respectively.

From the casuistic study performed it is possible to observe that there is a very high percentage of non required ethanol analysis in car accident fatal victims. However, over the years and consequent law implementation, the number of samples submitted for toxicological analysis (ethanol) from car accident autopsies increased

As also discussed by other authors [2], driving under the influence of alcohol is a persistent problem in our country. The problem of drinking and driving is of special relevance in Portugal. This is due, not only to the fact that alcohol consumption is frequent in our country (and, therefore, driving under the influence of alcohol), but also because of an alcohol-related culture; society is very permissive towards its consumption and tolerant towards alcohol-related problems.

Although comparison with data from other countries should be carefully conducted, the figures observed in our study were among the highest. In the UK [3], data accumulated from 1990 to 1994 revealed that in 45.5% of deaths alcohol was detected, and that this exceeded 0.8g/l in 20.25% of the victims. Alcohol has been frequently been detected in Italy (49.0%) [4], in France (45.7% > 0.7% g/l) [5] and in Belgium (28% over 0.49 g/l) [6], whilst figures for the Nordic countries (Sweden: 27% [7] and 20.3% [8], Norway: 28.3% [9]) are considerably lower, perhaps as a result of the fact that in these countries there exists a very strict policy with regard to drinking and driving.

Furthermore, Portugal is still one of the European Union countries showing highest figures for road accidents. Consequently, psychoactive drugs and driving are a matter of great interest for developing health policies.

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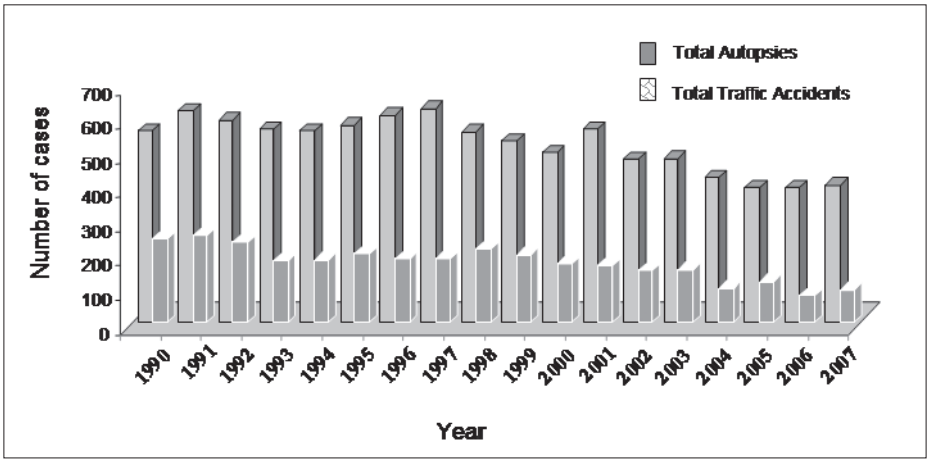

Figure 1 – Number of Traffic Accidents in the autopsies performed in the forensic pathology service between 1990 and 2007.

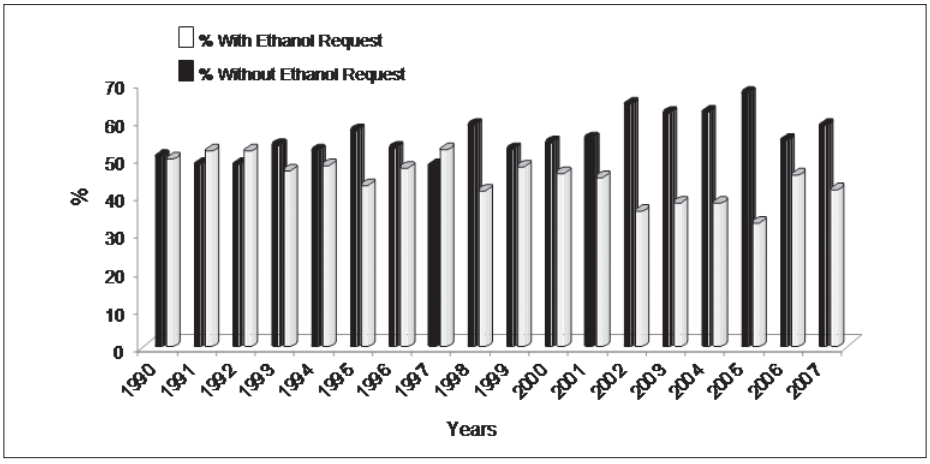

Figure 2 – Number of Traffic Accidents without Ethanol request (in the forensic pathology service).

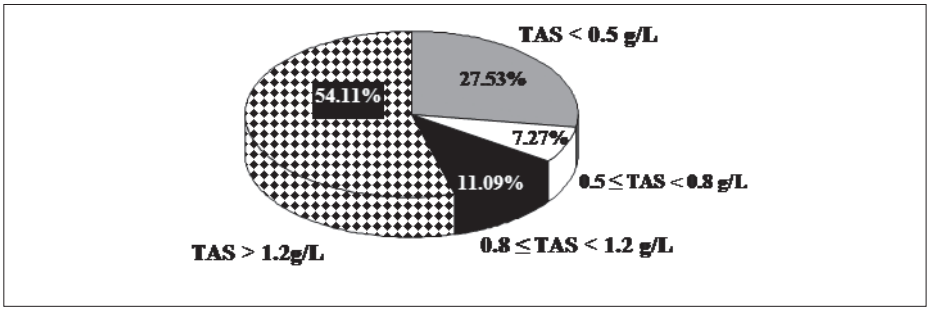

Figure 3 – Positive Ethanol Results distribution in the Centre Branch.

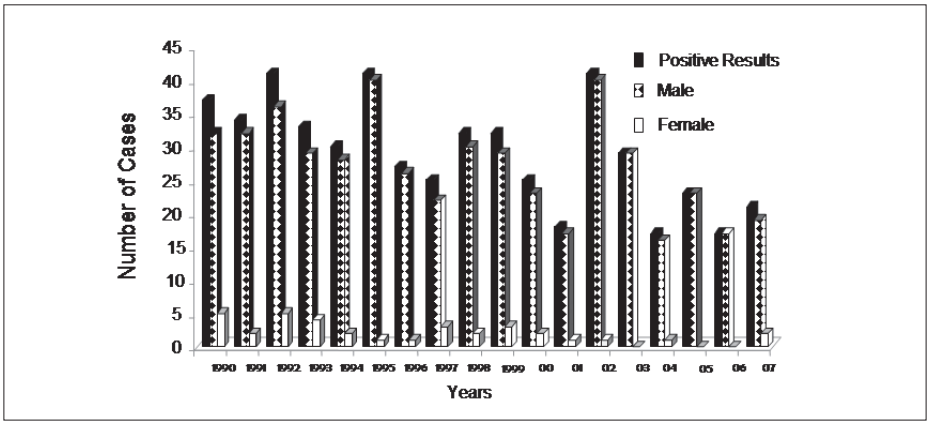

Figure 4 – Positive results distribution per gender/year (in the forensic pathology service).

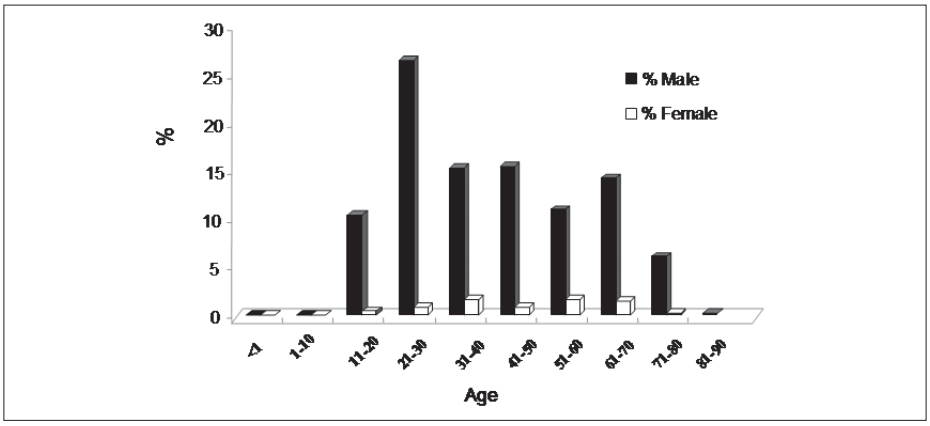

Figure 5 – Positive results distribution per gender/age (in the forensic pathology service).

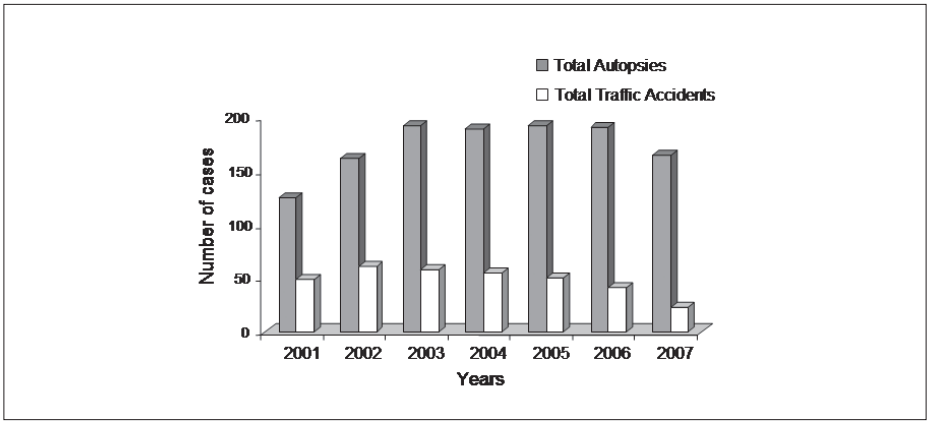

Figure 6 – Number of Traffic Accidents in the autopsies performed in the Legal Medicine Office of Figueira da Foz between 2001 and 2007.

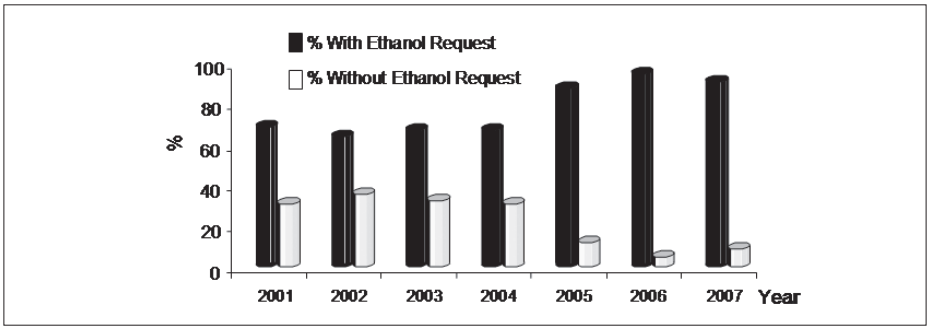

Figure 7 – Number of Traffic Accidents without Ethanol request (in the Legal Medicine Office of Figueira da Foz).

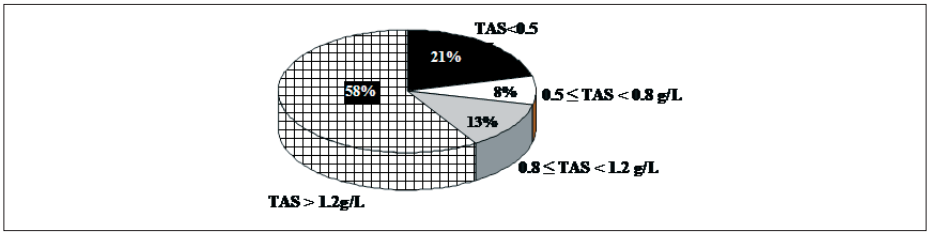

Figure 8 – Positive Ethanol Results distribution (in the Legal Medicine Office of Figueira da Foz).

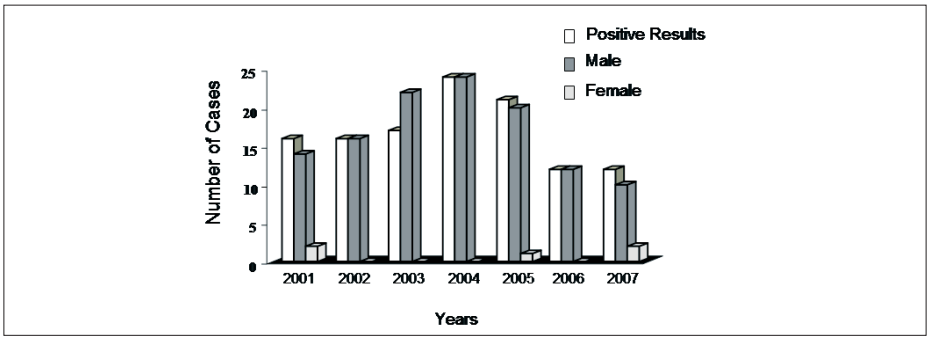

Figure 9 – Positive results distribution per gender/year (in the Legal Medicine Office of Figueira da Foz).

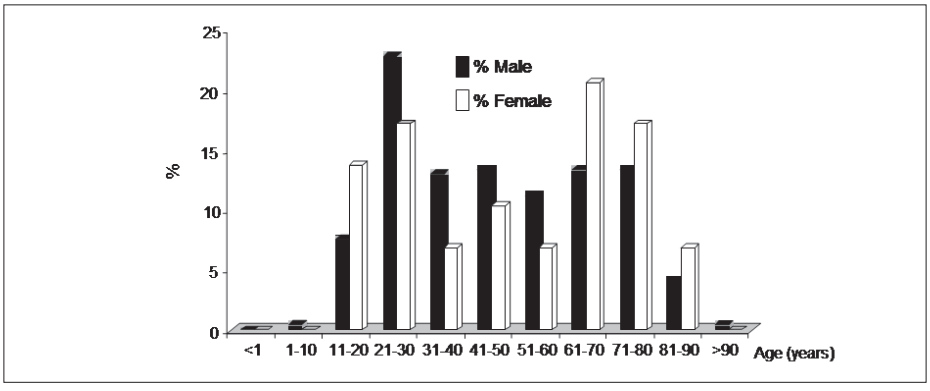

Figure 10 – Positive results distribution per gender/age (in the Legal Medicine Office of Figueira da Foz).

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ETHANOL MONITORING IN LABOUR FATAL ACCIDENTS: AN OVERVIEW BETWEEN 1990 AND 2007 IN THE CENTRE OF PORTUGAL

Abstract: No much information is available on workplace drug testing, including ethanol, being this substance often implicated in labour accidental deaths. The aim of this study was to assess the presence of alcohol among in labour fatal accidents between January 1990 and December 2007, by presenting the requests and toxicological results of corresponding autopsies, from the Pathology Service of the Centre Branch of the National Institute of Legal Medicine and from a Legal Medicine Office. In the Forensic Pathology Service, between 1990 and 2007, a total of 261 autopsies were performed on labour accidents victims. Ethanol analysis was requested in only 50% of the cases, with 25% positive results. From the positive studied cases, 24% presented ethanol concentrations above 1.2g/L and 55% under 0.5g/L, with all the cases related to men aged between 41 and 50 years-old (24%). In the Legal Medicine Office, from April 2001 to 2007, 1219 autopsies were performed, 28 to labour victims, with only one female victim. Ethanol analysis was requested in 75% of the cases, with 48% positive results, 70% under 0.5g/L, with all the cases related to men aged between 51 and 60 years-old (24%).

Keywords: Ethanol; labour accidents.

Introduction

According to WHO, approximately 10-30% of work accidents would be alcohol related. Alcoholic beverages at workplace may produce an even remarkable increase of direct risks, because of psychophysical alteration and indirect risks produced by added effect of alcoholic beverages with industrial toxic substances. Even with low alcohol rates, the reaction time becomes longer, error frequency in response to visual or hearing stimulations is higher and hence the risk of accidents becomes sensible. Besides, alcohol-related working performance problems may be caused not only by drinking at work but also by drinking a lot of alcoholic beverages outside working time [1].

Alcoholism is a growing medical and public health issue both in adult and in younger populations. It is a multi-aetiological phenomenon influenced by genetic, psychological, cultural and other factors. Alcoholic beverages have traditionally been prepared from various ingredients, such as grapes, malt, and rice. Drinking prevalence has varied and is more pronounced in women and the youth. Alcoholism is shown to be of neurophysiologic origin and may lead to the impairment of all human

body systems. The most frequent cause of death in alcoholics is the diseases of the cardiovascular system [2].

Fatal accidents in the workplace can be caused by work conditions, aggravation of a chronic disease or alcohol intoxication [3].

Material and Methods

This study has been carried out by the National Institute of Legal Medicine of Portugal. The target population consisted of individuals killed in labour accidents. The data available in each case was: year, sex, age and alcohol concentrations.

The authors present the requests and consequent alcohol results of autopsies performed on fatal victims of labour accidents from 1990 and 2007 in the Pathology Service of the Centre Branch of the National Institute of Legal Medicine (NILM) and performed from April 2001 (open date of the office) and 2007 in the Legal Medicine Office of Figueira da Foz (LMO). All alcohol analyses were performed in the Forensic Toxicology Laboratory of the Centre Branch of the NILM.

Several variables were studied and all the pertinent data was registered, separated and statistically treated with the SPSS program (Statistical Package will be Social Sciences).

Results and Discussion

As mentioned above, the main objective of our work was the analysis of all labour accident cases from autopsies performed in the Forensic Pathology Service of the Centre Branch of the National Institute of Legal Medicine (NILM) between 1990 and 2007 and performed from April 2001 and 2007 in the Legal Medicine Office of Figueira da Foz (LMO), in order to know the prevalence of alcohol in this cause of death.

It is important to be aware that there is no published data in Portugal, any work that could allow us to make any comparison with the results now obtained, and the results will be discussed based on a vision of our system and medical-legal reality.

Analyzing the cases performed in the Centre Branch, it can be observed that they do not differ significantly in the 90th decade, with about 55% of cases, compared to the 2000s with about 45%. Of all the cases analysed, we found that the vast majority were related to build construction, as also referenced by other authors [4].

In fact, we can assume that this can be explained within the framework of civil protection workers, as there is a greater awareness and obligation for the employer to address the risks inherent in the employee's tasks [5]. Examples include the construction workers, where it is required to obey certain security measures, such as the placement of security barriers in order to avoid possible precipitation, among other important and effective measures, such as compulsory use of helmets for protection, thus contributing to an improvement in working conditions in order to prevent possible this kind of fatal accidents [6]. The years 1994 and 2002 presented the highest number of cases, 22 cases each, accounting for 8.4% of the total analyzed.

Analysing the years 2000s in the Centre Branch, we observe that since 2003 the number of accidents decreased, excepted in 2007 with a very high percentage eventually

explained by the possible association of fatigue felt when working consecutively, sometimes more than 40 hours per week, and this behaviour can be the basis for many of these accidents and the possible association of alcohol and other substances, known to disrupt the dexterity necessary to perform their functions. In the LMO, the opposite is true, because despite a sharp increase in the number of accidents over the years, reaching its peak in 2006 with 8 cases (28.6%), there is a decrease in 2007 with only 1 case (3.6%), which may possibly be explained by the fact that Figueira da Foz is a smaller area and does not cover as many villages as the area covered by the Branch, where security measures are properly implemented.

In the Forensic Pathology Service, a total of 9409 autopsies were performed between 1990 and 2007, 261 on labour accidents victims, 97% in male victims (Fig.1), aged from 41 to 50 years-old. The number of cases in males can be explained by the fact that men are closely linked to the performance of heavy work, which, by their nature entails more health risk [4,6]. Presently, there is a behaviour changing, since there have been an increasing number of women workers, particularly in professions that were previously male safeguards, such as in petrol stations, driving taxis and even industrial.

In the Forensic Pathology Service, ethanol analysis was requested in only 50% of the cases, with 25% positive results (Fig. 2). But it is important to sate that these numbers correspond to 18 years analyzed.

In the Legal Medicine Office, from April 2001 to 2007, 1219 autopsies were performed, 28 to labour victims, aged from 61 to 70 years-old, with only one female victim (Fig.4).

The labour capability for certain works depend on the individual ability to perform each task being, sometimes, the age a very important and conditioning factor for accident risk.

In the Centre Branch, only 50% of the labour fatal accident cases included ethanol request analysis. In fact, with regard to the years in which this determination was not required, there was a clear predominance in the years 1990, 2001, 2005 and 2006. There was, however, an increase in this request as we move forward in time, too pronounced in the 2000s. In the LMO, we observed, over the years studied, an increase in the requests, and in 2007 almost all cases included the request of this psychotropic substance.

Of the positive cases analyzed, we observed that the majority of the cases were well below 0.5 g / L, the minimum legal limit allowed by law, with 13.7% (18 cases) for the Brach and 28.6% (6 cases) for the LMO (Fig. 3-6).

Analyzing the cases in this BAC (BAC $\leq$ 0.5 g / L), there has been a regular consumption through each year, with a predominance in March, in the Branch, and a regular consumption over the months in the LMO, highlighting again the male gender, with about 55% of the cases in the Branch and about 80% of cases in the office. The most incident group was aged between 51 and 60 years in the Branch, and aged between 31 and 50 and between 61 and 70 years-old in the office, with about 30% of the cases in both.

Portuguese law concerning labour monitoring significantly addresses the problem of alcohol and psychotropic substances, because workers can, by law, be subject to a random check by the employer at any time of their activity. However, its review or determination in postmortem cases is not fully informed or legislated, in relation to how

to act specifically in labour accident autopsies. Accordingly, the discrepancy of results concerns the practice carried out by experts who, more and more, are being aware and scientifically instructed to further research and evaluation of substance influence in labour accidents and consequent legal aspects implicated, such as life insurance, for example.

Conclusion

From the casuistic study performed it is possible to observe that over the years the pathologists increased the number of requests to alcohol in labour victims in order to better explain and/or interpret the presence of this substance and its possible interaction with psychomotor skills required in several activities.

As stated by some authors [2] alcoholism at workplace is a very important issue as it affects health, reduces productivity, and may lead to accidents, injuries and decreased working capacity. Alcohol-related difficulties develop much earlier than the clinical picture. The diagnosis of alcoholism includes early detection of alcohol-related problems, so it is necessary to orient the healthcare services towards primary prevention and early intervention.

Many studies have shown the role of alcohol intake, even in low quantities, as a risk factor in accidents, mainly road accidents, but also in accidents occurring in the home and at the workplace. For a blood alcohol level of 0.5 g per litre, the risk of accident is already two-fold. Accidents occur more often to persons who drink occasionally than to those who are alcohol-dependent. For a subject of average body weight, it is accepted that one glass of an alcohol-containing drink can raise the blood alcohol level by approximately 0.2 g per litre. One to two hours is required to eliminate this quantity of alcohol [6].

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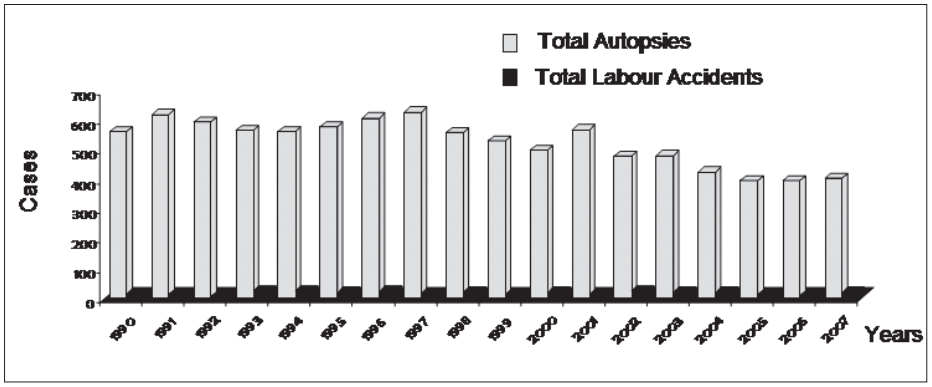

Figure 1 – Number of labour Accidents in the autopsies performed in the forensic pathology service between 1990 and 2007.

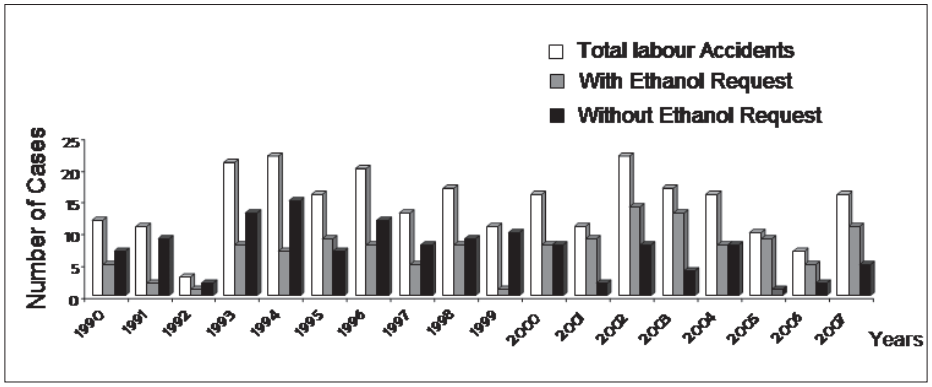

Figure 2 – Number of Labour Accidents without Ethanol request (in the forensic pathology service).

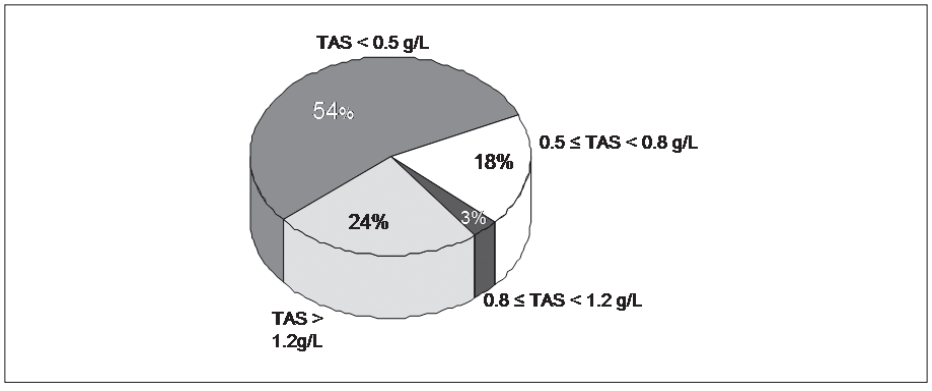

Figure 3 – Positive Ethanol Results distribution (in the forensic pathology service).

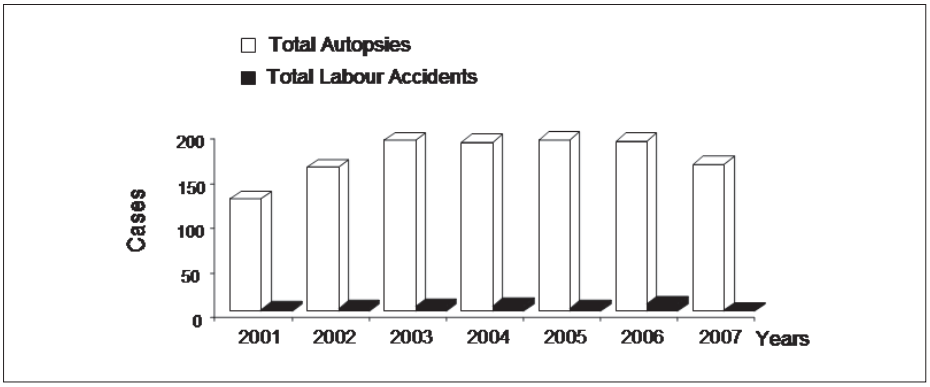

Figure 4 – Number of labour Accidents in the autopsies performed in the Legal Medicine Office (Figueira da Foz) between 2001 and 2007.

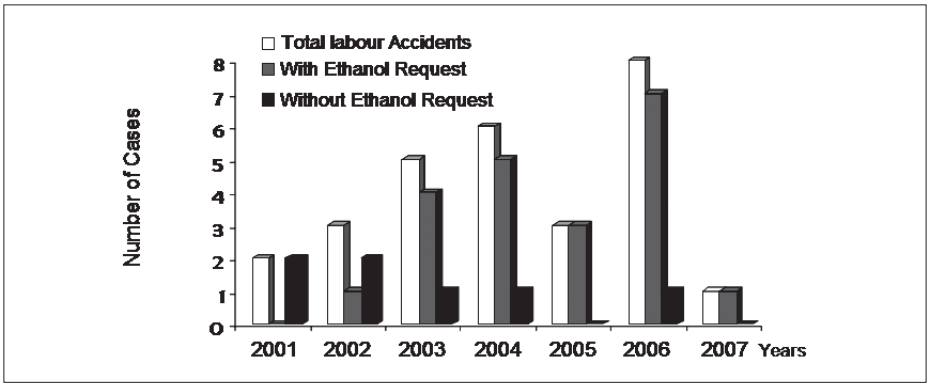

Figure 5 – Number of Labour Accidents without Ethanol request (in the Legal Medicine Office of Figueira da Foz).

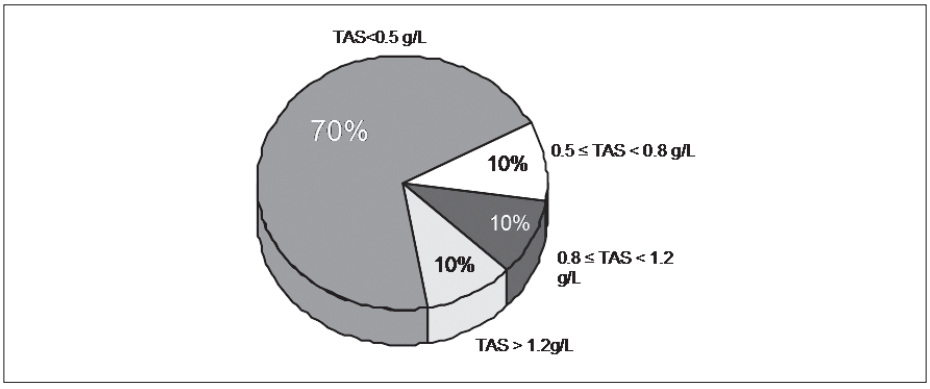

Figure 6 – Positive Ethanol Results distribution (in the Legal Medicine Office of Figueira da Foz).

Age	Male	Female
<1	0	0
1-10	0	0
11-20	11	0
21-30	16	2
31-40	27	0
41-50	35	1
51-60	25	0
61-70	11	0
71-80	3	0
81-90	0	0
>90	0	0
TOTAL	128	3
%	97.71	2.29

Table 1 – Positive results distribution per gender/age (in the forensic pathology service).

Age	Male	Female
<1	0	0
1-10	0	0
11-20	0	0
21-30	0	0
31-40	0	0
41-50	2	0
51-60	3	0
61-70	2	0
71-80	3	0
81-90	0	0
>90	0	0
TOTAL	10	0
%	100	0

Table 2 – Positive results distribution per gender/age (in the Legal Medicine Office of Figueira da Foz).

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LETHAL POISONING FROM OLEANDER

Introduction

Oleander (*Nerium oleander*), an evergreen shrub or small tree in the Dogbane family Apocynaceae, is widely cultivated as ornamental shrubs or hedges, but it's one of the most poisonous plants known, because of the presence of cardioactive glycosides in every part of the plant whose chemical structure is similar to those of digoxin. Despite its toxicity, Oleander has been used in the past for manufacturing herbal medicaments for the treatment of many different diseases (leprosy, malaria, ringworm, venereal infections).

Poisoning from Oleander are not common but a variety of cases are described in world literature, most of all referring to accidental ingestion, but in some instances concerning suicidal or homicidal purposes.

Case report

In December 2004, the bodies of two young people, one male and one female, were found in a pine forest near Cecina (Tuscany).

They were lying as asleep on the floor of a forester's storehouse, dressed with shabby clothes, very light for wintery season (Fig. 1,2). They had neither documents of identification nor money and there was no food near the bodies, but only a half-fully bottle of water.

The preliminary examination of the two cadavers showed no signs of traumatic injuries and there was no evidence of the previous presence of other persons on the scene; from the thanatologic data it was supposed that the death had occurred about two-three days before the discovery.

At autopsy the most important element was represented by the condition of extreme malnutrition and physical debilitation of both of them: the man was 171 cm high and weighed 37,5 Kg, the woman was 170 cm high and weighed 38 Kg (Fig. 3,4).

The autopsy confirmed the absence of traumatic injuries and didn't reveal significant disease. Vegetal remains, (leaf-like and fibres) were found in the stomach of both the

cadavers; in the male cadaver stomach content was dark-bloodish and semi liquid, while in the female it was brownish and liquid. It was also observed hemorrhagic pancreatitis, more severe in the male.

The circumstances of death obviously excluded the possibility of a death from natural causes and suggested that it was due to external factors, physical or toxic.

The preliminary hypothesis was about a perfrigeration-related death, considering the low winter temperature, the light clothes and the severe physical debilitation, but it was not fully credible because of the contemporaneity of deaths and the fact that the deceased have not made any attempt to protect themselves from cold, so that forensic investigation focused on toxic causes.

Materials and method

Toxicological analysis, performed with a ThermoElectron gas-chromatograph (GC) coupled to PolarisQ mass-spectrometer (MS), resulted negative for traditional drugs and many other chemical compounds.

A further hypothesis concerned the possibility of an intoxication from Oleander, a plant very common nearby the pine-forest where the bodies were found, so laboratory analysis was performed aimed at the detection of Digoxin in the blood, which cross-reacts with the molecule of Oleandrine in radioimmunoassay1. The test, performed with F. P.I.A. TDX Abbot® System resulted positive results for both the cadavers (1,4 ng/ml for the man, 0,7 ng/ml for the woman).

Discussion

The presence of Digoxin in the blood of both the cadavers had no other reasonable explanation other than that it was due to ingestion of Oleander. Some Authors (2,3,4) suggest the possibility of mistakes in blood-level post-mortem investigation of Digoxin due to the presence of endogenous compounds classified as Digoxin-Like Immunoreactive Substances (DLIS), which can be significantly elevated in specific clinical conditions. However these substances are not detectable with the method utilized in our case, confirming the exogenous provenience of Digoxin in blood.

Human intoxication from Oleander can occur via accidental exposure, ingestion by children, purposeful administration in food or drink, medicinal herbal products and criminal poisoning. The review of international literature points out that human intoxication from Oleander are not very common but, on the other hand, neither extremely rare (5): in U.S.A. Poison Control Centers listed 633 cases in 1988 (6). In Australia 27% of plant poisoning involve Oleander (7) while deliberate ingestion of Oleander seeds is a popular method of suicide in Sri Lanka (8).

All parts of the plants are poisonous due to the presence of Oleandrine, a toxic glycosides that can cause multiple arrhythmias as A-V blocks, bradicardia, ventricular excitability. It may cause also gastrointestinal symptoms as diarrhea, vomiting, gastric or intestinal hemorrhages (9). The mechanism of cardiovascular activity of Oleandrine

consists in inhibiting the activity of Na+ – K+ – ATPases that results in increasing intracellular Ca++, responsible of inotropic effect (10).

Reviewing world literature, it is possible to find also strange ways of intoxication from Oleander

a) two subjects (a female aged 43 and a male aged 66) presented gastrointestinal and cardiovascular symptoms (similar to those observed after acute digoxin intoxication) 8-10 hour after eating a meal which included escargot stew; the patients had found the snails near a Nerium Oleander plant in their garden and toxicological analisys showed significant level of digoxin in their blood and in snails tissue (11);
b) a 59-year-old man was admitted as a medical emergency for a severe degree of pulse rate (26/min) and AV block after having treated his psoriasis using a homemade lotion of Nerium Oleander blooms and leaves (12);
c) a 49-year-old man with known history of diabetes mellitum was admitted to the hospital with digoxin-like toxicity and died a few hours later; his wife referred that he had drunk an infusion of leaves provided from unknown person, which contained extract from Oleander (13).

Referring to our case, it's to notice that the identity of the two young people remained unknown, despite the efforts of Italian Police and Interpool, until 2008, when a married couple of Belgium recognized the daughter in a photo of the cadavers shown on television broadcast dedicated to missing persons. They told us that her daughter ran away from home in June 2004 with her friend, who belonged to Vegan movement, whose affiliated, for various reasons, choose to avoid using or consuming animal products, not only flesh foods, but also dairy and eggs, as well as fur, leather, wool, down, and cosmetics or chemical products tested on animals and they try to live exploiting the product of nature that they can find during their wandering.

This information reinforces the hypothesis that the two young has fed with vegetables, as confirmed also by the gastric contents, inadvertently eating Oleander that has caused their death.

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Figure – 1

Figure – 2

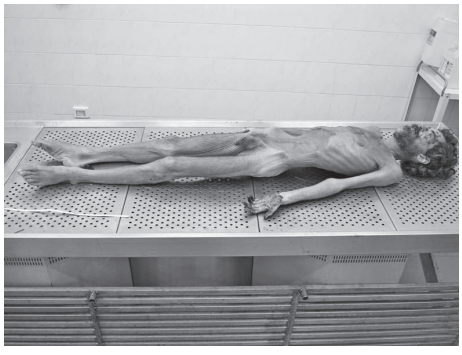

Figure – 3

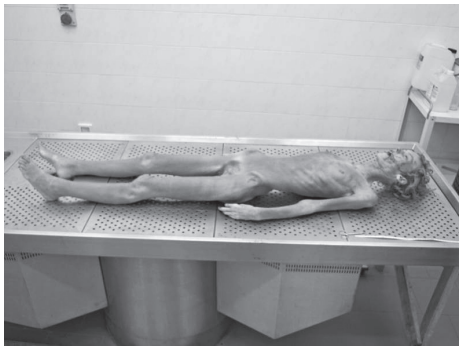

Figure – 4

M. Burrell[1], **S. Francese**[2], **D. D. Cameron**[1], **C. Steels**[1], **M. Bennett**[3], **D. Wells**[3], **T. Holman**[3], **J. Leake**[1], **M. Clench**[2]

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MALDI-MSI – A NEW TOOL FOR METABOLITE ANALYSIS IN FORENSIC SCIENCE.

Abstract: Imaging Matrix Assisted Laser Desorption Mass Spectrometry provides a new powerful tool to analyse the distribution of metabolites within plant or animal tissues and to compare with an untargeted approach different tissues or samples from different organisms. Metabolites may be mapped at a single cell resolution and the technique may be used to resolve the differences between two samples of tissue.

Introduction

The aim of the work reported in this paper was to develop a method, which would allow the cellular distribution of metabolites in plant tissues to be determined. Metabolic fingerprinting and profiling is an emerging technique and has recently been used to identify ecologically important genes and traits[1]. In a recent study of *Arabidopsis petraea* it was used to identify that individual plants of this species from different countries were significantly different. Plants from the same country were more closely related[2].

To understand the control of metabolism one needs to determine the amounts of metabolites present in the cell and the amounts of enzyme present[3]. Many recent studies with techniques such as immunolocalisation and *in situ* hybridisation have demonstrated that cells from the same tissue can have different patterns of gene expression[4,5]. With plant material the task of measuring metabolites is very large since there are in excess of 100,000 metabolites that can occur[6,7]. Thus homogenisation of a whole tissue can potentially reduce a high concentration of a compound present in a few cells to a level that is not detectable.

Matrix Assisted Laser Desorption Ionisation Mass Spectrometry Imaging (MALDI-MSI) offers potential as an approach to both analyse in a targeted way the distribution of metabolites across tissues, and to profile in an untargeted way individual samples. MALDI is a very versatile technique since it will ionise compounds from a wide range of chemical classes[8]. MALDI-MSI is a derivation of this technique where the sample on the MALDI stage is moved on the x/y axis and each sample position is assayed[9]. Therefore by recording the mass spectrum at each position a two dimensional image of the metabolic profile of a sample may be obtained. The diameter of the laser in modern

mass spectrometers is in the order of 100 µm or less. Thus at a spatial resolution of 100 µm it is possible to obtain a mass profile of those compounds present on the surface of a tissue. With modern high resolution mass spectrometers and careful selection of the matrix MALDI has the potential to allow analysis of metabolites and avoid too many interfering peaks.

