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Observation of Barr bodies in dental pulp tissue and its relevance for sex estimation in a forensic context.

Zacharie MARTINEZ-ALMANSA

Dissertação conducente ao Grau de Mestre em Medicina Dentária (Ciclo Integrado)

Gandra, 17 de junho de 2022

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Trabalho realizado sob a Orientação de Professora Doutora Alexandra Teixeira e Coorientação de Professor Doutor Daniel Mongiovi

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ACKNOWLEDGEMENTS

To my parents and grandparents who helped me to follow this path and always supported me.

To my little sister "poulet" Ezéchielle.

To my girlfriend, already had five years with you and more to come.

To my oldest friends Anthony and Francis. We have years to catch up.

To Elliott, the only man I saw more than my parents.

To my binomial since the second year JB my favorite Marseillais.

To my roommates Anatole, Pierre e Quentin and this crazy last year.

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RESUMO

Introdução: A identificação humana de pode ser realizada utilizando métodos comparativos como o ADN e as impressões digitais, assumindo que os dados antemortem estão disponíveis. Contudo, quando o corpo está demasiado danificado, como em casos de grandes catástrofes, o procedimento pode tornar-se mais complexo. Nestas circunstâncias, a odontologia forense pode ser uma ferramenta muito útil, nomeadamente na determinação do sexo da vítima.

Objetivo: O objetivo deste trabalho é desenvolver um protocolo simples para a determinação do sexo num contexto forense através da deteção dos corpúsculos de Barr em células da polpa dentária.

Material e Métodos: Foi feita uma pesquisa bibliográfica para contextualizar o tema. Depois, aplicados protocolos histológicos e citológicos com coloração H&E ou imunofluorescência (usando um anticorpo para uma variante de histona- MacroH2A) em polpa dentária proveniente de indivíduos do sexo masculino e feminino, obtidas por acesso endodôntico em dentes previamente extraídos.

Resultados: Foram observados corpúsculos de Barr em preparações citológicas e histológicas de polpa dentária feminina corada com H&E. Foi igualmente detetada uma marcação mais relevante por imunofluorescência nas amostras histológicas femininas, quando comparada com amostras do sexo masculino.

Discussão/Conclusão: Embora, tenham sido observados corpúsculos de Barr com a técnica de H&E em polpa dentária extraída de pacientes do sexo feminino, este método não é, por si só, suficiente para a diagnose sexual. A imunofluorescência utilizando um anticorpo anti- Macro H2A é uma ferramenta promissora para detetar os corpúsculos de Barr, no entanto são necessários mais estudos, bem como a otimização do protocolo, para validar estas observações preliminares.

Palavras-chave: Odontologia forense, Corpúsculos de Barr, determinação sexual.

ABSTRACT

Introduction: Human identification can be performed using comparative methods such as DNA and fingerprints assuming antemortem data is available. However, when the body is too much damaged, such as in cases of major disasters, the procedure can become much more complex. In these circumstances, forensic odontology can be very useful, namely, to determine the sex of the victim.

Objective: The objective of this laboratory research is to develop a simple protocol for sex determination in a forensic context through the detection of Barr bodies in dental pulp cells of extracted teeth.

Material and methods: Bibliographic research was conducted to contextualize the subject theme. Then, different protocols (histological e cytologic with H&E staining or immunofluorescence, using an antibody for a histone variant - MacroH2A) were applied to dental pulp samples obtained by endodontic access on teeth previously extracted from males and females.

Results: Barr Bodies can be observed in pulp cells as well as histological samples from female dental pulp, stained with H&E. Furthermore, the Immunofluorescence results, showed Increased labeling in female histologic samples, when compared to samples from male individuals.

Discussion/Conclusion: Although Barr bodies were observed in female dental pulp using H&E staining, we believe that alone, this is not a suitable method to discriminate between males and females. Immunofluorescence using an antibody against histone variant H2A is a promising tool to detect Barr bodies, but more studies as well as the optimization of the protocol, are needed to validate these preliminary observations.

Keywords: Forensic dentistry, Barr bodies, sex determination.

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INDEX OF ABBREVIATIONS

DNA: Deoxyribonucleic acid

H&E: Hematoxylin-Eosin

BB: Barr bodies

PBS: Phosphate-buffered saline

BSA: Bovine serum albumin

Ab: Antibody

FIG: Figure

1 INTRODUCTION

Forensic medicine is a branch of medical practice, between medicine and law, which consists of victim's remains with the main purpose of establishing facts in the context of criminal or legal cases. It comprises several areas, including forensic anthropology, traditionally associated with the *"identification of more-or-less skeletonized remains known to be, or suspected of being human."* (Stewart, 1979.) (1,2).

Nowadays, the field of forensic anthropology includes various areas of expertise: crime scene analysis, post-mortem interval determination, biological profile establishment (age, sex, ancestry, stature), and personal identification by using several factors of individualization, x-rays, dental records, DNA analysis and other methods. It also involves trauma analysis and mass disaster victim identification (2,3).

Human identification can be performed using comparative methods such as DNA, fingerprints, and dental records, assuming antemortem data is available. However, when the body is too much damaged, such as in cases of major disasters, the procedure can become much more complex. Bodies may be carbonized, dismembered, and bones may be broken, making a general physical examination alone, useless (4–7). In these circumstances, secondary or reconstructive methods can be applied to establish the biological profile, where the first and most important step is sex estimation since it reduces the candidates to human identification by half (6–9).

The most dimorphic bones, used for sex estimation are the pelvic bones, followed by the skull. However, in many circumstances, as previously mentioned, these elements may be absent or too damaged to be studied (1). In these cases, forensic odontology, "an area of dentistry concerned with the correct management, examination, evaluation, and presentation of dental evidence in criminal or civil legal proceedings in the interest of justice" (4) can be a very useful tool (10). Since of all human tissues, teeth are the most durable and are often preserved, which makes them valuable in the process of human identification (11–14).

The human dentition can present several variations in its morphological structures. The anatomical structures of the mandibular molars serve as data capable of characterizing a population (15). The structural characteristics of the teeth, especially of molars, are partially hereditary and do not change once formed (16). But sex estimation based on morphological characteristics of teeth has a debatable accuracy due to the continuous wear and tear of teeth

throughout life (17,18). Furthermore, the odontometric methods available, seem to be dependent on the population studied (15). Therefore, the development of alternative methods, technically simple and cost-effective is a valuable contribution to this field being the identification methods based on dental pulp analysis of particular interest.

When analysing dental pulp, several methods can be applied: first, molecular methods based on nuclear or mitochondrial DNA analysis show great accuracy but are expensive and time-consuming due to the complexity of the analysis and equipment required (17).

An intermediate histological method, which is useful for sex determination, involves the evaluation of the presence of sex chromatin, or Barr bodies, in somatic cells. There are hyper condensed, almost completely inactive X-chromosomes in the nucleus of female cells. These Barr bodies are found in about 40% of female cells, which are known as chromatin positive. Male cells are chromatin negative. The presence of Barr's body has also been registered in dental tissues (7,8,19–23). This is an important finding because as mentioned earlier, teeth are protected by oral tissues and dental pulp by the mineralized constituents of the tooth (11). This allows sustainable production and conservation of cell pulp to help sex determination in circumstances where other tissues cannot be used.