In this paper we have examined the usefulness of the method for metabolic profiling of different plant tissues. It has also been used to measure the distribution of drugs applied to skin and the metabolism of these drugs[10]

Materials and Methods

Wheat (*Triticum aestivum* L. var Axona) plants were grown as described previously[11]. Orchid material *Goodyera repens* was collected from the wild and maintained in a growth room. Longitudinal sections (60µm thick) were prepared, in a Leica Cryostat, from roots flash frozen in liquid nitrogen. *Arabidopsis thaliana* seedlings were grown as described previously[12] and roots taken from 7 d old seedlings. Sections were freeze dried prior to coating with matrix. Sections were coated using an airspray with a solution of 25mg ml^{-1} α-cyano-4-hydroxycinnamic acid in methanol containing 0.1% (v/v) trifluoracetic acid.

MALDI MS spectra were acquired with an Applied Biosystems/MDS Sciex hybrid quadrupole time-of-flight (Q-Star Pulsar-i), fitted with an orthogonal MALDI ion source and an Nd:YAG laser. The instrument conditions were Repetition rate: 1000Hz, Laser energy 20% (2.3µJ) and analysis time of 5 seconds per position. Images were created from the Analyst data files with Biomap 3.7.5.

Principal component analysis of mass profiles in images was performed using the program SimcaP supplied by UMetrics.

Results and discussion

An analysis of sections from wheat endosperm is presented in Fig 1. Some 40,000 masses were detected in these sections and taking into account that adducts of sodium and potassium as well as the protonated ion can occur then this figure could represent about 13,000 metabolites. It can be seen that the mass of 175 for arginine is not evenly distributed across the section at any stage of development (figure 1a). Thus an analysis involving extracting the whole grain would grossly under estimate the concentration present in certain parts. The mass of 175 was shown to be mainly arginine by a MS/MS analysis (data not shown). The distribution of arginine (175) is similar to the distribution of ornithine (133) but not sucrose (343) and aspartate (134) (figure 1b). Arginine is synthesized in the ornithine arginine cycle. The images therefore suggest that the cycle is operating in the seeds rather than representing just an accumulation of the arginine.

As demonstrated by Burrell et al. 2007[9] solution of less than 1µg ml^{-1} can be detected. This is equivalent to detecting approximately 1.2 pg of compound if it is all present in the area covered by the laser during one pulse. Several researchers have

developed methods to map metabolites in tissues[13-15]. These methods can be very sensitive and where fluorescence is involved certainly may be more sensitive than MALDI-MSI. However a direct comparison of the sensitivity of different methods has not been undertaken to date. MALDI-MSI offers two benefits as a mode of analysis. First it simultaneously measures many compounds and secondly it does not depend on chemical class it only requires the compound to become charged.

The diameter of the laser is approximately 100µm. It was of interest to determine whether the resolution of the images could approach the size of cells. Mycorrhizal roots (symbiotic interactions between plant roots and fungi where the plant exchanges carbon for mineral nutrients from the fungus partner) were chosen since they have cells some of which approach the size of the laser and one would expect the metabolism of fungal infected cells to differ from the host plant uninfected cells. It is clear from figure 2 that mass 184 is specifically localized to certain cells thus demonstrating the method is sensitive for near cellular resolution. The latest software for MALDI-MSI allows the horizontal resolution to be decreased below the diameter of the laser.

MALDI-MSI provides a method to achieve untargeted analysis of metabolites. With many thousands of masses detected one needs a method to separate those masses which are evenly distributed from those that are localized in areas. A PCA analysis (figure 3) of the data used to provide the image in figure 2 provides a method of identifying these masses. Mass 184 is well separated from most masses and from sucrose identifies as the potassium adduct (381). Similarly this approach may be used to determine whether two samples are from the same individual or species. Figure 4 shows an analysis of the surface of two plant roots from the species *Arabidopsis thaliana*. Each laser position used to acquire the image has been treated as a separate sample. It is clear that the wild type and putative mutant roots are different and that there is variation across the surface of the root.

Conclusions

MALDI-MSI provides a powerful targeted or untargeted method of analyzing small molecules such as endogenous metabolites or exogenously applied compounds. Sample preparation is simple and analysis time is rapid. The method can be used with many different tissues. It can be used to identify the presence of known compounds and associated metabolites and to identify where in a sample metabolism is occurring. Alternatively it may be used to identify whether two samples are from the same individual.

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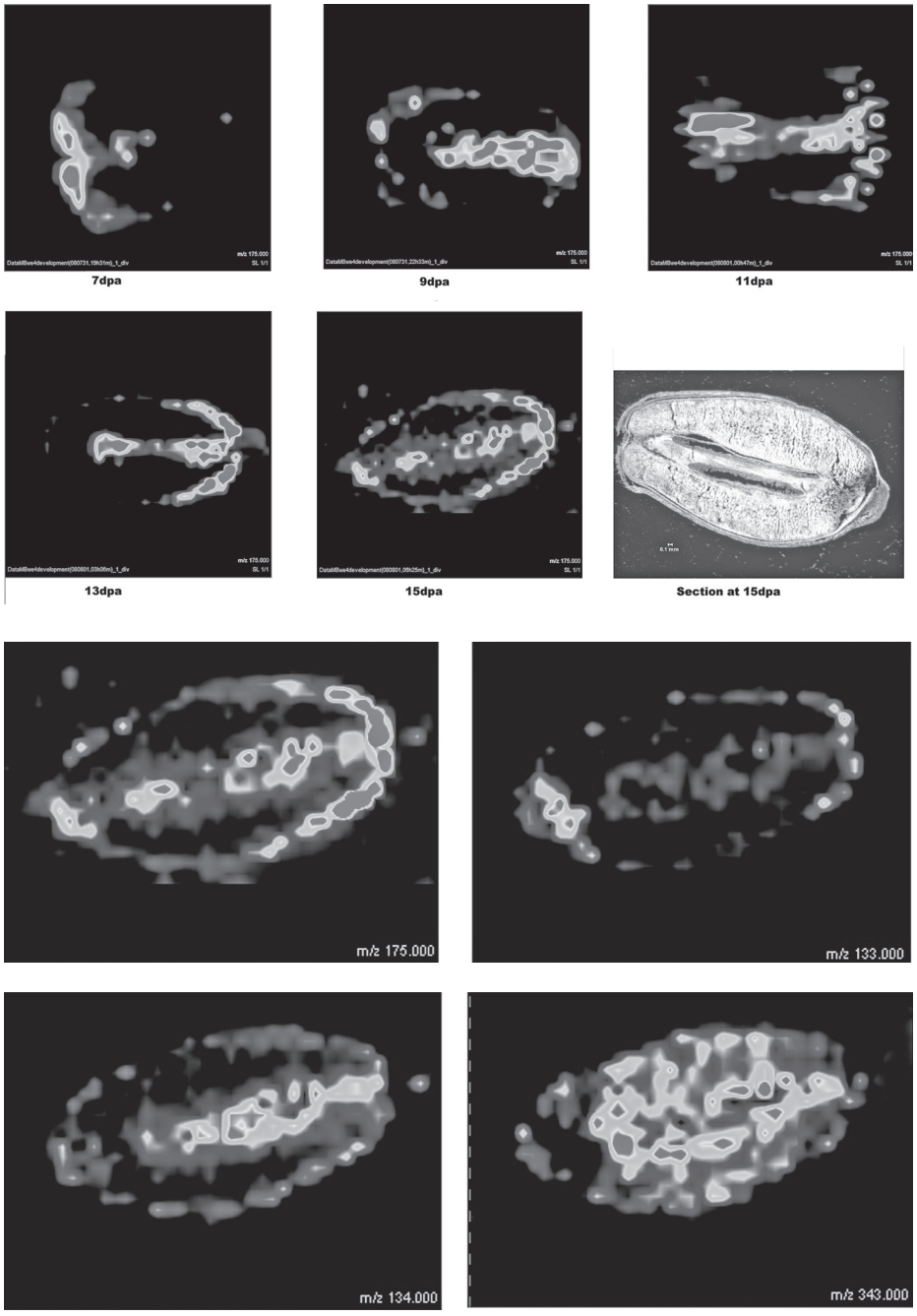

Figure 1 – Distribution of amino acids in developing wheat grains.

A. The distribution of the mass 175 (arginine) during different stages of development

B. The distribution of arginine (175), ornithine (133), members of the ornithine arginine cycle compared with aspartate (134), and sucrose (343) in a section of grain taken 15 days post anthesis. Red indicates a high intensity of signal, blue a low intensity.

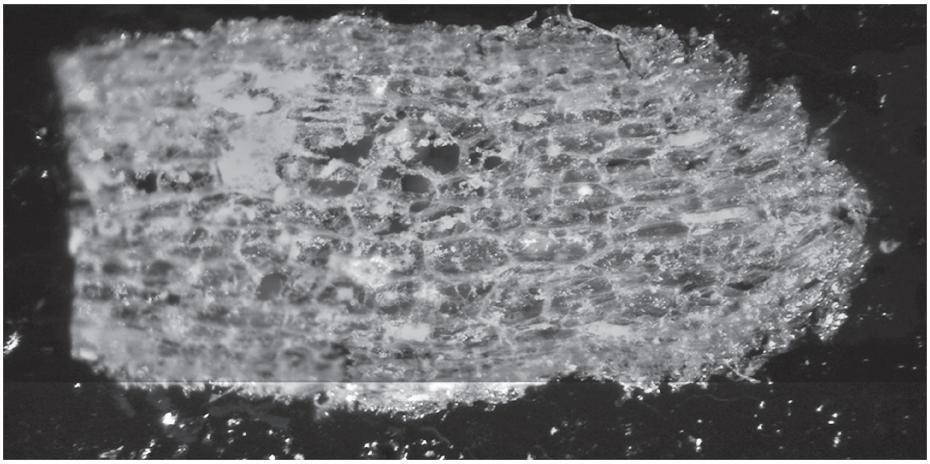

Figure 2 – The cell specific distribution of mass 184 in orchid mycorrhyza roots

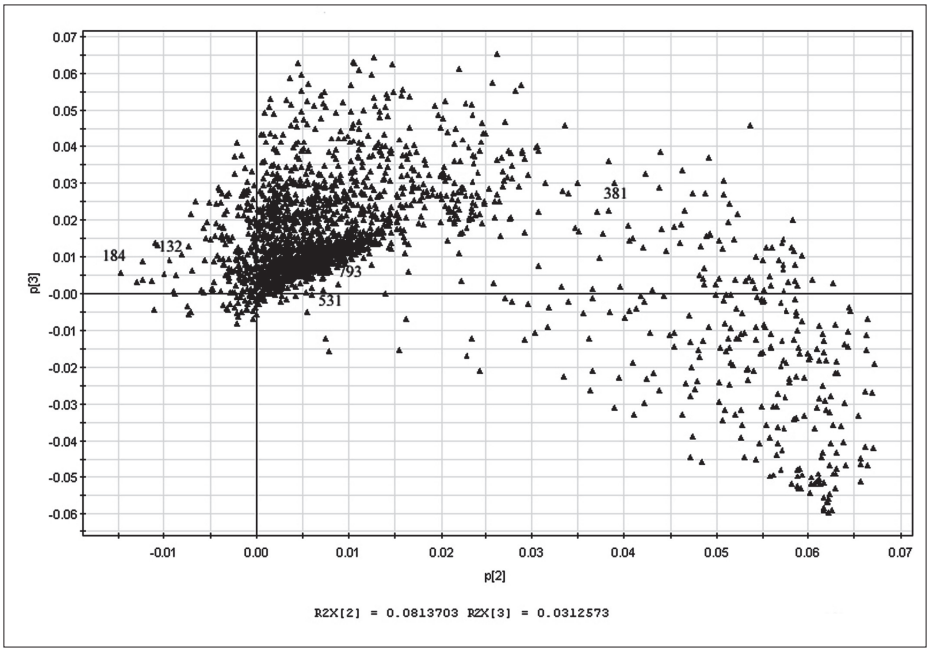

Figure 3 – PCA analysis of image from the analysis shown in figure 2.

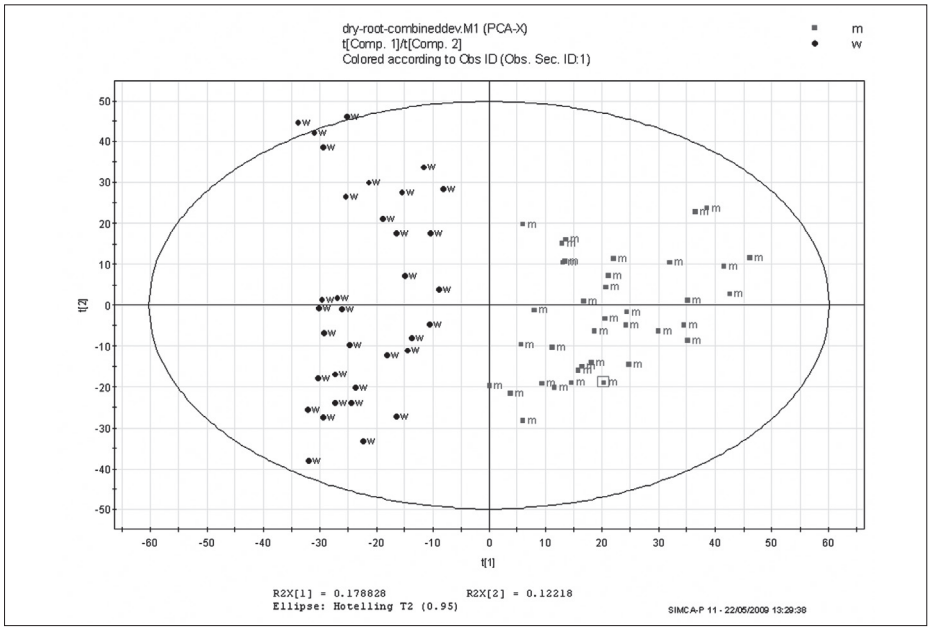

Figure 4 – PCA analysis of two images from roots of Arabidopsis thaliana.
The metabolic profiles obtained from the individual laser positions used to obtain an image of the two roots were used as separate data sets in the analysis. The roots from one sample were labeled w and those from the putative mutant root were labeled m.

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DEVELOPMENT AND VALIDATION OF HPLC-UV METHOD TO DETERMINE CREATININE AND METABOLITES OF XYLENE IN URINE

Abstract: A liquid chromatographic (LC) method for simultaneous determination of creatinine and o-, m-, and p-methylhippuric acids (metabolites of xylene) in urine is described. The analytical procedure is based on direct injection in system LC.

With this method, which does not require much time and handling, the different acids can be satisfactorily determined with high sensitivity and specificity. A statistical study shows a good reproducibility for the determination of creatinine, o-, m-, and p-methylhippuric acids. The coefficient of variation for 5 determinations in all cases was less than 2%.

Introduction

Xylene exists in three isomeric forms (ortho-, meta, and para-xylene). It is a colourless liquid with a typical aromatic odour, volatile and flammable, its vapour is explosive. The commercial product, commonly known as "xylol", is a mixture of all three isomers, with m-xylene predominating (usually 60-70%). It may contain a small amount ethylbenzene. The xylenes are extensively used as solvents for protective coatings, dyes, inks, and cements; as constituents of aviation gasoline blends and cleaning fluids; and as starting materials and intermediates for chemical synthesis (Ogata, Yamazaki, Sugihara et al., 1980). It´s the mayor chemical substance used in Pathological Anatomy Laboratory.

In occupational exposure, xylenes enter the body mainly through the respiratory tract. Pulmonary absorption of vapours is similar for all isomers of xylene and amounts to 60-70% (Šedivec and Flek, 1976). It remains relatively constant throughout the whole exposure period (Riihimaki, Pfaffli, Savolainen et al., 1979). The percutaneous absorption of xylene vapours is negligible in comparison with the liquid. In humans, xylene is efficiently metabolized. More than 90% is biotransformed to methylhippuric acid (MHA), which is excreted in urine. Xylene does not accumulate significantly in

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the human body, and are not normally present in the urine of non-exposed people (Šedivec and Flek, 1976).

Biological monitoring is very important to agreement health to workers. The exposure to organic solvents like xylene, is one of the highest potential risks for millions of persons in workplaces; they can generate substantial environmental pollution leading to outbreaks of public health problems.

The urine methylhippuric acid level has been measured by gas chromatography (Engström J and Bjurström R, 1978; Szőts, Tóth, Legoza et al., 2002), colorimetry (Ogata M and Hobara T, 1979), thin-layer chromatography (Bieniek, Palys and Wilczok, 1982; Bieniek and Wilczok, 1981) and high performance liquid chromatography (Antunes MV, Patuzzi ALM, Linden R. 2008; Ogata and Taguchi, 1987; Ogata, Yamazaki, Sugihara et al., 1980; Perlingov, Dabrowsk, Stránský et al., 2004).

This study describes a development of a rapid high performance liquid chromatographic with ultraviolet detector (HPLC-UV) method for estimation of metabolites of xylene (o-, m-, pmethylhippuric acid) in human urine. The aim of this work was to validate an appropriate chromatographic method for the simultaneous determination of creatinine and o-, m-, p-methylhippuric acid in urine samples.

Materials and Methods

Chemicals and reagents

Analytical standards of creatinine and xylene metabolites (purity 98%) were obtained from Sigma Aldrich®. Potassium phosphate monobasic anhydrous and orthophosphoric acid were obtained from Merck®. The acetonitrile was obtained from LiChrosolv® (HPLC grade). Ultrahigh purified water was obtained from Milli-Q water dispensing system by Millipore Corporation. HPLC grade solvents and reagents were previously filtered through Teflon filters with porosity of 0.45 µm Tracer (TR 200200) and degassed by ultrasound. We used disposable filters Tracer TR 200112, the polypropylene with 0.22 µm pore size for filtration of all solutions injected in LC.

Standard solutions and buffer

Stock solutions of creatinine (8 µg/mL) and each metabolites of xylene (30 µg/mL) standard were prepared in ultrahigh purified water and stored at –4ºC.

The solution of phosphate buffer at pH 2.3 (50 mM) was prepared by dissolving 6.66 g of potassium phosphate monobasic in 800 mL of ultrahigh purified water, followed by addition of 4.8 g of phosphoric acid 85% (v/v). The volume was completed to 1000 mL with purified water and pH was adjusted with the addition of phosphoric acid or potassium hydroxide, 0.1 M.

Instrumentation and analytical conditions

HPLC analysis was performed on a Varian® liquid chromatography system equipped with a Star 9010 Solvent Delivery System pump, an /visible detector (Star 9050

Variable Wave length), and a manual injector (10µL loop) was used. Chromatographic separations were carried out on a Varian® C-18 column (mesh size: 5 µm; length: 150 cm; internal diameter: 4.6mm). Creatinine and metabolites of xylene were separated by using a linear gradient elution program and the mobile phase was a mixture of phosfate buffer and acetonitrile (Table I). The flow rate was 1.5 mL/min. The UV detector was fixed at λ = 220 nm.

Urine samples, preserved by addition thymol, were collected in polyethylene bottles at the end of the work shift and stored as quickly as possible in a refrigerator at 5°C. The samples were stable for one week under these conditions, but could be kept for longer if frozen.

Urine samples preparation

The samples were prepared in glass tubes (5mL), only dilution with ultra-purified water (1:10) and centrifugation by 4100 rpm (5min). Before the injection, 1000µL of sobrenadant was passed through a 0.45µm-pore-size Millipore® filter.

Calibration curves

The standard calibration curves were obtained by the dilution of reference standard. The dilution was made in ultra-purified water to obtain the concentrations in Table II. Calibration curves were generated from the five calibration samples by analyte peak–area ratio against creatinine, o-mHA and m-, p-MHAs concentration. Linearity was assessed using a weighted least square regression ($1/x^2$ nominal).

Results and Discussion

Optimization of the method

A good separation was obtained, with all analytes being resolved and showing adequate peak shapes. Total time of analysis was of 10 min. The retention times were 0.8 min for creatinine, 7.4 min for o-MHA and 8.9 min for m-, p-MHAs. Under our experimental conditions, peaks from other urine components that could interference with the compounds of interest were observed. A typical chromatogram of creatinine, o- and m-, p-MHAs is shown in Figure I.

Linearity

The standard calibration curves in the investigated range were linear. The statistical analysis demonstrated that the standar curves in the investigated range were linear from 0.14-100 µg/mL (R^2 = 0.9991); 0.14-1750 µg/mL (R^2 = 0.9979); and 0.14-1500 µg/mL (R^2 = 0.9876) for creatinine, o-MHA and m-, p-MHAs, respectively. Table III summarizes the regression data of the calibrations curves, squares of correlation coefficients (R^2) and maximum squares of correlation coefficients (R^2max) of each analyte, for a statistical significance of 95%.

According to the t-test values obtained from t-Student (tobtained) are inferior to the statistical t-test values (tcritical) (tobtained < tcritical), then the adjustment

of the model is considered satisfactory. The results showed that the squares of the linear correlation coefficients were above 0.99 (Table III), evidencing the linearity occurrence. The regression of standard curves in ultra-purified water was statically significant since the statistical test did not exceed the critical value. The values of R^2 and maximum squares of correlation coefficients (R^2max) were similar. This indicates that the error due to the analytical procedure, validating the use of the linear model.

Sensitivity

Creatinine at a concentration of 12.64 µg/mL, o-MHA at a concentration 250 µg/mL and m-, p-MHAs at a concentration 250 µg/mL was injected on five different days (inter-assay). The limit of detection (LOD) and limit of quantification (LOQ) of creatinine, o-MHA and m-, p-MHAs in the mobile phase was also determined (Table IV).

The limit of detection was 0.077, 0.048 and 0.048 µg/mL, for creatinine, o-MHA and m-, p-MHAs, respectively. The results evidence that the HPLC system and the method are adequate for monitoring creatinine and metabolites of xylene. Precision was expressed as the coefficient of variation (CV, %). Values of inter-assay precision were described in Table V. Values below 5% demonstrate the precision of the method.

Conclusion

A highly selective HPLC assay with UV detection has been optimized for determination of creatinine and xylene metabolites in human urine.

This assay in urine samples without pretreatment, provides a specific and reproducible alternative to currently available methods and, to our best knowledge, offers the level of sensitivity required to study the toxicokinetics of inhaled and percutaneous absorption of xylenes.

Acknowledgments

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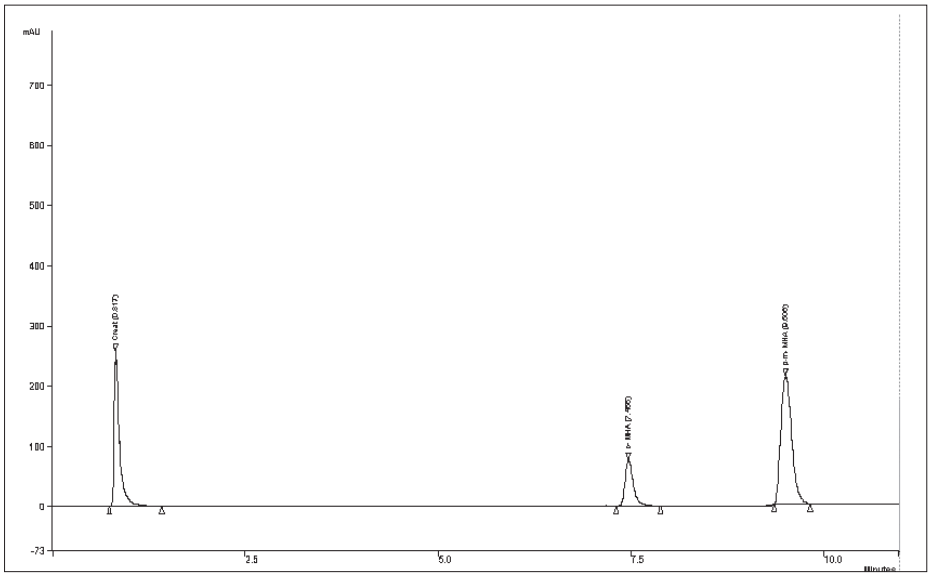

Figure 1 – Chromatogram (λ=220nm) of a standard mixture of g+creatinine (100 µg/mL), o-MHA (100 µg/mL), m-, p- MHAs (100 µg/mL).

Time / min	Phosfate Buffer (%)	Acetonitrile (%)
0	95	5
7.5	80	20
10	STOP	STOP

Table I – HPLC programmin showing the gradient of the mobile phase used to determine creatinine and metabolites of xylene

Analyte	Concentrations / ($\mu g mL^{-1}$)
Creatinine	10; 38; 66; 94; 100
o-MHA	0.3; 13; 26; 39; 52
m-, p-MHAs	0.3; 13; 26; 39; 52

Table II – Concentrations of analytes used in standard calibration curves

Analyte	t-Student Test		Linear Regression		
	$t_{critical}$	$t_{obtained}$	R	R^2	$R^2_{máx}$
Creatinine	2.3	1.85	0.9995	0.9991	0.9990
o-MHA	2.3	1.85	0.9989	0.9979	0.9976
m-, p-MHAs	2.7	2.13	0.9938	0.9876	0.9851

Table III – Results of linearity tests for statiscal significance at 95 percent

Analyte	LOD / ($\mu g\ mL^{-1}$)	LOQ / ($\mu g\ mL^{-1}$)
Creatinine	0.077	0.235
o-MHA	0.048	0.147
m-, p-MHAs	0.047	0.142

Table IV – Limit of detection (LOD) and limit quantification (LOQ) of analytes

Analyte	T_{ret}/min[a,b]	Precision	
		Pick Area[c]	CV (%)
Creatinine	0.82±0.01	71663.5±6.36	0.01-0.45
o-MHA	7.43±0.01	1250722±6074.05	0.24-1.08
m-, p-MHAs	8.98±0.02	4354776±32993	0.70-0.83

[a] Retention times are mean ± SD.
[b] Values obtined of standard calibrations curves (n=5).
[c] Pick area are mean ± SD.

Table V – Precision of the method

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APPLICATION OF ENERGY DISPERSIVE X-RAY FLUORESCE SPECTROMETRY (EDX) IN MEDICO-LEGAL AUTOPSY CASE

Abstract: We applied here energy dispersive X-ray fluorescence spectrometry (EDX) to medico-legal autopsy case of drowning in a river under the influence of hypnotics. Rapid elemental analysis using EDX identified bromide in blood, urine and cerebrospinal fluid of victim during autopsy. Subsequent toxicological analysis with a high performance liquid chromatography revealed bromovalerylurea in blood and other specimens. Present case shows that screening using EDX, ideal examination for non-destructive, rapid elemental analysis, provides useful information for identification of drugs.

Introduction

Rapid screening for drug overdose and the estimation of toxic substances are important in the fields of both emergency medicine and forensic toxicology. Such screening is usually performed using an immunological screening kit or color test paper [1].

Energy dispersive X-ray fluorescence spectrometry (EDX) is an easy and convenient way to identify various elements without special sample preparation [2]. Therefore, EDX is a quite useful tool for the primary identification of toxic compounds or pharmaceutical drugs including those such as arsenic and bromine.

We previously reported EDX was useful for screening of drugs in medico-legal autopsy cases [3]. Here, we report toxicological screening by EDX in another case of bromovalerylurea ingestion which was not revealed by police investigations.

Case history

A male in his thirties (height 188 cm, weight 115 kg) was found dead in the river below the bridge. He had told to his families about his attempt of suicide. Police investigation revealed that he was suffered from depression but the medications were unclear other than diphenhydramine, commercially available as over-the-counter hypnotics in Japan. The postmortem interval was estimated to be approximately 12 hours.

At autopsy, the lungs (left 550 g and right 370 g in weight) were edematous with marginal emphysema. White frothy fluid was found in the trachea and both bronchi. There was 500 ml of light-brownish stomach contents, and 4 ml of brownish liquid in sphenoidal sinus. Diatom test of lung, liver, kidney and sphenoidal sinus fluid were positive.

A drug screening test of the urine with Triage® (Biosite Diagnostic Inc., CA, USA) panel was positive for barbiturates.

Materials and Methods

The elemental screening tests and the quantification of bromide in blood, urine and cerebrospinal fluid were operated using EDX (JSX3200, JEOL, Tokyo, Japan) [3, 4]. The quantification limit for bromide ion using this method was 19.7 μg/ml.

Toxicological analysis was also performed using a high performance liquid chromatography (HPLC) drug analysis system (Class-VP system, Shimadzu, Kyoto, Japan) [5]. Quantification of ethanol was performed using a head-space gas-chromatography.

Results

EDX spectra of blood, urine and cerebrospinal fluid from victim showed the characteristic lines of bromide (Fig. 1). From the calibration curve, the concentrations of bromide in heart blood, urine and cerebrospinal fluid were calculated as 108.1, 41.2 and 38.0 μg/ml, respectively. Bromide was not detected from the river water using EDX, consistent with our previous report [4].

Subsequent toxicological analysis using HPLC identified bromovalerylurea, barbital and diphenhydramine within the concentrations of toxic levels (Table 1). Concentrations of ethanol in femoral blood and urine were revealed to be 0.08 and 0.22 mg/ml, respectively.

Discussion

From the autopsy findings and the results of diatom test, we determined the cause of death was aspiration of river water under the influence of sedative drugs, including bromovalerylurea.

Bromide concentration in blood from normal healthy subject was reported to be approximately 5.35 μg/ml [6]. We previously suggested that detection of bromide in blood using EDX could be an indicator in cases of drowning in seawater [4]. In present case, bromide was detected from each specimen regardless of freshwater- drowning. These data suggested that he took some chemicals containing bromide before his death. Bromovalerylurea, bromide-containing hypnotics, was identified by the subsequent detailed toxicological examinations using HPLC.

The concentrations of bromide in each specimen detected using EDX were different from those of bromovalerylurea by HPLC, especially in blood and urine. This may be because EDX can detect whole bromide including parent drug and its metabolites, while HPLC can detect only parent drug. Consistent with present case, Maguchi [7] also reported that blood concentration of bromide was higher than that of bromovalerylurea by 6 to 36 times.

It is reported the transfer of bromide ion into cerebrospinal fluid from blood is rarely occurred, whereas, bromovalerylurea easily invades into cerebrospinal fluid without being metabolized to bromide ion in this space [8]. Consistent with this report, in present case the concentration of bromide was more close to that of bromovalerylurea in cerebrospinal fluid than in blood.

EDX has been applied for the detection of some pharmaceuticals or metallic substances in medical fields [3, 9-11]. EDX enables non-destructive, non-degenerative analysis and is useful as a screening test when sample preservation is required for evidence.

Conclusions

We found EDX was useful as primary analysis of drug screening. Further applications in the field of forensic practice can be expected.

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	Barbital	Diphenhydramine	Bromovalerylurea	bromide
Heart blood	17.7	0.9	9.6	108.1
Femoral blood	16.9	0.9	9.1	118.6
Urine	62.1	5.2	Not detected	41.2
cerebrospinal fluid	15.0	0.4	5.2	38.0
Therapeutic levels in blood	10-26	0.03-0.11	10-20	less than 500
Fatal levels in blood	more than 100	more than 8	44-93	more than 3000
Reference	[12]	[12]	[13]	[10]

Table 1 – Concentrations of drugs and bromide in each specimen (μg/ml) with their fatal and therapeutic levels.

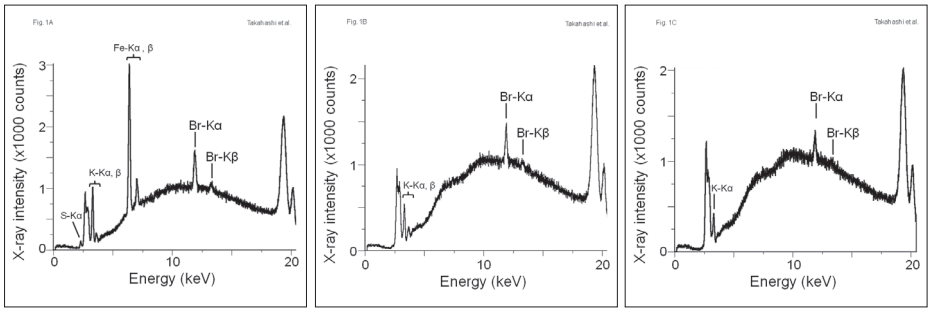

Figure 1 – EDX spectra of each sample.
The characteristic Kα and Kβ lines of bromide were identified in blood (A), urine (B) and cerebrospinal fluid (C). The concentrations of bromide in blood, urine and cerebrospinal fluid were calculated as 108.1, 41.2 and 38.0 μg/ml, respectively.
S-Kα; Kα line of sulfur: K-Kα, β; Kα and Kβ lines of potassium: Fe-Kα, β; Kα and Kβ lines of iron: Br-Kα; Kα line of bromide: Br-Kβ; Kβ line of bromide.

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DETERMINATION OF PARAQUAT IN BLOOD AND URINE BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY-MASS SPECTROMETRY.

Abstract: Paraquat (PQ) is a toxic quaternary ammonium compound widely used in agriculture. Numerous cases of paraquat intoxication have been reported either accidentally or intentionally as suicidal attempts.

A method for the determination of the herbicide PQ in blood and urine samples was developed using liquid chromatography-(electrospray ionization) mass spectrometry (LC-ESI-MS), following extraction with Oasis® WCX solid-phase cartridges. Chromatographic separation was achieved using an Atlantis® HILIC silica column, eluted isocratically with acetonitrile and ammonium formate (200mM) buffer, pH 3.8, at a 300 µL/min flow rate. Quantitation was achieved by the addition of ethyl paraquat as internal standard (IS). The compounds were detected monitoring two ions for PQ (*m/z* 185 and *m/z* 171) and *m/z* 213 for the IS. The method was applied to determine PQ in two cases: a non fatal case, a 42-year-old female with 0.13 µg/mL PQ concentration in blood and 6.29 µg/mL in urine and a lethal case, a 51-year-old male with 0.27 µg/mL PQ concentration in blood.

The authors developed a specific, sensitive and rapid assay for the identification and quantification of PQ, very important for monitoring suspected paraquat intoxications in hospitals and subsequently help in the treatment of these patients.

1. Introduction

The herbicide paraquat (1,1'-dimetyl-4,4'-dipyridyl cation, PQ), has been encountered in several cases of accidental and suicidal poisonings. The dichloride salt of paraquat or methyl viologen is well known under the trade name *Gramoxone*® (a 20% aqueous solution). Concentrated liquid formulations have been responsible for most (and more severe) poisonings than granular forms, which contain less PQ. Although normal use of the herbicide does not present a serious health risk, many successful suicide attempts are often the result of the ingestion of concentrated forms of PQ [1-2].

Paraquat has low but rapid gastrointestinal absorption (5-10%). Peak plasma concentrations appear in less than 2 h. Following ingestion, PQ is actively transported to all major organs, especially to the lung, where it is reduced to form highly reactive free radicals. It is slowly excreted unchanged in urine and feces [3-4].

A wide variety of analytical techniques have been reported to determine PQ in biological samples, including gas chromatography (GC) [5-6], GC/mass spectrometry (MS) [7], high-performance liquid chromatography (LC) [8-13], LC/MS [14-15] and LC/MS/MS [16-19]. However, most of them require a complicated and a time-consuming sample pretreatment.

Because this substance is ionic, solid-phase extraction (SPE) and HPLC usually have been accomplished with the aid of an ion-pairing reagent such as an alkyl sulphonic acid. LC-MS provides the analyst with a more sensitive and highly selective analytical method for paraquat. Strong cation exchange-based SPE methods that do not require ion-pairing reagents have been employed successfully, but the strong salts or strong acids used for elution are difficult to remove and are serious impediments to optimal LC-MS analysis. In order to overcome these problems, a new type of sorbents has been developed [20] for the retention of quaternary ammonium compounds and strongly basic organic compounds. The Oasis WCX sorbent incorporates a weak cation-exchanger bound to a polymeric reverse-phase particle. An Atlantis HILIC column was utilized for LC using no ion-pairing reagents.

This work presents two cases due to oral ingestion of *Gramaxone*®, and describes a sensitive, specific, and rapid LC-ESI-MS method used to detect, confirm and quantify PQ in blood and urine samples.

2. Case reports

The first report describes a successful clinical case regarding the intoxication of a 42-year-old woman by a presumed lethal dose of paraquat. After hospital treatment this patient has shown a gradual return to normal spirometry values from the marked reduction that occurred at the time of paraquat intoxication. PQ was detected in blood and urine at levels of 0.13 µg/mL and 6.29 µg/mL, respectively. Blood and urine samples were sent again to our laboratory 7 days after intoxication. PQ was not detected in blood and PQ urine concentration found was 0.18 µg/mL. She survived.

The second case was a 51-year-old man admitted to the hospital with the information of a suicide attempt with intentional intake of *Gramaxone*®. Treatment with repeated activated charcoal hemoperfusion was attempted (total time 14h). The patient developed acute renal and respiratory failure. He died from multiple organ failure 8 days after intoxication.

At autopsy, the internal examination revealed that both lungs were solid with acute hemorrhagic edema and both kidneys were deeply congested. Histological findings showed marked pulmonary congestion with numerous hemosiderin-laden macrophages.

The paraquat blood concentration found was 0.27 µg/mL.

3. Materials and methods

3.1. Chemicals and reagents

Paraquat and ethyl paraquat (used as internal standard) were supplied by Sigma-Aldrich-Chemie GmbH (Steinheim, Germany). Each standard solution was prepared

in methanol (1 mg/mL) and stored in plastic bottles at +4°C. Acetonitrile and methanol were HPLC-grade and were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid was from Riedel-de Haën (Seelze, Germany). Deionised and purified water was obtained using a Milli-Q system (Millipore, Molsheim, France). Solid-phase cartridges Oasis® WCX, (60 mg, 3 cc) were purchased from Waters (Milford, MA). The phosphate buffer (pH 7.0) was prepared by dissolving 0.12 g of NaH2PO4 into a 100 mL volumetric flask and brought to volume with deionized water. Ammonium formate buffer 200 mM (13.0 g/L) was prepared with deionized water, and the pH adjusted to 3.6 with formic acid. The mobile phase was filtered through a 0.20 µm filter (Schleicher & Schuell) and degassed in an ultrasonic bath for 15 min just before use.

3.2. Instrumentation

The chromatographic system (LC) used was a Waters 2695 Alliance System and Atlantis® HILIC silica column (2.1x150 mm, 5 µm). The mobile phase consisted of acetonitrile and 200 mM ammonium formate buffer, pH 3.6, (30:70, v/v) at a 300 µL/min flow rate. The column temperature was maintained at 35°C. The injection volume was 10 µL.

A Waters 996 photodiode array detector operated on a 210-400 nm wavelength scan with a 1.2 nm resolution. The UV absorbance was measured at 258 nm.

Mass spectrometry detection (MS) was carried out on a Waters ZQ 2000 single quadrupole mass spectrometer with an electrospray ionization (ESI) performed in positive mode. Full-scan spectra were recorded from *m/z* 130-500, at a scan time of 0.5 s and an interscan delay of 0.1 s. The other main instrument settings were: capillary voltage 3.5 KV; cone voltage 40 V; extractor 4 V; ion energy 0.5; source temperature 120°C; desolvation temperature 400°C; cone gas (N2) flow rate 0 L/h and desolvation gas (N2) flow rate 600 L/h.

Instrument control, data acquisition and processing were achieved using Waters Empower software (Milford, MA).

3.3. Sample preparation

Controls and calibration samples were prepared by spiking drug-free whole blood and urine samples with standard solutions.

A 1 mL aliquot of whole blood or 1 mL of urine was spiked with 50 µL of internal standard (10 µg/mL) and diluted with 2 mL of acetonitrile. Then the samples were vortex mixed and centrifuged for at 2500 rpm for 10 min. Extraction cartridges (Oasis® WCX, 3cc) were conditioned with 1 mL of methanol followed by 1 mL of deionized water. Each sample was loaded through a cartridge. It was then washed with 1 mL of phosphate buffer (pH 7) followed by 1 mL of deionized water and 1 mL of methanol. After drying under vacuum for 10 min, elution was carried out with 1.5 mL of acetonitrile/water/TFA (84:14:2, v/v). The eluate was evaporated to dryness under a nitrogen gas flow at 40°C. The residue was dissolved in 100 µL of methanol and an aliquot (10 µL) was injected into the LC-ESI-MS system.

4. Results and discussion

Calibration curves for paraquat in blood and urine samples were linear from 0.010 to 2.0 µg/mL in blood (y= 0.0142x+0.1497 with r^2 = 0.9994) and from 0.025 to 10.0 µg/mL in urine (y= 0.0141x+1.347 with r^2 = 0.9994).

The detection limit of PQ in blood and urine samples was 0.004 µg/mL and 0.007 µg/mL respectively (*LOD*, S/N=3) and the lower limit of quantification (*LOQ*, S/N=10) was 0.012 µg/mL in blood and 0.024 µg/mL in urine. For intra-day and inter-day precision determinations, five replicate analyses were performed at each of the three studied concentrations. Relevant validation data for recovery and precision are presented in Table I. The method proved to be precise for paraquat, both in terms of intra-day and inter-day analysis, with coefficients of variation (CV) less than 20%. In selectivity study, an analysis of blank blood samples showed there were no interfering peaks at the elution time of paraquat or the internal standard (ethyl paraquat).

Quantitation employed the selected ion-recording mode (SIR) using the *m/z* corresponding to the most abundant product ion $[M+H]^+$ at *m/z* 185 for paraquat and *m/z* 213 for the internal standard. Paraquat fragment ions, *m/z* 171 and *m/z* 144, were due to the loss of the methyl group corresponding to $[M+H-CH3]^+$ and due to the loss an HCN molecule $[M+H-CH3-HCN]^+$, respectively. Both SIR and Scan acquisitions were performed in centroide mode. SIR mass chromatograms and mass spectrum in SCAN mode (*m/z* 185) of the paraquat detected in the blood sample (case 2) are shown in Fig. 1.

The proposed solid-phase extraction procedure and LC-ESI-MS method provided an accurate assay for the determination of paraquat in blood and urine.

The authors developed a specific, sensitive and a rapid assay for the identification and quantification of paraquat, very important for monitoring suspected paraquat intoxications in hospitals and consequently help in the treatment of these patients. The procedure has also been applied to a fatal death case involving paraquat poisoning.

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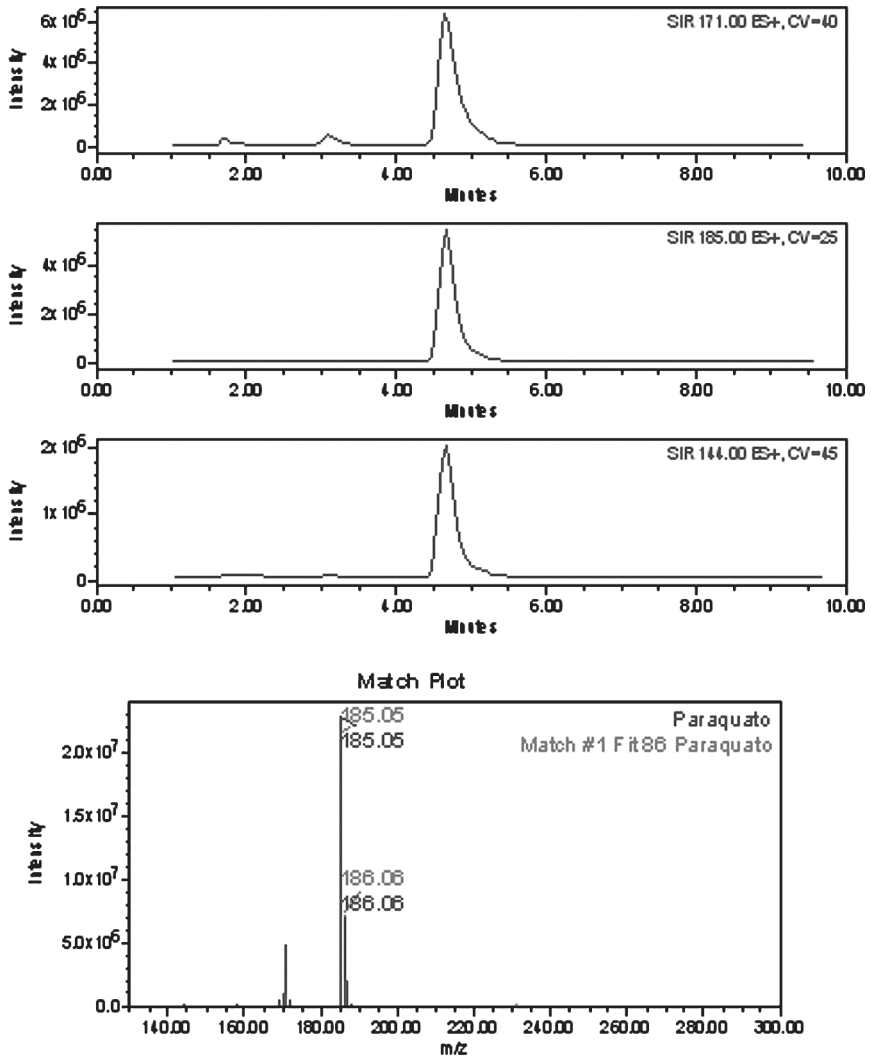

Figure 1 – SIR mass chromatograms and mass spectrum in SCAN mode (m/z 185), of paraquat in postmortem blood sample (case 2).

	Concentration level (µg/mL)	Recovery (%)	Intra-day CV (%)	Inter-day CV (%)	n
BLOOD	0.025	60 ± 8.7	10.4	4.2	5
	0.25	58 ± 7.7	6.0	6.7	5
	1	67 ± 1.8	10.2	17.3	5

Table I – Validation data of recovery and precision for paraquat.

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PROTEOMIC INVESTIGATION OF TISSUES OF MEDICAL INTEREST BY MALDI MSI

Abstract: MALDI Mass Spectrometry Imaging (MALDI MSI) enables images of the distribution of endogenous/xenobiotic compounds to be obtained directly from intact tissue sections. The ability to localise thousand of ions in a single analysis, without using radioactive probes or antibodies, makes it a very powerful tool in biomarker discovery. Technological improvements of *in situ* proteomic protocols have recently enabled MALDI MSI to be used for the identification of up/down regulated species in both snap frozen and archived tissues such as formalin fixed paraffin embedded (FFPE) samples. A further improvement of these protocols, the incorporation of a surfactant in the enzymatic digestion step, is discussed for the investigation of protein distribution and expression from frozen and FFPE adenocarcinoma tissue sections.

Introduction

MALDI Mass Spectrometry Imaging (MALDI MSI) is an advanced MS technique first reported by Richard Caprioli in 1997 [1]. This technology enables the visualization of the spatial distribution of endogenous and xenobiotic compounds directly from intact tissue sections. A variety of molecules can be investigated ranging from drugs, lipids and peptides to proteins. Typically, in a direct MALDI MSI approach, snap frozen tissues are mounted and sectioned in a cryostat at a thickness varying between 10 and 20 µm. The section is then either spotted or sprayed with an UV absorbing matrix. In MALDI Imaging mode, the laser (UV or solid state) is automatically fired on a raster of points over the section. Each ion within the mass spectra acquired can be visualised as a two-dimensional image by using its m/z ratio (Fig. 1). The closer the raster points, the higher the image resolution. This technology can operate in both Imaging and Profiling mode and the choice depends on the goal to be achieved. In Imaging mode, high resolution images of the ion distribution are desired and consequently the matrix is applied by homogeneously spray coating the section. In profiling mode, rather then images, the aim is to obtain and compare molecular profiles (as mass spectra) in specific tissue regions. This is accomplished by depositing discrete spots of matrix on the regions of interest and recording mass spectra from each spot separately. This is very quick and helpful when differential proteomics studies are undertaken on tissues of medical

interest. In a biomarker discovery experiment therefore, instead of tedious extraction methods, pre-purification and separation through 2D electrophoresis or bidimensional HPLC followed by MS, this technology gives ready access to an enormous amount of information and, at the same time, retains the spatial information [2].

In 2004 Chaurand and co-workers reported one of the first demonstrations of the potential of MALDI-MSI in medicine [3]. In an attempt to demonstrate the feasibility of the technology as a diagnostic and prognostic tool for assessment of cancer staging, they were able to distinguish between gliomas and non-tumor brain tissue as well as to sub-classify grade IV gliomas from grades II and III at a molecular level, by observing in the spectra profiles an up-regulation of the protein S-100β in the high versus low grade tumours. This was confirmed by the mass images of distribution of the protein and validated by immunohistochemistry. These results suggested an important role for MALDI MSI which could in fact be crucial in those borderline cases where the diagnosis relies uniquely on the personal judgment and experience of the histopathologist. Moreover, simultaneous immunochemical localization of proteins/peptides in one experiment generally provides information on two to three markers at the same time, thus suggesting that immunohistochemistry is more amenable for biomarker validation rather then for biomarker discovery.

A major source of tissue samples are potentially the formalin-fixed, paraffin-embedded (FFPE) tissues found in hospital libraries. However, because of the predictable ion suppression events, MALDI MSI was initially thought only to be applicable to frozen tissues. This prevented access to huge tissue archives and information from tissues which are already analysed and diagnosed. Indeed, for FFPE tissues, the older they get, the more intricate protein network the formalin promotes, thus significantly lowering signal intensity and resolution. Nonetheless in 2007, Lemaire and collaborators developed a protocol showing feasibility of MALDI MSI for the investigation of FFPE tissues [4]. The authors demonstrated that for less than 1 year old FFPE tissues, the use of 2,4 DNPH as matrix, which blocks unreacted formalin, combined with on tissue digestion, for up to less than 2 years old FFPE tissues, followed by MS and MS/MS analysis, is a valid experimental approach to access archived tissues. Proteomic investigation by *in situ* tryptic digestion was first introduced by the group of M. Setou [5] and it is now a rather consolidated methodology but it had never be applied to FFPE tissues before the work of Lemaire and colleagues. Despite this revolutionary approach, sensitivity can still be an issue, especially for older FFPE tissues. Here, taking stock from all the previous work, a four step methodology is suggested to improve proteomic investigation of both frozen and FFPE tissues. This study has been recently published [6] and used, as models, xenograft breast tumours and FFPE breast tumour sections. The major novel elements were the inclusion of a non ionic detergent (octylglucoside, OcGlu) for a more efficient *in situ* proteolysis, and the use of Ion Mobility Mass Spectrometry (IMS) to retrieve MS/MS spectra of isobaric ions, thus improving protein identification. This articles focuses on these two aspects of the experimental procedure.

2. Materials and Methods

Modified sequence-grade trypsin was purchased from Promega (Southampton, UK). All other materials were purchased from Sigma-Aldrich (Dorset, UK). MCF7

breast tumour xenografts were obtained from the Institute of Cancer Therapeutics, Bradford, UK. *Ex vivo* human breast tumour tissue samples were obtained following fully informed patient consent and local ethical committee approval.

2.1. Tissue preparation

Frozen MCF7 xenograft tissue samples were cut in a cryostat operating at –20°C. Five 10 µm sections were obtained and thaw-mounted onto either an aluminum foil, or an ITO glass slide. Rinsing procedures, involving washings in ethanol at different percentages and in chloroform, were performed to increase the MS data quality as described elsewhere [7]. Sections were then submitted to enzymatic digestion and matrix deposition. For FFPE tissue sections, paraffin wax was first removed according to procedures described previously [8].

2.2. In situ *digestion*

In situ digestion was performed using a 0.05 mg/mL trypsin acqueus solution containing 0.1% of OcGlc. 100–150 nL of trypsin solution were deposited on the section using a Sun-Collect MALDI-Spotter (SunChrom). The spot-to-spot distance was set at 400 µm. The section was then incubated for 2h in a humid chamber at 37°C. Following enzymatic digestion, matrix deposition was performed using a robotic printer. An ionic matrix, consisting of CHCA mixed with ANI (CHCA/ANI), was used for MALDI MS analysis of the resulting peptides.

2.3. In situ *peptide analysis by MALDI-MSI and direct protein identification with IMS MALDI-MS/MS*

MALDI-MSI data were acquired in the reflector and positive mode using either a MALDI SYNAPT™HDMS system (Waters Corporation, Milford, MA) operating with a 200 Hz Nd:Yag laser, or an Ultraflex™II MALDI-TOF/TOF instrument (Bruker Daltoniks, Bremen, Germany) equipped with a Smartbeam™ laser. Standards for spectral calibration consisted of poly(ethylene glycol) ranging between m/z 400 and 3000 Da and a peptide mixture ranging between 900 and 3500 Da. Full scan mass spectra were recorded from 600 up to 5000 Da. Images were generated and reconstructed using Biomap 3.7.5 and FlexImaging™ 2.0 software. MALDI MS/MS analyses were acquired using the MALDI SYNAPT™HDMS operating in IMS mode directly from the digested tumour tissue sections. MS/MS spectra were submitted to a MASCOT query search and searched against the Swiss-Prot database. *De novo* sequencing was performed manually and using the PepSeq™ *de novo* interactive MS/MS sequencing tool.

3. Results

3.1. *In situ* protein identification by MALDI-MSI

In order to improve the detection of low abundant and high mass proteins, a main experimental step was introduced consisting in the use of a non ionic detergent such

as OcGlu in the trypsin solution. This proved to be a crucial step to improve peptide yield. Fig. 2 shows a comparison of spectra profiles obtained after *in situ* digestion performed with trypsin in either water (Fig. 2A) or in a solution containing 0.1% of the detergent at 37°C for 2h (Fig. 2B). It is clear that in the latter case, many more peptide signals were generated. These were later identified and attributed to low abundant proteins. Histones and stress proteins such as Grp75 were identified through MS/MS analysis and confirmed by direct protein analysis (profiling and imaging) [6]. The same methodology was applied to FFPE breast tumour sections with equally successful results.

3.2. Ion Mobility Mass Spectrometry

In order to address ion signal overlap from isobaric peptides generated by the use of OcGlu, IMS was used as a mass separation step prior peptide fragmentation. Figure 3A shows the IMS of two isobaric species of m/z 850 arising from the direct MS/MS analysis. When a MASCOT search was performed on the entire MS/MS spectrum, without taking into account the mobility separation, no significant protein identification was obtained. However the IMS data can be processed thus extracting individual MS/MS spectra corresponding to each species (Fig 3B and 3C). In this way the species at m/z 850.47 could be fragmented and the fragments imported into MASCOT allowing the identification of the histone H2A. Localisation of this protein in the tumour areas was successful for both frozen and FFPE tumour sections (Fig. 4a).

4. Discussion

An improved methodology for *in situ* proteomic investigation of tissues of medical interest (both frozen and FFPE), is reported. In particular, the inclusion of OcGlu as a non ionic detergent in the proteolytic step has shown to increase the number of signals detected relating to low abundant proteins. To overcome the overlap of peptide signals deriving from the improved proteolysis, one approach is to use a high resolving power mass spectrometer allowing accurate mass detection. In the present work a MALDI instrument has been used allowing IMS, thus enabling separation of isobaric peptides on the basis of their collisional cross section. This opportunity allows the obtainment of two separate MS/MS spectra for the isobaric species and has been found to improve the selectivity and facilitate database search, as shown for the species at m/z 850. Numerous protein signals were detected and some proteins including histone H3, H4 and Grp75 present in the tumour region were identified [6].

A more detailed and comprehensive MALDI based proteomic workflow for improved proteomic investigation and validation of biomarkers in tissues of medical interest is shown in Fig 5. Briefly, this strategy ideally includes the use of high resolving mass spectrometry as well as the use of IMS to potentiate protein detection, profiling, imaging and identification. A parallel analysis by microextraction, digestion and nanoLC MS MS analysis could be simultaneously performed. The peptide maps generated are submitted to database search for protein ID supported by their fragmentation spectra, imaged

and submitted to statistical analysis to pinpoint biomarkers. Immunohistochemistry is used as last methodological step for biomarker validation.

5. Conclusions

The present work demonstrates that the incorporation of a non ionic detergent in the proteolytic solution, combined with the use of IMS greatly improves protein identification in both frozen and FFPE tissues.

The recent past, the present work and further improvement in the development of a more and more efficient MALDI MS based proteomic methodology, leave little doubt about the role and the potential of this technology for the diagnosis of medical conditions at an early stage as well as prognosis of the treatment.

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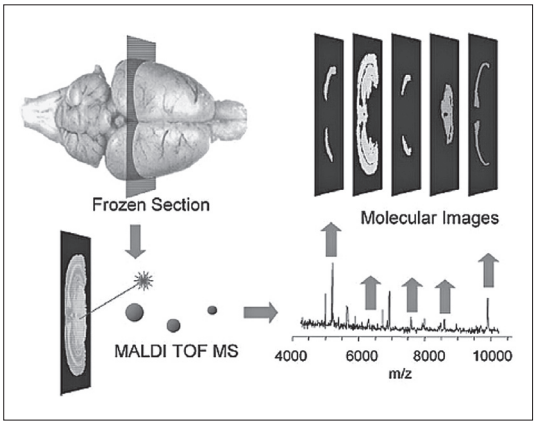

Figure 1 – Schematics of MALDI MSI from sample preparation to data analysis[1].

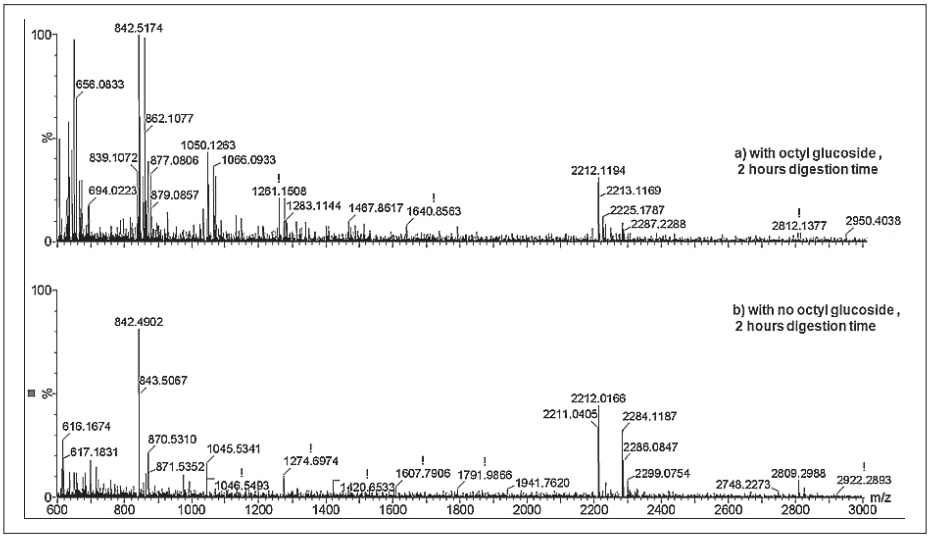

Figure 2 – Improved in situ tissue digestion. Panel A displays the observed peptide profiles obtained using trypsin in water compared to the one generated by a trypsin solution containing 0.1% of OcGlc (Panel B)[2].

[1] Reprinted from Stoeckli, M.; Staab, D.; Staufenbiel, M.; Wiederhold, K.H. Molecular imaging of amyloid ß peptides in mouse brain sections using mass spectrometry. Anal. Biochem., 2002, 311, 33-39, with permission from Elsevier.

[2] Reproduced and adapted by permission of John Wiley & Sons from Djidja et al, Proteomics, 9, 1–15 (2009).

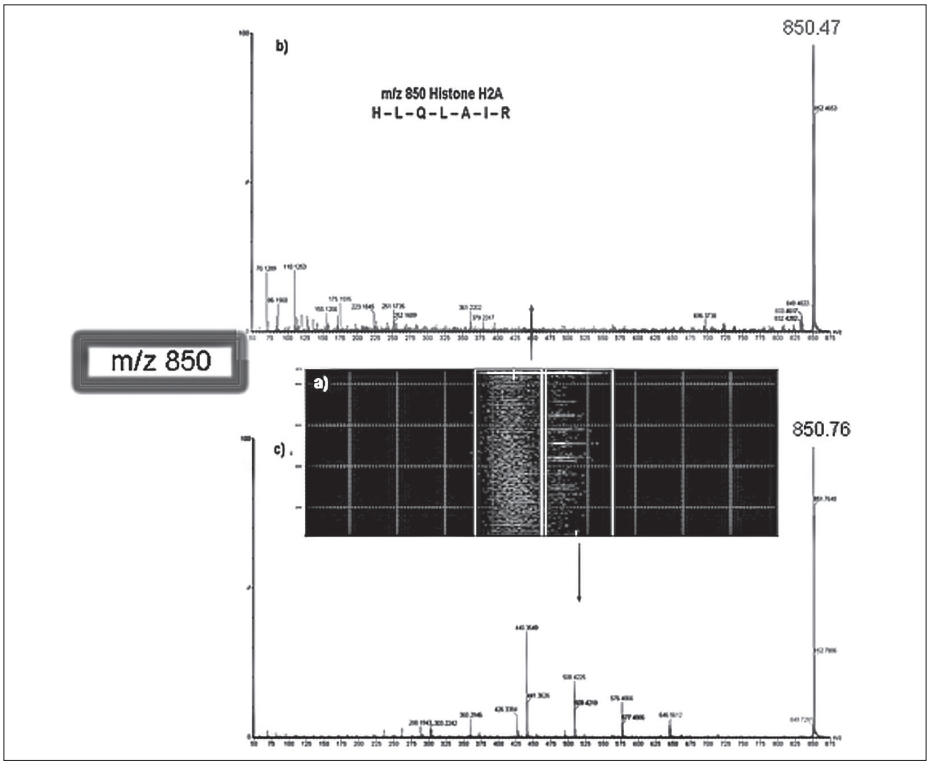

Figure 3 – IMS of the ion signal at m/z 850. The observed "Driftscope" plot (A) displays interference between species in MS/MS analysis mode. These species can be separated using their mobility. Different MS/MS spectra (B and C) can be extracted using the IMS.[2]

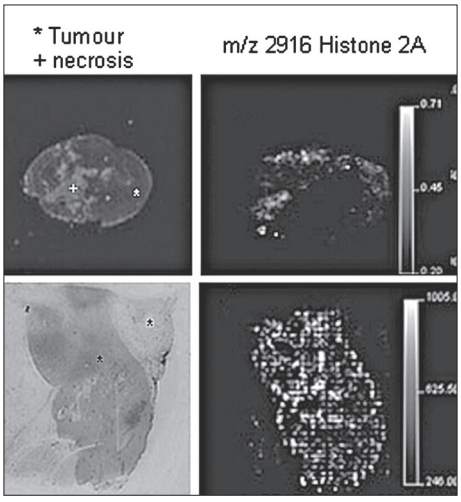

Figure 4 – Localisation maps of histone H2A. The protein was identified and localised in the tumour region through the peptide at m/z 2916 in frozen and FFPE sections. [2]

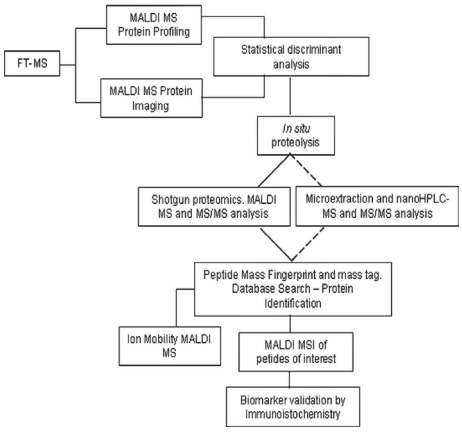

Figure 5 – Proposed MALDI MS based proteomic workflow for the analysis of intact tissue sections. Dashed lines indicate parallel non *in situ* analyses that can be performed.

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CASE REPORT

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GENETIC ANALYSIS OF RHABDOMYOLYSIS-ASSOCIATED GENES: AN AUTOPSY CASE OF METHAMPHETAMINE-RELATED HYPERTHERMIA AND ACIDOSIS.

Abstract: In an autopsy case of methamphetamine (MA)-related hyperthermia and acidosis, we investigated the genetic background of hyperthermia and muscular hyperactivity in relation to rhabdomyolysis. We examined mutations in the *ryanodine receptor1* (*RYR1*) gene, which is associated with malignant hyperthermia, the *very long-chain acyl-CoA dehydrogenase* (*VLCAD*) gene, which is associated with rhabdomyolysis, the *carnitine palmitoyltransferase II* (*CPT II*) gene, which is the most common cause of recurrent rhabdomyolysis in adults and the *cytochrome P450* (*CYP)2D6* gene which encodes MA-metabolizing enzyme. There were two homozygous and three heterozygous silent mutations in the three hot-spot regions in the *RYR1* gene. There was no mutation in the *VLCAD* gene. In the *CPT II* gene, the subject was found to be homozygous for two amino acid substitutions, ^{352}Phe>Cys and ^{368}Val>Ile in exon 4. In the *CYP2D6* gene, the subject was heterozygous for ^{100}C>T, ^{1661}G>C and ^{4180}G>C causing ^{34}Pro>Ser, a silent mutation and ^{486}Ser>Thr, respectively, in the *CYP2D6*10A* allele.

Introduction

We report an autopsy case of methamphetamine (MA)-related hyperthermia and acidosis. It was recently reported that MA causes rhabdomyolysis, myoglobinuria, and acute renal failure[1]. There is a possibility that rhabdomyolysis can be triggered by fragility of muscular cells or a reduction in the metabolism of the causative agent, which are caused by genetic background. The *ryanodine receptor1* (*RYR1*) is mainly expressed in skeletal muscle where it mediates Ca^{2+} release from the sarcoplasmic reticulum, following depolarization of the plasmalemma. Mutation in the *RYR1* gene have been found in association with malignant hyperthermia[2]. The *very long-chain acyl-CoA dehydrogenase* (*VLCAD*) gene is an enzyme catalysing the dehydrogenation of long-chain fatty acids in the first step of mitochondrial fatty acid oxidation. Recognized heritable causes of rhabdomyolysis are defects in fatty acid oxidation[3]. The *carnitine palmitoyltransferase* (*CPT*) enzyme system plays an important role in the transfer of long chain fatty acids from the cytosolic compartment to the mitochondrial matrix, where beta-oxidation occurs. CPT II deficiency is an important cause of recurrent rhabdomyolysis[4]. MA is metabolized in the liver by the *cytochrome P450* (*CYP)2D6*[5].

Materials and methods

Examined case

A man in his twenties was found dead in a rice field with his clothes scattered around.

Autopsy findings: He was 177 cm tall and weighed 74 kg. The rectal temperature was 40°C at the postmortem examination. There were many small abrasions and subcutaneous bleeding on the body. Rigor mortis was relatively advanced. The brain was edematous, and various organs were congested. There was a blood-like solution and bubbles in the trachea and bronchus.

His cause of death was diagnosed by histological, toxicological and other examination. Especially, to diagnose the acute renal disorder followed by rhabdomyolysis, immunostaining of kidney was performed with antibodies against myoglobin, 70 kDa heat shock protein (HSP70), 8-hydroxy-2'-deoxyguanosine (8-OH-dG), 4-hydroxy-2-nonenal (4-HNE), superoxide dismutase Cn/Zn enzyme (SOD), and 50kDa oxygen regulated protein (ORP-150). Immunostaining of skeletal muscle was also performed with antibodies against myoglobin.

Genetic analysis

For the genetic analysis, genomic DNA was isolated from blood samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The mutational analysis was performed using direct sequencing. The *RYR1* gene contains 106 exons. Intronic primers for amplification from genomic DNA were designed for each exon within the three mutational hot-spot regions. The *VLCAD* gene contains 20 exons and we designed primer pairs for all exons of the *VLCAD* gene. Mutation in the *CPT II* gene was analyzed according to the method of Kaneoka et al[4]. CYP2D6 was analyzed all exons using designed primer pairs specific to the *CYP2D6* gene. All PCR products were sequenced directly on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Results and Discussion

Cause of death

Histologically, severe congestion was found in all organs, and was especially marked in the lung, liver, kidney, and spleen. The lungs were also edematous. In the heart, there were no abnormal findings. In the proximal tubules, the epithelia were swollen and their nuclei were enlarged. Immunohistochemical findings are shown in Table 1. In the kidney, myoglobin and 8-OHdG were negative. HSP70, 4-HNE, SOD and ORP-150 were positive. Myoglobin immunoreactivity was decreased in the skeletal muscle.

In blood from the heart, 0.75 μg/ml MA was detected by GC/MS analysis, and was 16.8 μg/ml in the urine, and 6.2 μg/ml in the stomach contents. Amphetamine was also detected in the blood by GC/MS analysis.

Autopsy findings, histological findings, immunohistochemical findings and toxicological analysis revealed his cause of death was MA-related death, such as hyperthermia and metabolic acidosis caused by muscular hyperactivity.

Genetic analysis

So, we performed a mutational analysis of 4 rhabdomyolysis-associated genes (Table 2). In the *RYR1* gene, there were two homozygous and three heterozygous silent mutations in the three hot-spot regions, but there was no mutation causing an amino acid substitution. Mutation of the *VLCAD* gene was not found. In the *CPT II* gene, the subject was found to be homozygous for two amino acid substitutions, ^{352}Phe>Cys and ^{368}Val>Ile in exon 4 (Fig. 1). However, it has been reported that the ^{352}Phe>Cys and ^{368}Val>Ile substitution alone did not affect enzyme activity in vitro[6 7]. The *CYP2D6* gene is highly polymorphic, causing no, decreased, normal or increased enzyme activity. A relationship between increased drug concentrations and rhabdomyolysis has been reported. In the *CYP2D6* gene, the subject was heterozygous for ^{100}C>T, ^{1661}G>C and ^{4180}G>C causing ^{34}Pro>Ser, a silent mutation and ^{486}Ser>Thr, respectively, in the *CYP2D6*10A* allele (Fig. 2). The subject was heterozygous for the *CYP2D6*1* allele and *CYP2D6*10A* allele. The *CYP2D6*10* allele, which includes both the *CYP2D6*10A* and *CYP2D6*10B* variants, is widely observed in Japanese. CYP2D6*10 encodes an unstable enzyme with reduced catalytic activity[8]. It is possible that an alteration of CYP2D6 activity changes the metabolism of MA.

Conclusions

His cause of death was considered to be hyperthermia and acidosis caused by muscular hyperactivity. There was no mutation which causes the amino acid substitution in the *RYR1* and *VLCAD* genes. In the *CPT II* gene, the subject was found to be homozygous for two amino acid substitutions, ^{352}Phe>Cys and ^{368}Val>Ile in exon 4. It has been reported that these substitutions did not affect enzyme activity in vitro. In the *CYP2D6* gene, the subject was heterozygous for ^{100}C>T, ^{1661}G>C and ^{4180}G>C causing ^{34}Pro>Ser, a silent mutation and ^{486}Ser>Thr, respectively, in the *CYP2D6*10A* allele. CYP2D6*10A is associated with a decreased metabolic clearance of CYP2D6 substrates.

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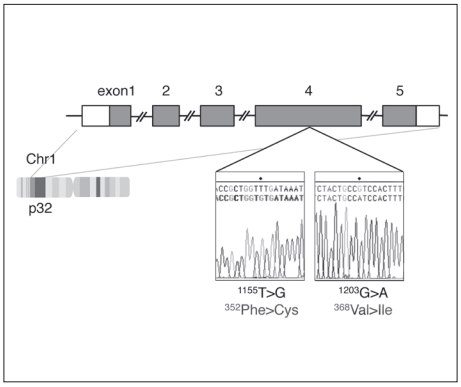

Figure 1 – Partial nucleotide sequences of the *CPT II* gene.

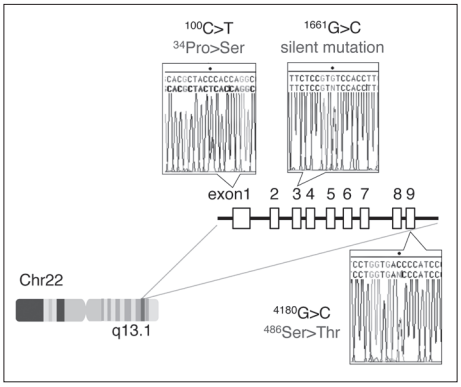

Figure 2 – Partial nucleotide sequences of the *CYP2D6* gene.

Immuno-reaction	Glomerulus	Proximal tuble	Distal tuble
Myoglobin	–	–	–
HSP70	+	–	–
8-OHdG	–	–	–
4-HNE	–	–	+
SOD	–	+	–
ORP-150	–	+	+

Table 1 – The results of immunohistochemical staining (+; positive, –; negative)

Gene	Nucleotide change	Exon (s)	Amino acid change	Zygosity
RYR1	^{1668}G>A	15	Silent mutation	Homo
	^{7281}C>T	45	Silent mutation	Hetero
	^{7584}C>T	47	Silent mutation	Homo
	^{11754}T>A	85	Silent mutation	Hetero
	^{14256}A>C	98	Silent mutation	Hetero
VLCAD	none	–	–	–
CPT II	^{1155}G>A	4	^{352}Phe>Cys	Homo
	^{1203}G>A	4	^{368}Val>Ile	Homo
CYP2D6	^{100}C>T	1	^{34}Pro>Ser	Hetero
	^{1661}G>C	3	Silent mutation	Hetero
	^{4180}G>C	9	^{486}Ser>Thr	Hetero

Table 2 – Identified mutations

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SIMPLE FRACTURE = GOOD CLINICAL EVOLUTION? REGARDING A CASE ASSESSMENT IN CIVIL LAW

Abstract: Fractures of the limbs are one of the most common injuries resultant from road accidents, and so, the assessment of these situations is also very probable in civil law. Normally, they have a good evolution but the development of complications can, in some situations, extend the disease periods for several years and lead to severe handicapping. The authors underwent a forensic bodily damage expert assessment under civil law of a male, 24-years-old who suffered a car accident in 2001 with trauma of the left lower limb and resulting fracture of the leg bones. Following the event and during the next 6 years he was subjected to several surgeries (10 procedures) due to the large number of complications who demanded a very large incapacity period (2432 days) with the consolidation date being established several years after the traumatic event. The objective of this study is to add to the body of information on the forensic assessment of similar cases and also seek to call attention to the treatment of closed leg fractures.
Keywords: Leg fracture; handicap; civil law.

Introduction

Fractures of the limbs are one of the most common injuries resultant from road accidents, and so, the assessment of these situations is also very probable in civil law. Normally, they have a good evolution, manly in cases where joints are not affected, with almost any resulting sequelae and not very long periods of incapacity. On the other hand, even when those fractures are not complex, the development of complications [1] can, in some situations, extend the disease periods for several years and lead to severe handicapping. When situations like this take place in young people, it results in spending a great period of time between treatments which can be decisive for the future of the victim. This study is intended to add to the body of information on the forensic assessment of similar cases especially regarding the long incapacity periods [3]. We also seek to call attention to the treatment of closed leg fractures, that despite of being common injuries, sometimes remain challenging to treat [2].

Case report

The authors present a case of a male, 24-years-old, locksmith by trade, who underwent a forensic bodily damage expert assessment under civil law in 2008 in the

Department of Clinical Forensic Medicine of the Centre Branch of the National Institute of Legal Medicine Coimbra – Portugal). It reports to a hit and run car accident on July of 2001, when he was 17-year-old, with trauma of the left lower limb and resulting fracture of the tibia and fibula (Figure 1). Initially he was treated with a conservative approach using a cast immobilization (Figure 2), but due to vicious consolidation had to be submitted to intramedullary nailing during 2002 (Figure 3). One month after the surgery, the nailing material fractured (Figure 4) and had to be replaced by plate and screws (Figure 5). In the following months he was subjected to more surgeries with a 3rd in intramedullary nailing (Figure 6). In February of 2005, because of the development of a large number of complications (tibial pseudarthrosis and osteomyelitis) (Figure 7, 10 & 11) he was submitted to the application of *Illizarov* fixations (Figures 8 & 9). In January 2007 the X-ray showed the following aspect (Figure 12).

The medical examination revealed a 5 cm shortening of the left limb and a substantial amyotrophy of both the thigh and leg. Furthermore we observed multiple scars in the area corresponding to the fracture, as well as trophic changes and chronic oedema of the leg (Figures 14 – 16). The X-ray showed a fibrosis pseudarthrosis (Figure 13). The victim was assessed with the help of the Table of Evaluation of Permanent Disability in Civil Law [3].

Discussion and conclusions

Closed leg fractures are common injuries that remain challenging to treat because of the wide spectrum of fracture patterns and soft-tissue injuries. Good outcomes depend of a good understanding of indications for surgical and nonsurgical treatment of these fractures. Although cast treatment of stable tibial fractures has traditionally been successful and continues to be widely used, recent clinical studies have shown that intramedullary nails may be more advantageous for fracture healing and function than casting [2, 4]. Regarding our case, the large number of complications and the performance of 10 surgeries demanded a very large incapacity period (2432 days) with the consolidation date being established several years after the traumatic event, in 27-02-2008. Due to the age of the victim we would like to highlight the importance of the expended time, stuck between surgeries and treatments. The impairment parameters were also assigned, with high values for quantum doloris – 7/7, aesthetic damage – 5/7 (due mostly to claudication) and permanent incapacity – 27 points. So, despite having a common occurrence, due to his abnormal evolution, this situation configures a condition of particular seriousness which should not be neglected in cases of forensic examination.