Presently, although this method is known, very few studies have been done either to test its efficiency or to find a simple and reliable protocol to detect Barr bodies.

2 OBJECTIVE

The objective of this laboratory research is to develop a simple protocol for sex determination in a forensic context through the detection of Barr bodies in dental pulp cells of extracted teeth.
The expected results are the development of a simple, accurate, and economical protocol for sex determination from dental pulp.

3 MATERIAL AND METHODS

The bibliographic background for this dissertation was carried out using a search in the Google Scholar and PubMed databases. Full-text articles concerning research in humans, written in English from the period between 2000 and 2022 were searched, using the combinations with the following keywords: "Forensic dentistry" AND "Barr bodies" AND "sex determination". No articles were found in PubMed but a total number of 103 articles was obtained in the Google scholar database search. A preliminary evaluation of the abstracts was carried out to determine whether the articles met the objective of the study. The selected articles were read and evaluated individually. A total of 16 articles were considered relevant to this study. Furthermore, to contextualize the subject theme, a total number of 1 book and 7 articles from other sources were also consulted and included in this work, comprising a total of 24 publications. The last inclusion of article was done on the 25TH of February 2022.

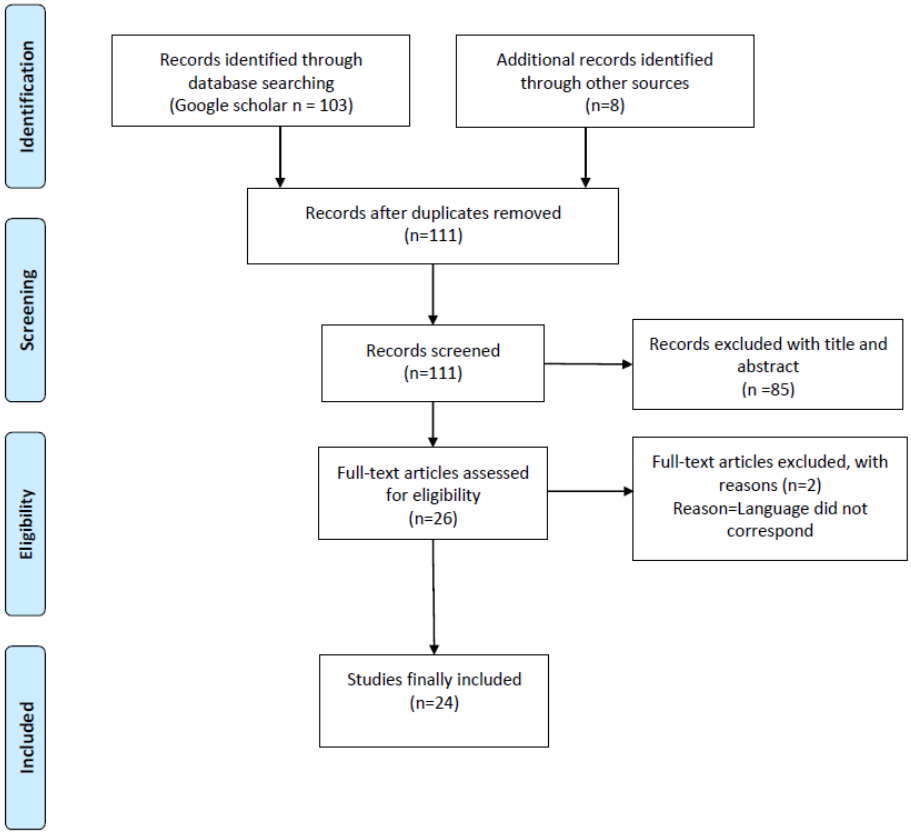

FIGURE 1: PRISMA FLOW CHART OF STUDIES SEARCH AND SELECTION

For this study, teeth extracted at the university clinic Filinto Baptista were recovered, having obtained a favorable opinion from the Ethics Committee of the Instituto Universitário de Ciências da Saúde (Annex 1). All participants signed an informed consent where they were explained the objective of the study and guaranteed the confidentiality of the information collected (Annex 2). Teeth were selected based on the presence of the whole pulpal canal and extracted for periodontal reasons (dental mobility). They were healthy or with only superficial caries or restorations where the pulp was not affected.

First, the teeth were washed in sterilized water after extraction. The pulp was conventionally obtained through the normal access cavity on the occlusal surface of the teeth. In the case of caries or previous restorations on the crown, a transversal groove was made through the root to prevent any contamination. This part is done with the help of a dental turbine and endodontics drills (spherical drill, Endo-Z). Next, the pulp tissue was separated from the pulp cavity with the help of standardized endodontics files (K-files), washed with saline to remove any debris and

finally kept in an Eppendorf tube immersed in 1 mL of fixative. All the Eppendorf tubes were then protected from light and heat.

Then we set up different protocols.

3.1 Procedures 1 and 2: Staining of dental pulp cells with Hematoxylin-Eosin

The following materials were used in this procedure:

-Dental pulp in fixative	-Microscopic slides
-Glass tube and rod	-Drying oven at 57°C
-20% acetic acid	-Thermo Shandon Cytospin 4
-Cytoclip slide clip	-Micropipette
-Cytofunnel sample chambers with caps	-Microscope
-Disposable filter cards	-Carrying rack
-Reagent containers	-Eosine
-Haematoxylin	-Ethanol 96%
-Tap water	-Absolute ethanol
-Xylol	Plastic pipet
-Histofluid mounting medium	-Paper towels
-Cover slip	-Micropipette and tips
-Tweezers/forceps	

Table 1: Material for staining of dental pulp cells.

3 samples, 2 males and 1 female, were used. The supernatants were discarded, and the dental pulp tissues were transferred to a glass tube and washed twice with 20% acetic acid. The separation of cells was carried out by crushing with a glass rod until the suspension was turbid. Slides were prepared according to 2 different procedures:

- **Procedure 1**: Slides were prepared by centrifuging the cell suspension for 5 minutes at 6000 rpm, followed by a cytospin for 5 minutes at 1250 rpm to spread the cells onto the center of a chilled microscopic slide.

-**Procedure 2**: Cell suspension gently centrifuged using spin pulse for 4 minutes. The supernatant was collected and submitted to a cytospin for 5 minutes at 1250 rpm to spread the cells onto the center of a chilled microscopic slide.

Then, staining of the slides of the two different procedures has been achieved.

Protocol for Hematoxylin-Eosin (H&E) staining

The slides were placed into a carrying rack that was immersed in the reagent containers according to the steps shown in table 2.

	Time	Reagent
Step 1	3'	Haematoxylin
Step 2	1'	Rinse in tap water
Step 3	3'	Ethanol 96%
Step 4	1'	Ethanol 96%
Step 5	1'	Eosin
Step 6	1'	Ethanol 96%
Step 7	1'	Ethanol 96%
Step 8	1'	Absolute ethanol
Step 9	30"	Xylol
Step 10	30"	Xylol

Table 2: Protocol for staining cells with H&E.