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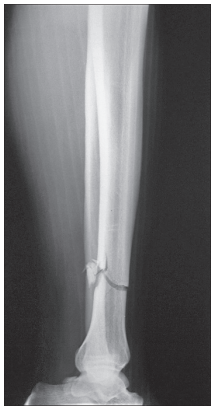


Figure – 1
Day of the accident
X-ray (01/07/2001)

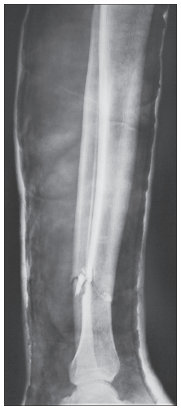


Figure – 2
Cast
immobilization
one month after
the accident

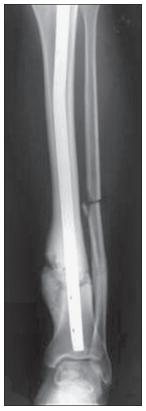


Figure – 3
X-ray showing
intramedullary
nailing of
the tibia
(06/03/2002)

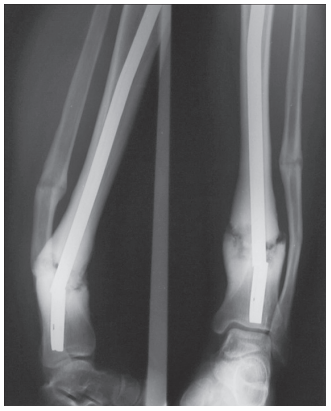


Figure – 4
Intramedullary nailing fracture
(10/01/2003)

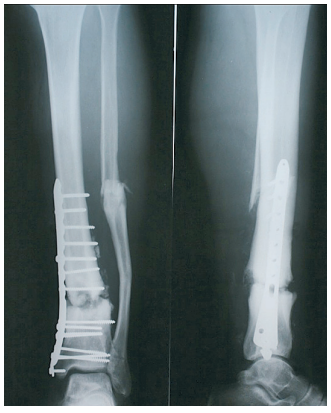


Figure – 5
X-ray showing application of plate
and screws (14/02/2003)

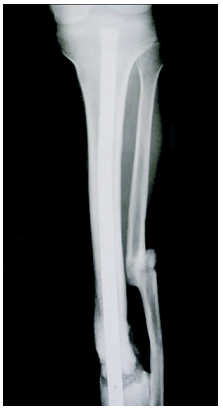


Figure – 6
X-ray after the 3rd
intramedullary nailing
(14/08/2003)

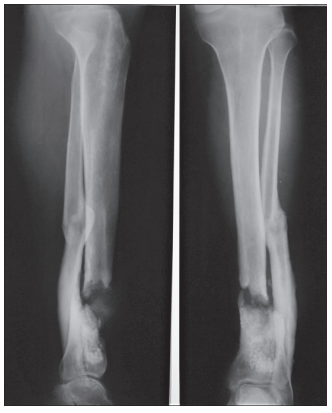


Figure – 7
X-ray showing tibial
pseudarthrosis (09/02/2005)

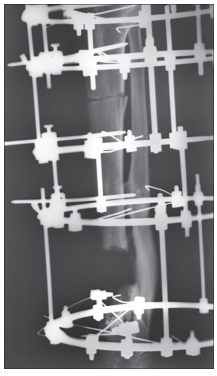
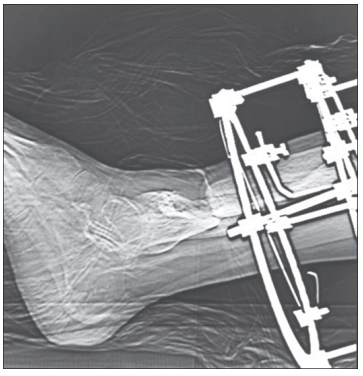
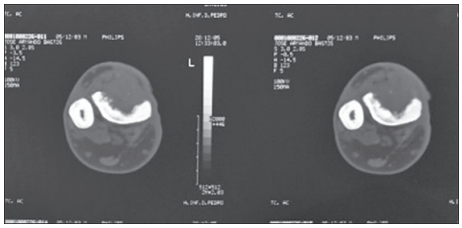


Figure – 8
X-ray after external
fixation (30/03/2005)

Figure – 9
CT Scan after external fixation
(20/12/2005)





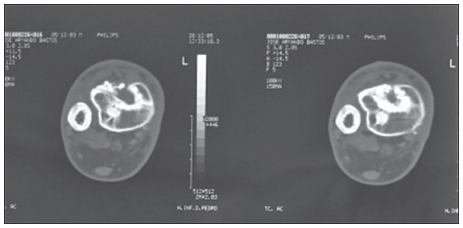


Figure 10 & 11 – CT Scan details

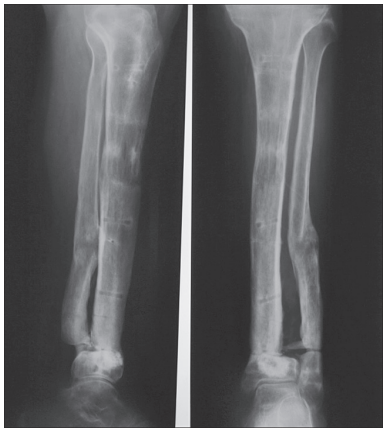


Figure – 12
X-ray after removal of the external fixation (20/01/2007)

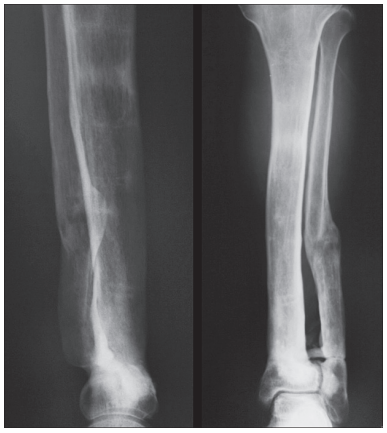


Figure – 13
X-Ray showing the resultant sequelae (18/07/2008)

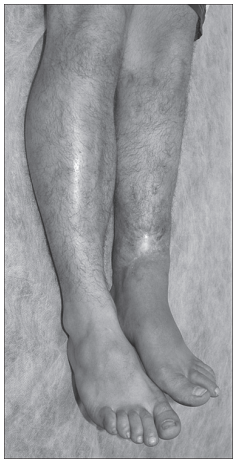


Figure – 14
Photo showing shortening of the left limb (July 2008)

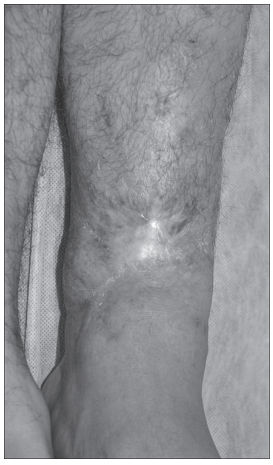


Figure – 15
Photo showing scars and oedema of the leg (July 2008)

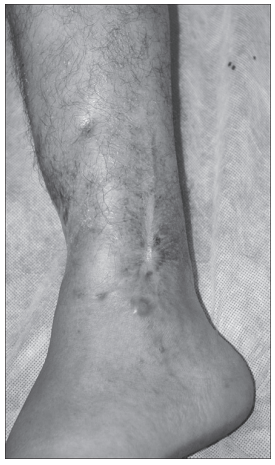


Figure – 16
Left leg scar (July 2008)

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INBORN HEART DEFORMATION AS A CAUSE OF SUDDEN DEATH IN A 7-YEAR OLD GIRL

Abstract: We report the case of a 7-year old girl who is treated with antibiotics because of an angina tonsillaris. Five days later, vomiting and diarrhea develop and the child is admitted to a hospital. Shortly after admission, cardiac arrest occured, and rescuscitation attempts proved to be unsuccessful. Autopsy shows an inborn heart deformation of the right and also of the left, ventricle: basal trabecular hypertrophy and outflow problems such as a subvalvular pulmonary stenosis in the right ventricle. Additionally, hints for a chronic heart overload were seen in both ventricles. Histologically, multiple necrotic areas in the inner muscle layers in various stages of organization appear besides a multifocal, irregular course of hypertrophic muscle cells as well as fatty inclusions in the myocardial cells. The described heart deformation can be classified nearest as a primary cardiomyopathy with consecutive ventricular noncompaction. It remains questionable, how the girl could reach an age of almost eight years, even without showing signs of cardiac insuffiency.

Case

A 7 year old girl suffers from angina tonsillaris, and amoxicillin is prescribed. 5 days later, the child is admitted to a hospital as during the antibiotic therapy, repeated vomiting and diarrhoeas occurred. An antiemetic drug is prescribed, but the girl vomits repeatedly during the following night. The child is briefly unconscious and cerebral convulsions take place. Because of the possible diagnosis of volvulus/invagination, an abdominal sonography is performed. During sonography, the child suddenly shows tachyarrhythmia, generalized stretching cramps and finally a cardiac arrest. Immediate resuscitation measures are performed but are cancelled unsuccessfully after more than 1 hour. The sudden death is unexplainable. As now inappropriate medication as cause of death is assumed, a forensic autopsy is ordered.

Autopsy findings

Autopsy shows a heart [150g] deformation in both ventricles: In the lower half of the right ventricle partially over-crossing, clearly thickened trabecular muscles limit the full development of the ventricle strongly. The right blood flow course is narrowed

by a bulge-like trabecular muscle. Below the pulmonal valve, the endocardium is fibrotic. There is a strong dilatation of the basal ventricular half by general myocardial thickness. In the left ventricle, one mitral valve segment is split, the strong musculature overlaying itself partly. The endocardium is changed below the aortal valve fibrotically, the left atrium is moderately extended. The coronary arteries are inconspicuous. The environment of the tonsils does not exhibit any inflammatory changes. The suspected diagnosis volvulus / invagination cannot be confirmed.

Histology

The hypertrophied myocardial fibres are bizarrely over-crossing each other (Fig. 3b). Multiple necrotised areas in the subendocardium in different stages of organization predominate (Fig. 4a + 4b). The necrotic muscle cells present themselves in the immunohistochemical staining (C9) noticeably and can be differentiated from autolytic cells (Fig. 4b). There is a histological gradient of increasing necroses from outer to inner myocardium; arterialisation of the subendocardial myocardium is rarified. There are multiple, partly cord-like lined-up fat intracellular inclusions in some myocardial cells which partly dissolve the muscular continuity (Figure 4c).

Bacteriology / Virology

Microbiological investigations of the lungs show α-haemolysing Streptococci (physiological upper lung flora). In the blood culture, Clostridium species, Citrobacter freudii and Staphylococcus aureus can be proven. These findings contain no signs of sepsis. Viral DNA (Cytomegaly, Herpes simplex, Epstein-Barr, Varizella, Enterovirae, Adenovirae, Parvovirus B-19) was not detected.

Cause of Death

Acute heart failure by arrhythmia.

Discussion

The splitting of the left mitral valve muscle is no disease – here a large range of physiological variants exists [5]. The myocardial hypertrophy leads towards the diagnosis of primary cardiomyopathy (PC) with dilatative, hypertrophic, obstructive and obliterative components. The hypertrabeculation narrowing the right flow course can be added in terms of a subvalvular pulmonal stenosis to PC [7,8]. The subaortal endocardium fibrosis permits the conclusion that also in the left ventricle a developmental disturbance with in vivo muscular stenosis (during contraction) was present. Etiology of PC is various: hamartoma, genetic arrangement, myocardial metabolic disturbances, unnoticed hypertonia, etc. are discussed. With PC, a generalized

greasing of the myocardial cells and generalized fatty degeneration is known. The extent of the diffuse, fine-dropped fatty degenerations of myocardial cells with a gradient from the outside inward is too small pronounced and not generalized, so we interpret this as a hypoxia-caused degeneration of chronic myocardial ischemia [5]. The histological overall view is also not to be explained by the protracted frustrane resuscitation, as the necroses are already in different stages of organization. The prognosis of PC is bad, therapy attempts exist in the administration of ß-blocker and/or myectomia [8]. An already intrauterine developing PC may lead to ventricular noncompaction (VNC) and myocardial hypertrabeculation [2,3,6,9,10,11]: During embryogenesis, the loose network of myocardium fibres which are supplied via sinusoidal recessus directly by the ventricle clearing "compacts" and a connected muscle is formed [10]. A lack of this "compaction" leads to abolition of the myocardial texture in the inner muscle layer [1,2,3] and thus causes a morphologic hypertrabeculation with endocardium-covered sinusoids communicating with the ventricle clearing ("sponge heart"/"spongy myocardium"). VNC is considered to be a rare, innate and usually left-sided arising disturbance of the myocardial morphogenesis, but seldom cases report an affection of the right ventricle. Etiology is not enlightened yet. An underlying pathologic mechanism consists of a restricted flow course – in this case due to subvalvular pulmonal stenosis – with intrauterine intraventricular hypertonus. This may handicap the fusion of the loose myocardium fibres, the heart muscle is altogether unsatisfactorily consolidated. Such hypertonus also obstructs the angiogenesis in the ventricle, so that besides vascular rarifications also ischemic necroses and hypoxic fatty inclusions may result. As histological findings, these necrotic areas impress with minimal inflammatory reaction. Therapy is heart transplantation [4]; prognosis is bad, fatal are cardiac arrhythmias [1,2], heart failure and thrombembolic events [11]. It is remarkable that the girl never showed any signs of cardiac insufficiency. Typically, children concerned with heart deformations are in the last third of their age [7]. It must be assumed that in the described case sufficient compensation mechanisms were available, which possibly failed later in the context of an infection. Possibly a syncope could have taken place and was misinterpreted as cerebral convulsion. This case shows the fateful process of a non-diagnosed cardiomyopathy with consecutive VNC of the right ventricle.

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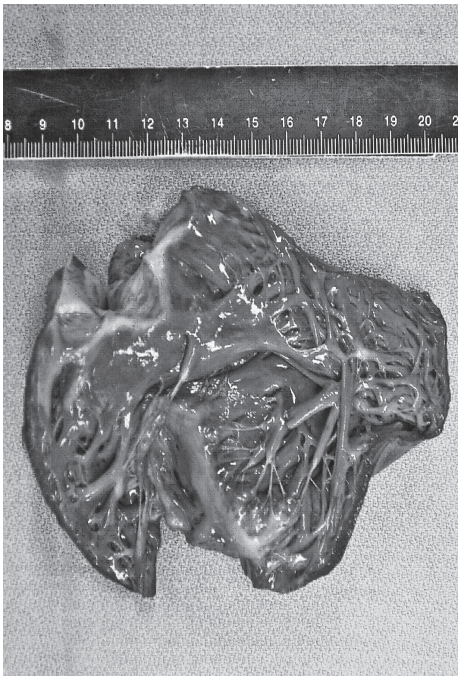

Figure 1 – Right heart: trabecular hypertrophy and endocardial fibrosis

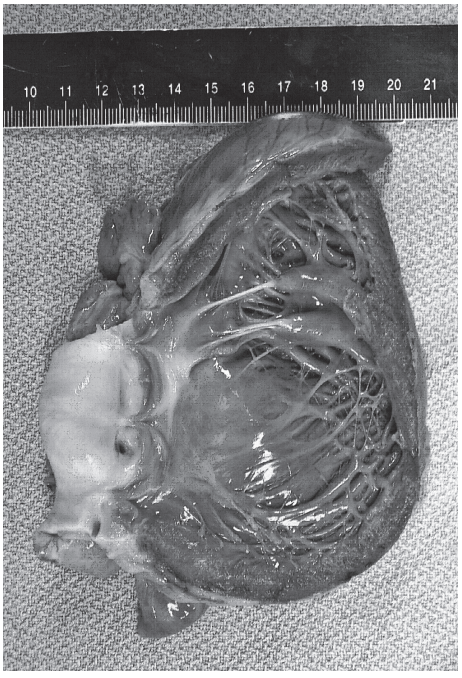

Figure 2 – Left heart: subaortal fibrotically changed endocardium and amplified trabeculation

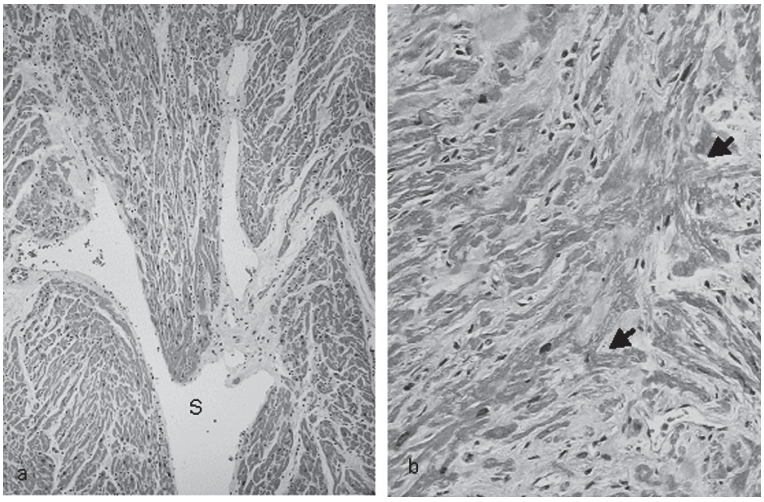

Figure 3 – shows the internal layers of the ventricle myocardium.
3a. VNC with sinusoids reaching deeply into the myocardium and communicating with the ventricle clearing on reduction of the normal vessel supply. HE x 25.
3b. Hypertrophic myocardium with hyperchromatic cells. Irregular, partly star-shaped myocyte arrangement (fiber disarray) [arrow]. HE x 50.

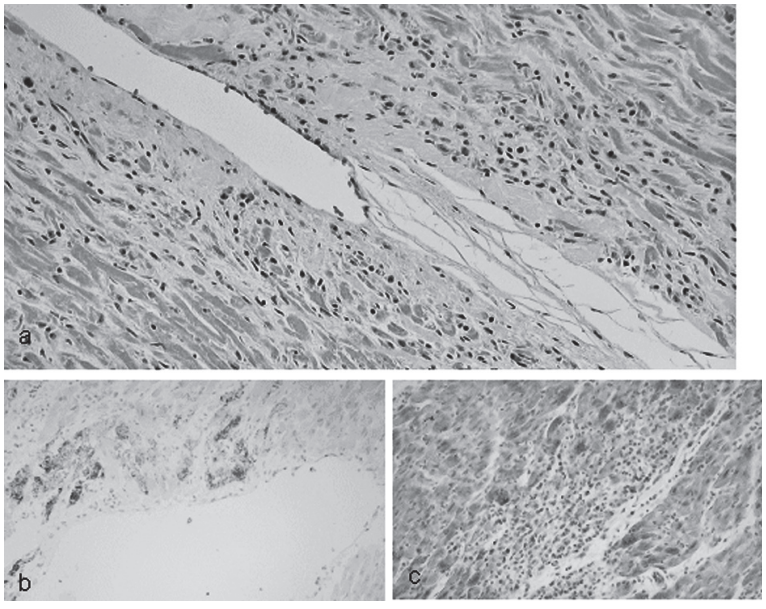

Figure 4 – shows the internal layers of the ventricle myocardium with small, oven-like necroses.
4a: Myocardial necroses with loose cellular clearing reaction. HE x 50.
4b: Brown colouration of necrotic myocardial cells; non-coloured vital myocardial cells in the right corner. Immunohistochemical on C9 x 50.
4c: Red colouration of fatty degenerated myocardial cells around a necrosis interspersed by absorptive cell infiltrates. Fat red 7 B x 50.

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AESTHETIC DAMAGE VS. COSMETIC SURGERY IMPLICATIONS IN CIVIL LAW EVALUATION

Abstract: Taking as an example the case study presented in this paper, we intend to discuss whether the availability of plastic surgery, as a form of improvement of traumatic lesions, should be taken into account during clinical assessment of aesthetic damage, or whether each patient is entitled to refuse this specific therapeutic, considering the risks it involves, like any other form of treatment.

Introduction and objectives

Nowadays, society is consumed by stereotyped images that media, with the help of numerous advertising and marketing campaigns, diffuse all around the world. As a result, appearance has actually become vital to its individuals that are devoured by the need of compulsive consumption in order to correspond to those creations and please others.

Hence, it is not at all surprising, that aesthetic damage is considered susceptible of monetary compensation following trauma in civil responsibility cases.

Medical experts are thus, expected to be familiar with this field so as to produce precise clinical evaluation that will be the foundation on which the judge can determine each individual compensation.

In the case study presented, we intend to discuss the problematic of plastic surgery as a form of correction of traumatic lesions and whether or not its availability should be taken into account during clinical assessment.

Case Study

In this case, we studied an 18 year old, female student, victim of a car accident in 01-04-2001. She suffered dislocation of the right shoulder, bifocal fractures of the right humerus (figs. 1, 2 and 3) and pelvis (fig. 4), second degree burn of the left thigh (fig. 5) and generalized abrasions throughout the body. The fractures of the arm were surgically treated, whilst the ones of the pelvis were treated conservatively.

As for the lesions on the thigh, the patient was submitted to a skin graft, derived from the ipsilateral leg, which then formed a queloid (figs. 5, 6, 7 and 8).

One year later, two expandable prostheses were placed beneath the healthy skin of the left thigh and were periodically filled, consequently increasing the amount of normal skin (figs. 9, 10 and 11).

They were then removed and the scared segment was excised, leaving a much narrower scar on the external aspect of the knee and lower thigh. The scar was surgically revised two more times measuring in the end, 25cm longitudinally per 5cm in its widest portion (figs 12, 13 and 14).

In the end, aesthetic damage was evaluated as 4 in a scale of 7 degrees, of increasing severity.

Discussion and Conclusion

When observed by the medico-legal expert, the patient had by far, a more discreet scar then initially, a fact that positively influenced the end result of the evaluation. Taking this example into consideration, two main questions arise. Firstly concerning the time elapsed between the traumatic event and the moment of observation. It is essential, for final conclusions, that the sequels are considered consolidated before examination. And secondly, one could be tempted to argue that in all cases, in which cosmetic surgery intervention could be of assistance in the resolution or minimization of the deformities produced by the traumatic event, it should be compulsory for the patients to accept such treatment. However, like any other form of medical management, it involves specific risks and each individual is entitled to determine which therapeutics presented should or not be accepted.

Therefore, would it be reasonable to slightly devaluate the sequels presented in cases of refusal of further cosmetic treatment?

Due to its controversy, it was concluded that it depends on each individual evaluator to determine whether in any given case, it would be reasonable to slightly devaluate the sequels presented when the patient refuses further cosmetic treatment at disposal.

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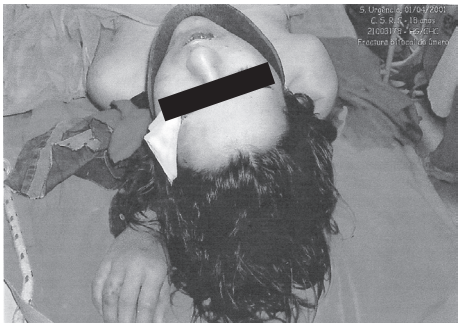

Figure 1 – Patient in the emergency room

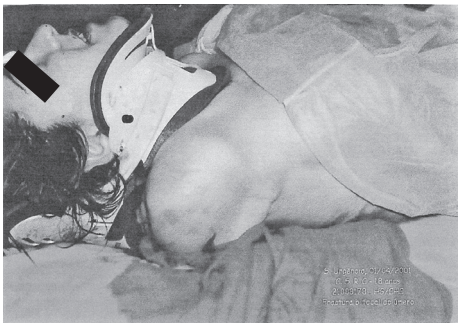

Figure 2 – Closer view of the dislocation of the shoulder in the emergency room

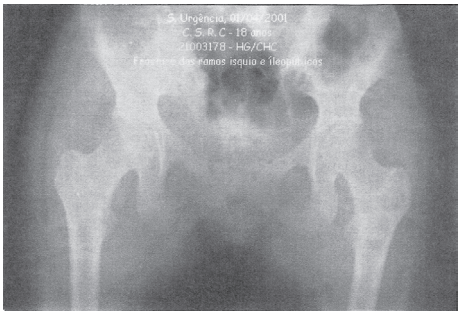

Figure 3 – X-ray of the fractured pelvis

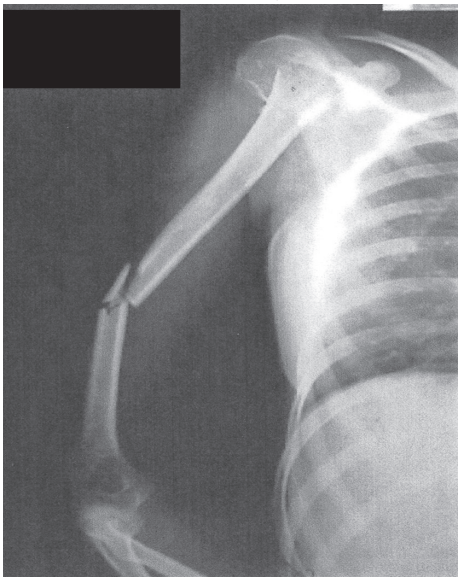

Figure 4 – X-ray revealing the bifocal fracture of the right humerus

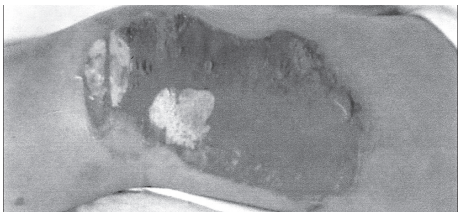

Figure 5 – Close view of the second degree burn of the left thigh

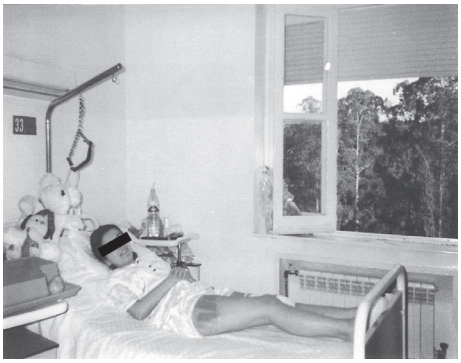

Figure 6 – Donor region of the skin graft on the right thigh.

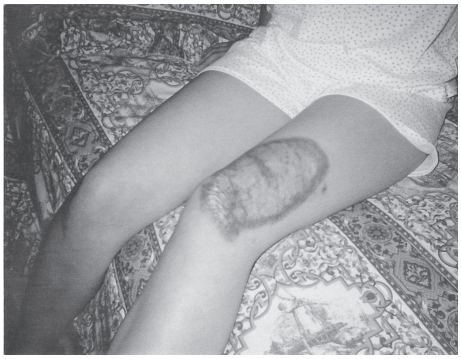

Figure 7– Healing of the lesion after the receiving the skin graft

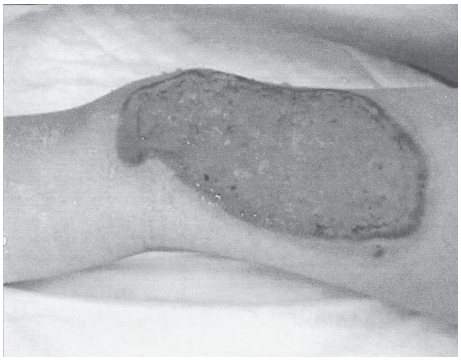

Figure 8 – Closer view of the healing tissue

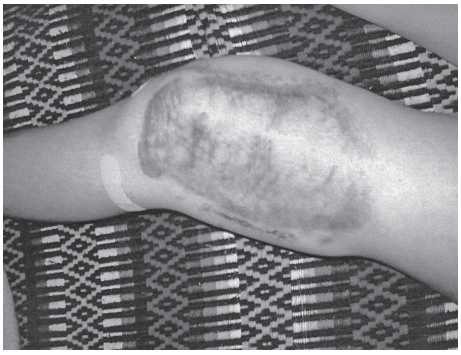

Figure 9 – View of the expandable prostheses placed beneath the healthy skin

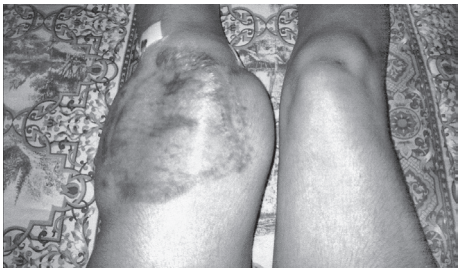

Figure 10 – Expandable prostheses fuller than before, increasing the amount of normal skin

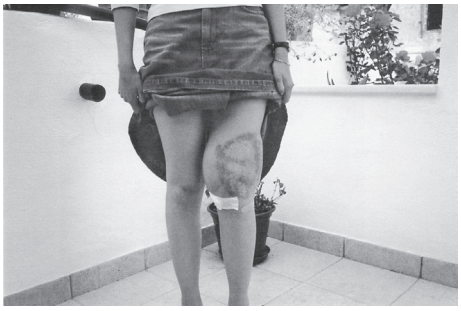

Figure 11 – Prostheses at maximum volume

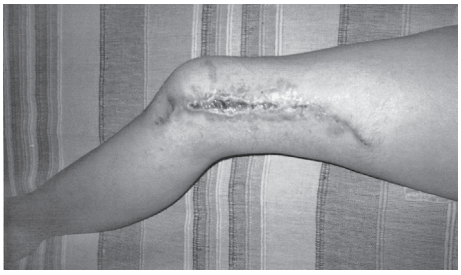

Figure 12 – Scar after the first corrective surgery

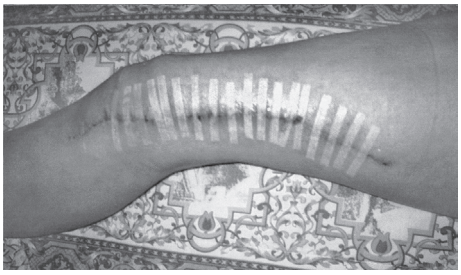

Figure 13 – Status after the second corrective surgery

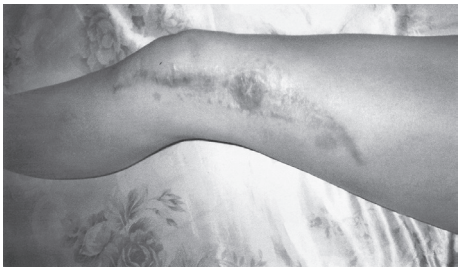

Figure 14 – Final result after plastic surgery intervention

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ESTABLISHING IN CRIMINAL LAW THE CORRELATION BETWEEN A TRAUMATIC EVENT AND A SEPTAL PERFORATION OF THE NOSE A CASE STUDY

Abstract: Septal perforation is a known complication following facial trauma. Despite being an uncommon aetiology, in the case study presented, the patient's information and clinical history seems to acknowledge it to be the only likely explanation, thus the particular interest of the following case discussion.

Introduction and objectives

The nasal septum is a structure composed of cartilage and bone, which separates the two nasal cavities. Its blood supply is assured by the overlying mucoperichondrium. When circulation is disrupted, perforation occurs.

There are many underlying causes for septal perforation, which can be classified into four main categories: Traumatic (most commonly self-induced from nose picking or resulting from facial trauma); Iatrogenic (surgical procedures and nasal intubation or nasogastric tube placement); Inhalation of irritants (such as cocaine, decongestive sprays, chromic or sulphuric acid fumes, glass dust, mercurials, phosphorous and others) and Inflammatory/Malignant causes (including tuberculosis, syphilis, Wegner granulomatosis and sarcoidosis), that should always be considered in the differential diagnosis. More specifically, the most common are: previous nose surgeries, cocaine use and chronic use of nasal steroid and decongestive sprays.

The aetiology of septal perforation is usually sex related and its determination is essential in establishing the treatment and prevention methods to be applied in each case.

Other important elements to be considered are characteristics such as the type and size of the perforation. Posterior perforations are typically asymptomatic and do not need any repair, whereas anterior ones commonly cause increasing crusting, recurrent epistaxis, nasal obstruction, whistling, malodorous discharge and saddle nose deformity (when the external nose collapses).

Treatment lies mainly in prevention in high risk patients, using local ointments, nasal emollients and estrogens, topical lavage and constant humidification. Surgically, different techniques can be used in accordance with the type and size of perforation,

being the mucopericondrial bipediculed flap with cartilage interposition, the most used for any type of perforation and the one that has the best results.

As disclosed above, some aetiologies have obvious medico-legal implications, for example as in this case discussion, in the interpretation and differentiation of traumatic septal perforation.

Case Study

A 49 year old female, who reported falling in a bus and hitting her face on the seats ahead and floor of the vehicle. She referred immediate and abundant epistaxis and pain on her nose and right shoulder, breast and knee. She was observed at the location by an emergency team and then in a Hospital, where she was X-rayed. There were no apparent fractures, namely in the nose. There was also no active bleeding in the emergency room and thus, the patient was discharged.

One week later, the persistence and increase of pain, oedema of the nose, difficulty breathing and daily epistaxis led her to see an Otorhinolaringologist. She was submitted to an anterior rhinoscopy and diagnosed with a significant perforation of the cartilaginous septum.

Subsequently, one month after the accident, the patient was subjected to a CAT scan where it was possible to visualize a discontinuity of the cartilaginous septum of the nose measuring 33 millimetres horizontally and 15,5 millimetres vertically (Fig. 1- 6). Given the large size of the perforation, the patient was informed that surgical reconstruction was not possible.

During the medico-legal examination, in the investigation of past medical history, she denied the use of any type of nasal sprays, inhalation of other irritant substances or the use of drugs such as cocaine. She also denied having been submitted to any previous nasal surgery or medical procedures such as cauterization, nasogastric tube placement, intubation and/or perinasal oxygenation. She also had no history of disease which could cause nasal perforation.

Discussion and Conclusion

It is well known that the cartilage of the septum relies upon the overlying mucoperichondrium for its blood supply and nutrients. Therefore, most traumatic perforations result from mucosal lacerations on corresponding sides of the septum with exposure of the underlying cartilage or from its fracture which impede the blood flow, ultimately leading to necrosis of the tissues and perforation.

In this specific case, although a rhinoscopy was promptly carried out by a qualified Otorhinolaringologist, merely one week following the alleged traumatic event, no comment was made concerning the state of the mucous membrane or of the margins of the perforation. This information would have been most helpful when determining the compatibility with an injury of recent traumatic origin.

Despite not having this important macroscopic description and even though the patient was submitted to a CAT scan one month subsequent to the accident, conclusions could be drawn from the patients information reported during the medico-legal examination, as well as from the documentation referent to the patient's medical history sent by her general practitioner.

As a result, given the exclusion and/or lack of confirmation of all the most frequent causes of this lesion, already described above, one was left to admit to its traumatic aetiology and succeeding rapid evolution.

Accordingly, in this particular case, the medico-legal experts included the septal perforation presented, as having contributed to the increase of the period of temporary general incapacity, as well as of the period of incapacity for work. And although it was assessed as being a permanent consequence of the traumatic event described, it was also stated in the final report, that it was not a cause of severe disfiguration to the patient, nor did it severely impair her respiratory function.

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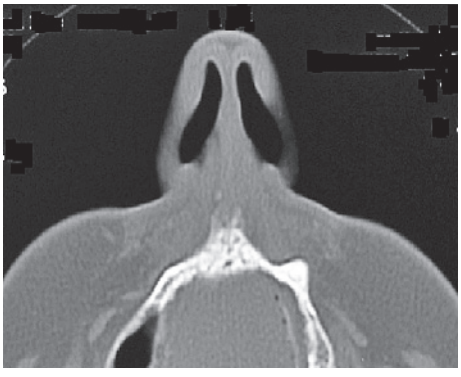

Figure 1 – First image from the CAT scan of the nose

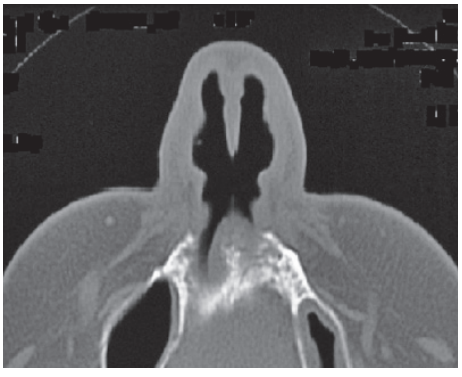

Figure 2 – Inferior aspect of the perforation of the septum

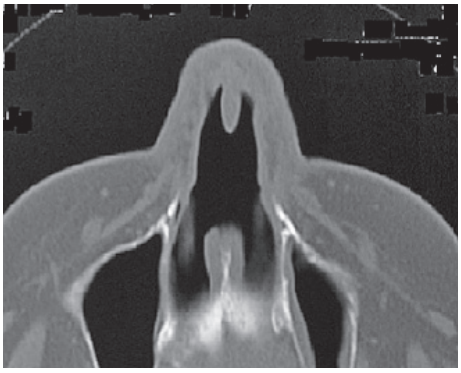

Figure 3 – Central part of the septal perforation

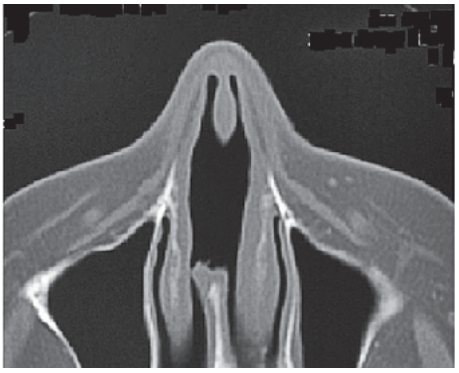

Figure 4 – Widest parte of the septal perforation

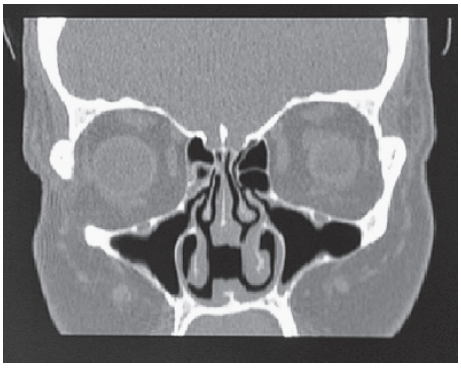

Figure 5 – Coronal view of the septal perforation of the nose

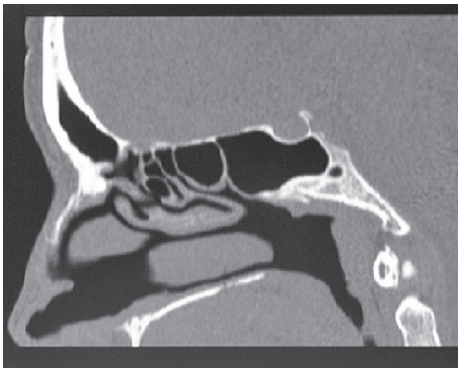

Figure 6 – Lateral view of the septal perforation of the nose

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ONE CASE OF DROWNING AFTER CYFLUTHRIN INGESTION

Abstract: This work reports a case, involving a 47-year-old Portuguese male found dead in a river. Near the victim clothes the authorities found two bottles containing a toxic product identified as cyfluthrin. Cyfluthrin is a synthetic pyrethroid insecticide that exerts its poison action through contact or ingestion.

Toxicological analyses were performed in blood, gastric content and in the bottles. Samples preparation was achieved using solid phase extraction and the extracts obtained were analysed by GC/MS.

Cyfluthrin was found in the gastric content and in the extract obtained after washing the bottles with a solvent.

Keywords: Cyfluthrin; suicide; blood; gastric content; GC/MS.

Introduction

Cyfluthrin is a pesticide belonging to the class of synthetic pyrethroids, which are a major class of pesticides, a group of chemicals that entered the marketplace in 1980 but, by 1982, accounted for more than 30 percent of worldwide insecticide usage [1,2]. These synthetics arise from a much older class of botanical insecticides, pyrethrum, a mixture of six insecticidal esters extracted from dried pyrethrum or chrysanthemum flowers. The ever-increasing demand for this product has far exceeded the limited world production, leading chemists to focus attention on the synthesis of new analogs, hopefully with better stability in light and air, better persistence, more selectivity in target species, and low mammalian toxicity [2,3]. In addition to extensive agricultural use, the synthetic pyrethroids are components of household sprays, flea preparations for pets, plant sprays for home and greenhouse use, and other applications. Natural pyrethrum consists of a mixture of six esters derived from two acids (chrysanthemic, pyrethric) and three alcohols (pyrethrolol, cinerolol, jasmolol), producing an effective contact and stomach poison mixture having both knockdown and lethality [2-5].

Pyrethroids' action as insecticide includes effects in sodium channel mechanism in the nervous system leading to disturbance of membrane polarization and abnormal discharges in neurons. Insects become paralyzed and die by dehydration and starvation. Pyrethroids can also act on isoforms of voltage-sensitive calcium channels, contributing to the discharge of neurotransmitters. This can lead to salivation and excitability in

the central nervous system. Also chloride channels and possibly potassium channels can be involved [6,7]. Pyrethroids are divided in Type I, with T-syndrome (tremors), and Type II, with CS-syndrome (choreoathetosis with salivation). Cyfluthrin is a Type II pyrethroid, based on functional observatory battery data. In humans, pyrethroids' intoxication effects can include nauseas, loss of appetite, paresthesias, irritations of the skin and mucosa and headaches. Neurological effects can be seen after consuming foods with residues of cyfluthrin or exposed to applications [7-12]. Animal studies showed that symptoms can include beyond salivation, unusual hindlimb movements and body tremors. Cyfluthrin has a nearly full and quick absorption by ingestion or inhalation. Possibly because cyfluthrin does not accumulate in chronic exposures, the neurotoxicity is reversible and the effects are similar at maximum blood concentrations, the WHO/FAO established a chronic acceptable daily intake based on an acute dosing study [12].

Cyfluthrin is a (*RS*)-α-cyano-4-fluoro-3-phenoxybenzyl-(1*RS*)-cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropane carboxylate (figure 1), being metabolized by esterases in cis- and trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA) and 4-fluoro-3-phenoxybenzoic acid (FPBA), which are partially conjugated and eliminated by the kidneys [13].

Case History

A 47-year-old Portuguese male was found dead under water in a river. Authorities collected the victim clothes and two plastic bottles from the river bank, one containing a white pink liquid and the other identified as Baythroid® - cyfluthrin (figure 2). At the autopsy the samples collected were femoral blood and gastric content. Requested analyses included ethanol, drugs of abuse and pesticides.

Material and Methods

Reagents and materials

Ethion, used as internal standard (IS), was purchased from Sigma Aldrich (St. Louis, MO). Methanol and hexane were obtained from Merck (Darmstadt, Germany). Stock solution of internal standard was prepared in hexane at 1000 µg/mL. Working solutions were prepared at 100 µg /mL by diluting stock solution with hexane. All solutions were kept protected from light and stored at 4ºC until use. Other reagents used were all analytical grade.

Solid-phase extraction (SPE) Oasis® HLB (60 mg) columns were obtained from Waters (Milford, MA). Deionized water was obtained using a Milli-Q system from Millipore (Molsheim, France).

Sample preparation

To a 2 mL blood sample and 0,5 mL of stomach content it was added 5 µg of the IS. Samples were diluted with 3 and 5 mL of deionized water, respectively, after being homogenized and centrifuged at 3000 rpm for 10 minutes.

Extraction procedure

SPE columns were conditioned with 2 mL of methanol and 2 mL of deionized water. After samples were applied the columns were washed successively with 2 mL of 5% methanol:water (v/v), and then dried under vacuum for 30 minutes. The substances of interest were eluted with 2 mL of methanol and 2 mL of ethyl acetate. Subsequently, the eluate was evaporated to dryness under a gentle stream of N2 at 40°C. The residue obtained was reconstituted in 100 µL of methanol.

Instrumentation and chromatographic conditions

Instrumental analysis was performed using a Hewlett-Packard 6890 Series gas chromatograph coupled to a 5973 mass selective detector (Waldbronn, Germany). Chromatographic separation was achieved using a capillary column (30 m × 0.32 mm id × 0.25 µm film thickness) with 5% phenylmethylsiloxane from Agilent. Carrier gas was helium with a flow rate of 1 mL/min. Volume injection was 1 µL (split ratio 1:5). The oven was programmed to an initial temperature of 120 ºC held for 2 minutes, increased by 5 ºC/min to 240 ºC and then 30 ºC/min to a final temperature of 270 ºC, held for 20 minutes. The detector was operated in the EI mode (70 eV), and the monitored ions were *m/z* 163, 206 and 226 for cyfluthrin and *m/z* 231 and 153 for IS.

Results and Discussion

Upon reception the samples were screened for ethanol and drugs of abuse in blood and pesticides in blood and in the gastric content. A positive result was obtained for cyfluthrin in the gastric content (figure 3).

Along with the samples, two bottles were received for analysis. The inside of the bottles was washed with methanol which was then analyzed by GC/MS giving also a positive result for cyfluthrin at both bottles (figure 4).

In the chromatograms (figures 3 and 4) we can observe the four diastereoisomers peaks obtained after analysis of the active substance as well as their mass spectrum.

As it can be observed from the analysis of chromatograms, the quality of the results (e.g. separation, capacity of detection) can be significantly improved with the use of a chromatographic column which is more appropriate to the characteristics of the substance involved. However, the variety of substances that can be detected in the routine of a forensic toxicology laboratory recommends the use of universal analytical columns, which obviously can not produce the best results with certain compounds in particular.

Due to the low mammalian toxicity, the mortal intoxication by cyfluthrin is not a common situation. Considering the casework of the Forensic Toxicology Service from

the Centre Branch – National Institute of Legal Medicine (which includes the central area of Portugal), no cases were observed in the last 5 years (2005-2009). Nevertheless, the study of these substances has particular aspects to be considered by the pathologist.

The analysis of pyrethroids in different organs and tissue samples can be important to understand particular cases, due to its complex distribution. However, the toxicological analysis is limited usually to the samples collected by the pathologist which, sometimes, are not sufficient to a complete evaluation. Furthermore, the determination of different pyrethroids usually involves more complexity than organophosphats or carbamats, considering their characteristic cyclopropane rings which can cause the appearance of stereoisomers. Due to the possible binding of pyrethroids to erythrocytes and because no additional chromatographic peaks seems to appear when compared with serum analysis, the whole blood study represents a satisfactory way of pyrethroid testing [14].

The cyfluthrin acceptable daily intake of 0.02 to 0.04 mg/kg body weight per day is rapidly metabolized, by hydrolytic cleavage of the ester bond, followed by oxidation and mainly glucuronization. The cis-/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA) and 4-fluoro-3-phenoxybenzoic acid (FPBA) are almost totally excreted (93%) within 24 hours after cyfluthrin inhalation exposure of 160 μg/m3, with peak excretion rate at 0.5-3h, depending on interindividual variation [13,15]. However, other studies observed an excretion of 35-50% of administered dose of type II pyrethroids in the first 5 days, with a urinary peak excretion at 24 hours. Due to the high excretion rate, urine samples from the first 3 hours after exposure can be useful, if creatinine correction is considered. The biomonitoring of cyfluthrin can be done directly in blood (or plasma) or by measuring the metabolites DCCA (common to other pyrethroids) and FPBA (specific to cyfluthrin) [12,15,16].

Conclusions

Considering the pathologist report and the toxicological analysis, the results obtained highly suggest the occurrence of a suicide by drowning with the concomitant use of a toxic substance. As in other similar cases, the information collected by the authorities or experts on site has proved crucial to help the interpretation of the case.

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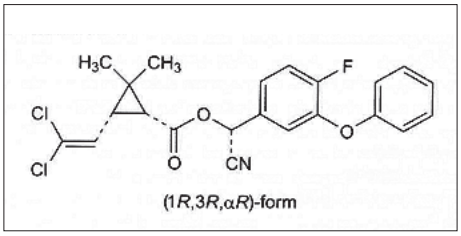

Figure 1 – Structure of cyfluthrin [5].

Figure 2 – Bottle with a white pink liquid; Bottle of Baythroid® .

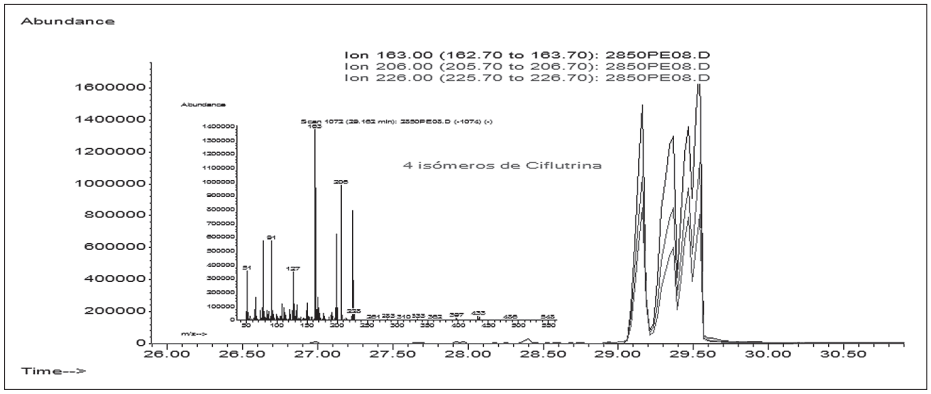

Figure 3 – Chromatogram obtained from the analysis of gastric content.

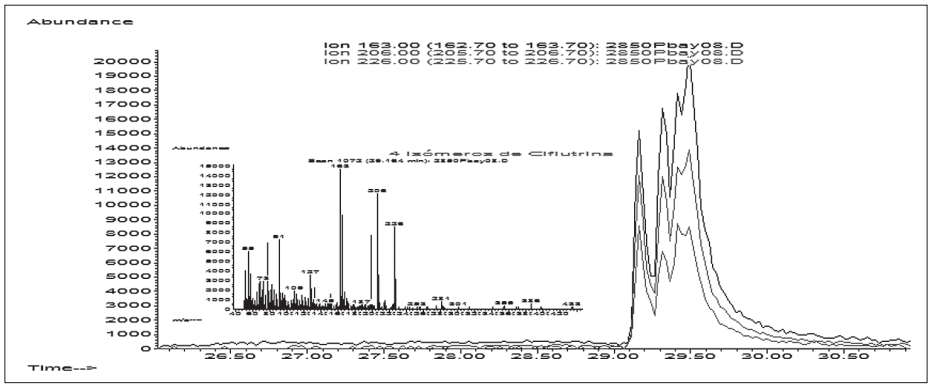

Figure 4 – Chromatogram obtained from the analysis of bottles content.

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"STRYCHNINE POISONING – A CASE REPORT"

Introduction

The use of strychnine as a mean of committing suicide is currently extremely unusual, as its commercialization, formerly as a pesticide, has been strictly forbidden. As a matter of fact, no other cases were found in the database of the Centre Branch of the Portuguese National Institute of Legal Medicine in the last 5 years.

Case Report

The authors report a case of a 72 year old Caucasian male with background of prostatic illness and other unspecified diseases, suffering from extremely intensive pain, that had frequently expressed his wish to commit suicide.

One morning, while his wife left the house for a moment to buy some food, he decided to carry out his idea by way of ingesting strychnine. When the wife came back home, she found him very nervous, sitting on the bed. He told her what happened and pointed out the place where the package containing strychnine was kept.

When the medical emergency team arrived to the scene, the victim was already in a severe agonic condition. No convulsions where registered in the police report, which solely mentioned that the victim became weaker and weaker.

At the autopsy room, external examination of the body showed merely facial cyanosis, puncture marks in right arm, related to medical procedures, and an urine catheter.

Concerning internal examination of the body, the most remarkable findings were enlarged lymph nodules surrounding the carotids vessels (the most enlarged measuring up to four centimetres along its longer axis). Abdominal aorta, inferior vena cava and its pelvic ramifications, urine bladder and prostate were invaded by white hard tissue masses. In the liver there were several hard round nodules, the largest with a diameter of fifteen millimetres. An *ante mortem* fracture of the sternum's body was found, related to medical procedures. Lungs were oedematous, a significant left ventricular hypertrophy was detected and the stomach contained about ten cubic centimetres of a pink odourless fluid. There was also a generalized organic congestion.

Histopathological investigation confirmed prostatic adenocarcinoma of a low degree differentiation (score 10 in Gleason's ranking), with local and distant metastatic proliferation.

Toxicological ancillary report confirmed the presence of strychnine in blood, urine, gastric contents samples, as well as in the contents of the package retrieved from the house.

Discussion and Conclusions

Strychnine affects primarily the nervous systems by acting as an antagonist of the inhibitory neurotransmitter glycine at receptors in the spinal cord, brain stem and higher centres. Therefore, by lowering the neuronal excitatory threshold it leads to increased muscular activity and convulsions. Death can occur in as short as two minutes but usually takes one or two hours and is due to severe breathing impairment that occurs during each consecutive convulsive wave.

Because of its bitter taste and less availability, strychnine is currently seldom used as a mean of suicide. Since there was no mention about convulsions in the information given before autopsy and, aside from the evidence of neoplasia, the most relevant findings in the internal examination, were pulmonary oedema, generalized organic congestion and significant left ventricular hypertrophy (which carries an increased risk for arrhythmic events), which in an aged individual could point towards a natural cause of death, it is understandable the challenge that could be set if the victim had decided to hide the poison and had been found already dead by his wife.

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SUDDEN DEATH OF CARDIOVASCULAR AETIOLOGY IN INDIVIDUALS YOUNGER THAN 45 YEARS OLD

Introduction

Sudden death in young individuals has always a significant social and economic impact. Nowadays, it is generally accepted that death occurring in young people seems to be more associated with cardiovascular pathology.

Methods

A retrospective study of cases of sudden death of cardiovascular aetiology in individuals less than 45 years old, from January 2006 until December 2008, was performed at the Centre Branch of the Portuguese National Institute of Legal Medicine. Data were analysed according to age and gender, types of fatal cardiovascular events and results of histological and toxicological ancillary investigation.

Results

During such period 979 autopsies were performed, 33 of which concerned sudden death in individuals younger than 45 years old; among these, just 9 were related to cardiovascular pathology (0.9% of the total number of autopsies; 27.3% of sudden death cases), 40% were related to other pathologies mostly pulmonary and in 24% cases the cause of death was undetermined. In cases related to cardiovascular pathology, the victims' age ranged from 19 to 45, with an average of 37 +- (SD- 8.4) years; the majority were male (77.8%; n=7) and 55.6% were married. In all cases, there was no information about occurrence of symptoms previously to death and, in fact, they were found already dead.

Histological samples were collected and processed in each and every case; inclusively, in five cases, sino-auricular and atrioventricular nodules were acutely analysed. The major causes of death (all with relative frequency of 22.2%) were obliterative coronariopathy, ischemic cardiopathy and acute heart infarction. There was also one

case of coronary thrombosis, one case related to aortic dissection and one case of acute miocarditis (in a 19 year-old male).

As for toxicological ancillary investigation, results were negative for alcohol and narcotics in all except two cases (1,59mg/dL and 2,62mg/dL alcohol level).

Discussion and Conclusions

The definition of sudden death accepted by most medical examiners includes not only instantaneous deaths but also those occurring up to one hour after the onset of symptoms, without the establishment of any definitive diagnose. In our study, we found that the nine individuals were found already dead with no information regarding symptoms or medical assistance previous to death. It could be expected a greater number of deaths due to cardiovascular disease and, as such, it could be reasoned those individuals with cardiovascular pathology that didn't die as quickly or that presented symptoms and received medical assistance, even if the final outcome was death, were not autopsied and, therefore, not included in our study.

As expected, most cases were male individuals, being cardiovascular pathologies evenly distributed over obliterative coronariopathy, ischemic cardiopathy and acute heart infarction.

Since there were only nine cases related to sudden death from cardiovascular disease in this period, it wasn't possible to determine a predominance relative to others causes of death. A study considering a range of more years, and therefore, including more cases, should be considered to further evaluate the impact of cardiovascular disease in the death of young individuals submited to autopsies.

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ALTERNATIVE MATRICES: A CASE REPORT

Abstract: A 36-year-old Portuguese male hit by a train was found dead on the tracks, with his limbs partially separated and several parts of his body exhibiting severe injuries. The precise circumstances of death were unknown. As in many other similar cases, blood and urine were unavailable. A toxicological study for drugs of abuse in the liver and kidney was performed by solid phase extraction after sonication and homogenization of the samples. The organic phases obtained were evaporated until dryness and derivatized with a mixture of MSTFA/TMCS for the analysis of opiates, cocaine and canabinnoids, and with MBTFA for amphetamines. Derivatized extracts were analyzed by gas chromatography/mass spectrometry in selected ion monitoring mode. Morphine, cocaine and its metabolite benzoilecgonine were found in the liver and kidney. No alcohol was found in the vitreous humor. Although blood and urine are the most common and preferred matrices used for toxicological studies involving drugs of abuse, sometimes the choice of specimen is dictated by the case being investigated.
Keywords: Drugs of abuse; gas chromatography-mass spectrometry; alternative matrices.

Introduction

Forensic toxicology includes an understanding of drug use in the immediate ante mortem setting, analytical methodologies and interpretation of results [1].

In recent years, there has been a growing interest in the development of methodologies for detecting drugs of abuse in alternative biological matrices, although the analysis of these specimens is limited by many factors including putrefaction, sample homogenization and complexity, time-consuming techniques and analytical and chromatographic problems [2-19]. Tissues, such as the liver and kidney have been long used in post mortem toxicology analysis, especially in those cases where blood is unavailable. The extraction of the compounds from within these matrices is the major problem in forensic toxicology, due to potentially interfering substances. The liver and kidney are suitable tissues to prepare homogenates but they contain high concentrations of lipids, which may interfere in analytical procedures [12, 14-19].

The liver is the largest organ in the human body and has been used extensively as an important specimen in postmortem toxicology analysis. As a specimen, the liver has the advantage of being relatively unaffected by postmortem redistribution compared to

blood, but drug concentrations in the lobe proximal to the stomach may be affected by post mortem diffusion in cases of oral overdose [3, 10, 14, 15]. The great impediment to using liver for the interpretation of routine positive drug findings is the lack of database information of liver concentrations. As with all drugs and specimens, the process of interpretation should include consideration of all aspects of the investigation into the death, including the analysis of multiple specimens [10, 20, 21].

This work presents a particular case where the liver and kidney are the only available matrices to perform the toxicological analyses of drugs of abuse.

Case Report

A 36-year-old Portuguese male hit by a train was found dead on the tracks, with his limbs partially separated and several parts of his body exhibiting severe injuries.

The precise circumstances of his death were unknown. At the scene of the accident there was a car with the key in the ignition. The local authorities managed to identify its owner and the family made a statement saying that he had not been seen for over 3 days. One of the parents mentioned the man's drug addiction.

Like in many other similar cases, blood and urine were unavailable. The vitreous humor, liver and kidney were collected for toxicological analysis of alcohol and drugs of abuse.

Materials and Methods

The toxicological study for drugs of abuse was performed in the liver and in the kidney according to the validated procedures for the analyses of opiates, cocaine, cannabinoids, amphetamines and related compounds in blood, routinely used in our Laboratory of Forensic Toxicology.

Ethanol was tested in vitreous humor, using a gas chromatograph/flame ionization detector system, model 6890N (Hewlett-Packard, Waldbronn,Germany) equipped with a headspace autosampler.

Portions of the tissues (2g) were placed in disposable plastic tubes, sonicated and centrifugated at 3000 rpm for 5 min. Appropriate trideuterated internal standards, purchased from Cerilliant (Round Rock, TX, USA), were added to the pre-treated samples and subsequently the extraction of the drugs of abuse were performed with a Vac-Elut system assembled with columns Oasis® MCX (3 mL, 60 mg) purchased from Waters (Milford, MA, USA). The obtained extracts were dried at 40ºC under a gentle stream of N2 and dissolved in the derivatization reagent (N-Methyl-Bis (trifluoroacetamide) for amphetamines and related compounds, and with a mixture of N-Methyl-N-(trimethylsilyl) trifluoroacetamide/chlorotrimethylsilane for cocaine and metabolites, opiates and cannabinoids). The extracts were transferred to autosampler vials, and a 1 µL aliquot was injected in a HP 6890 gas chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a 5973 mass-selective detector (Hewlett-Packard), and a capillary column (30mx0.32mm i.d., 0.25mm film thickness) with 5% phenylmethylsiloxane (HP-5 MS) from J&W Scientific (Folsom, CA, USA).

The split injection mode was used at a ratio of 6:1, and helium was used as the carrier gas with a constant flow rate of 1.2 mL/min.

The mass spectrometer was operated with a filament current of 300 μA at electron energy of 70 eV in the electron ionization (EI) mode. The temperatures of the injector and detector were set at 250 and 280°C respectively. The chromatographic conditions were as follows: initial oven temperature was 90°C for 2 min, which was increased by 20°C/min to 300°C and held for 3 min.

Confirmation was done in the selected ion monitoring (SIM) mode, and the ions were monitored at *m/z* 236, 429 and 414 for morphine, at *m/z* 82, 182 and 303 for cocaine, and at *m/z* 82, 240 and 361 for benzoilecgonine. For the internal standards, only one ion was monitored for each compound, at *m/z* 432 for morphine-*d3* and at *m/z* 243 for benzoilecgonine-*d3*. The retention times were 12.24, 10.96 and 11.25 for morphine, cocaine and benzoilecgonine respectively.

Results and Discussion

Ethanol was not detected in the vitreous humor. Cannabinoids, amphetamines and related compounds were not detected either in the liver or kidney.

Morphine, cocaine and its main metabolite benzoylecgonine were detected and confirmed in the liver and kidney. It was not possible to determine 6-acetylmorphine in the liver or kidney, probably due to its instability and transformation to morphine in the liver. The results obtained in this study revealed evidence of cocaine intake [9-13]. Since both the illicit drug heroin and the prescription drug codeine are metabolized to morphine there is no evidence of heroin intake, which tends to complicate the interpretation of opiate-positive specimens in similar cases [15, 16, 18-20].

Conclusions

This work demonstrates that our validated methodologies used in the routine analysis to determine drugs of abuse in whole blood are suitable for application in other matrices such as the liver and kidney. However, in relation to the complexity of these alternative matrices, the samples must be pre-treated and homogenized before SPE cartridge application and thoroughly cleaned so that chromatographic analysis can be performed.

The lack of information provided both from the place where the victim was found and the circumstances of his death indicate the need for a toxicological analysis despite blood and urine not being available.

Lastly, as demonstrated by the results obtained, alternative specimens can provide important information about the intake of drugs of abuse. Forensic toxicologists must bear in mind that in some special circumstances the selection of matrices is dictated by the case under investigation.

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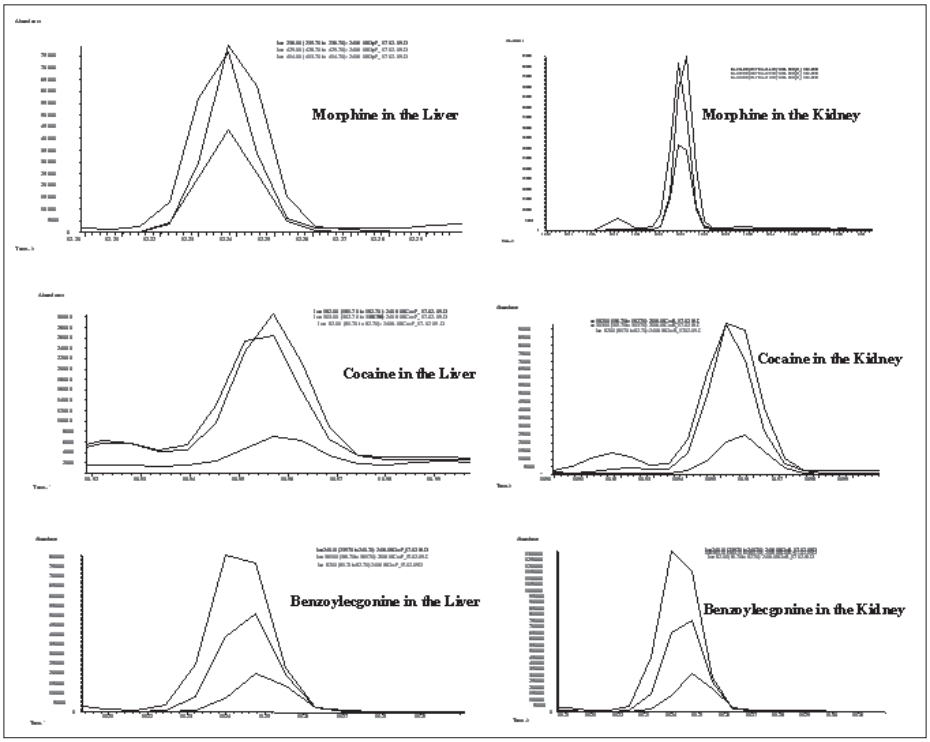

Figure 1 – Merged extracted ions chromatograms for morphine (*m/z* 236, 429, 414), cocaine (*m/z* 82, 182, 303) and benzoilecgonine (*m/z* 82, 240, 361) of liver and kidney.

	Morphine	Cocaine	Benzoilecgonine	Ethanol
Liver	D	D	D	
Kidney	D	D	D	
Vitreous Humor				ND

D – Detected
ND – Not Detected

Table 1 – Toxicological results of postmortem tissues.

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MURDER AFTER TORTURE BY SIDE-ARM: A CASE REPORT

Abstract: The authors present a case of a middle age man, who was found dead in his house. The examination of the crime scene and the forensic autopsy revealed several external injuries caused by a side-arm and a blunt instrument with different modalities. Autopsy revealed several external injuries due to a side-arm. The main wounds were superficial, mainly grouped in two different sites of the body: abdominal region and left leg. The fatal wound has caught up the heart through the thorax. A blunt injury was also found on the head. All the lesions had haemorrhagic infiltration; histology confirmed the vitality of all injuries. The interpretation of the medico-legal findings led to the hypothesis that the superficial wounds were inflicted to cause pain, while the only one, affecting the heart, was the final one. This hypothesis was confirmed by the reconstruction of the events in Court, where the suspects were sentenced for life imprisonment for inflicting lesions in order to gain information from the victim, and for murdering.

Introduction

The crime scene revealed the presence of a dead middle-aged man in the bathroom of his home. The human body was lying on the floor in supine position with his crura flexed on the thighs and his upper body – shoulders and head – lying on the wall; the lower extremity binding together by a plastic string (Fig. 1).

The inspection of the places showed the presence of a large pool below the body, in addition to several areas of bloodstain patterns located on the wash-basin, on the tiled wall above the head and on the tiles of the floor; there were also bloodspatters on the walls of the adjacent passage.

Materials and methods

We performed a medico-legal autopsy followed by histological examination of skin samples of regions interested by lesions. Toxicological analyses of blood, urine, gastric content, and bile samples were also conducted.

Results

The detected thanatological phenomena were hypostasis, located to the back and to the lower limbs, difficult to discern, blanched by compression, and rigor mortis fully established, absence of decomposition changes.

The external examination found numerous wounds by side-arm:

A) three cutaneous cuts, with clean non-abraded edges, on the face: one linear, at the right eyebrow, over the supraorbital ridge, with oblique direction from top to bottom and from left to right; one on the right ear, semicircular shape, with lower concavity; one on the upper left lip, near an ecchymotic area of the buccal side (Fig. 2).

B) five to the chest: one at the left shoulder, superficial; one in the middle of the breadbone, linear, superficial; one in precordium, near to the left nipple, lozenged shape, with dull upper corner and lower acute, deep, and two on the left side, respectively 14 and 26 cm below the ipsilateral axilla, both lozenged shape, with acute angles above and below dull (Fig. 3, Fig. 4).

C) fifteen to the abdomen, below the left costal arch, lozenged shape, confined in an oval area, wide 18x6 cm, with obtuse angles higher and lower acute, deep (Fig. 5).

D) six cutaneous cuts, confined on the lateral surface of the left thigh, 14 cm long, parallel each other, of which the central penetrates soft tissue underneath (Fig. 6, Fig. 7).

E) two defense wounds of both hands (palm of the right hand and first finger of the left hand) (Fig. 8, Fig. 9).

At the occipital and parietal region, near the vertex (Fig. 10), there was an undermined laceration, 8 cm long, produced by a bottle, inside an oval area of abrasion, wide cm7x5 and, at the back of the neck (Fig. 11), there was an abrasion, with slight indentation of the skin between parallel top and bottom line of demarcation, 14.6 cm long, thickness of 0.8 cm on the left and 0.2 cm on the right (Fig. 12, Fig. 13).

The autopsy revealed that the cutaneous sharp force injuries, penetrating into cavities that drew vital organs, were the following: the precordial one, interesting pericardium and anterior surface of the left ventricle, the upper left latus penetrating into the chest cavity and cutting the lower edge of the upper lobe of the left lung and eight stabs penetrated in the abdominal cavity, three of which pierced the stomach full-thickness and one draws the left lobe of the liver.

The toxicological investigations, performed on blood, urine, bile and gastric content were negative. Histology confirmed the vitality of all injuries.

Discussion

The United Nations (UN) Convention defines torture as: “any act by which severe pain or suffering, whether physical or mental, is intentionally inflicted on a person for such purposes as obtaining from him or a third person information or a confession, punishing him for an act he or a third person has committed or is suspected of having committed, or intimidating or coercing him or a third person, or for any reason based on discrimination of any kind, when such pain or suffering is inflicted by or at the instigation of or with the consent or acquiescence of a public official or other person

acting in an official capacity. It does not include pain or suffering arising only from, inherent in or incidental to lawful sanctions". The UN Convention against torture, adopted in 1984, is one of the least ratified major human rights treaties.

There is clear evidence for widespread use of torture among political prisoners throughout the world. Medical personnel frequently become involved, sometimes directly, often peripherally, for example, examining or treating victims.

In our case, the crime scene showed the immobilization of lower extremities binded together by a plastic string and the autopsy revealed different modality of injuries: stab and cut wounds by side-arm, laceration on the head by blunt trauma, abrasion on the neck by a tentative of strangulation. All cuts and stabs wounds in the skin were produced by a single blade side-arm with typical mechanisms of sharp force injury.

The multiple clustered stab and cut wounds were confined to three areas of the body (root of the left thigh, abdominal area, left lumbar side) and were characterized by the uniformity of production, implemented with low power, by the spatial concentration and by the homogenous depth. All the lesions had haemorrhagic infiltration of the margins; therefore histology examination confirmed the vitality of all injuries.

Most of the wounds was superficial, while the only one, affecting the hearth, was the fatal one. In fact, the cause of death can be ascribed to the stab wound to the heart.

For what concerns the determination of the time of death, the interpretation of the crime scene and autopsy findings have helped us in identifying a postmortem interval of about 12-16 hours.

Conclusion

In many instances, the autopsy appearances of lethal torture are not different from those by any other homicide and the confirmation must depend upon circumstantial and other corroborative evidence.

In our case, the crime scene examination, the immobilization of lower extremities, the mechanism of injury and the characteristics of the lesions led us to suppose that the purpose of the murderer(s) was to injure but not to cause death immediately and so the superficial wounds were inflicted to cause pain, while the only one, affecting the hearth, was aimed to be fatal.

Our hypothesis of reconstruction of the homicide – immobilization and torture of the victim – was then confirmed by the Court, where the suspects were recognized to be guilty and sentenced for life imprisonment for inflicting lesions in order to obtain information from the victim and then for murdering.

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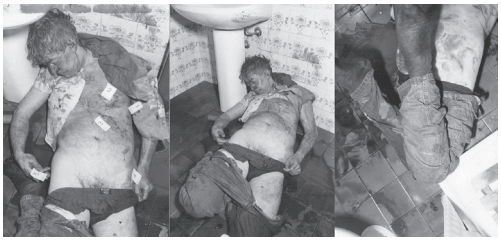

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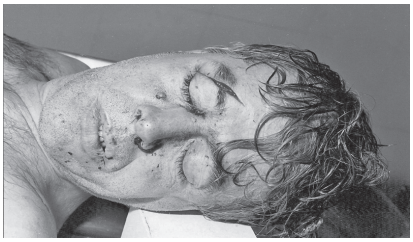

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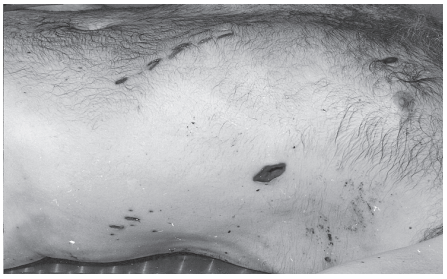

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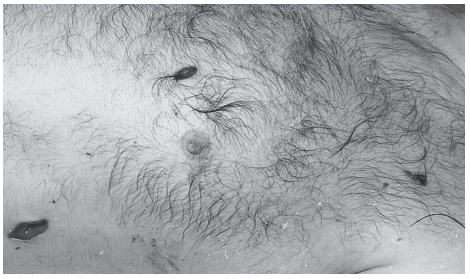

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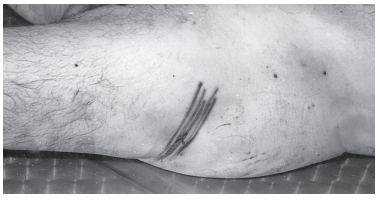

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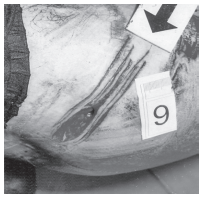

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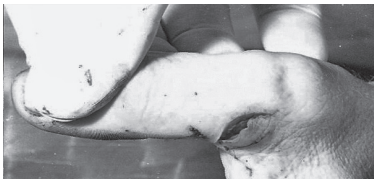

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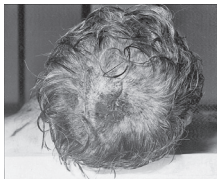

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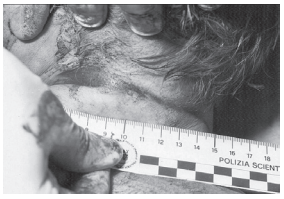

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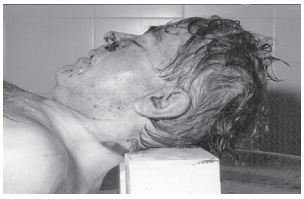

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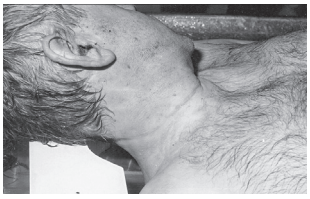

Figure – 13

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UNEXPECTED DEATH FOLLOWING FEMORAL CATHETERIZATION.

Abstract: Investigation of unexpected deaths related to diagnostic or therapeutic procedures is one of the most difficult fields of Legal Medicine. Technical and clinical knowledge is crucial to enable the relatives of the deceased to perceive the independence and neutrality of the investigative process

We report a case of an unusual complication following a catheterism in the lower limbs, which ended with the death of a patient.

Introduction

Investigation of unexpected deaths related with diagnostic or therapeutic procedures is one of the most difficult fields of Legal Medicine. The role of clinical and forensic pathologists is not well established and while one can think that is into the field of the later, others can argue that they do not have the independence necessary to carry out such type of procedures. Technical and clinical knowledge is crucial as it is the perception of the independence of the investigative process by the relatives or the deceased.

We report a case of an unusual complication following a therapeutic catheterism in the lower limbs that ended with the death of a patient. We expect to contribute to a better understanding of the difficulties inherent to an investigation like this and we conclude that the knowledge of the risks following diagnostic and therapeutic techniques is helpful for all pathologists, whatever their preferential dedication may be.

Case report

A 70-year old man is admitted to the hospital for pain in the lower limbs of sudden onset. He had a previous episode the previous week for a respiratory infection that was completely resolved. This new episode is considered independent form the later and he is diagnosed of arterial ischemia. An emergency catheterism is decided to remove an arterial obstruction in both femoral arteries (Fig. 1). The procedure results in complete disappearance of the pain, but the condition of the patient worsens progressively.

Since there is no clinical explication for that, an extensive clinical and analytical investigation is performed. Blood analyses reveal a persistent and progressive drop of red cells count and hemoglobin. Hemoglobin levels are 16,1 gr/L in the morning before surgery and 15,9 immediately after surgery. Since then, they fall to 7,9 and 4,4 two and three days after surgery, respectively. Hematocrit values are 47,9, 43,8, 25 and 14%, for the above-mentioned hemoglobin figures. Other analytical findings were not relevant. Abdominal ultrasonography is suspicious for splenic rupture and CT scan cannot rule out this possibility (Fig. 2).

With these data, the patient is submitted to an exploratory laparotomy that rule out abdominal bleeding. The surgeon can visualize satisfactorily the spleen, which is not ruptured or bleeding. The condition of the patient continues worsening and death occurs one week later without a firm clinical diagnosis. Clinical autopsy is authorized.

External examination shows an extensive hemorrhage in the lower right limb and the right and posterior abdominal wall. Internally, massive hemoperitoneum (about 2,5-3 L of blood were collected at autopsy) and an extensive bleeding in the retroperitoneum and the right inguinal soft tissues are the most noticeable findings. The later is considered responsible of the analytical alterations prior to the exploratory laparotomy. Although, the precise amount of blood accumulated in the soft tissues cannot be estimated accurately, a considerable volume can be inferred from the gross examination and it is later confirmed histopathologically. Moreover, histopathological examination of the right femoral artery disclosed persistence of the hemorrhage in the arterial injury related with the catheterism (Fig. 3).

The hemoperitoneum was caused by spleen rupture that was originated in multiple infarctions.

The remaining findings of autopsy were related to the shock developed along the last days of life, mainly Adult Distress Respiratory Syndrome and liver centrozonal hemorrhage.

Comments

Unexpected deaths following diagnostic or therapeutic procedures may arise the possibility of medical malpractice and, sometimes, such cases may be submitted to medico-legal autopsy. Though, it is not clear if this kind of deaths should be investigated by forensic or clinical pathologists, both of them may be involved occasionally in this kind of investigation. Consequently they should be familiar with the risks of most common medical and surgical procedures and be able to lead a postmortem medical examination under these difficult circumstances.

This patient presented an unusual complication resulting from the catheterism performed in the right femoral artery to resolve an acute ischemia. The local problem was eliminated successfully, but the condition of the patient did not improve but persistently deteriorated with any clear symptoms except for the blood analysis abnormalities. These consisted in a progressive fall in the red count values as well as in the hematocrit and hemoglobin figures. Due to ultrasonography and CT scan images, he was submitted to exploratory laparotomy with the suspicious of abdominal bleeding for spleen rupture. But surgery demonstrated that he did not have hemoperitoneum

and that the spleen was not bleeding. Externally, it was considered non-pathological by the surgeon, who could satisfactorily visualize it during laparotomy. No other source of bleeding in the abdomen was seen, either. The patient shocked and died one week later. Autopsy revealed a recent massive hemoperitoneum that, obviously, was not present in the moment of surgery and cannot be considered responsible for the loss of blood reflected by blood analysis. No other cause of bleeding was found except for the accumulation of blood in the soft tissues of the right thigh ant the abdominal wall. This was already noticeably externally, and autopsy confirmed a large amount of blood was present in this location. Retrospective review of the CT-scan showed that it was present prior to surgery. Histopathological examination of the right femoral artery showed that the injury caused by the introduction of the catheter was not resolved and bleeding persisted. Taking into account all this information, it was concluded that this was the cause of the progressive and persistent loss of blood responsible for the poor situation of the patient. The massive hemoperitoneum, ultimate cause of the death, was a final complication resulting from ruptured splenic infarctions. Its cause may be attributed to the atherosclerotic disease with multiple embolisms, one of them resulting in the lower limb ischemia.

Femoral catheterization is not exempt of complications, both systemic and local, the later much more common. Thrombosis, new emboli and hematomas are the most frequent, but the real incidence of them is not well known. Massive hematoma has been reported in about 0.9% of cases and it may be a serious complication. Many risk factors for hematoma development have been identified, including advanced patient age, hypertension, large-bore catheters, operator inexperience, poor groin compression after catheter removal, high puncture site, abnormal vessel or graft, and anticoagulant-thrombolytic therapy. Except for the age, we have not been able to determine any other risk factor since the technique had any complication.

In summary, we report an unusual complication of a common therapeutic procedure that highlights the difficult inherent to postmortem investigation of intrahospitalary unexpected deaths.

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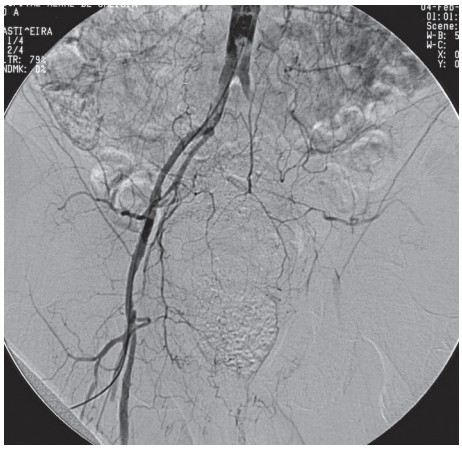

Figure 1 – Arterial ischemia as shown in the lower limb arteriography

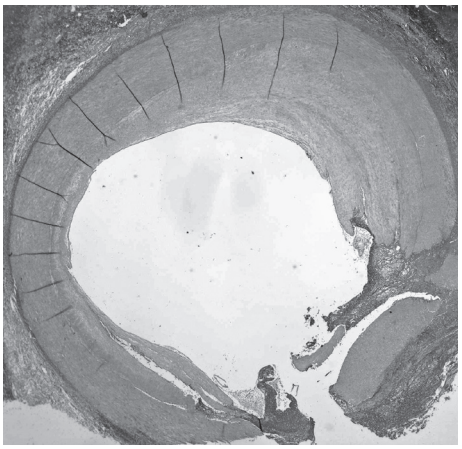

Figure 3 – Histopathological examination of the right femoral artery

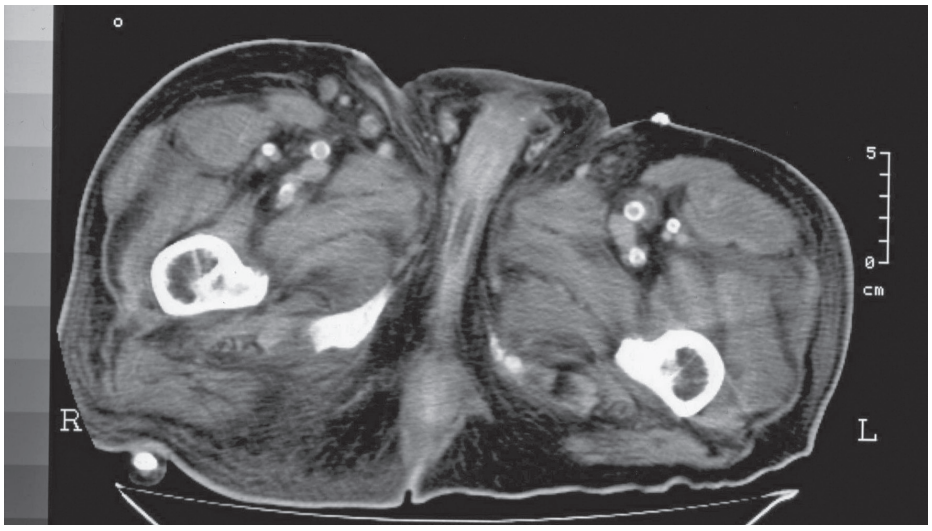

Figure 2 – Extensive hemorrhage in the soft tissues

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SCLEROSING LIPOGRANULOMA CAUSED BY ACCIDENTAL INJECTION OF INDUSTRIAL OIL

Abstract: Accidental injection of oily substances causes lesions with severe sclerosis such as those in a 28-year old male agricultural worker case presented, which needed surgical treatment and left after-effects.

Introduction

Sclerosing lipogranuloma is a tissue response to exogenous oily materials, as it happens after repeated injection of substances like paraffin or liquid silicon. This is more usually seen in the male genitalia as a consequence of injection of foreign materials into the scrotum or the penis in the mistaken belief that this would enhance erections [1,2,3].

Case Report

Two years previous to our consultation, a 28-year old male agricultural worker was accidentally injected with high-pressure hydraulic oil in the ball of his right thumb, first commissure and thenar eminence when the coupling of his tractor burst.

After surgical and medical treatment the after-effects included hard tumefactions on the thenar eminence and dorsal of the first commissure as well as retraction in flexing of the articulations of the metacarpal phalange and the proximal phalange of the thumb (Fig. 1a-1b).

Surgical reconstruction was carried out and sample fragments of the soft parts of the tumefactions were taken (Fig. 2). Histologically there was a substitution of normal dermis and hypodermis by a sclerotic tissue, with pseudoangiomatous slit spaces, and an infiltrate of lipophages, lymphoid cells and foreign body giant cells (Fig. 3-5).

Conclusions

Accidental injection of oily substances causes lesions with severe sclerosis such as those in the case presented which needed surgical treatment and left after-effects.