After staining the slides with H&E, they were placed on a paper towel; 1 ml of histofluid mounting medium was added and a cover slip carefully over the area with cells and mounting medium.

3.2 Procedure 3: H&E staining of paraffin-embedded dental pulp tissue

The following materials were used in this procedure:

-Dental pulp in fixative	-Tweezers/forceps
-Shandon HistoCentre 2	-Microscopic slides
-Cassette and cassette lid	-Drying oven at 57°C
-Mould	-Microtome
-Knife	-Cutting blade
-Freezer	-Brush
-Tissue floatation bath	-Micropipette
-Reagent containers	-Microscope
-Haematoxylin	-Carrying rack
-ethanol 80%	-Eosine
-Ethanol 50%	-Ethanol 96%
-Tap water	-Absolute ethanol
-Xylol	-Plastic pipet
-Histofluid mounting medium	-Paper towels
-Cover slip	

Table 3: Material for H&E staining of paraffin-embedded dental pulp tissue.

The pulpal tissue of 2 female samples and 2 male samples were embedded in paraffine using the Histocentre 2 from Thermo Shandon. 1 female and 1 male sample were from dental pulp obtained within 24 hours after tooth extraction. The two other samples were from dental pulp obtained from teeth 7 days after tooth extraction. Teeth were maintained in a saline solution during this time.

Protocol for embedding tissue in paraffin

The dental pulp sample was placed in the cassette (cut side down). A small amount of molten paraffin (at 61°C) was placed in the mould. Using warm forceps, the tissue was transferred into the mould, which was then transferred onto the cold plate, and the tissue was pressed flat in the middle of the mould. When the paraffin was solidified in a thin layer, the labelled tissue cassette was added on top of the mould. Hot paraffin was added to the mould until the plastic cassette

was covered in paraffin and the mould with the cassette was placed onto the cold plate until solidification. After 15 minutes of cooling, the sides of the paraffin block were cut with a knife and the block was placed in the freezer at 0°C for additional 15 minutes before cutting the block with a microtome.

The paraffin blocks were cut using a Thermo Shandon Finesse 325 microtome. The sections were cut by operating the microtome manually after adjusting the thickness to 4 µm. The sections are picked from the cutting blade and carefully placed in a container containing water and 1 ml of absolute ethanol and made to float in a tissue floatation bath at 50°C to remove folds. The sections were picked up using a clean glass slide and placed in an oven at 57°C for 15 minutes.

Protocol for H&E staining paraffine sections

The slides were placed into a carrying rack that was immersed in the reagent containers according to the steps shown in table 4.

	Time	Reagent
Step 1	10'	Xylol
Step 2	10'	Xylol
Step 3	5'	Absolute ethanol
Step 4	2'	Ethanol 96%
Step 5	2'	Ethanol 80%
Step 6	2'	Ethanol 50%
Step 7	5'	Haematoxylin
Step 8	1'	Rinse in tap water
Step 9	2'	Ethanol 96%
Step 10	2'	Ethanol 96%
Step 11	2'	Eosin
Step 12	2'	Ethanol 96%
Step 13	2'	Ethanol 96%
Step 14	2'	Absolute ethanol
Step 15	2'	Absolute ethanol
Step 16	30"	Xylol
Step 17	30"	Xylol

Table 4: Protocol H&E staining microscopic slide with paraffine sections.

3.3 Procedure 4 and 5: Immunofluorescence of paraffin-embedded dental pulp tissue

The tissues were first embedded in paraffin with the same protocol as above. 4 samples, 2 male and 2 females, were used. 1 female and 1 male sample are from dental pulp obtained within 24 hours after tooth extraction. The two other samples are from dental pulp obtained from teeth 7 days after tooth extraction. Teeth were maintained in a saline solution during this time.

- Procedure 4:

Deparaffinization and rehydration

First step, deparaffinize and rehydrate by immersing the slides through the following wells:

	Time	Reagent
Step 1	5'	Xylene
Step 2	5'	Xylene
Step 3	5'	Xylene
Step 4	10'	Ethanol 100%
Step 5	10'	Ethanol 100%
Step 6	10'	Ethanol 95%
Step 7	10'	Ethanol 95%
Step 8	10'	Ethanol 70%
Step 9	10'	Ethanol 70%
Step 10	10'	Ethanol 50%
Step 11	10'	Ethanol 50%
Step 12	5'	PBS
Step 13	5'	PBS

Table 5: Protocol for deparaffinization and rehydration

Next, draw a circle on the slide around the cells with a hydrophobic barrier pen.

Antigen retrieval

The slides were maintained at a sub-boiling temperature (98°C) in 10mM sodium citrate buffer (pH 6.0) for 20minutes. Next, the slides were cooled on the bench-top for 30 minutes and washed by immersion in Phosphate-buffered saline (PBS) for 5 minutes.

Permeabilization and blocking non-specific binding

The slides were quenched with 3.0% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity. Then they were washed in PBS two times for 5 minutes. To permeabilize the cells, we washed the slide twice for 10 minutes with 1% Bovine serum albumin (BSA) in PBS with 0.4% Triton X-100 (PBS-T). Non-specific bindings were blocked by incubating the sections with 1% BSA in PBS-T for 60 minutes at room temperature.

Antibody staining

The primary antibody anti- MACRO H2A SC-377452 (200 µg/mL) by Santa Cruz Biotechnology, was first diluted in PBS-T (1:50) and added to the slides. They were then incubated at room temperature for 2 hours. The sections were then washed twice with 1% serum PBS-T for 10 minutes each. In a second phase, a secondary antibody was added to the slides, with a dilution of 1:1500 serum PBS-T and incubated for 1 hour. The second antibody used was Goat anti-Mouse IgG Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488, Molecular Probes. Finally, slides were washed again two times with 1% PBS-T for 10 minutes each.

- Procedure 5:

It follows the same protocol as procedure 4 but includes at the end double labelling with DAPI fluorescent staining. DAPI was included in VECTASHIELD[R] Antifade Mounting Medium, H-1000, Vector, to label cells.

3.4 Microscopes for the observation and image capture of the prepared sections

For all the sections under H&E staining, we used a Nikon TE 2000-U microscope (Nikon, Amsterdam, The Netherlands), coupled with a DXM1200F digital camera, and Nikon ACT-1 software (Melville, NY, USA) with a 60x objective.

For the sections under immunofluorescence, we used an Axio Observer Z.1 SD microscope (Carl Zeiss, Germany), coupled to an AxioCam MR3, and with the Plan Apochromatic 63×/NA 1.4 objective. Image analyses were performed with the software AxioVision Release 4.8.2 SPC and processed using ImageJ version 1.51.

4 RESULTS

4.1 Cytologic preparations: Procedures 1 and 2

Cells from dental pulp were obtained using two different procedures and stained using H&E (FIG. 2 and 3A). Immunofluorescence labelling using DAPI for cell nuclei and an antibody anti-histone H2A was also tested in cells obtained using procedure 2 (FIG.3B).

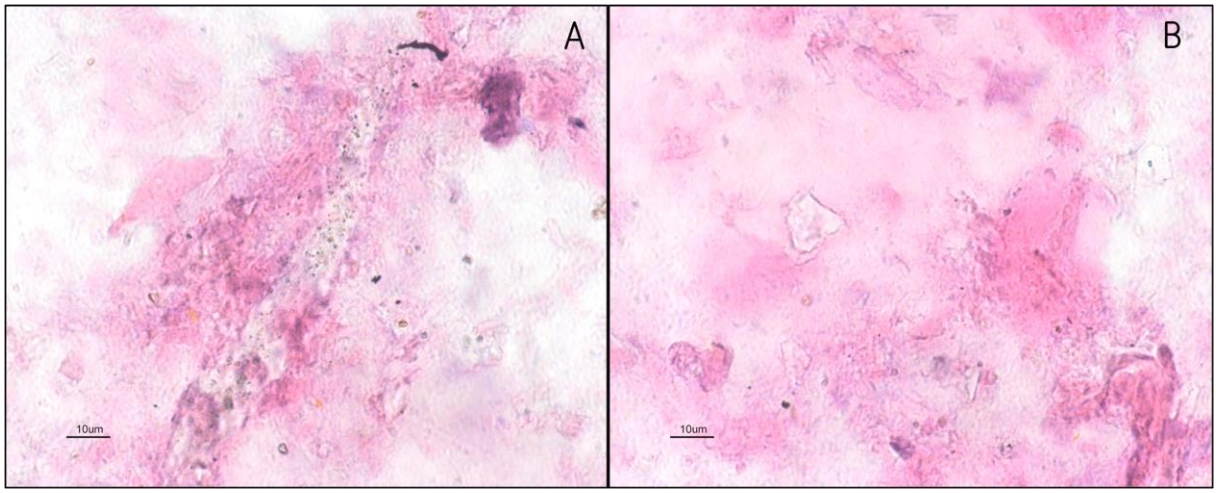

FIGURE 2: Cytologic sections of 2 males (A and B) dental pulps under H&E staining, using a 60x objective (procedure 1).

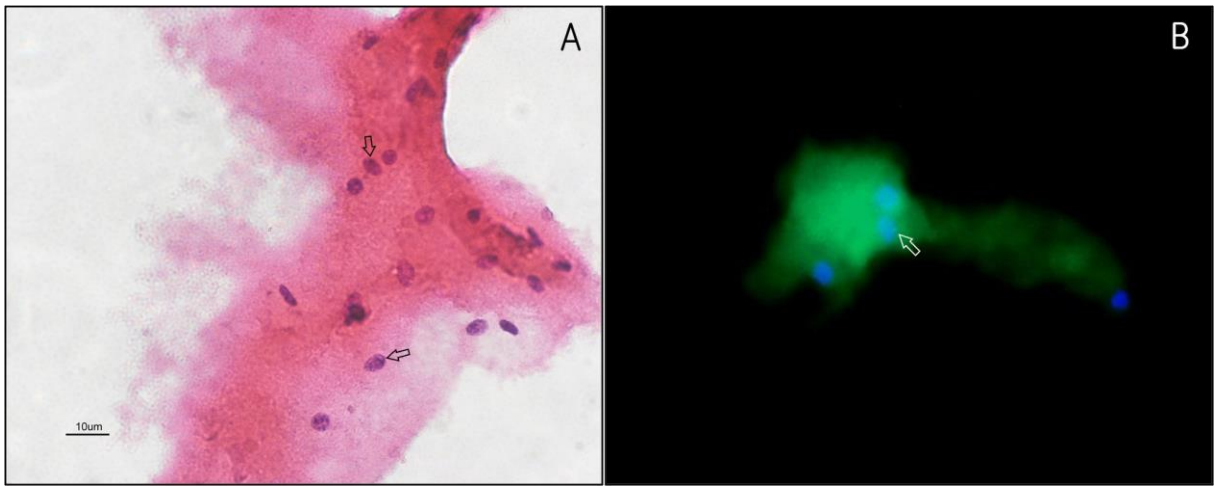

FIGURE 3: Cytologic section of a female dental pulp under H&E staining, with a 60x objective (A) and immunofluorescence DAPI + Ab anti- Macro H2A with procedure 2. Barr bodies are indicated by arrows. (Objective 63X)

4.2 Histologic sections: Procedures 3 and 4.

4.2.1 Fresh pulps obtained within 24 hours after teeth extractions

Cells from fresh dental pulp were obtained using procedure 3 and stained with H&E (FIG.4). Immunofluorescence labelling with an antibody anti-histone H2A (procedure 4) was also tested in cells previously embedded in paraffin (FIG.5).