A peculiar histological pattern of sclerosis, morphologically similar to pseudoangiomatous hyperplasia of the breast [4], a type of stromal proliferative lesion, is reported for the first time.

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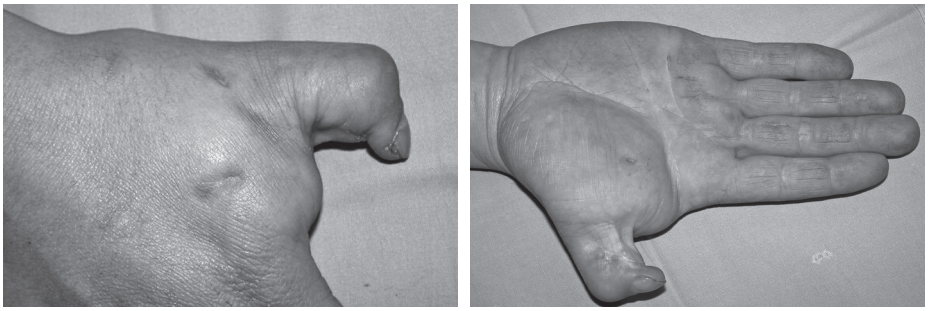

Figure 1 – Tumefactions on the thenar eminence and dorsal of the first commissure and retraction in flexing of the articulations of the metacarpal phalange and the proximal phalange of thumb

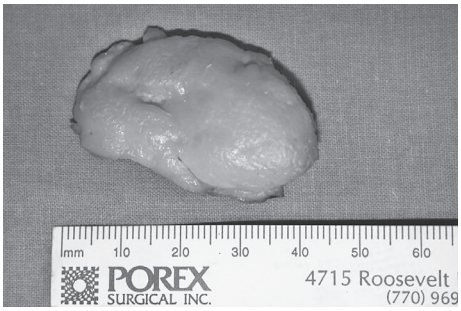

Figure 2 – Grossly, the excised tissue was whitish and elastic intermixed with fat tissue.

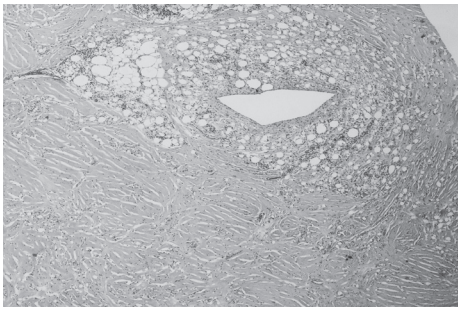

Figure 3 – Low power view: optically empty spaces surrounded by inflammatory infiltrate (top) and sclerosis with irregular clefts (bottom).

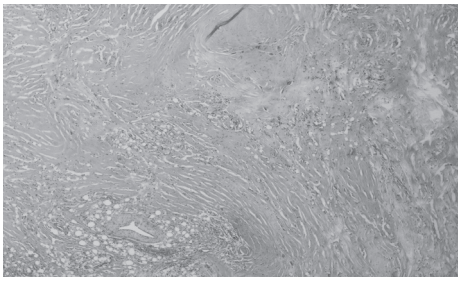

Figure 4 – Sclerosis produces artefactual capillary-like irregular spaces (left) similar to pseudoangiomatous hyperplasia of the breast.

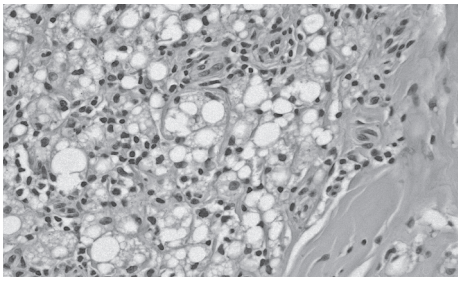

Figure 5 – Numerous hystiocites with fat vacuoles (lipophages).

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ESOPHAGO-PULMONARY ARTERIOUS FISTULA BY INGESTION OF A FOREIGN BODY: A CASE REPORT

Abstract: This is a case report of a 92-year-old woman, suffering from dysphagia and retrosternal pain, caused by an oesophageal foreign body that was found during an endoscopic examination. Few days after unsuccessful attempts of removing the woman suddenly died. We present the anatomical and histopathological study of the anatomical part, preceded by Computed Tomography images acquisition. All data substantiate a fistula development between oesophagus and pulmonary artery caused by a foreign body (during the post-mortem examination we found out it was a chicken bone).

The presentation of this extraordinary event wants to point out that high definition radiodiagnostic techniques have key role as an essential preparatory aid to face peculiar problems of forensic pathology and can be extremely useful to lead a much more targeted macroscopic examination and histological verification.

Introduction

Oesophageal perforation by foreign body, for its diagnostical and terapeutical implications and for the correlated severe prognosis is often a situation of forensic medicine interest.

As well knew, the majority of oesophageal perforations occurs because of impacted foreign bodies at site of anatomical narrowing (cervical-thoracic or diaphragmatic).

Most severe complications are sepsis or lethal hemorrhage caused by perforation of most important mediastinal vessels after fustulas formation.

In medical literature a lot of cases of dead by haemorrhage caused by oesophageal-arterious fistula, between oesophagus and aorta or one of its major branches are described.

A singular case of oesophageal-arterious fistula produced by a foreign body, between pulmonary-artery and oesophagus is presented. In this particular case imaging diagnostic techniques were used in order to acquire suggestive elements for corpse section procedure and for physiopathological proceeding reconstruction.

Case Report

A 92-year-old woman attended to the Emergency Department with a two months history of dysphagia, post-prandial regurgitation and retrosternal chest pain.

Oesophagoscopy revealed a foreign body embedded in the oesophageal wall, 35 cm from the incisor. An unsuccessful attempt of retrieval of the foreign body was made. A thoracic Computed Tomography (CT) revealed: an oesophageal wall inspissation in correspondence of tracheal bifurcation; a thin foreign body of bone density; no mediastinal troubles. A new unsuccessful attempt of retrivial was performed, followed by a moderate bleeding which was controlled by Sengstanken-Blakemore tube insertion. A new urgent Computed Tomography showed thin air-bubbles into mediastinal tissues and in correspondence of Aortic bulb and a para-oesophageal haematoma.

In absence of unequivocal clinical and radiological signs of iatrogenic perforation, and because of her steady clinical conditions, the woman was recovered for further management. 5 days after the woman suddenly died.

During autopsy, on the basis of clinical and radiological findings, mediastinal structures were *en bloc* removed and showed: at the lower third of oesophageal lumen, at 11 cm from aditus ad laringem, a foreign body (Fig. 1). This consisted in a sickle-shaped chicken bone with pointed tips (Fig.2), one of which embedded into the anterior oesophageal wall, where there was a 1.3 cm length ulcera-like lesion, with rounded edges (fig. 3). The whole pulmonary artery wall was interested by a 1cm length irregularity (Fig.4).

During post-mortem examination were also found: left ventricle concentric hypertrophy and wall inspissation and calcification of coronary arteries.

Mediastinal structures were fixed with formalin buffered solution and after few days a Computed Tomography was carried out. Images acquired indicated a whole thickness wall oesophageal perforation (Fig. 5) and some empty spaces into soft tissues of mediastinum (Fig. 6).

A following targeted anatomo-pathological examination of the specimen was performed. This permitted to demonstrate, and confirm what CT images revealed: a fistula passage (Fig. 7) which connected the oesophageal lesion with the pulmonary artery lumen, surrounded by an organized haematoma (Fig.8).

Subsequent histological findings were:

Oesophagus: irregularity of all the 4 layers of the oesophageal wall, preceded by Red Blood Cells and granulocyte cells infiltration that produced squamous epithelium loss and swelling of mucosal layer. The edge was infiltrated by a close exudates of granulocytes, that penetrates into the wall as far as muscolaris propria, where also lymphocities and hystiocities were presented. Deeply the passage result in an haematoma.

Haematoma: Coagulated blood of different chronology: the recent one consisted in platelets, fibrin and granulocityes; and the oldest one consisted in fibrin and hyaline.

Pulmonary artery: the tunica adventitia was reached by flogosis elements, around haemorrhagic focuses. The vessel wall presented a reduction of elastic elements. Deeply the vessel wall appeared deteriorated and in continuity with the haematoma.

Heart: several areas of scar tissue; coronary arteries with diffuse stenotic atherosclerosis.

Lung: chronic emphysema, edema areas, congestion.

The cause of death was ascribed to a terminal cardio-respiratory failure.

Discussion

Unlike aorto-oesophageal fistula, in this case the cause of death wasn't due to a massive haemorrhage and then cardiovascular collapse. With reference cardio-respiratory pathological findings, it appeared very difficult to find a causal relationship between mediastinal haematoma and the exitus. Mediastinal haematoma formation and grew, on the basis of imaging, anatomical and histological data must be referred to the interruption of pulmonary-artery wall, progressively reached by the fistula passage. The fistula evolution was, evidently, conditioned by complex mediastinal dynamics, such as respiration, circulation and oesophageal transit.

The case suggest some consideration.

Surely imaging techniques, and in particular CT, should be considered as a methodics of large aid that can give accurate indication for post-mortem dissection. In this case, in fact, multiplanar images acquisition, allowed to evidence and put in relation the mediastinal empty spaces with the oesophageal wall interruption and the chicken bone, foreseeing anatomo-pathological data and suggesting in a correct and precise post-mortem examination.

Otherwise, on fixed tissues CT has some technique limits, correlated to pathological or traumatic modification underestimation. This limits could be overcome by usual forensic pathology methods support, such as anatomical and histological research

Application and utility of imaging techniques seem to be unquestionable, above all in case of lesions placed in particular anatomical regions, difficult to approach and to evaluate, such as mediastinum, and especially on isolated organs or on anatomical parts.

We also think that the forensic pathologist should frequently use imaging techniques as a propedeutical aid, for an accurate approach to the anatomical parts dissection and so for their macroscopical and microscopical assessment.

The use of these many different methods and techniques will make easier the possibility of a correct interpretation of any singular case.

We hope in a continuous increase of high resolution imaging application in forensic pathology, both on corpses and their parts. This will request an higher cooperation between forensic pathologist and radiologist.

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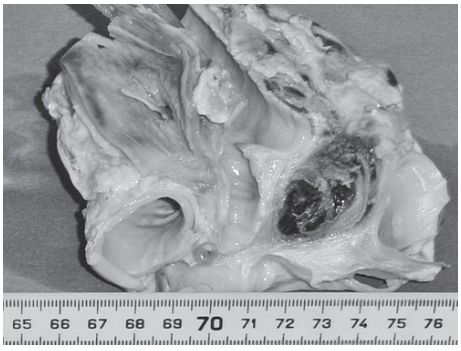

Figure 1 – Mediastinal structures removed *en bloc*. An oesophageal foreign body and a mediastinal haematoma are visible.

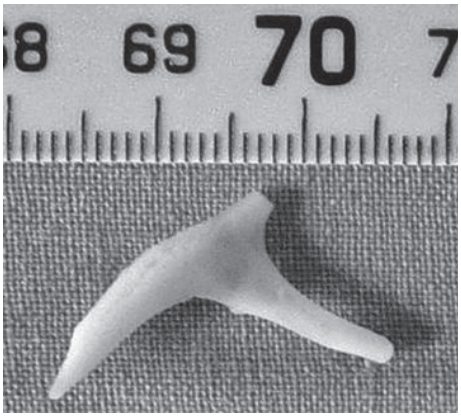

Figure 2 – Foreign body: a sickle-shaped chicken bone with pointed tips.

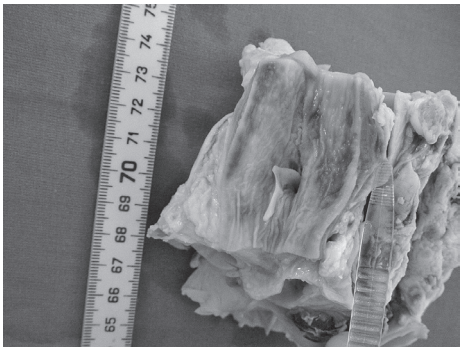

Figure 3 – Foreign body embedded into the anterior oesophageal wall. An ulcera-like lesion of mucosal layer is visible.

Figure 4 – Pulmonary artery injury: a 1 cm length irregularity of all the pulmonary artery wall layers.

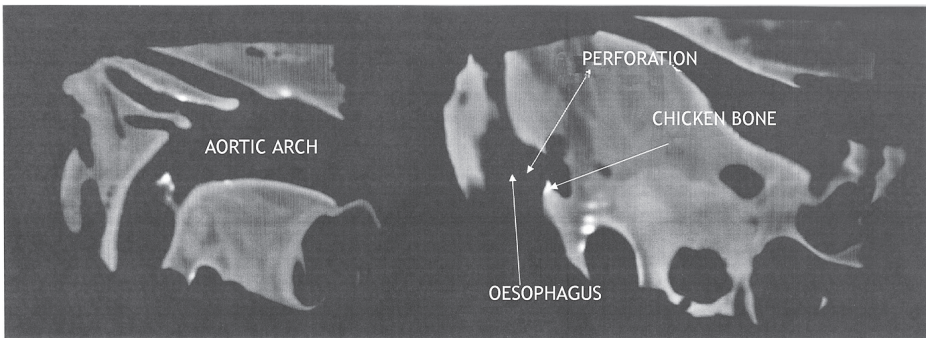

Figure 5 – Computed Tomography findings. Esophageal perforation and a foreign body of bone density are recognizable.

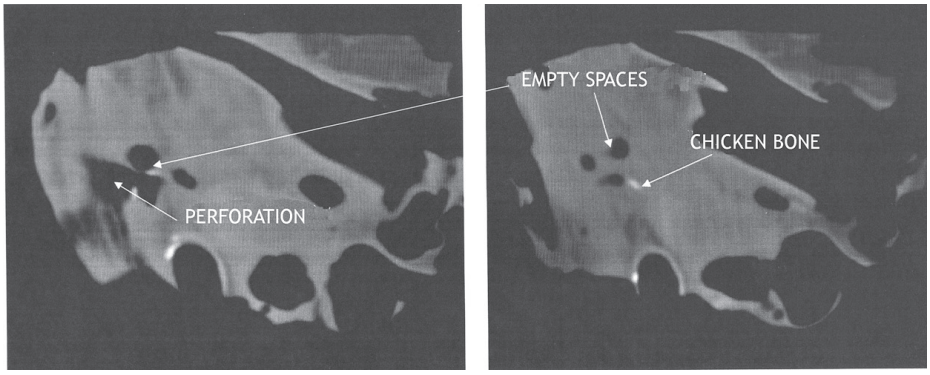

Figure 6 – Computed Tomography findings. Empty spaces into mediastinal soft tissues in relation with chicken bone and esophageal perforation.

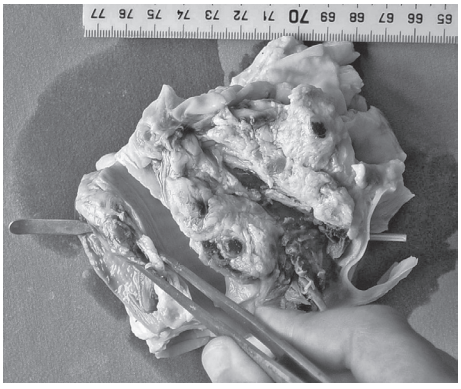

Figure 7 – Fistula passage. Oesophageal lumen and pulunary artery lumen are connected.

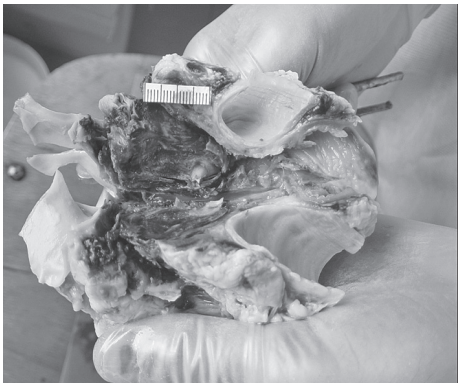

Figure 8 – Oesophageal-pulmunary artery fistula surrounded by a mediastinal haematoma.

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SUDDEN DEATH FROM CRYPTOCOCCAL ENCEPHALITIS

Abstract: We describe a case of sudden death from Cryptococcus-related encephalitis of a young man with misdiagnosed AIDS-Syndrome. The patient was admitted twice in Hospital in the weeks before the death and he was also visited at home just the same day he died, but clinical suspect of encephalitis was completely failed, despite he'd complained neurological symptoms, with subsequent medico-legal implications concerning medical malpractice.

Introduction

Cryptococcal meningo-encephalitis (CME) is generally uncommon (1/1000 per years in U.S.A.) but AIDS outbreak has caused its dramatic worldwide increase in recent years. CME represent a typical opportunistic infections associated with AIDS, occurring about in 6% to 8% of AIDS patients (1,2) even if rare cases are observed also in immunocompetent individuals (3,4,5,6).

Therapy is based on antifungal drugs but the prognosis is dramatically poor.

Case report

A 35-year-old man, addicted to heroin in the past, was found dead in bed while he was spending a few days at friend's home. For many weeks before, he had been complaining of disease with nausea, vomiting, headache, dizziness.

He went to the Hospital in E.R. twice in the early period before death; the first time (13 days before the death) no signs of meningism were found at medical examination; during the admission to the Hospital the patient had a lipotymic crisis, but he was discharged a few minutes later with diagnosis of lypotimia flu-syndrome related. After six days he was transported again to the Hospital, complaining of lost of consciousness, vomiting, asthenia and cold sweat: it was performed a physical and electrocardiographyc examination, negative for abnormalities, and the patient was discharged with the same diagnosis.

During the following week he has been continuously staying in bed for a severe weakness; after the onset of a convulsive crisis the doctor who examined the patient at

home attributed the symptom to oppioid withdrawal syndrome and did not arrange hospitalisation. The day after the man was found dead in the bed.

The medico-legal investigation revealed remarkable brain swelling (Fig.1,2) associated with pulmonary congestion. Histological examination of brain specimens showed micotic clusters in meninges, cortex and cerebellum (Fig. 3,4), not associated with significant inflammatory cells response. Laboratory tests indicating a meningo-encephalitis from Cryptococcus neoformans and virologic examination from liquor and brain specimens revealed also the presence of Epstein-Barr and BK viruses while blood examination revealed HIV.

Discussion

Cryptococcal meningo-encephalitis is the most common fungal infection of the central nervous system. It is rare in immunocompetent hosts, but it represents the most important life-threatening fungal disease in patients affected by AIDS (7), with a prevalence of 2-10% among HIV-positive patients in Western Europe and U.S.A. Cryptococcosis is often the initial AIDS-defining illness (about 60% of patients in Italy (8) and France (9), 30-40% in U.S.A.(10)).

Clinical manifestations evolve chronically with severe headache, deficit of cranial nerves (aphasia, hearing loss, visual deficit), seizures, cerebellum's signs, and increasingly intracranial hypertension, but signs of neurological infection are manifest only in one third of patients.

The impact of increased intracranial pressure on early mortality of patients with AIDS-associated cerebral Cryptococcosis has been stressed and the guidelines of the Infectious Diseases Society of America recommended that patients with an opening pressure > 250 mmH20 must be treated with mechanical (11) and pharmacological cerebrospinal fluid drainage (12). From a Pathological point of view, AIDS-related CME differs from other neurological infections for the lack of inflammatory response and mortality is due to intracranial hypertension, with subsequent brain swelling and herniation produced by mechanical barrier in the arachnoids' villa caused by clusters of mycetes.

Cryptococcus is generally considered difficult, if not impossible, to eradicate in AIDS patients, thus requiring life-long antifungal therapy, based essentially on amphotericin B and fluconazole (13). Most of the available evidences suggest that amphotericin-B-base therapy represent the gold standard and the combination of an amphotericin B preparation plus flucytosine should be regarded as the best initial treatment, being the more effective treatment to obtain rapid clearance of the fungus from cerebrospinal fluid, which is probably the best surrogate marker for ultimate successful therapy (14). Because of the toxicity of amphotericin, after initial treatment, it is necessary to modify therapy switching to fluconazole, which instead is less efficient in eradicate the fungi.

Untreated Cryptococcal meningitis is uniformly fatal within a relatively short period of time and the prognosis of Cryptococcal meningitis is still poor even in patients who receives early and correct therapy (15), considering that mortality rate is about 50%. In our case, the pathology affecting the central nervous system has been further

aggravated by the simultaneous infection from Epstein-Barr and BK viruses, opportunist agents often responsible for meningo-encephalitis in AIDS patients (16,17,18).

Even considering these dramatic prognosis, the lack of adequate instrumental and radiographic investigations despite the severe symptoms that affected the man before the death, has determined medico-legal implication concerning the case submitted to our study, in order to asses medical responsibility in failing the correct diagnosis.

Cryptococcal encephalitis represents a common complication among patients infected with the human immunodeficiency virus and it must be kept in mind the possibility of a Cryptococcal neurological infection in those patients with evidence of AIDS, being an early treatment the only resource for a life-threatening outcome.

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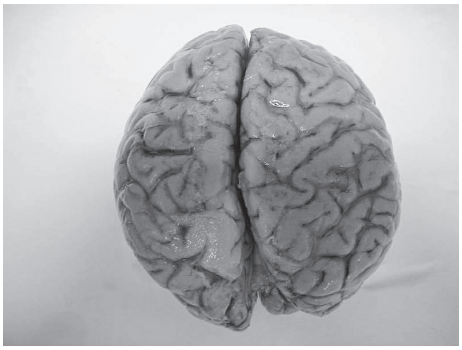

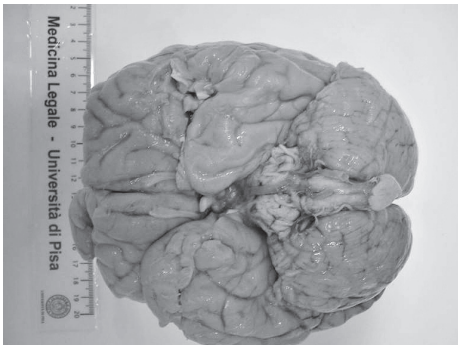

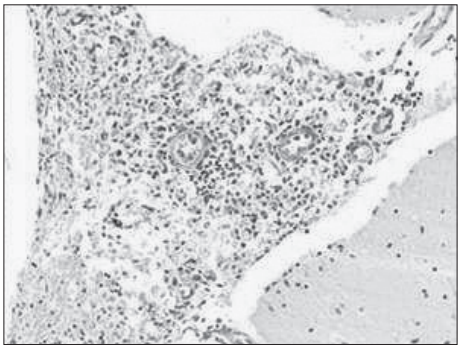

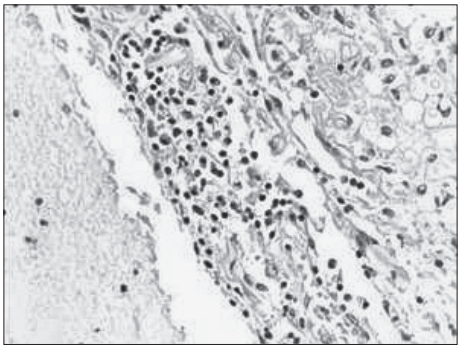

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LETHAL INHALATION OF POPPER

Abstract: We present a case about an unexpected death occurred during inhalation of Popper, a recreational drug constituted by alkyl nitrates, which is generally considered safe for life, despite its activity on cardiovascular system. Fatal inhalation of nitrates are extremely rare in world literature; the dangerousness from nitrate are primarily related to the synthesis of methaemoglobin, the oxidative form of haemoglobin, which cannot carry oxygen causing hypoxia. In some instances death from nitrates can derivate from lethal arrhythmias induced by a sensibilisation of myocardium to cathecolamine. In our case toxicological analysis were hampered by advanced putrefaction, but nevertheless it was possible to asses in biological samples the presence of Butyraldehyde dibutylacetal, a molecule originated during the chemical synthesis of nitrates but more stable, that confirmed the assumption of Popper just before the death.

Introduction

Popper is a recreational drug constituted by various alkyl nitrates, as amylnitrite, butyl nitrite, isopropyl nitrite and isobutyl nitrite, that has begun gradually to widespread since 60's years. Nowadays it is usually sold in sexy-shops (somewhere as a room deodorizer) in little plastic bottles or imbued in cotton or other adsorbent materials. Popper, (the name derivates from the period when the drug was sold in small glass tubes, covered with fishnet, that made a popping noise when broken to open and inhale) is known as a recreational drug, traditionally abused in male homosexuals activity (1) because of the induction of a vigorous excitant effect associated to anal muscle relax and anesthetization.

It is assumed by inhalation and the effects begin within few seconds and last for a very short period (from 30 seconds to 2 minutes), and the effects consist in relaxation of smooth muscle surrounding the blood vessels resulting in an immediate increase in heart rate and blood flow throughout the body, producing a sensation of heat, euphoria and excitement; at the same time, the relaxation of the sphincters of the anus and vagina make penetration easier so it's commonly said that poppers can enhance and prolong orgasm.

Case report

A 48-years old man, who was working as a musician on a cruise-ship, was found dead inside his cab, after he had been seen alive 36 hours before the discovery of the body, when he entered into his cabin for the last time, as recorded by the magnetic-key.

Some days before he underwent a medical control to receive topical cream to treat a rush around his mouth, which he alleged as a reaction to dental surgery. No significant pathology was noticed in his clinical folder.

The body was found in advanced state of decomposition, with severe swelling of face and limbs. He was sitting on a chair, lining on his right side, dressing only a t-shirt and a pair of socks (Fig. 1). He was in front on desk where a DVD-monitor was showing an hardcore movie of sadomasochist genre. The man had a mask on his face (Fig. 2), with a wad of cotton inside emanating a sweetish smell. On the desk, near the body, there were three little bottles, whose labels was that of a room odorizer named "Jungle Juice". In the refrigerator there were others equal and more bottles with the label "Rush" (Fig. 3). In the cab there were also Viagra package and some tablets of smart-drug, containing caffeine and other natural components. The room has been locked from inside and the cracks of the door and air conditioned-grid were sealed with adhesive taped (Fig. 4); a friend referred that the deceased alleged he was used to did so to prevent noise disturbing him while he was asleep.

Autopsy findings just consisted in severe coronarosclerosis of the descendant anterior coronary artery, with histological evidence of mild miocardiosclerosis, pulmonary congestion and hepatosteatosis. A thorough inspection of the nose, lips and neck didn't reveal any traumatic injuries, although we must consider that putrefaction could have made little abrasion or bruise impossible to be noticed.

Toxicological analysis

Toxicological tests, performed with Thermo Fisher Scientific (GC) coupled to DSQII (MS), revealed that the liquid inside the bottles contained isobutyl-nitrite.

Although the research of this substance in biological samples resulted negative because of the extreme volatility and the effects of putrefaction, that affected also the research of methaemoglobin for the endogenous production of this molecule during blood decomposition, the GC/MS showed the presence of an organic compound in Rush, named Butyraldehyde dibutylacetal, that was discovered also in sample of tissue from cadaver (blood, hair, lung, brain, liver and kidney) and in the wad of cotton found inside the mask. This molecule is produced during chemical syinthesis of nitrates and it was possible to found it in biological samples because of it's more stable than vapours of nitrate.

Discussion and conclusion

Isobutyl-nitrate and amyl-nitrate are the active molecules of Popper, whose assumption is still increasing, especially in male homosexuals contest (2.) Once exposure to substance has occurred, usually by inhalation, the effects are rapid and brief, so multiple intakes during sexual activity could be required.

The nitrate is rapidly absorbed and vasodilatation can result in palpitation, skin flushing, hypoxia and dizziness. Headaches, nausea and syncope are also described.

Toxic effects arise from production of methaemoglobin, the oxidative product of haemoglobin, that is incapable to carry oxygen, determining generalized hypoxia.

Fatal cases related to assumption of Popper are extremely rare, especially by inhalation intake. Bradberry et al. (3) reported a case of fatal methaemoglobinemia due to inhalation of Popper, while a few other reports of clinically significant (but not lethal) methaemoglobinemia, related to the assumption of nitrates, are described (4,5·6). Besides, isolated lethal cases has been documented when alkylnitrites have been taken orally (7,8).

For these reasons, in suspect of Popper's assumption, methaemoglobinemia determination is necessary, in addition to the dosage of nitrite and nitrate in blood and stomach contents.

Some Authors suggest that frequently death can occur suddenly for arrhythmias, as a result of myocardial sensibilisation to catecholamine induced by inhalants, consisting in the so-called Sudden Sniffing Death (SSD), firstly described by Bass in 1970 (9).

Referring to our case, all the evidence from technical survey and autopsy were strongly indicative of an assumption of Popper with exclusion of a possible involvement of other persons in causing the death. Although toxicological analysis aimed to the research of nitrites and methaemoglobin have been not useful by putrefaction, laboratory investigation showed the presence in biological sample (blood, hair, lung, brain, liver and kidney) and in the cotton wad found inside the mask, of Butyraldehyde dibutylacetal, a molecule produced in chemical synthesis of nitrates but not as volatile, offering the evidence of intra-vitam assumption of Popper. From the discovery of nitrate inhalation, there are no doubts that perioral skin rush and abrasion were due to multiple contacts with irritant nitrate vapours and that the hermetic sealing of doors and grid was performed to avoid vapours getting out from the cab and being perceived by staff members of the ship.

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HISTORICAL-ANTHROPOLOGICAL-FORENSIC ANALYSIS OF A SKELETON FROM A GRAVE DATABLE ABOUT YEAR 1000 WITH TRAUMATIC LESIONS THAT INDICATE CAUSES AND MODALITIES OF THE DEATH

Abstract: The archaeological finding of an isolated buried skeleton in the neighbourhood of a small medieval church has made to date, by the archaeologists, the grave approximately about the year 1000. The study of the skeleton with forensic-anthropological techniques has allowed to obtain many marks about the physical features of the subject; but, above all, very thorough forensic-pathological examination, also using of optical and electronic microscopical techniques, has allowed to characterize meaningful injuries at the head, evocative of their intra-vitam production, indicative of some features of the productive mean and much evocative of causes and modalities of the death, probably identifiable in an execution. The purpose is to suggest the comparative use of macroscopic, microscopic and ultra-microscopic morphologic analysis compared can suggest solutions of cases happened in far historical ages, cases of which we only have the skeletal substrate.

Introduction

The church of San Biagio is situated higher than the town of Cittiglio, to which it belongs. From the ecclesiastical viewpoint, Cittiglio's area borders with the Ambrosian diocese of Milan and the Roman one of Como (fig 1). Church's origins are undoubtedly Romanesque if not even paleochristian and its bell-tower, which dates around 1.000 a.C., distinguishes for the archaic shape of its mullioned window with crutch capital.

The on-going excavation has brought to light at least 3 different floor layers laid down in the past under the more recent floor belonging to the '70s (one layer of brick dated 1630, one of red-coloured mortar dated 1200 and one of trodden mortar dated around 1000).

Several architectonic structures belonging to an older church – smaller than the present one – were found out; among them there are the remains of a church's front demolished during Medieval Age – in place of the medieval apse there is the door entering the present churchyard, which was the graveyard at the time.

Together with the wall remains many old manufactured objects have been found out and they date between XII and XVI centuries.

A first and still temporary stratigrafic analysis of the fossil documentation of the site, carried on by Doctor R. Mella Pariani, dates the burial between 1000 and beginning 1200 a.C.

In addition, these data are enhanced by the dating of the floor, below which the grave containing the bones of the subject was located and which belongs to the same historical period of time.

The grave was a lithic burial niche with obliquely laid stones and mortar was not used to bind them. Characterized by a very narrow and anthropomorphic shape, it was placed along the narthex of the church with east-west direction and the head of the dead looked towards west (that was a privileged position reserved to aristocrats and founde (fig 2,3).

Materials and methods

Biological profile.

Race diagnosis: Caucasian. Orbits of quadrangular shape with particularly rounded corners, long and narrow nasal fossae, ogival palate, typical morphology of the incisors and of the first molar tooth (fig 4).

Gender diagnosis: Male. According to criteria identified by Ascàadi and Nemeskéri for studies concerning cranial bones.

Age diagnosis: between 20 and 30 years old. According to the observation of cranial sutures on the points indicated by Meinland and Lovejoy and basing on the study of dental elements.

Height calculation: about 174 cm. Use of Trotter and Gleser method (on the right femor 172.08 cm. and on the right tibia 172.36 cm.). Use of Meadows and Jantz formula on metacarpus (176.42 cm) and use of the Byers and team's members on the metatarsus (174.53 cm.).

Dental evaluation (fig 5,6). Teeth are generally very well preserved. All dental elements are present except for 46, taken ante-mortem (within 6 months before death) (fig 20,21,22,23). Arches are wide and dental elements are in line. The inter-arch relation is I Class of Angle with normal OVJ (overjet) and OVB (overbite). Worn areas are evident on palatine cusps of first molars and upper premolars bilaterally and on vestibular cusps of the first left lower molar, of the second left molar – though less seriously – and of both lower right premolars (fig 11,12,13,14). The features of the dental wear are indicative of a peculiar mastication or of particularly hard food or of the use of the denture for somewhat activity; there's no relation with the methods to establish the age of the subject. The marked periodontal disease of the lower frontal area can be attributed to the juvenile periodontal pathology, associated with life and food habits of the subject (fig 17,18,19). The loss of element 46 ante-mortem – within 6 months before death – does not seem to be due to chronic dental caries, since the periapical bone structure is intact and the post-extraction regeneration is effective. The third molars are at different phases of eruption (fig 15,16); this is a very useful

detail in order to establish the age of the subject together with radiographic images of the incomplete apical closure of the elements (about 20).

Lesion 1 (Fig. 24,25,26). It involves the right parietal bone and part of the occipital bone and it ends on the left branch of the lambdoid suture. Length 11,4 cm. Cranio-caudal inclination of 27,5° towards the sagittal plane and of about 10° in back-frontal direction towards the frontal plane. Sharp cut area in correspondence of the external bony lamina and of part of the diploe.

Lesion 2 (Fig. 27, 28 ,29) It starts from the squama of the occipital bone, 2 cm left of the right branch of the lambdoid suture and it ends in correspondence of the squamous suture. It has a front-back inclination of 40° towards the sagittal plane and creates a cranio-caudal angle of few degrees towards the frontal plane. It involves the whole thickness of the external bony lamina, of the diploe and of the internal bony lamina.A huge number of big and parallel sulci, which are perpendicular to the bony plane, can be seen with the naked eye.

Lesion 3 (Fig. 30,31,32). It is placed over the occipital protuberance and, with oblique course, it extends laterally and right towards the crest (direction: cranio-caudal and back-front) till it touches the area of the lesion 2 with an inclination of 40° towards the frontal plane and of 35° towards the plantar plane. It involves the whole thickness of the external bony lamina, of the diploe and of the internal bony lamina.

Results

At a first rough observation of the neuro-cranium of the dead in the grave T13-us 168b some lesions due to naked steel were noticed and they became more and more evident after the cleaning of the bones. Therefore, an additional research was requested to analyze lesions, which – if vital considering their place – could have caused death through a peculiar and violent action bound to the death.(Fig. 33)

The cranium was then recomposed and that allowed to evaluate the real wideness of the lesions and to calculate their size and inclination.

From the very beginning the extremely sharp characteristic of the cut sections was amazing. For that reason, it was decided to go on studying the lesions at the epimicroscope to better analyze the morphological features of the holes and of the crests, which were already visible by the naked eye, and to possibly reconstruct the profile of the cutting item, which was likely to be a saw, considering that lesions are indented. It seems more and more reliable the hypothesis that lesions were inflicted in perimortem.

The observation by growing enlargements of the cut sections shows thick holes of different size and with course reciprocally parallel and perpendicular to the bony lamina at level of all lesions. This last consideration excludes the use of a saw as a cutting item due to grounds of dynamic type.

Additional analysis at the scanning electronic microscope (SEM) are carried out, because it offers a higher definition level of details by means of its higher in-depth analysis capacity.

These data indicate that lesions were caused by naked steel used like a cutting item. Even if it is not absolutely sure, the type of inhumation and the topographic situation of the lesions enhance the hypothesis that lesions were caused in the perimortal period of time.

At this stage of the study, the following conclusions can be drawn: he cutting item can reasonably be a quite long blade, which was very sharp (so that to be able to cut a fragment of occipital bone without causing any sign of fracture) and not too much heavy (otherwise, it would have acted like a contusive mechanism causing secondary fractures); the holes visible at every enlargement were not due to the teeth of a saw but to the irregularity of the profile bound to the sharpening of the blade.

Discussion

At that time the problem of the historical compatibility of so clean cut lesions arose: the circumstance when lesions were made, the type of cutting item and the technical possibility bound to the production of such sharp blades. Trajectories of the blows of naked steel were reconstructed on a model (Fig 34,35). The reconstruction of the direction of the trajectories have made to hypothesize a case of decapitation.

At first the presence of multiple lesions on the cranium led to think that it was a decapitation happened during a fight: in literature the presence of multiple lesions often indicates an excited and chaotic event happening during a battle rather than an execution.

Conclusion

Some experts in medieval history and in blades were asked for advice in order to better frame the facts from the historical viewpoint and to identify the cutting item probably used. Doctor Mario Scalini of the Soprintendenza Speciale of Polo Museale in Florence contributed to elaborate the following possible reconstruction of the facts.

Death dynamics seem to be that of a capital execution: the condemned person knelt and the executioner was on his right.

The first blow caused lesion 2 and a second blow followed, while the condemned person was staggering, and caused lesion 3 – this dynamic was given value by the observation of the prosecution of lesion 2 on the bone fragment cut by lesion 3; finally, when the person lay on earth, the third blow was given and caused lesion 1 (the fact that this is the last lesion is deducible by the growing irregularity of the cut surface, like due to the wear of the blade).

The reconstruction is based both on the topography of the lesions as well as on the fact that the lesions by naked steel do not seem compatible with a war sword – particularly heavy and that could have caused bone lesions with sinking and certainly secondary fractures – but with an execution sword – lighter, extremely sharp, with a blade with lenticular section and without central veining (Fig 36).