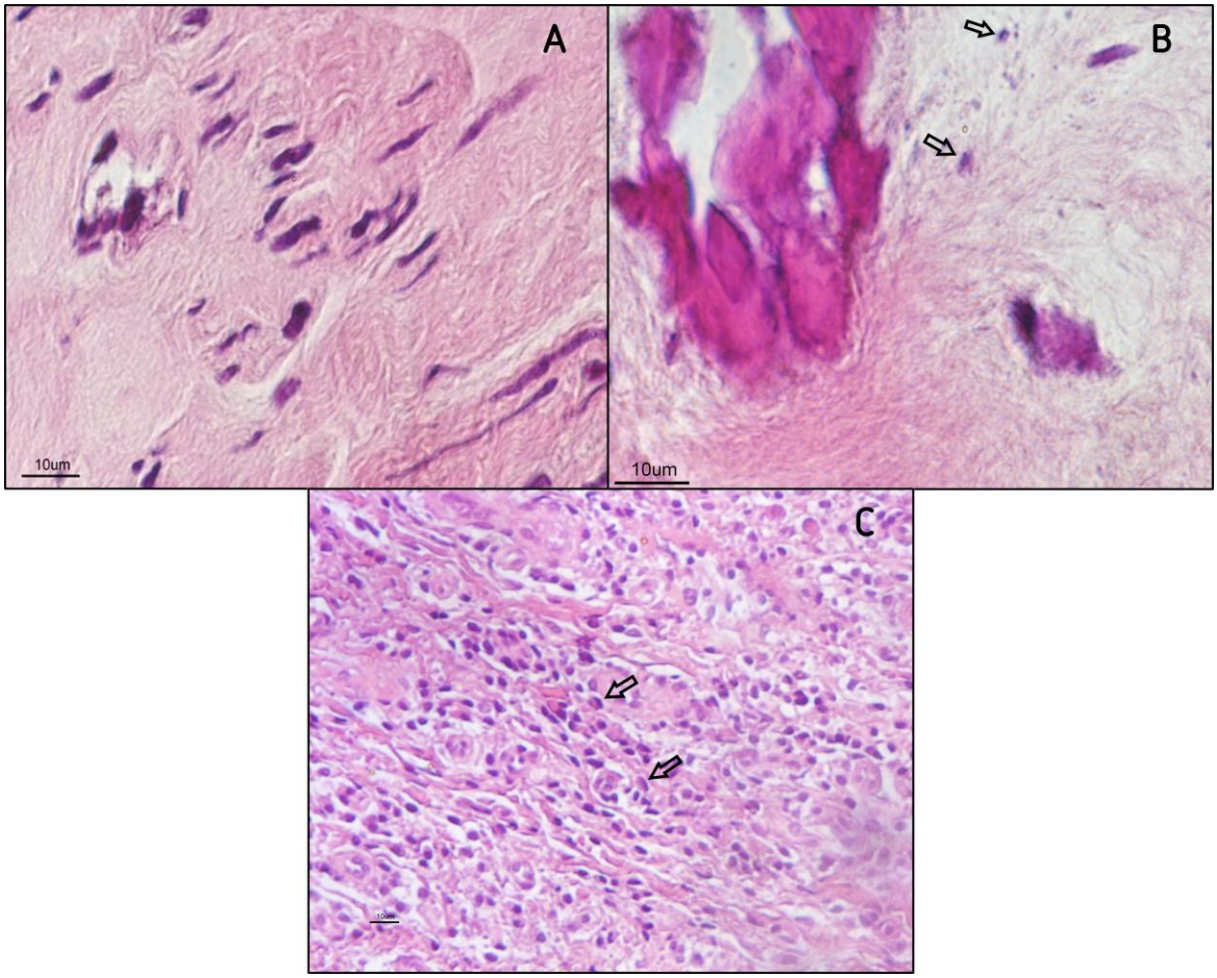

FIGURE 4: Histologic sections of paraffin-embedded dental pulp tissue of a male(A) and a female (B) under H&E staining using a 60x objective. (C) is a positive control of female rat gingiva tissue (procedure 3), using a 40x objective. Barr bodies are indicated by arrows.

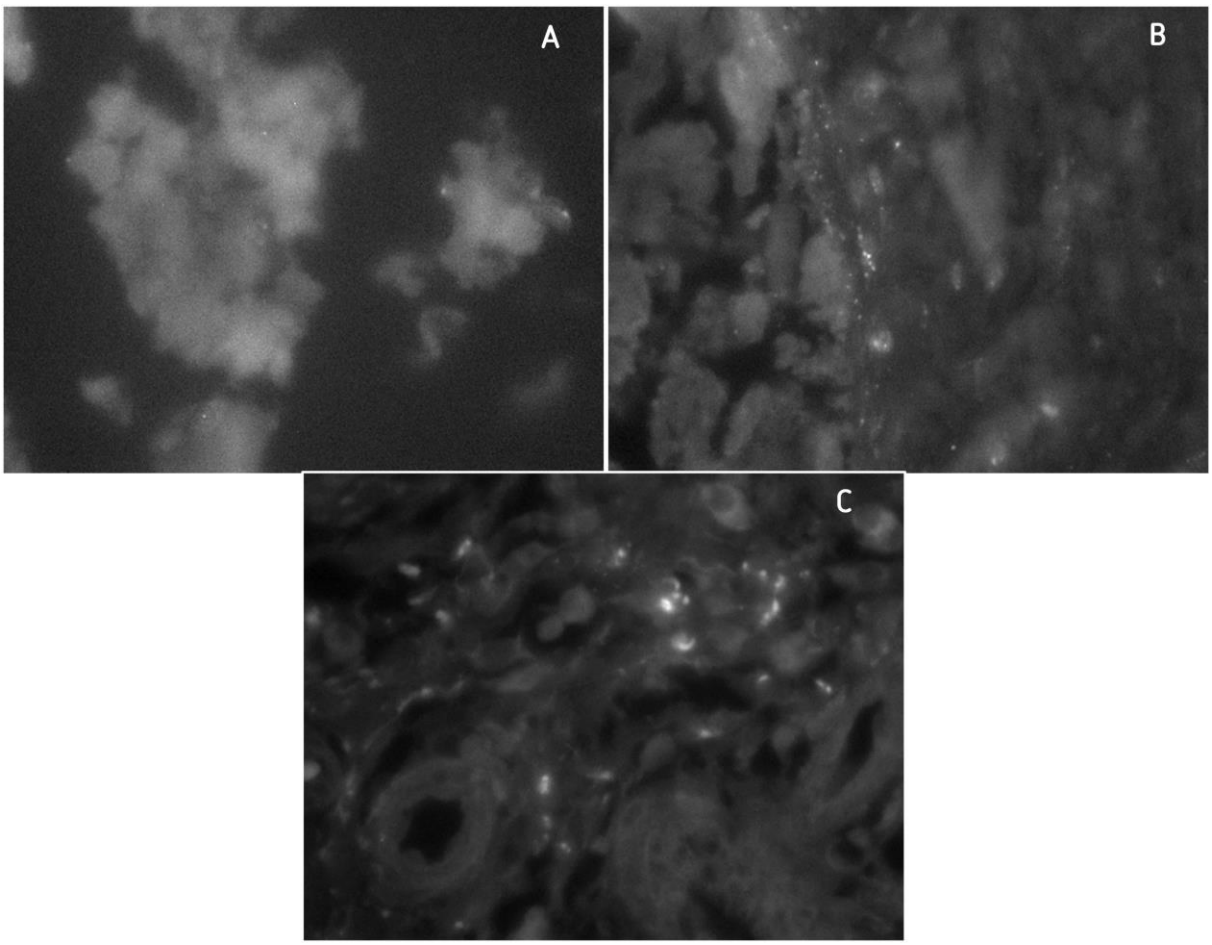

FIGURE 5: Histologic sections of paraffin-embedded dental pulp tissue of a male (A) and a female (B) under fluorescence. (C) is a positive control of female rat gingiva tissue (procedure 4). Macro H2A labelling is indicative of Barr bodies (bright spots). Objective 63x.

4.2.2 Dental pulp obtained from teeth 7 days after teeth extractions

Cells from dental pulp obtained 7 days after teeth extractions were obtained using procedure 3 and stained with H&E (FIG.6). Immunofluorescence labelling with an antibody anti-histone Macro H2A (procedure 4) was also tested in cells previously embedded in paraffin (FIG.7).

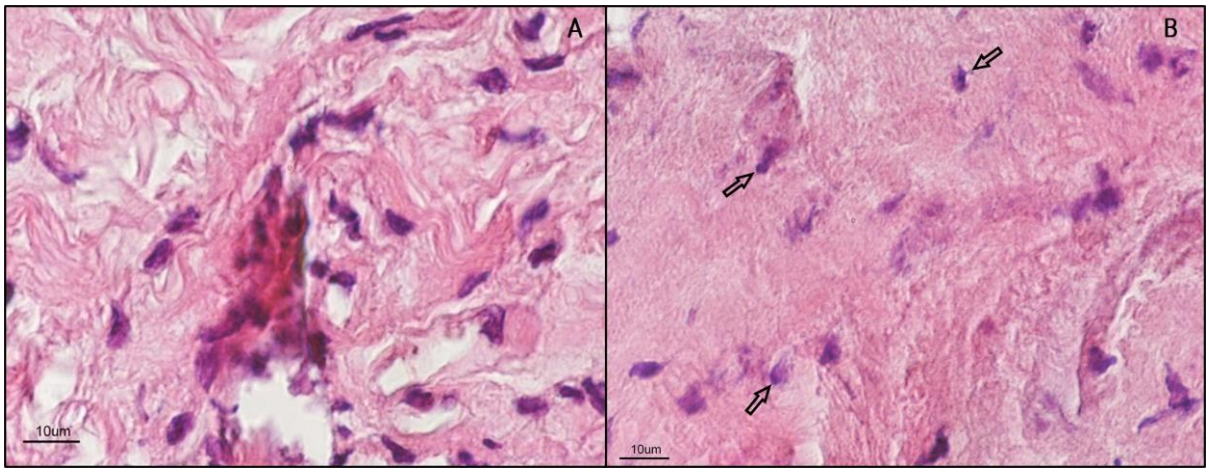

FIGURE 6: Histologic sections of paraffin-embedded dental pulp tissue of a male (A) and a female (B) under H&E staining after 7 days post-extraction (procedure 3), using a 60x objective. Barr bodies are indicated by arrows

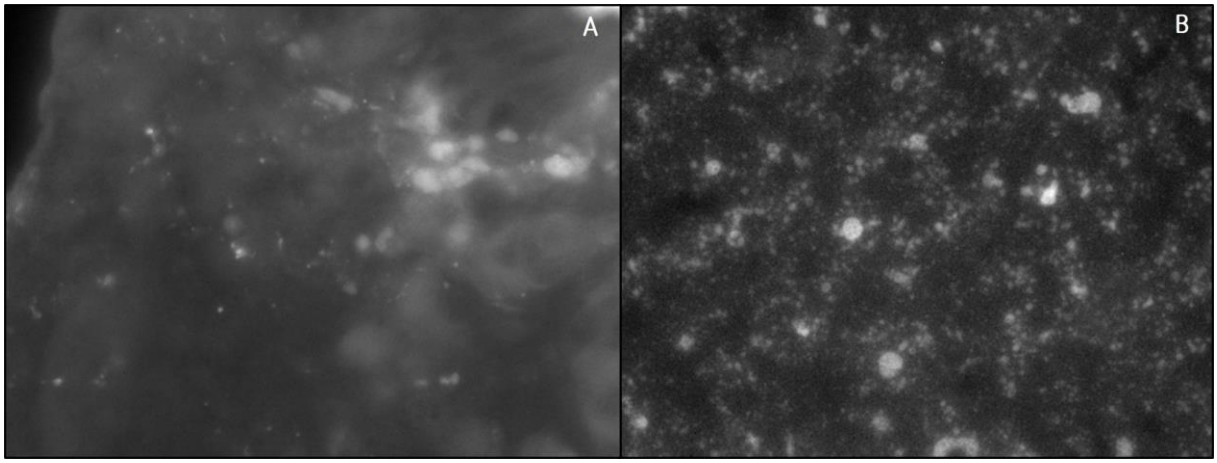

FIGURE 6:Histologic sections of paraffin-embedded dental pulp tissue of a man (A) and a woman (B) under fluorescence after 7 days post-extraction (procedure 4). MacroH2 labelling is shown (bright spots), using a 63x objective.

4.3 Histologic sections under Double labelling (procedure 5)

4.3.1 Fresh pulps obtained within 24 hours after teeth extractions

Cells from fresh dental were obtained using procedure 5 and stained with a double labelling DAPI+Ab Histone H2A (FIG.8).

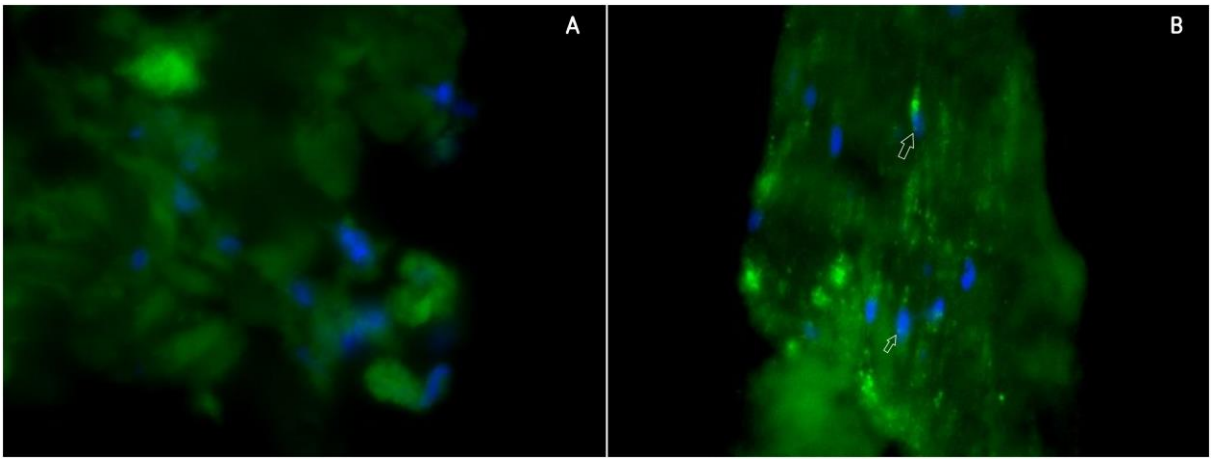

FIGURE 8: Histologic sections of paraffin-embedded dental pulp tissue of a man (A) and a woman (B) under fluorescence with DAPI+ ab anti-Marcro H2A (procedure5) using a 63x objective. Barr bodies are indicated by arrows.

4.3.2 Dental pulp obtained from teeth 7 days after teeth extractions

Cells from dental dental pulp obtained 7 days after teeth extractions were obtained using procedure 5 and stained with a double labelling DAPI+Ab Histone Macro H2A (FIG.9).

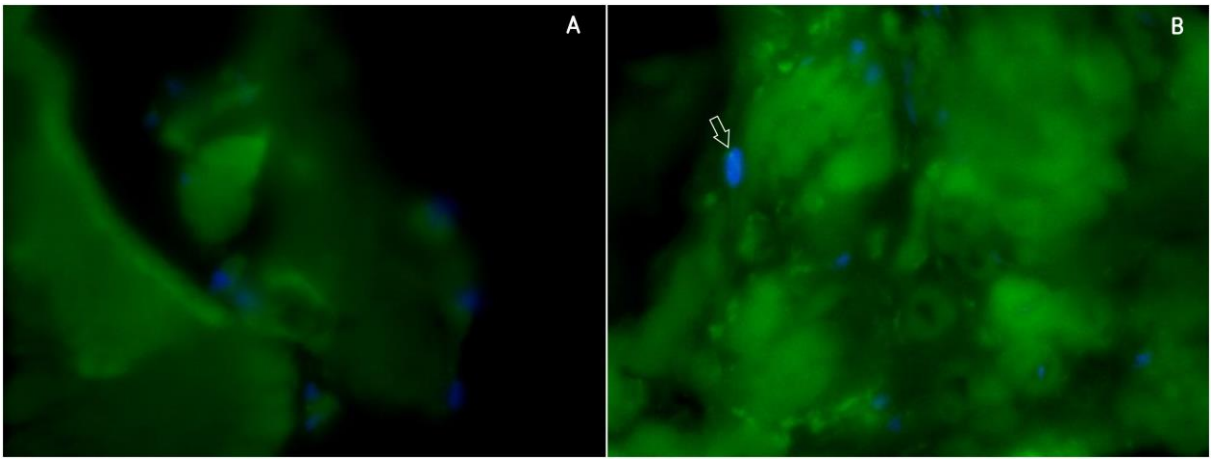

FIGURE 9: Histologic sections of paraffin-embedded dental pulp tissue of a man (A) and a woman (B) under fluorescence with DAPI + Ab anti-MacroH2A (procedure 5) after 7 days post-extraction, using a 60x objective. Barr bodies are indicated by arrows.