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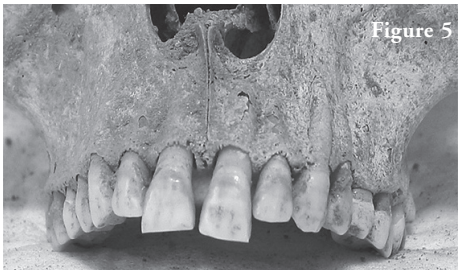
Figure 1 – The San Biagio Church in Cittiglio
Figure 2, 3 – The grave T13 US168b
Figure 4 – The skull
Figure 5 – maxillary arch
Figure 6 – mandibular arch
Figure 7,8 – Palatine suture: the ossification of the palatine suture is not complete, especially at the level of the first third of the suturein the intra-alveolar section; it indicates that cartilage was present there when he was alive and it's a useful detail for the age calculation.
Figure 9,10 – Right maxillary sinus: wide pneumic cavity without thickening of the floor (this radiographic detail excludes sinus chronic inflammatory diseases).
Figure 11,12 16, 17 and 18 dental elements – the upper right third molar looks partially included (mucosa) with well-developed radicular apparatus, with very wide canals and without visible apexes. It is evident that palatine cusps of 16 are particularly worn, while palatine cusps of 17 are unworn.
Figure 13,14 25, 26 and 27 dental elements – the third molar is nearly completely erupted with the third apical of the roots developed and still open apexes. It is evident the relation between the roots of 16 and the floor of the left maxillary sinus.
Figure 15,16 37 and 38 dental elements – the third molar is in the eruption phase but it is still partially included, third apical in the development phase, pulp chamber, wide radical canals and open apex. By applying Kulmann method, the man should be aged around 20(+/- 2).
Figure 17,18,19 41, 42, 43 and 44 dental elements – despite the wideness of the arch and the excellent inter-cusp, there is a mesial inclination and a partial rotation of the 43, there's an evident periodontal disease of the frontal portion with a bones reabsorption both vertically and horizontally, also visible in the following radiography of the elements 41, 31, 32 and 33.
Figure 20.21 45 and 47 dental elements – the alveolar site of 46, which was taken ante-mortem, is empty and there is a thin post-extraction regenerative bony reticulum. No signs of periapical disease are present. The inter-radical sedimentation is still recognizable and it indicates that the tooth was taken within 6 months before the death.
Figure 22,23 47 dental element – the recent loss of element 46 did not cause either the shift of close dental elements 45 and 47 nor the extrusion of the antagonist 16.
Figure 24 lesion 1 – macroscopic view
Figure 25 lesion 1 – epimicroscpic view
Figure 26 lesion 1 – electron microscopic view
Figure 27 lesion 2 – macroscopic view
Figure 28 lesion 2 – epimicroscpic view
Figure 29 lesion 2 – electron microscopic view
Figure 30 lesion 3 – macroscopic view
Figure 31 lesion 3 – epimicroscpic view
Figure 32 lesion 3 – electron microscopic view
Figure 33 – three lesions at the skull
Figure 34, 35 – the lesions reproduced on a model
Figure 36 – the measurement of the lesion 1
Figure 37 – fac-simile of a miniature on wood in the "Cosmographie Universelle" of Munster. In folio, Sale 1952
Figure 38 – the possible used weapon

Figure 1

Figure 2

Figure 3

Figure 4



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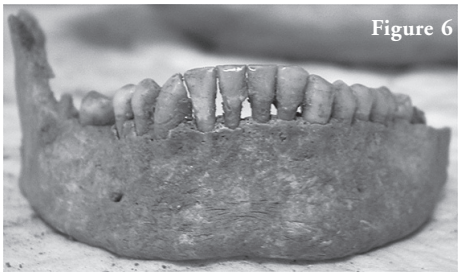
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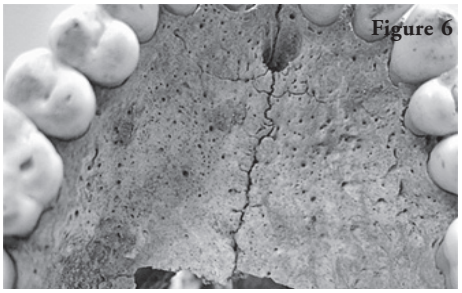
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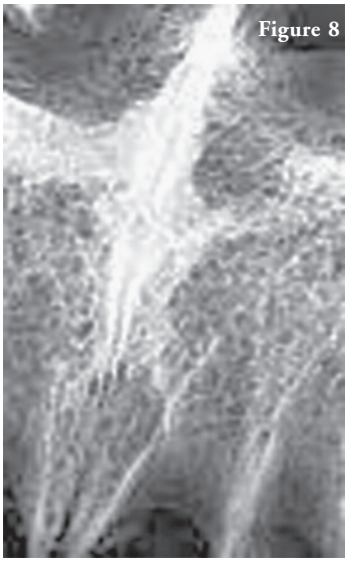
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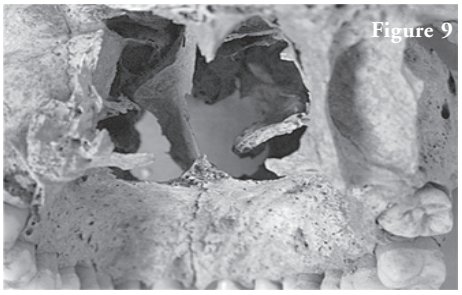
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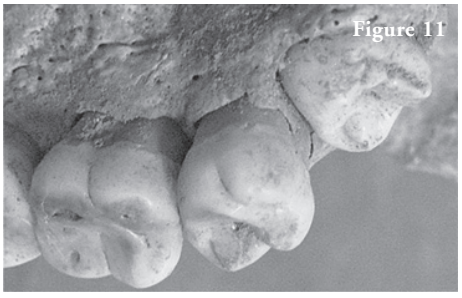
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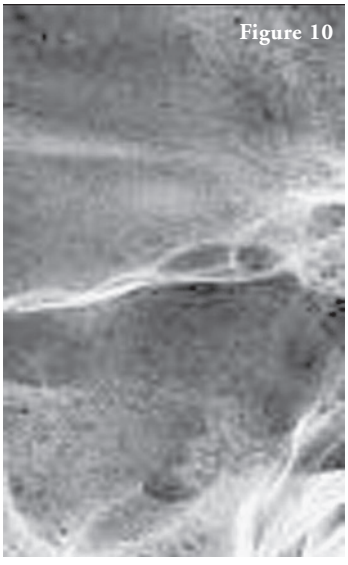
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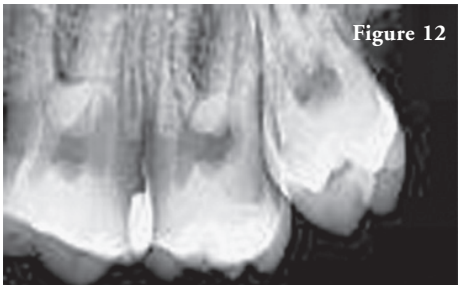
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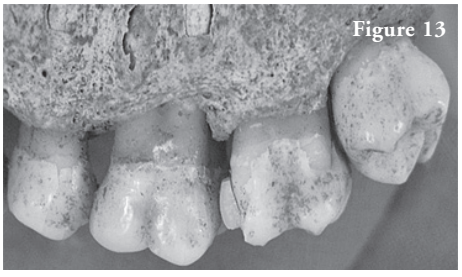
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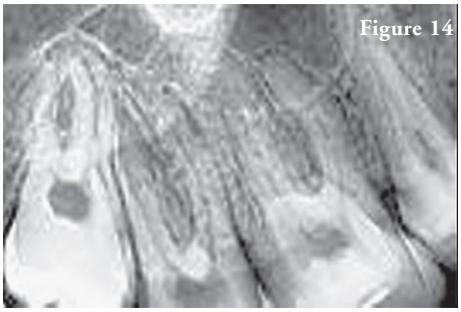
Figure 14


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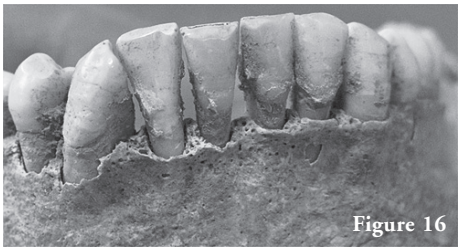
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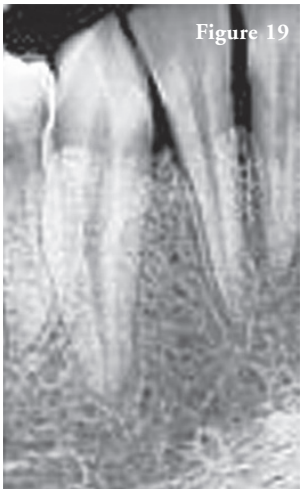
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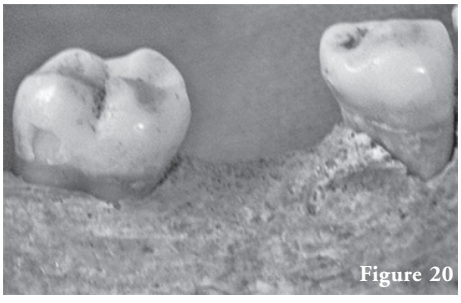
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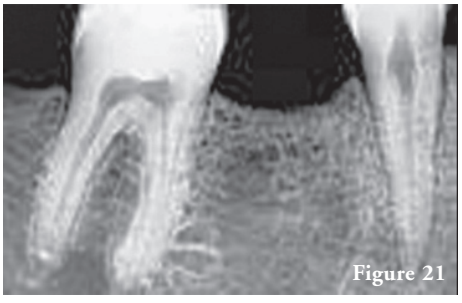
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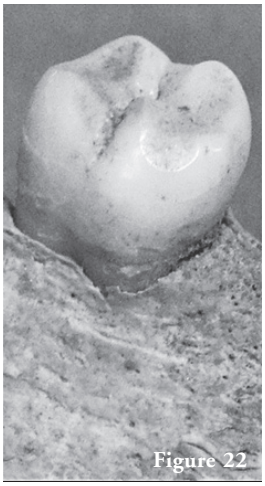

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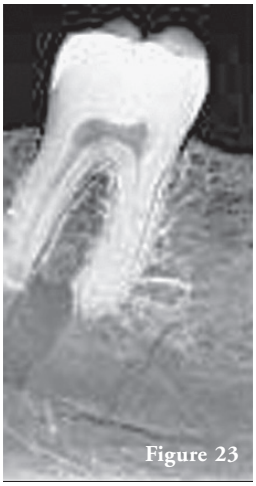

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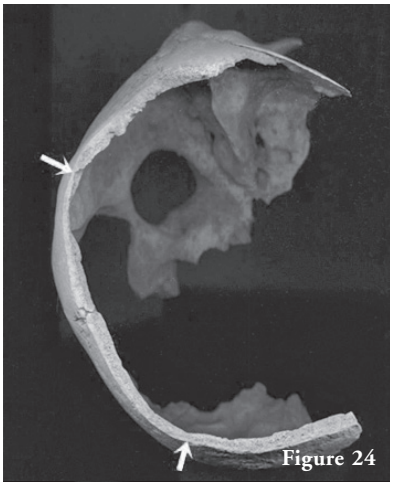

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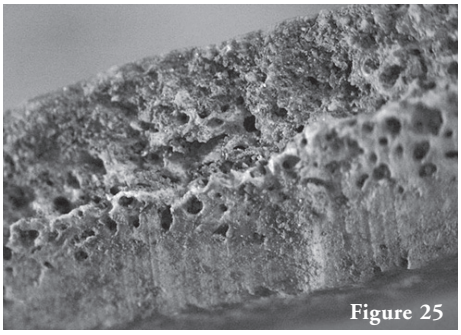

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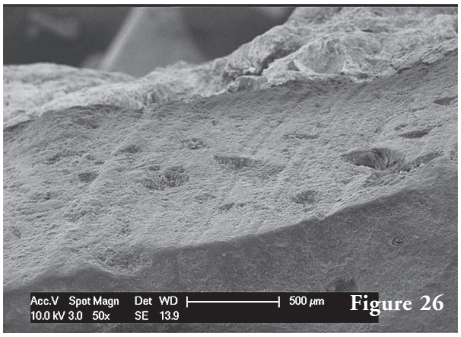

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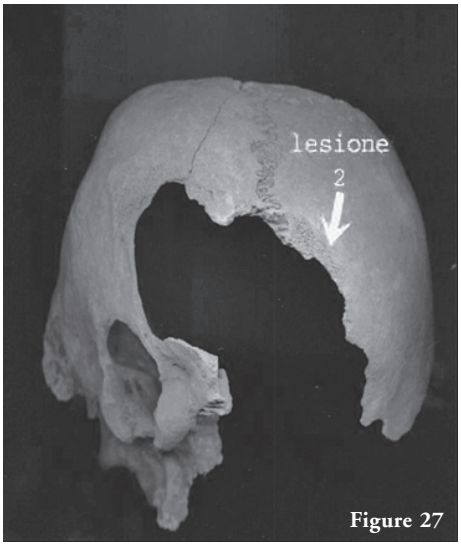

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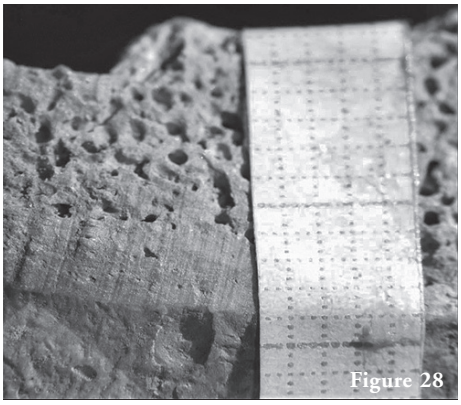

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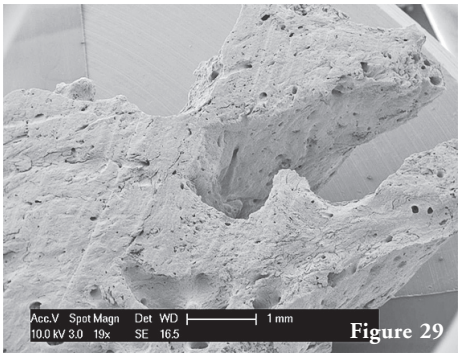

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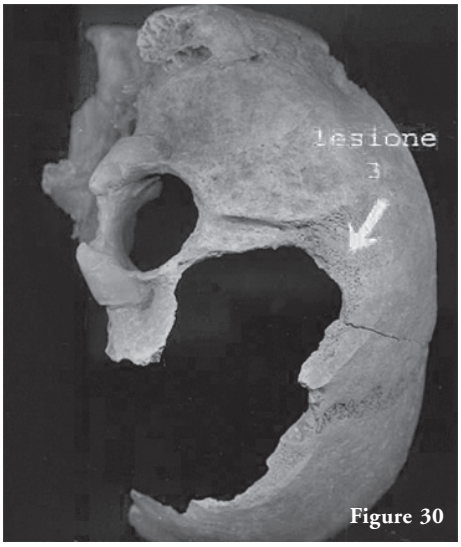

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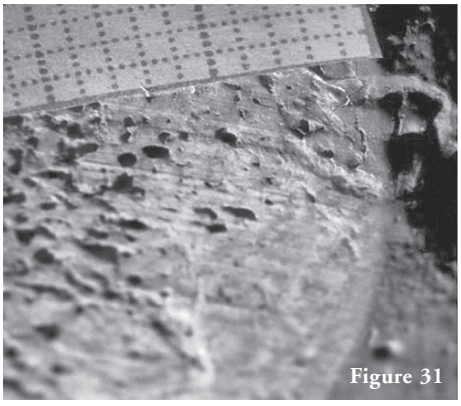
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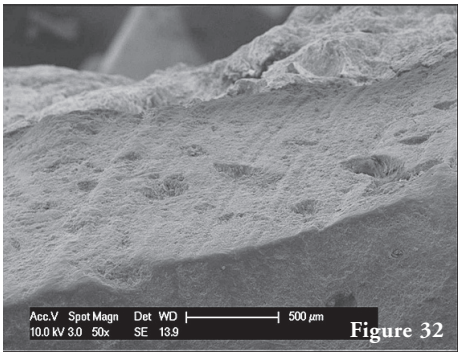

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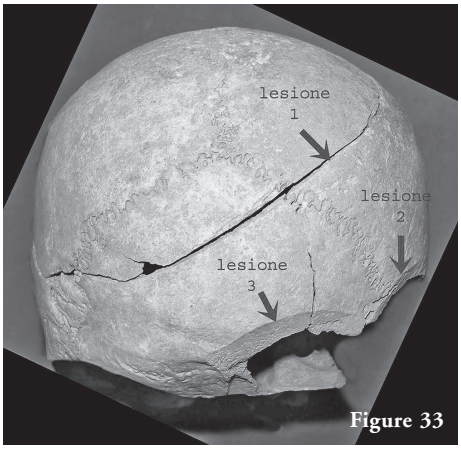

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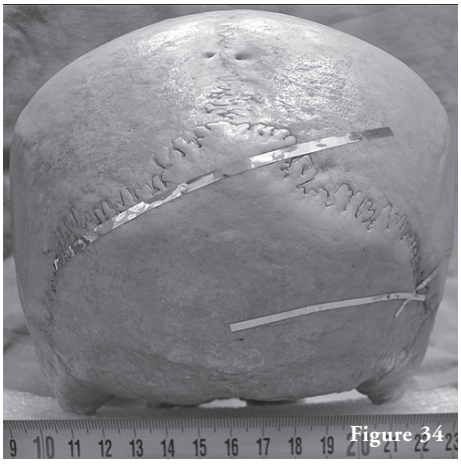

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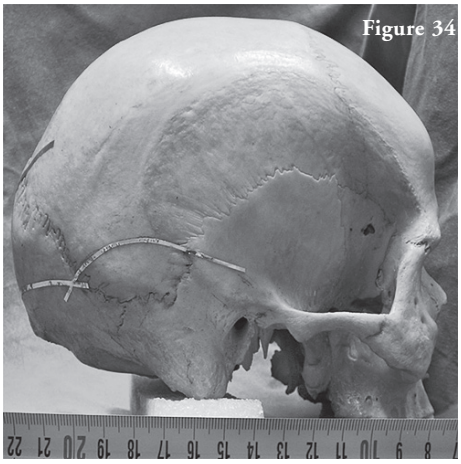

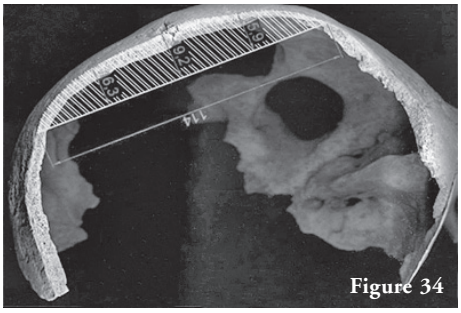


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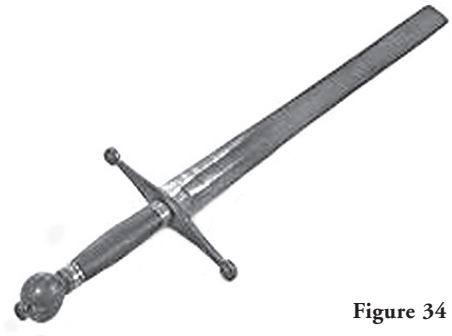
Figure 34

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A RARE CASE OF TRAUMATIC LACERATION OF INTRACRANIAL VERTEBRAL ARTERY IN ABSENCE OF PATHOLOGIES AND DIRECTED TRAUMAS TO THE HEAD

Abstract: We present a rare case of death of a young healthy man, due to massive cerebral haemorrhage from the breaking of the intracanical vertebral artery, followed to a scuffle in which there were not produced directed traumas of such violence to explain the laceration of the vessel with a direct bruising mechanism.

A very thorough study of the case allowed us to exclude structural weaknesses of the part of the interested artery and obliged to assume a mechanism of abnormal mobilization of the head by traumatic origin that determined an abnormal stretching of the vessel with consequent laceration of the same one with lethal outcome. We presented the intra-vitam cerebrovascular imaging study and the post-mortal MRI examination and one possible reconstruction of the dynamic. Only the combined use of many radiological and histopathological techniques can assumed complex aetiopathogenetic mechanisms in the genesis of intracranical vascular injuries, excluding predisposing natural factors.

Case report

On the second of February 2008, in the Saturday morning a prosecuting attorney of Swiss Canton Ticino Procuration asked us to visit a twenty-two years old boy at Bellinzona's Hospital. The boy was assaulted by three young boys, around midnight, during citizen Carnival's celebration.

When we arrived at the hospital, around ten a.m., the doctor told us that the cerebral death observation period has already began. Patient brain CT scan pointed out a massive brain haemorrhage, with the discharge of the contrast liquid from left vertebral artery with very abundant cerebral aedema. (Fig 1,2)

The neurological examination demonstrated the absence of brain and brainstem reflexes, and a Glasgow Coma Scale of three.

We proceeded to visit accurately the young boy, who presented:

- at the left fronto-temporal region a slender reddish abrasion; (Fig 3)
- at the chest, at right parasternal and at left lateral region two slender erithematous lesions few centimetres wide;

- at the left elbow a 1 centimetre exchoriation;
- at the right hand, in correspondence of the 3rd, 4th and 5th metacarpalphalangeal articulation, other little exchoriations; (Fig 4)
- at the front face of the left leg, three little abrasions of 3 centimetres maximal dimension.

In the evening of the same day was declared the death of the boy and we gave positive opinion for the organs explantation, after previewing all done examinations. So, at Sunday morning, heart, lungs, liver, pancreas and kidneys were explanted.

In the Monday morning, we performed the autopsy (Fig 5). During external examination, besides the lesions pointed out the day before, we saw:

- at the distal third of the left arm, on the posterior face, an irregular 8 x 5 centimetres wide ecchymosis (Fig 6);
- at the right gluteal region an irregular 5 x 3 centimetres ecchymosis.

The autopsy revealed, beside the absence of intrathoracic and intra-abdominal organs:

- a slender haematic infiltration of the left deep scalp tissues and the left temporal muscle, under the bruise described before (Fig 7);
- a very abundant sub-arachnoid haemorrhage. The whole brain was taken and fixed in formalin buffered solution in order to do other specialistic examinations (Fig 8,9);
- at the neck region, in absence of visible injuries, beside the routinary section, we did a postero-lateral section of the skin, that pointed out a slender haematic infiltration of superior fascicles of left trapezius muscle, of splenius capitis and the semispinal capitis;
- another skin postero-median section pointed out a slender haematic infiltration of median muscles deep fascicles and of its contiguos left portion (Fig 10).

After other two weeks, we proceeded to the exposition of brainstem and cerebellum vascular structures, removing the coagulated blood to locate the cerebral haemorrhage source.

This research was done with the help of a neuroradiologist, who ri-edited the CT examination done at the Bellinzona's Hospital and he indicated us the probable point of the vertebral artery laceration (Fig 11,12,13).

So, we catheterized the distal tract of the left vertebral artery and we injected water with slow pressure and we saw the discharge at the intracranial tract of the left vertebral artery, about two centimetres distally to the basilar artery. We observed a laceration in the lateral slope of the artery wall of 0.3 centimetres length. We observed also an hypoplasia of the right vertebral artery, an antomical variant widely described in literature (Fig 14).

After this study we took same samples of the vessel's wall to perform histological examinations by an expert pathologist. These examinations pointed out no alterations of vessel structures, due to both genetic and acquired diseases that could cause a reduced tensil strength of the wall to moderate trauma (Fig 15,16).

Conclusions

In conclusion the cause of death was certainly the cerebral haemorrhage from intracranial left vertebral artery laceration. The slender injuries found on the corpse, bring us to assume that the vessel lesion was caused by a very quick and abnormal movement of neck and head, determining extreme traction on the artery wall behind the tension maximum limit of the vessel and so its laceration.

This hypothesis was supported by the signalling of same similar cases published in international journals, in which the intracranial vertebral artery laceration was caused by minor traumas (also the whiplash), in absence of genetic diseases (such as Marfan and Elher-Danlos syndrome) or acquired diseases (such as aneurysm) of the vessel.

In this case the movement was determined, like to demonstrate in court, by three boys who landed some punches and lashed out at the victim. The sentence of first degree acknowledged boys' guilt, sentencing them to ten years' imprisonment.

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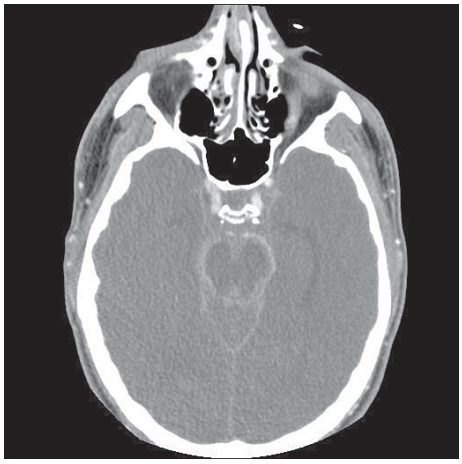

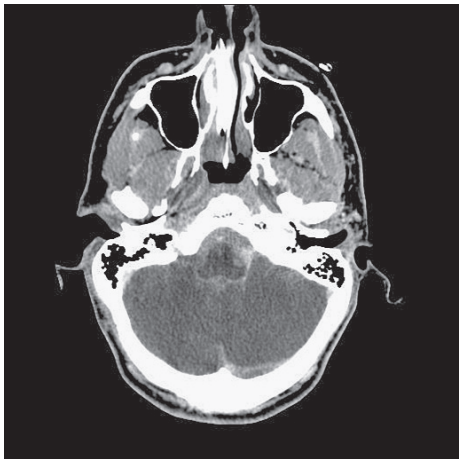

Figure 1,2 – brain CT scan: a massive brain haemorrhage, with the discharge of the contrast liquid from left vertebral artery with very abundant cerebral aedema.

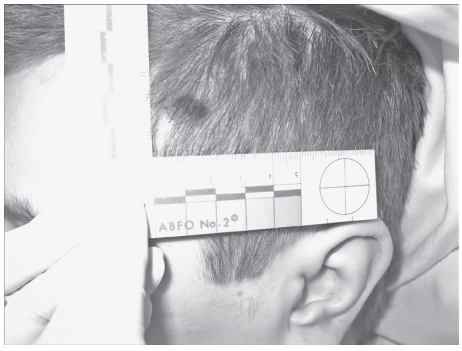

Figure 3 – a slender reddish abrasion at the left fronto-temporal region.

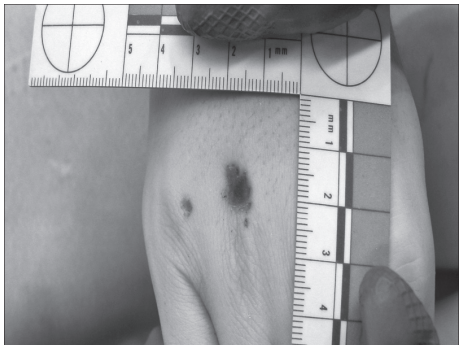

Figure 4 – little exchoriations at the right hand, in correspondence of the 3rd, 4th and 5th metacarpalphalangeal articulation

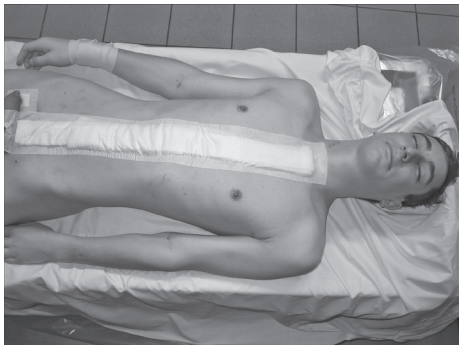

Figure 5 – the cadaver before the autopsy.

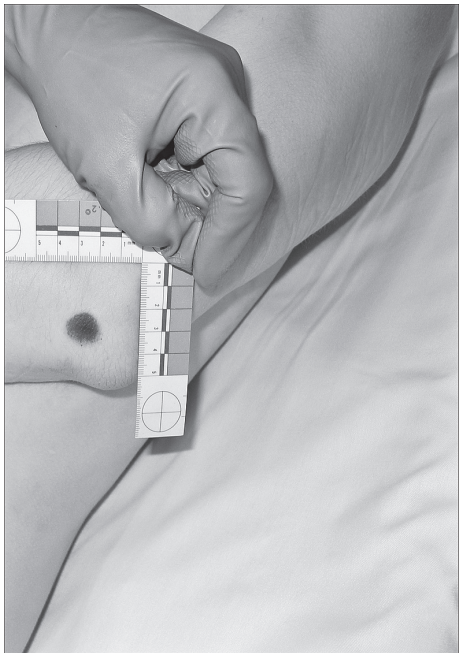

Figure 6 – irregular 8 x 5 centimetres ecchymosis at distal third of left arm, on posterior face.

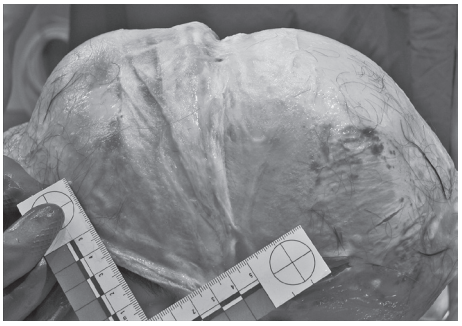

Figure 7 – a slender haematic infiltration of left deep scalp tissues and left temporal muscle.

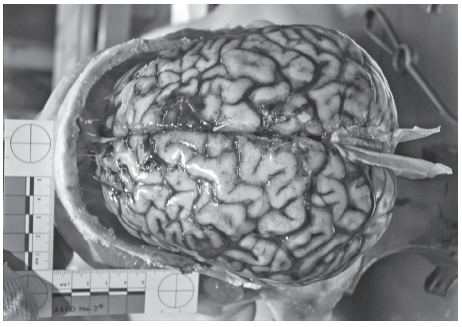

Figure 8 – in situ brain: a very abundant sub-arachnoid haemorrhage.

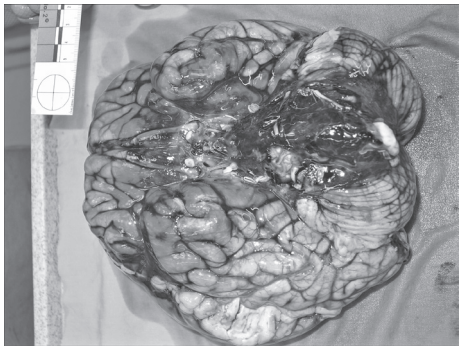

Figure 9 – isolated brain: cerebellum and brainstem haemorrhage.

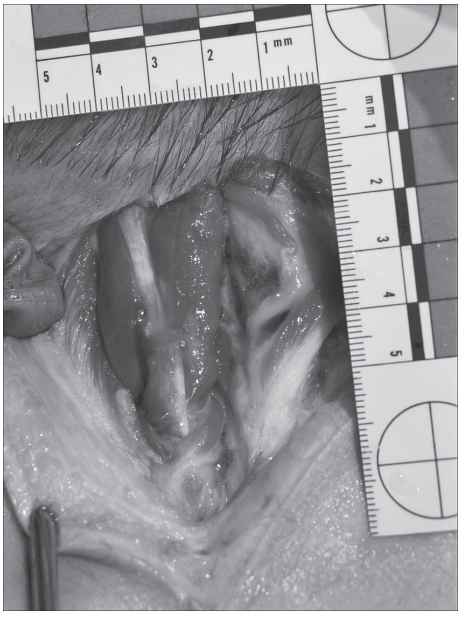

Figure 10 – skin postero-median section: a slender haematic infiltration of median muscles deep fascicles and of its contiguos left portion.

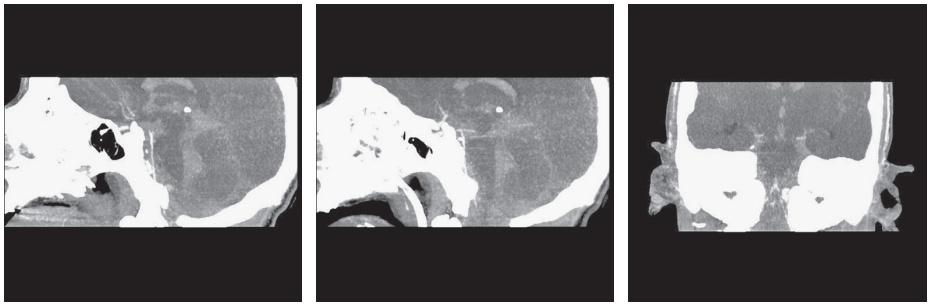

Figure 11-12-13 – ri-editing by neuroradiologist of CT scan done at the Bellinzona's Hospital; the specialist indicated us the probable point of vertebral artery laceration.

Figure 14 – the injection of water in left vertebral artery: the discharge of liquid from laceration.

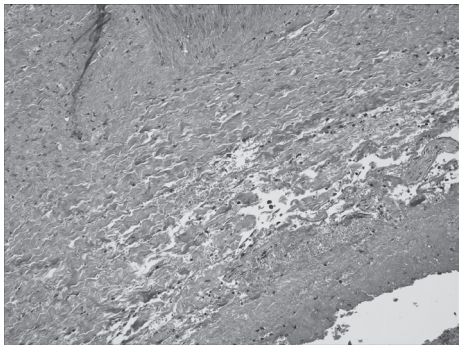

Figure 15 –histological examination (200x): normal vessel's wall of left vertebral artery.

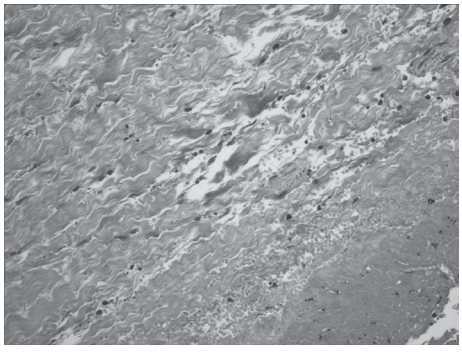

Figure 16 – histological examination (400x): further magnification of vessel's wall of left vertebral artery.

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OSTEOMYELITES IN NEWBORD: A CASE REPORT

Abstract: The Authors report a case of medical malpractice tied up the missed asepsis during a new born screening and the following bad management of Staphylococcus bacterial infection.

Introduction

The practice of screening the neonatal population for certain diseases (Phenylketonuria and Congenital hypothyroidism) by biochemical testing of a dried blood spot has been introduced by Italian Government in the 1992.

The public health activity aimed at the early presymptomatic identification of infants who are affected by certain genetic, metabolic or infectious conditions (1), these requirements are particularly challenging.

Heel punctures have provided an easy method of obtaining blood from neonates for biochemical screening, but this procedure has a low risk rate.

In fact, in literature the superficial infection at the puncture site is a rare occasions in small infants (2).

We report a case of osteomyelitis after heel punctures during neonatal screening program.

Case report

A 2600 kg infant born at 37 weeks' gestation had any right heel punctures during the first 48 hours of his life.

On the ninth day, the right heel was erythematous, swollen and tender.

On a day after admission, the infant developed fever of 38,4°C and he had a convulsive episode.

The patient's physical examination was remarkable for temperature of 38°C and rubor, calor, fuctio laesa of right food was showed.

There was no history of local trauma. There were no cardiac, thoracic and abdominal pathology in clinical diary. Inflammatory indices such as erythrocyte sedimentation rate and C-reactive protein were normal. Electrolytes were normal.

Cultures of blood and spinal liquid grew Staphylococcus aureus that was sensitive to cefazolin, levofloxacin and oxacillin. Antibiotic therapy was started.

After two days of the admission an X-ray revealed erosion of the posterior portion of the right calcaneus compatible with osteomyelitis.

A bar was positioned on the right heel. After 45 days he came back to home. Now, the child right foot continues to grow smaller. An X-ray of the right food shows an osteolytic lesion in calcaneus.

Discussion

Osseous infection can be caused by haematogenous spread of organisms to bone (haematogenous osteomyelitis) or by direct local invasion by bacteria and the haematogenous spread of organisms follows bacteremia due to, for example, urogenital infections, enteritis, cholangitis or endocarditis; often the infective focus is not identified (3).

Osteomyelitis is defined as inflammation of bone and marrow cavity (4). Acute haematogenous osteomyelitis occurs predominantly in the paediatric age group, but the newborn disease is rare.

Usually precipitating conditions are infections of the umbilicus, ear, nose and threat often by streptococci (3).

In new born less than four months the Staphylococcus aureus, Gram- bacilli, group B Streptococcus are the most common organisms in Osteomyelitis (6).

In adult, the diagnosis of Osteomyelitis is first suspected on clinical grounds; beside, the leukocyte count may be elevated, but is often normal in chronic cases. Both erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are elevated, and usually return to normal during the course of treatment. Confirmation of the presence of Osteomyelitis usually entails a combination of radiologic, microbiologic, and histopathologic tests.

Cross-sectional imaging modalities such as computed tomography (CT) scanning and magnetic resonance imaging (MRI) are now considered standard in the diagnosis of Osteomyelitis (7).

In newborn, Osteomyelitis is usually an acute disease which has a predilection for the proximal and distal femoral metaphyses.

In this work we report a case of Osteomyelitis by Staphylococcus aureus infection of heel after puncture.

In fact the legal medical aspect of this case is relative to correct screening procedure. The health care provider who showed the infant with systemic signs of sepsis excluded the presence of the other pathology.

The little time among heel puncture and systematic signs of sepsis and the exclusion of other pathology open to liability for health care provider who made the screening procedure.

Conclusion

We think that the missed activation of a possible search of Staphylococcus bacterial infection, despite the characteristics clinical demonstrations in the newborn, represents a connected forgetfulness with an inexcusable defect of professional technical behavior.

The behavioral defect was stamped to inexperience and negligence because the sanitary had to set attention to the evident characteristics manifested by the newborn and to worry about to verify its motive through a simple blood investigation before the resignation from hospital.

It subsequently needs to specify that there was also an inadequacy in the management of the newborn screening from which the Staphylococcus bacterial infection was baited.

In fact, the asepsis of screening procedure in infant is very important.

Besides, the delay with which the aforesaid infection was underlined then in hospital environment was extremely prejudicial for the child; in fact Osteomyelitis was activated with following anatomic-functional repercussion to load of the left foot.

In this case the health care provider shows a neglect approach and medical malpractice negligence is serious stuff, especially when a patient is harmed unnecessarily.

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SUICIDE BY DROWNING AFTER BROMAZEPAM INTOXICATION: A CASE REPORT

Abstract: The authors report a case of a floating body recovered from a well. A 62-year-old woman committed suicide by drowning herself in a well. According to information given by her relatives she had a 13-year history of depression and she wanted to commit suicide. Liquid chromatography with electrospray ionization mass spectrometry (LC-ESI-MS) was used to determine bromazepam in blood. Samples were extracted using Oasis® HLB solid-phase cartridges, and separation and quantitation was done using positive–mode electrospray ionization in the single ion monitoring (SIR) mode. Chromatographic separation was achieved using an Atlantis® T3 column (2.1x150 mm, 5 µm), eluted in a gradient system with acetonitrile and formic acid 0.1%, at a flow rate of 300 µL/min. Quantitation was achieved by the addition of diazepam deuterated (DZP-d5) as internal standard. The compounds were detected monitoring two ions for bromazepam (*m/z* 318, and *m/z* 288) and *m/z* 290 for the deuterated, DZP-d5.

Toxicological results revealed in blood a toxic concentration of bromazepam of 418 ng/mL. This finding might be important for the interpretation, not of the cause of death (since the drowning was confirmed by autopsy), but for the state of mind of the victim that had, somehow, helped her to committ suicide (concomitant existence of a psychotropic substance).

1. Introduction

Bromazepam (*Bromalex*®, *Lexotan*®, *Ultramidol*®) is an intermediate-acting 1,4-benzodiazepine and is widely prescribed as an anxiolytic, but it also exhibits sedative and hypnotic properties [1]. The literature shows that following a single administration of 12 mg to 10 subjects, an average peak plasma concentration of bromazepam of 131 ng/ml was achieved between 1 and 4 h, declining with an average half-life of 11.9 h [2].

Bromazepam is metabolised primarily by 3-hydroxylation and cleavage of the seven-membered ring, followed by glucuronide conjugation of the hydroxylated metabolites. Intact bromazepam is a major blood constituent, about 2% of dose is excreted in the 72h urine as unchanged bromazepam, 0.4% as the ring cleavage product, 27% as conjugated 3-hydroxybromazepam (3-HOB) and 40% as the hydroxylated and

conjugated cleavage product [3]. Serum bromazepam therapeutic concentrations are in the range of 80 – 170 ng/mL. Levels higher than 250 ng/mL are considered as potentially toxic [4].

Deaths caused by benzodiazepines alone in the absence of other xenobiotics or pathology are uncommon, although some fatal cases have been reported in the literature [5-6].

The authors present a drowning case with bromazepam and an LC-ESI-MS method to detect, confirm and quantify this benzodiazepine in blood samples.

2. Case report

A 62-year-old woman committed suicide by drowning herself in a well. She was found by her sister who first saw a bench and her slippers near the well. She lived alone since her parents' death, 13-years ago and according to information given by her relatives, she was under a severe depression and wanted to commit suicide.

At autopsy, an external examination revealed eye congestion. On internal inspection, abundant white foam was observed in the trachea, larynx and bronchi. Swollen lungs, presence of Paultauf's spots, fluid in the stomach, pulmonary edema were observed. The postmortem findings also indicated generalized visceral congestion. No signs of violence were observed.

Blood, liver and kidney samples were submitted to toxicological analysis.

3. Materials and methods

3.1. Chemicals and reagents

Bromazepam and diazepam-d5 were obtained from Cerilliant (Promochem, France) at a concentration of 1 mg/ml in methanol. Separate working solutions of bromazepam and diazepam-d5 were prepared in methanol after appropriate dilutions and were stored at +4 ºC. Formic acid, methanol and acetonitrile were HPLC grade and were purchased from E. Merck (Darmstadt, Germany). Deionized and purified water was obtained using a Milli-Q system (Millipore, Molsheim, France). Oasis® HLB, 3 cc, solid-phase cartridges were purchased from Waters (Milford, MA). The mobile phase was filtered through a 0.20 µm filter (Schleicher & Schuell) and degassed in an ultrasonic bath for 15 min just before use.

3.2. Instrumentation

Liquid chromatography (LC) was performed using a Waters Alliance 2695 separation mode. A 20 µL aliquot of the extract was injected onto the column (Atlantis® T3 5 µm, 2.1x150 mm) (Waters). Each 20-min chromatographic run was carried out with a gradient (10% acetonitrile, 90% formic acid, 0.1% to a ratio 90-10% at 14 min) at a flow rate of 300 µL/min. The column temperature was maintained at 35°C.

Instrument control, data acquisition and processing were achieved using Waters Empower software (Milford, MA).