5 DISCUSSION

5.1 Contextualization

Forensic identification is generally easy if the body to identify is in good condition. But in case of mass disasters or war conflicts where the bodies can be extremely damaged, the process is difficult (12). Sex determination becomes then a priority step human identification, because the number of candidates is approximately decreased by half (17,22). This is where forensic odontology is useful. Teeth are the *"hardest and chemically the most stable tissue in the body"* (Monali, 2011) (16), which allows for maintaining viable cells with the pulp protected by enamel and dentin (14).

Different dental techniques can be used to evaluate sex including morphological and odontometric methods although its efficacy is variable due to the wearing on the occlusal and proximal surfaces of teeth over the years and may be affected by the population studied (15). Soft tissues namely dental pulp analysis may be an alternative technique. Pulp is a completely vascularized and innervated tissue, composed of fibroblasts, histiocytes plasma cells, mesenchymal cells, and stem cells (11). Due to its anatomical characteristics, it's a very interesting tissue for sex evaluation in a forensic context. In this case, DNA analysis and histological methods can be used (1). DNA testing shows great accuracy, but it requires special equipment and it's a time-consuming and expensive technique (17).

An intermediate and easier method is the histological and cellular techniques, especially the utilization of X-chromatin also referred to as Barr bodies. The Barr bodies were initially discovered by Barr and Bertram in 1948 in the nuclei of the nerve cells of the cat and have been studied then by a lot of researchers who have detected its presence in various cells of the body. Barr chromatin comes from the inactivation of the X chromosome in female cells and is present as a mass lying against the nuclear membrane (10). The process of inactivation of the X chromosome is known as Lyonization and was discovered by the researcher Lyon in 1961. He defined that on both chromosomes X present in the female, only one is genetically active and the other is rendered inactive that it comes from the father or mother. Also, the inactivation of either the maternal or paternal X occurs at random among all the cells of the blastocyst on or about the 16th day of embryonic life. Finally, the inactivation of the same X chromosome persists in all the cells derived from each precursor cell. The origin of this inactivation involves a unique gene called

Xist, which initiates a "gene-silencing" process. Xist is inactive in the active X-chromosome(9,10,23).A normal human female will have only one Barr Body per cell (the X-inactive) while a normal human male will have none. Sex determination by observation of the presence of Barr bodies could therefore be an accessible, rapid, and less expensive method that might allow its observation with most of the nuclear stains (7,12,17,19). However, few studies have been done using this method and difference between Barr bodies and condensed chromatin in male cells it is not always very clear. Interestingly, a histone variant, Macro H2A, seems to be associated with inactive X-chromosomes (24)

The objective of this work was to search for an effective method to find Barr bodies in dental pulp. We used a classic staining technique (H&E) to evaluate the cells and pulp tissue integrity as well as the nuclear staining. We then tested whether an antibody against Histone Macro H2A could provide an effective method to detect Barr bodies. If this method could be optimized and applied in a forensic context, it would provide a quick, simple and Inexpensive sex evaluation method, without the need for major laboratory reagents and equipment.

5.2 Analysis of the results

Cytologic preparations

To evaluate if procedure 1 was adequate to obtain pulp cells to undergo H&E staining, we used pulps extracted from two male subjects. Our results show that pulp tissue was quite fragmented, and the cells obtained were broken. We concluded that this protocol was not suitable for our studies and no further tests were performed (FIG.2). We then tested procedure 2 using an extracted pulp from a female patient. In this case, we could obtain cells showing correct H&E staining (FIG.3A) and Barr bodies could be detected. There is a limited number of studies that have already been conducted to analyse Barr bodies in forensic circumstances. Previous investigations claimed the viability of cytological preparations and H&E staining to detect Barr bodies(10,17,20). However, the images are not always very clear or representative of male and female samples (10,17) and in one investigation there were no images of the stained cells (20), which exemplifies the lack of strong data that would support the application of this method in a practical forensic setting. Our results are encouraging but very limited and need to be further developed, in the future, using both male and female samples.

We then tested double labeling under immunofluorescence using DAPI for nuclear labelling and an antibody anti-histone Macro H2A to evaluate if this might be a good marker for Barr bodies, since it has nuclear localization and is enriched in inactive X chromosome chromatin (24) (FIG.3B). DAPI-labeled cellular nuclei as well as chromatin condensation (labeled with both DAPI and anti-Macro H2A antibody), could be seen in some, but not all cells (FIG. 3B). Therefore, we suggest that this method (procedure 2) is suitable to collect cells from dental pulp, in a very simple way, so that further studies can be performed to evaluate Bar bodies and other cellular structures using conventional staining, such as H&E as well as immunofluorescence. However, more experiments are needed to validate the H&E results and to optimize the immunofluorescence protocol, as detection of anti-histone macro H2A was not very strong and not present in all DAPI-labeled nuclei.

Histological sections

In different studies, histological preparations of dental pulp with Hematoxylin-eosin staining have been used (5–7,12,14,19) even in teeth subjected to high temperatures up to 400°C (5,6). The fact that Barr bodies can be observed in histological sections of pulp tissue from female patients are consistent with our observations. We can see on FIG.4 several cells from female pulp (FIG.4B) with the staining of the condensation of Barr chromatin, however when analysing the male pulp (FIG.4A) sometimes the chromatin condensation that is labelled more intensely can be seen and the difference not as striking as we hoped. Since H&E is not the only nuclear stain that can be used, other reagents such as Feulgen, giemsa or aceto-orcein for example (7). In a 2015 study by Khanna (7), It was shown that Feulgen is a better stain than H&E to specifically label Barr bodies and it would be interesting to repeat these studies, using a male control sample to confirm and validate the results. Concerning Papanicolaou staining, Barr bodies observation was very difficult, according to Murugesan and Balakirishnan (10). The same results had been found in 2017 by Kaur et al. but on buccal mucosal scraps (23). This experiment showed that Papanicolaou staining is not very reliable to differentiate Barr bodies, particularly because it can sometimes tint other cellular components (23).

To evaluate the possible usefulness of H&E staining and MacroH2A labelling in a forensic setting we repeated the study but using pulp from both male and female patients 7 days after tooth extraction. Analysing our results, it seems more difficult to observe cells and BB with H&E after

7 days when compared to fresh pulp (FIG.6); the labelling appears less specific because we observe a dissemination of the staining (FIG.6A) and is more difficulty to differentiate BB in the female pulp cells (FIG.6B). The most likely hypothesis for this dissemination is that there is a degradation of the nuclear membrane after 7 days. We then analysed our histological samples by immunofluorescence, using the anti-histone Macro H2A antibody (procedure 4). The observation of the microscopic photographs shows that the labeling seems stronger in female cells whether in fresh pulp (FIG.5) or after 7 days (FIG.7). In both cases, slides of female pulp appear with an increased number of Macro H2A labeling (bright spots), demonstrating the possible use of this technique for sex determination. The hypothesis of nuclear degradation is further suggested by the presence increased fluorescent spots compatible labeling dissemination of fragmented condensed chromatin in both male and female pulp tissue after 7 days. Two different studies using fluorescence microscopy to detect Barr bodies, have been published, using acridine orange (7,9). In both studies, whether with buccal mucosal scraps for Reddy D. (2012) (9) or with dental pulp tissue for Khanna KS. (2015) (7), acridine orange staining detected condensed chromatin/ Barr bodies.

The combination of procedures 3 (H&E) and 4 (immunofluorescence) allows us to conclude the following: first, H&E staining on its own, is not sufficient for sex determination in our experience, especially when the pulp is not recovered soon after extraction. Although it is necessary as a first approach to analyze the cells. Immunofluorescence using an antibody anti-macro H2A labels the female nuclei much more than males, with the same difficulties after 7 days as discussed previously, even though in these circumstances the increased level of staining in females is still verified. We also used a double-labelling using DAPI in conjunction with our antibody macro H2A, but these experiments need to be repeated ads the results were inconclusive with fresh pulps or after 7 days, and further studies are needed to evaluate the labelling of histone variant Macro H2A as a marker for Barr bodies.

5.3 Limitations of this research

Of course, forensic odontology cannot be applied when the person has been dead for a long time or edentulous. But the principal limitation is that we can observe false negative or false positive results with patients having chromosomal alterations like Turner's or Klinefelter's syndrome, or SRY translocation (8,9,14,17,19). A patient with Turner's syndrome while have one of the X chromosomes missing or partially missing. It can affect only women, resulting in the diminution or absence of Barr bodies in cells. Klinefelter's syndrome is when men while an extra chromosome X (XXY). BB can then be observed, even if the subject is a man.

For the difficulty to surely distinguish BB in the procedures, the failure can be explained in various ways: delay in sample staining, storage difference, cellular debris and bacteria can alter the results for a given procedure of sex determination (20,23). That can also be explained by a limitation of histological method described by Suazo (2010). Indeed, we can have superposition between chromatin and nuclear membrane causing the impossibility to observe Barr bodies (19). Finally, the forensic/laboratory expert must have experience in the sex determination with Barr bodies, as an incorrect interpretation of the sections would bring an incorrect diagnosis (9).

Similarly, there were several limitations in the present study: the sample analyzed was small and we needed to repeat the experiments to confirm our observations. The fact that in the cytology we did not have a male sample to compare with the female sample analyzed, limits the interpretation of the results.

Concerning the histologic sections, the optimization of the immunofluorescence protocol was not possible due to the lack of samples and a limited time.

5.4 Future perspectives

In the future we need to repeat the cytology studies in male and females to compare H&E staining as well as test other stains that might be better to discriminate sex, such as Feulgen. The immunofluorescent using anti-macro H2A to label Barr bodies is quite promising but further studies are to confirm its usefulness. Also, quantification of the labeled spots in both males and females should be performed, as it may provide a tool for sex determination even when teeth are analyzed several days after extraction. Ideally, we should be able to use this technique in cells, using the more complex histologic sections as a backup. To address the rare circumstances of chromosomal abnormalities that may interfere with sex determination, we would like to teste a double labeling using an antibody anti-SRY, characteristic of the Y chromosome, together with Macro H2A, as together they might provide a stronger basis for sex evaluation.

Finally, once the best protocols are in place, we should test them under different conditions such as temperature, pH or humidity as they are important in a forensic context and can change the diagnostic value (10–12,19).

6 CONCLUSION

In this study we successfully obtained cells from a female dental pulp, where Barr bodies could be detected by H&E and fluorescence labelling.

Histological sections from both male and females were stained with H&E and although Barr bodies were observed in females, we believe that alone, this is not a suitable method to discriminate between males and females.

Immunofluorescence using an antibody against histone variant H2A is a promising tool to detect Barr bodies, but more studies are needed to validate these preliminary observations.

If our protocols could be optimized and applied in a forensic context, they would provide a simpler and less expensive method for sex determination.

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ANNEX

Annex 1 – Acceptance of the ethics committee

CESPU
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Comissão de Ética

Exmo. Senhor Investigador
Zacharie Martinez-Almansa

N/Ref.ª: CE/IUCS/CESPU-11/22 **Data:** 2022/março/15

Assunto: - **Parecer relativo ao Projeto de Investigação:** 6/CE-IUCS/2022
- **Título do Projeto:** *"Observation of Barr bodies in dental pulp tissue and its relevance for sex estimation on a forensic context"*
- **Investigador responsável:** Zacharie Martinez-Almansa
- **Orientadora:** Prof Doutora Alexandra Sofia Pereira Teixeira

Exmo. Senhor,

Informo V. Exa. que o projeto supracitado foi analisado na reunião da Comissão de Ética do IUCS, da CESPU, Crl, no dia 10/03/2022.

A Comissão de Ética emitiu um parecer **favorável** à realização do projeto tal como apresentado.

Com os melhores cumprimentos.

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Presidente da Comissão de Ética do IUCS

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Annex 2 – Informed consent

DECLARAÇÃO DE CONSENTIMENTO INFORMADO

_____ (nome completo), compreendi a explicação que me foi fornecida, por escrito e verbalmente, pelo Investigador responsável, tendo-me sido dada oportunidade para colocar as perguntas que julguei pertinentes, e para as quais obtive resposta satisfatória.

Compreendo que o presente estudo pretende fazer uso dos meus dentes, após a sua extração, para fins de investigação, onde se procura analisar por métodos histológicos as células da polpa dentária e avaliar a sua aplicação forense, não estando previstos riscos associados.

Tomei conhecimento de que, de acordo com as recomendações da Declaração de Helsínquia, a informação que me foi prestada versou os objetivos, os métodos, os benefícios previstos, os riscos potenciais e o eventual desconforto. Além disso, foi-me afirmado que tenho o direito de decidir livremente aceitar ou recusar a todo o tempo a sua participação no estudo. Sei que posso abandonar o estudo e que não terei que suportar qualquer penalização, nem quaisquer despesas pela participação neste estudo.

Foi-me dado todo o tempo de que necessitei para refletir sobre esta proposta de participação. Nestas circunstâncias, consinto participar neste projeto de investigação, tal como me foi apresentado pelo investigador responsável sabendo que a confidencialidade dos participantes e dos dados a eles referentes se encontra assegurada, e que os dados deste estudo serão irreversivelmente anonimizados.

Gandra, ____ de _____de 20__

Assinatura do participante:

_____

Investigador responsável:

Assinatura:_____

Dados de contacto: Telemóvel _____;

Email: _____

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