Mass spectrometry detection (MS) was carried out on a Waters ZQ 2000 single quadrupole mass spectrometer with an electrospray ionization (ESI) performed in positive mode. Full-scan spectra were recorded from *m/z* 200-450, at a scan time of 0.5 s and an interscan delay of 0.1 s. The other main instrument settings were: capillary voltage 3.5 KV; cone voltage 60 V; extractor 4 V; ion energy 0.4; source temperature 120°C; desolvation temperature 350°C; cone gas (N2) flow rate 0 L/h and desolvation gas (N2) flow rate 600 L/h.

Quantitation employed the selected ion-recording mode (SIR) using the *m/z* corresponding to the most abundant product ion $[M+H]^+$ at *m/z* 318 for bromazepam and *m/z* 290 for the internal standard (diazepam-d5). Both SIR and Scan acquisitions were performed in centroide mode.

3.3. Sample preparation

Control and calibration samples were prepared by spiking drug-free whole blood samples with standard solutions.

A 1 mL aliquot of whole blood was spiked with 25 µL of internal standard (10 µg/mL) and diluted with 2 mL of deionized water. Then the samples were vortex mixed and centrifuged for at 3000 rpm for 5 min. Extraction cartridges (Oasis® HLB, 3cc) were conditioned with 2 mL of methanol followed by 2 mL of deionized water. Each sample was loaded through a cartridge. It was then washed with 2 mL of 5% methanol in water. After drying under vacuum for 15 min, elution was carried out with 2 mL of methanol. The eluate was evaporated to dryness under a nitrogen gas flow at 40°C. The residue was dissolved in 250 µL of mobile phase and an aliquot (20 µL) was injected into the LC-ESI-MS system.

4. Results and discussion

Blood alcohol concentration was measured by Headspace GC-FID. A systematic toxicological drug screening was carried out in blood with a combination of immunoassays and CG-MS analysis. Benzodiazepinas were positive by immunoassays and identified in blood by LC-ESI-MS. Bromazepam was detected, confirmed and quantitated in blood samples. Toxicological results revealed in blood a toxic concentration of bromazepam of 418 ng/mL. No other drugs were found in the postmortem blood samples of presented fatal case. No alcohol was found.

The calibration curves for bromazepam in the blood samples were linear, ranging from 5 to 1000 ng/mL (r^2=0.9995, seven calibration points, in triplicate). The detection limit of bromazepam in blood was 1 ng/mL (*LOD*, S/N=3) and the lower limit of quantification (*LOQ*, S/N=10) was 5 ng/mL (Table I).

Quantitation employed the selected ion-recording mode (SIR) using the most abundant characteristic ion, *m/z* 318 and the fragment ions, *m/z* 288 and *m/z* 209 for confirmation. SIR mass chromatograms of the bromazepam detected in the blood sample are shown in Fig. 1.

Bromazepam and its main metabolite (3-HOB, *m/z* 332, *m/z* 315 and *m/z* 303) were detected in liver. Kidney sample only revealed the presence of bromazepam.

Although several cases of acute bromazepam intoxication have been reported, only few were lethal [2, 7-8]. The blood concentration of bromazepam found in this fatal case was higher than the reported therapeutic level (80 – 170 ng/mL). Levels higher than 250 ng/mL are considered as potentially toxic.

This finding might be important for the interpretation, not of the cause of death since the drowning was confirmed by autopsy, but for the state of mind of the victim that had, somehow, helped her to commit suicide (concomitant existence of a psychotropic substance).

5. References

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Selected ions (*m/z*) and cone voltage		Limits (ng/mL)		Linearity (µg/mL)	R^2
Quantitation	*Confirmation*	*LOD*	*LOQ*		
318	288 ; 209	1	5	0.005 - 1	0.9995
30 V	50 V	S/N <3	S/N <10		

Table I – Selected ions, *LOD*, *LOQ* and linearity range of bromazepam in blood samples.

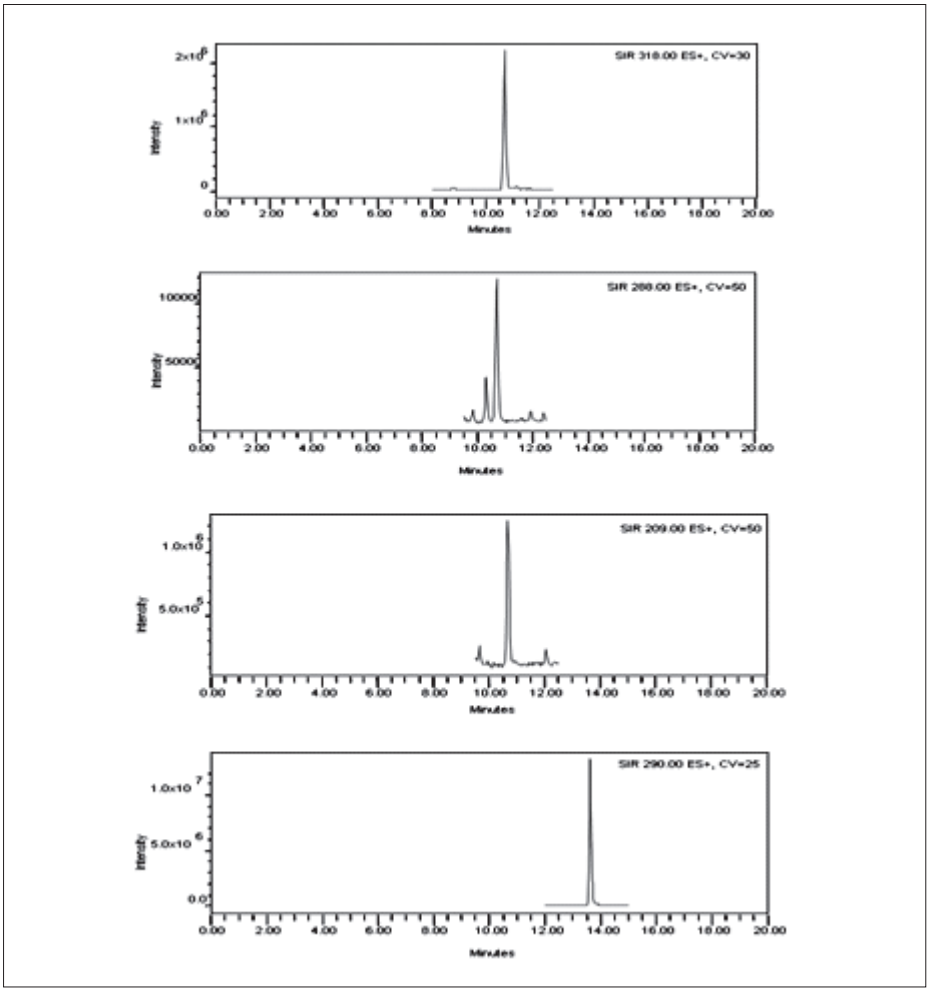

Figure 1 – SIR mass chromatograms of bromazepam in postmortem blood samples.

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SUDDEN CARDIAC DEATH
A CASE WITH TWO DISTINCT AND UNRELATED CAUSES

Abstract: Cardiovascular diseases are the most frequent causes of Sudden Death in the adult. The authors report the case of a 44-year-old man who died sudden and unexpectedly when driving from Lisbon to Coimbra. A forensic *postmortem* examination revealed not only hypertensive and ischemic cardiopathy but also hypersensitivity non-illicit drug-induced myocarditis, with morphological evidence of both mechanical and arrhythmic mechanisms leading to death. It highlights the relevance of histological examination in the investigation of sudden death and emphasizes the etiologic complexity underlying cardiac sudden, unexpected deaths.
Keywords: Sudden death; myocardial infarction; drug-induced myocarditis.

Introduction

Sudden Death is a worldwide important problem, not only due to its non-suspected nature, but also in virtue of the multiplicity of possible underlying causes. Cardiovascular disorders account for the highest rate (90%).[1]

Case report

The present case refers to a 44-year-old male with family history of diabetes mellitus and personal antecedents of systemic hypertension, who died suddenly when driving from Lisbon to Coimbra. He was found parked at the side of the motorway without evidence of trauma / accident. The family reported that lately he was living under a great professional stress, for what he used to take benzodiazepines and that he complained of a muscular / osteo-articular pain on the shoulder, for which he self-medicated with a non-steroid anti-inflammatory analgesic. Four month earlier, he had an episode of syncope, which he neglected.

Death circumstances required a forensic *postmortem* examination.

The autopsy presented minor alterations of the other organs, being the heart the target-diseaded organ. It weighed 600g; had concentric hypertrophy of the left ventricle, whose myocardium showed anomalous grey and bright foci; and the three

coronary arteries contained major occlusive atherosclerotic plaques, some complicated with thrombosis. Microscopic examination confirmed the aforementioned lesions and characterized them as myocardial hypertrophy, severe and complicated (with erosion, thrombosis and lumen occlusion from 50% to ≥75%) coronary atherosclerosis – type VI of the "American Heart Association" classification – (Figures 1, 2, 3), as well as acute myocardial infarction with an evolution of 7-10 days (that is, in the phase of late granulation tissue and recent, still cellular fibrotic scar tissue – Figure 4) in the setting of hypertensive and ischemic cardiopathy. Yet, microscopy also disclosed unexpected pathology, consisting in hypersensitivity myocarditis (since it presents interstitial œdema and eosinophils-rich inflammatory infiltrate, inducing focal myocardial fibers' necrosis or contraction bands – Figures 5, 6).

Toxicology was negative for alcohol and illicit drugs, but positive (within therapeutic concentrations) for benzodiazepines and metabolites – bromazepan, nordiazepan, diazepam –, as well as for the non-steroid anti-inflammatory analgesic ibuprofen (traces).

Discussion

Sudden Death is defined as a non-traumatic fatal event, occurring instantaneously or within one hour after the onset of complaints[2] and, if unwitnessed, when the deceased was in good health 24 hours before death happens.[3] The incidence of the underlying causes varies with age group[4], and if cardiac, coronary heart disease is the major cause in adults (60%).[1] Yet, other causes attain different ages in a more uniform way, like for example myocarditis.[5] The causes may act *per se* or in a combined manner, each contributing with its pathological share to the mechanism which will lead to the final outcome. Ultimately, the lethal mechanism is either mechanical, arrhythmic or both.[5] Death, in the case here reported, results of a combination between those two mechanisms. In one hand, they are underlain by hypertensive and ischemic cardiopathy – with its increased myocardial mass, scarring / remodeling areas and decreased coronary blood flow. On the other hand, there is the drug-induced hypersensitivity myocarditis – with its inflammatory / immunological alterations. Increased myocardial mass requires additional contraction effort and higher blood flow, leading to a mechanical burden. Scaring / remodeling areas may contribute to the mechanical burden due to its non-contractile nature, which creates an obstacle to the normal heart dynamics. Furthermore, this obstacle is also rhythmic, since the granulation / fibrotic tissue is not conductive. Atherothrombotic decreased coronary blood flow leads to further ischemia of an hypertrophic myocardium with already altered functional reserve, favoring mechanical and arrhythmic distress. Myocarditis inflammatory constituents comprise interstitial œdema and inflammatory cells. The former enlarges the myocardium interstitial compartment and compresses the muscle fibers, disturbing both mechanical and conduction function. The presence of inflammatory cells – lymphocytes, macrophages, plasma cells and eosinophils – not only further enlarges the interstitium and directly induce muscle fibers hypercontraction and/or focal myocyte destruction / necrosis, but also lead to indirect myocardial hypercontraction / destruction / necrosis due to the production and release of immunological reaction mediator factors, like for example cytokines, conditioning arrhythmic events and mechanical instability. Hypersensitivity

myocarditis occurred despite drug concentrations being therapeutic and/or in a trace amount. Evidence of ventricular arrhythmia is demonstrated by the contraction bands. In this case, arrhythmia is *cum materia*, meaning that it is caused by pathology with morphological lesions partially recognized on macroscopic examination of the heart and partially through histology.

Conclusions

This case (1) highlights the importance of systematic microscopic examination in the investigation of Sudden Death; (2) emphasizes the fact that multiple noxa may converge and contribute to the final event; and (3) shows the complexity of the interactions among potential causes of death and the challenge of knowing the real influence of each contribution. It also draws attention to the individual immunological answer to different drug concentrations, to the meaning and interpretation of the toxicological results and to their integration with the morphological lesions (4).

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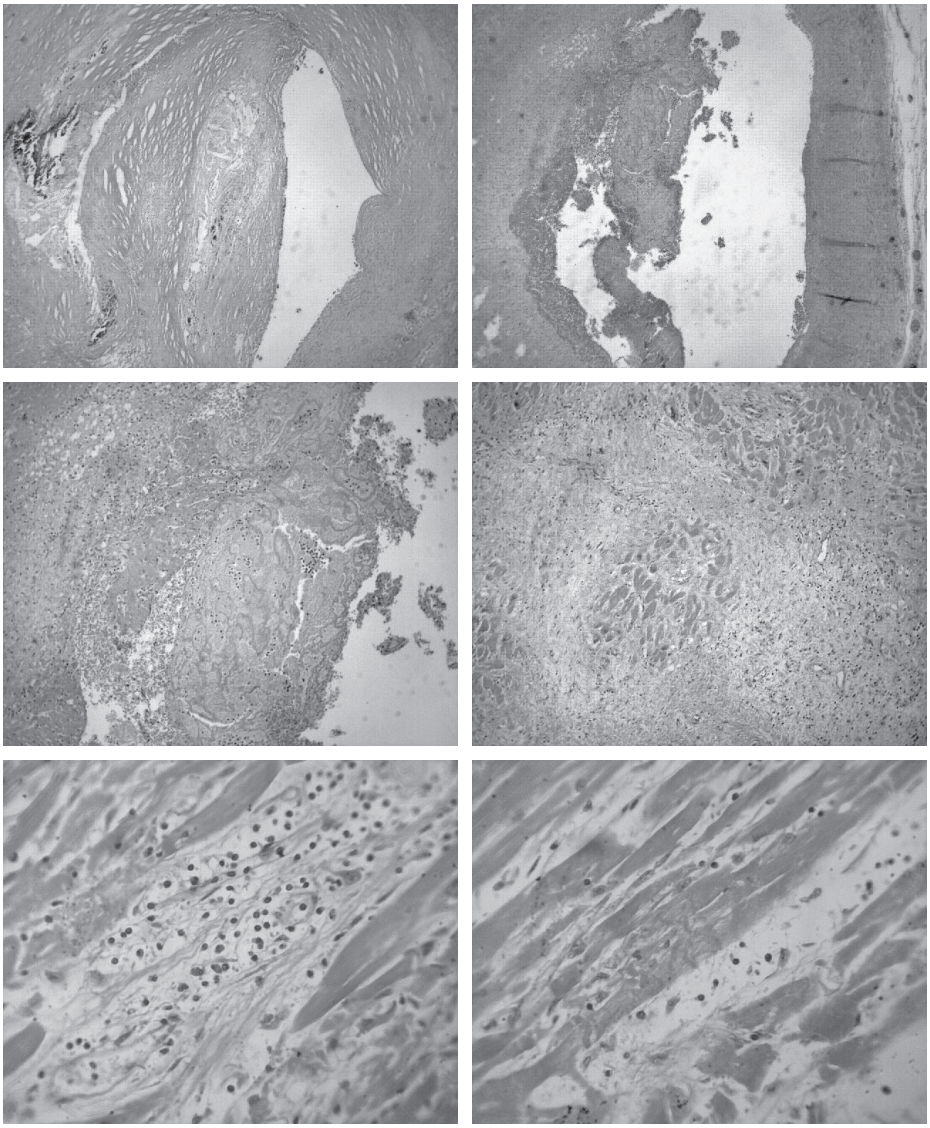

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SUDDEN CARDIAC DEATH OF A YOUNG FOOTBALL PLAYER

Abstract: The overall estimated risk of Sudden Cardiac Death in the young is 1/100,000. It may occur during physical activity and the underlying possible causes are multiple. The case here reported concerns an apparently healthy 29-year-old male that dropped unconscious during a recreational football game. Reanimation manœuvres were not successful. The autopsy revealed anomalous left coronary artery arising from the pulmonary artery (ALCAPA), associated to hypoplasia of the circumflex and to tortuosity and acute thrombosis of the right coronary artery. Congenital Anomalies of Coronary Arteries are a relevant cause of sudden and unexpected death, often during childhood and adolescence.
Keywords: Sudden death, young, sports, coronary malformations

Introduction

The overall estimated risk of Sudden Cardiac Death in the young is 1/100,000[1]. It may occur during physical activity[2,3] (in some series around 10.8%) and the underlying possible causes are various.[4]

Material and methods

The authors report a case of an apparently healthy 29-year-old male that dropped unconscious during a recreational football game. Reanimation manœuvres were immediately performed without success and he was declared dead at the Hospital emergency room soon afterwards.

A *postmortem* examination was done.

Results

The autopsy revealed – on macroscopic examination – generalized congestion of the organs, lung œdema and a heart weighing 480g and presenting markedly elongated, tortuous and dilated right coronary artery, which in section is acutely thrombosed (Figures 1, 2), anomalous origin of the left descending branch of the coronary artery

from the pulmonary trunk (Figures 3, 4) and hypoplasia of the circumflex branch. Microscopic examination disclosed slight atherosclerosis (type II of the "American Heart Association" classification) in the right coronary artery, but with erosive features, underlying the fresh thrombus (Figure 5). It also showed myocardial hypertrophy and adaptative changes of intra-myocardial coronary arteries (Figure 6).

Discussion

Congenital anomalies of coronary arteries are not frequent (0.2-1%), but are a high risk factor for arrhythmias, angina, infarction and sudden death.[5] They display a considerable number of morphology variety (in origin, orientation, etc), some being rarer and/or more life-threatening than others.[5] In fact, ALCAPA – Anomalous Left Coronary Artery arising from the Pulmonary Artery – is an unusual type, to which few persons survive childhood without surgical intervention and of those who do, up to 90% die suddenly before 35 years.[6,7] It was first described in 1933, as Bland-White-Garland syndrome.[6] The anomalous origin of the coronaries from the pulmonary artery is especially prone to cardiomegaly, myocardial hypertrophy, myocardial scars and adaptative remodelling of intra-myocardial coronary branches. These morphological changes plus eventual extrinsic compression of the anomalous vessel are mostly responsible for the clinical outcome. The case reported also presented a hypoplastic circumflex and a distorted right coronary artery. The erosive pattern of the atherosclerosis, that is: the presence of very superficial aggregates of foam cells, just beneath the endothelium, favours its erosion in the setting of the vessel "ondulation" and of the increased blood flow – due to exercise –, leading to thrombosis.[4] Through the years, two clinical classifications have been put forward, in order to better deal these patients. The first in 1968 by *Wesselhoeft et al* and the second in 2003 by *Rigatelli*.[8,9] Due to the clinical presentation, this case is included in group IV of the former (sudden death in adults / young adults) and in group III – IV of the latter (related to sudden death, but with atherosclerosis).[8,9]

Conclusions

1) Congenital anatomic anomaly of coronary arteries is an important cause of sudden death during physical activity and sports. 2) Anomalous origin of the left coronary artery from the pulmonary artery is rare but usually lethal. 3) When present at autopsy, it accounts with *certainty* to the sudden death final event; complemented, in this case, with acute right coronary artery occlusion due to thrombosis.

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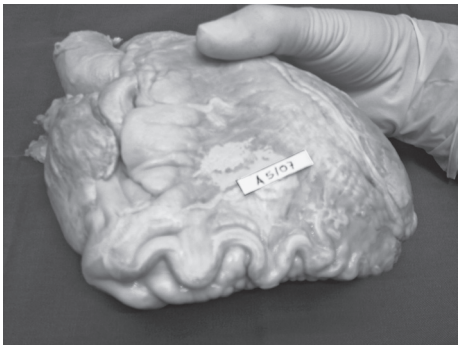
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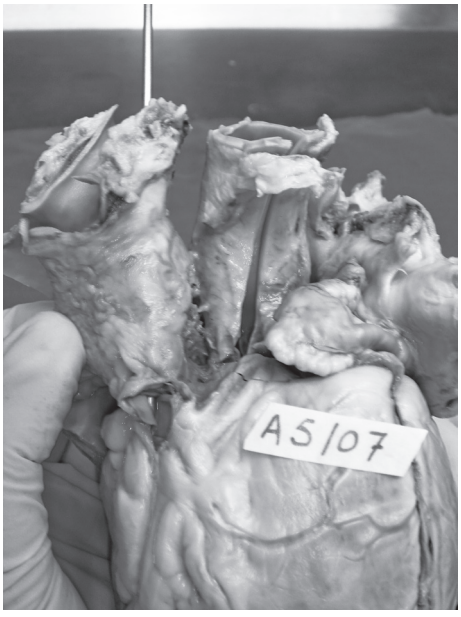
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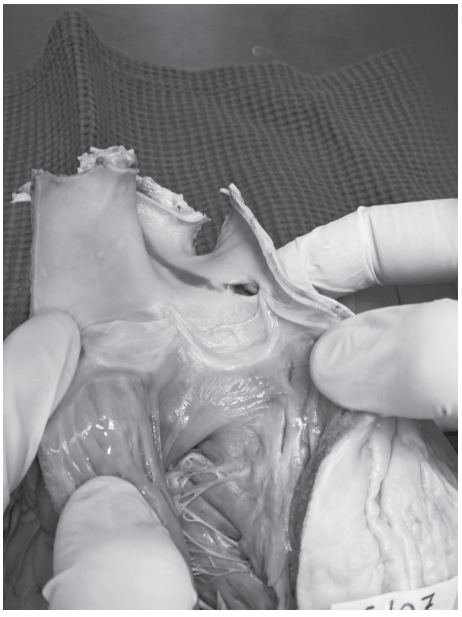

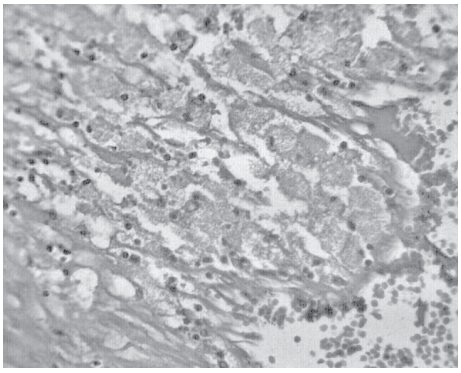

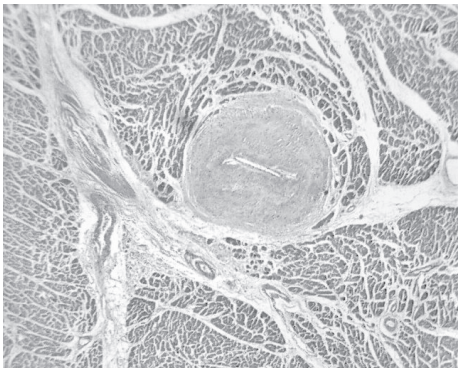

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SUDDEN DEATH DURING SPORTS ACTIVITY AN UNUSUAL CAUSE

Abstract: Sudden Death may occur during exercise due to cardiac or extra-cardiac causes. This case concerns an apparently healthy 49-year-old male that died during athletics training, immediately after complaining of chest pain. The *postmortem* examination revealed massive pulmonary œdema and multiple necrotic liver granulomas, eosinophil-rich (with degranulation), some of which contain foreign bodies whose morphology is consistent with *Schistosoma mansoni*'s eggs. Death resulted from an anaphylatic reaction with acute lung œdema as a consequence of parasitic allergic hepatitis aggravated by the effort of sports activity. Schistosomiasis is an unusual cause of sudden and unexpected death, mainly in Europe.
Keywords: Sudden death; sports; schistosomiasis.

Introduction

Sudden Death is a universal everlasting concern, since it occurs unexpectedly and as a consequence of a broad *spectrum* of causes. Some are unusual and extra-cardiac (10%). Characterized by a non-traumatic nature, occurring instantaneously or within one hour after the onset of complaints1, it may take place during sports activity.

Material and methods

The authors report a case of an apparently healthy 49-year-old male that dropped dead during athletics training, immediately after complaining of chest pain. There was no history of trips to foreign countries.

A *postmortem* examination was performed.

Results

The autopsy revealed diffuse pulmonary œdema (Figure 4), œdema of the myocardial interstitium and signs of acute "functional" heart dilation, coronary atherosclerosis,

generalized organ vascular congestion, fibrous thickning of splenic and hepatic capsules, as well as multiple necrotic liver granulomas, eosinophil-rich (with degranulation), some of which contain foreign bodies whose morphology is consistent with *Schistosoma mansoni*'s eggs (Figures 1,2,3).

Toxicology was negative.

Discussion

Schistosomiasis2, also named bilharzia, bilharziosis or snail fever, is a parasitic disease caused by several species of the genus *Schistosoma*. First described by Theodor Bilharz in 1851, it affects 200 million people in the world, mainly in Asia, Africa and South America. Some are assymptomatic and other present either acute or chronic disease.

Schistosoma mansoni is hepatotropic. Trapped parasite's eggs secret antigens that elicit a vigorous immune response. It is the resultant cellular infiltration, rather than the eggs themselves, that are responsible for the pathology.2 Macrophages, lymphocytes and eosinophils are proeminent and involved in cytokines (interleukin 4, 5, 9, 10, 13) and antibodies (IgE, IgG) production.3-5

Conclusions

Death may result from different underlying mechanisms.2,6 In the present case, data suggests that the final, sudden and unexpected event may have supervened from anaphylatic reaction with acute lung œdema as a consequence of parasitic allergic hepatitis aggravated by the effort of sports activity.6

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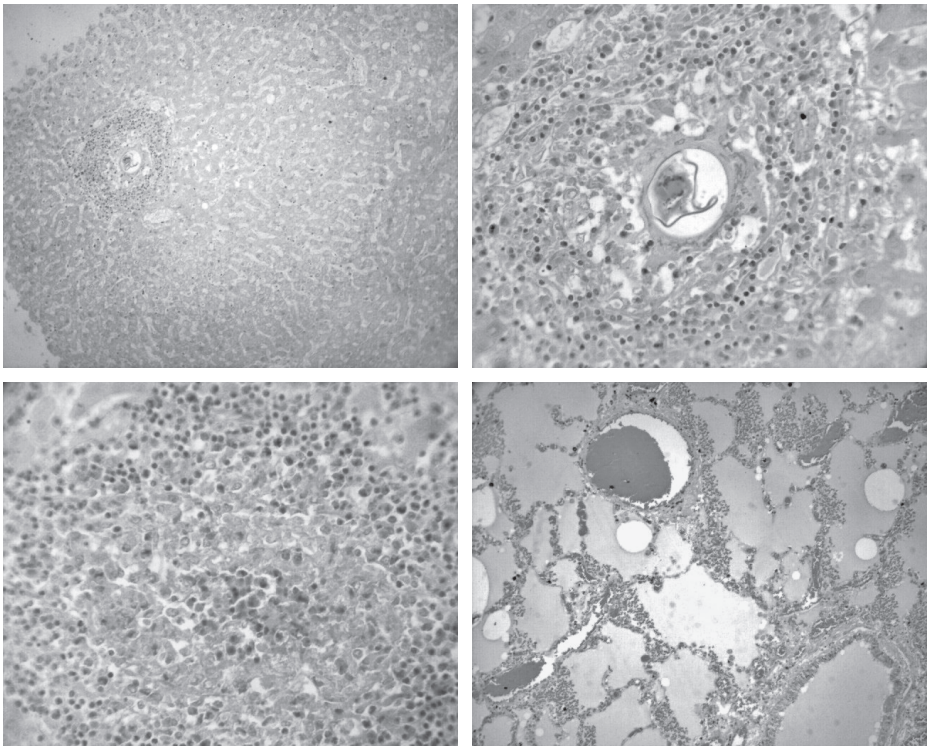

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FALL FROM A HEIGHT WITH HEAD TRAUMA: DUE TO OR COMPLICATED BY CARDIAC DYSFUNCTION? ONE CASE STUDY

Abstract: This paper refers to a case of clinical forensic evaluation, in terms of labour law, the patient evoking the existence of cardiac sequelae supervening head trauma, due to a fall from a height. Beside a brief reflexion on this pathological entity, the authors also pretend to illustrate the importance of an accurate analysis of all available data, from patient interview to clinical documentation, in order to establish the causal nexus.

Introduction and objectives

A wide variety of cardiac changes following acute head injury have been described, mainly concerning heart rate and rhythm, traducing it selves by EKG abnormalities, and are believed to be due to autonomic nervous system dysfunction (table 1), prevailing the sympathetic system activation, in the context of the acute stress response to trauma, with rising of circulating cathecolamine levels. Therefore, taquiarrythmias are more frequent than bradiarrythmias, the most commonly described being the supraventricular tachycardia. These changes have been reported even after minor head injuries and are usually reversible, showing a parallel course within the patient' neurological status. In the present case, the main question to answer was whether the accident was due to atrio-ventricular blockade (as evidenced by the EKG performed at the moment of medical emergency assistance), followed by syncope, or if the accident itself, resulting in head trauma, caused the atrio-ventricular blockade and could therefore be responsible for the present clinical condition of the patient.

Case Study

A 49 years-old man fell from a machine, 3 metres high, while working, which was followed by immediate loss of consciousness. He was promptly assisted by emergency medical professionals and immediately admitted to an intensive care unit. A temporary pacemaker was implanted and the patient went under a tracheostomy and mechanical ventilation, among other therapeutic measures, including suture of a blunt trauma

wound of the frontal region. Physical and imaging evaluation revealed the absence of any neurological disorder, namely of traumatic origin, reliable to justify the patient clinical status. In fact, fracture of the left side of the frontal bone and an isolated, small dimensioned, focal brain lesion (in the basal aspect of the frontal lobe), were the only traumatic lesions evidenced by CTScanning.

Concerning the circumstances of the accident, no witnesses were around and the patient mentions immediate lost of consciousness and no memory of feeling ill previously to the fall. However a careful analysis of the clinical reports disclosed that the patient referred, in a previous consultation, that he fell over after stopping the machine because he was actually not feeling good and was bleeding from the nose. Furthermore, it is referred, in the nursery clinical notes, during the patient's hospitalization, that about three days before the accident he began feeling ill, with the onset of dyspneia and orthopnea, with no clinical signs of an respiratory infectious disorder.

Finally, the patient revealed his clinical history, dominated by prior cardiac disorder, under pharmacological therapy.

In fact, full documentation on cardiac previous history, revealed dilated cardiomiopathy of unknown/non-defined aetiology and cardiac heart failure with ventricular arrhythmia and dysfunction. There was indeed described the necessity of temporary pacemaker implantation, during an surgical procedure, in order to prevent bradycardia.

Presently, the patient has a permanent pacemaker and is medicated with anti-hypertensive and anti-arrhythmic drugs. A brief neurological physical examination revealed no abnormalities, and the actual symptoms the patient complains about include episodes of headache, dizziness and irritability as well as fatigue.

Discussion and Conclusion

The establishment of causal nexus between a traumatic event and a given clinical condition, claimed to represent its sequelae, depends on several assumptions, which include, among others, the absence of other putative causes for that clinical condition as well as the absence of it's pre-existence.

In the present case, the analyses of the data concerning the history of this patient's case, by one hand, and, on the other hand, the acquaintance of the scientific literature, permits the establishment of elements voting in favour of and against the existence of causal nexus between the traumatic event suffered by the patient while working, resulting in head trauma, and the atrio-ventricular blockade, as well as his present cardiac dysfunction, as he reclaims.

So, voting in favour of this hypothesis is the occurrence of an atrio-ventricular blockade, immediately after a documented head trauma, being that condition one of the most frequently cardiac arrythmias reported with Central Nervous System pathology. However, stronger factors, such as the patient previous clinical condition and the persistence of cardiac dysfunction after the recovering and until the present time, vote against the patient's postulated causative theory.

In fact, and despite the above mentioned scientifically proved relation between cardiac rhythm disturbance and head trauma, including minor traumatic events, one could never neglect the patient's previous clinical history of complex and long-term cardiac disorders, to be the main suspect of being responsible for the occurred event, and so the more plausible scenario being the occurrence of a syncope followed by a fall, which in its turn caused the traumatic minor head lesions reported. Furthermore, there is a strong possibility that the epistaxis episode mentioned in the clinical reports, as preceding the traumatic event, was related with a sudden rise of arterial blood pressure, i.e, an hypertensive crises, which is in turn, a frequent condition associated with cardiomiopathy and heart failure. Another feature, in this case, pointing towards this theory versus the hypothesis of cardiac arrhythmias caused by head trauma, was the patient clinical outcome, with the maintenance of the disrythmia, in contrast with the reversible character of heart dysfunction due to head trauma, which tends to disappear once the acute phase of brain injury is solved. It is indeed established, in the literature review, the reversibility of cardiac disrythmic status as a main feature, when of traumatic origin.

Beside alerting to the probability of cardiac dysfunction succeeding to head injury, which is in fact an unusual documented condition, and therefore displacing a delicate matter in terms of causal nexus establishment, namely when no previous cardiac condition is reported, this paper emphasizes the importance of an accurate medical interview, and mostly of a complete clinical report of the event as well as concerning patient's clinical history, when determining the causal nexus between a traumatic event and the present clinical condition of the patient.

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ECG changes	*Cardiac arrythmias*
High-amplitude P waves	Sinus bradicardya
Increased CRS voltage	Supraventricular tachycardia
Q waves	Atrial fibrillation
Prolonged QT interval	Atrioventricular blockade
Shortened QT interval	Ectopiventricular contractions
Depressed ST	Multifocal ventricular tachycardia
Elevated ST segments	Ventricular flutter
T wave flattening	Ventricular fibrillation
T wave inversion	Left axis deviation
Tall, peaked T waves	
Notched T waves	
U waves	
Pulsus alternans	
U waves alternans	

Table 1 – EKG changes and Cardiac Arrythmias with CNS Pathology

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GIANT HYDATID CYST: AN UNSUSPECTED POSSIBLE CAUSE OF SUDDEN DEATH

Introduction

Hydatid cyst (*Echinococcosis multilocularis*) is an uncommon human parasitic infestation in the so-called developed countries, seen most usually in Mediterranean areas, Australia and South America where it is endemic.[1,2,4,5] Signs and symptoms vary, depending on the anatomical location and dimension of the cyst.[2] Usually it is located in the liver (55-75%)[2,3,5,6] and the clinical diagnosis requires a high level of suspicion.[3,6,7]

Material and methods

The present case refers to a Caucasian 93-year old female with a non-natural cause of death, due to severe craneo-encephalic lesions after an accidental fall. A postmortem examination was performed.

Results

The autopsy showed blunt force trauma to the right side of the head with cranial fractures and brain contusion. Unexpectedly, an hepatic mass was found (Figure 1). It occupied most of the abdominal cavity and extended to the pelvic area. Macroscopically, it weighed 9500 g, measuring 33 cm of diameter (Figure 2). The cut surface was cystic and contained a yellowish amorphous material with multiple vesicles resembling "daughter cysts" (Figure 3).

Microscopic examination disclosed the presence of a surrounding fibrous capsule (Figure 4), an outer laminated and an inner germinative membranes (Figure 5), as well as scolices amidst necrotic material and neutrophilic infiltrates (Figure 6), thus confirming the macroscopic hypothesis of hydatid cyst of the liver.[8] *

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* FC = fibrous capsule, LM = laminated membrane, GM = germinative membrane, S = *scolices.*

Discussion and conclusions

Hidatid disease is a parasitic infection most frequently caused by the larval form of the tapeworm *E. granulosus*, which uses the dog as the definitive host.[9,10]

After ingestion, the larvae go through the duodenal wall to the portal blood system and into the liver, where they are found in about 60% of cases.

Some of them may escape hepatic filtration and continue to the pulmonary circulation. A small percentage may reach the systemic circulation, resulting in infection and cyst formation in any organ.[9,10]

There may be symptoms of a feeling of pressure, but generally the cyst does not cause any clinical symptoms for a long time.[2] The cysts increase slowly in size, commonly reaching a sizeable mass over several years.

The fluid inside the cyst has highly antigenic properties, which may result in anaphilactoid reactions due to leakage either into a vessel or body cavity.

In Portugal the incidence of hydatid disease is very low (79 cases between 2000 and 2004), probably due to underreporting of the disease. It is in Alentejo (agricultural region) that the highest incidence can be found – 80%.

This case highlights the importance of histopathological exams even in the context of violent deaths.

It's relevance, apart from the gigantic dimensions (the 2nd largest9 in the literature, to our knowledge), lies in the possibility of spontaneous or traumatic rupture - with legal implications, consequently causing anaphylactic shock, which may lead to sudden death.

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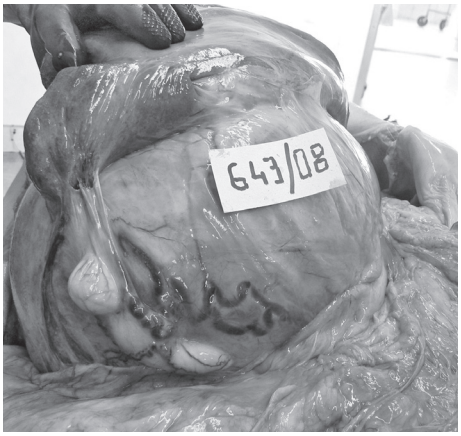

Figure 1 – Hepatic mass found at autopsy

Figure 2 – Hepatic hydatid cyst

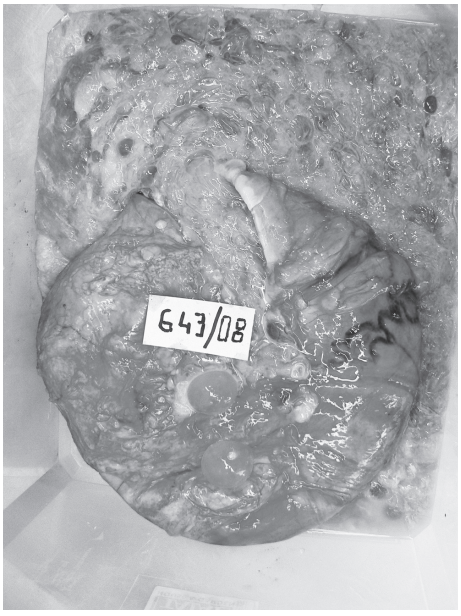

Figure 3 – Content of hydatid cyst

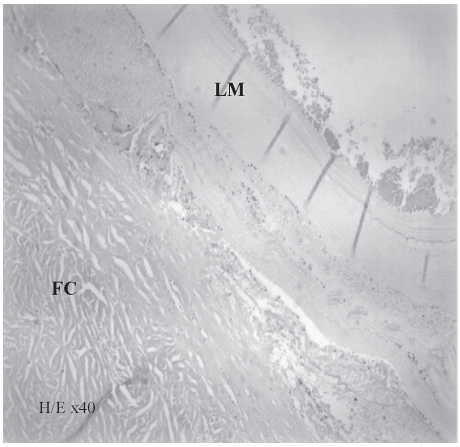

Figure 4 – Microscopic aspect of fibrous capsule and laminated membrane

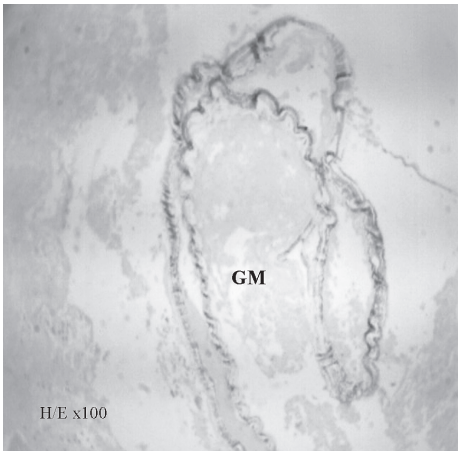

Figure 5 – Microscopic aspect of germinative membrane

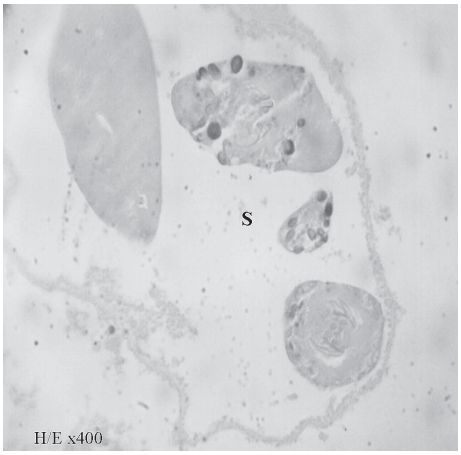

Figure 6 – Microscopic aspect of *E. multilocularis* scolices

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Documentos

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