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Histological transformations of the dental pulp as possible indicator of post mortem interval: a pilot study

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ABSTRACT

Background: The correct estimation of the post mortem interval (PMI) can be crucial on the success of a forensic investigation. Diverse methods have been used to estimate PMI, considering physical changes that occur after death, such as mortis algor, livor mortis, among others. Degradation after death of dental pulp is a complex process that has not yet been studied thoroughly. It has been described that pulp RNA degradation could be an indicator of PMI, however this study is limited to 6 days. The tooth is the hardest organ of the human body, and within is confined dental pulp. The pulp morphology is defined as a lax conjunctive tissue with great sensory innervation, abundant microcirculation and great presence of groups of cell types.

Aim: The aim of this study is to describe the potential use of pulp post mortem alterations to estimate PMI, using a new methodology that will allow obtainment of pulp tissue to be used for histomorphological analysis. The current study will identify potential histological indicators in dental pulp tissue to estimate PMI in time intervals of 24 h, 1 month, 3 months and 6 months.

Materials and method: This study used 26 teeth from individuals with known PMI of 24 h, 1 month, 3 months or 6 months. All samples were manipulated with the new methodology (Carrasco, P. and Inostroza C. inventors; Universidad de los Andes, assignee. Forensic identification, post mortem interval estimation and cause of death determination by recovery of dental tissue. United State patent US 61/826,558 23.05.2013) to extract pulp tissue without the destruction of the tooth. The dental pulp tissues obtained were fixed in formalin for the subsequent generation of histological sections, stained with Hematoxylin Eosin and Masson's Trichrome. All sections were observed under an optical microscope using magnifications of 10× and 40×.

Results: The microscopic analysis of the samples showed a progressive transformation of the cellular components and fibers of dental pulp along PMI. These results allowed creating a chart of qualitative and quantitative parameters to be used on the estimation on PMI based on microscopic degradation of dental pulp.

Conclusions: The histological transformations of dental pulp as a function of time can be used as PMI indicators.

1. Introduction

The tooth is the hardest organ of the human body. It is formed by pulp tissue, which is within a rigid layer of mineralized dentin, covered by enamel on the crown and cementum on the root [1]. This characteristic of dental pulp gives it a high mechanical resistance against the aggressions of the environment and the surrounding microorganisms. Dental pulp is a non-mineralized oral tissue composed by soft, vascular, lymphatic connective tissue and nerve elements that occupy the central pulp cavity of each tooth. The pulp has a smooth and gelatinous consistency, has great sensory innervation, rich microcirculation and cellular and fibrous variety (loose connective tissue) that makes it a unique tissue on the human body [2]. Some of the properties of the pulp are given by the rigid and mineralized dentin that surrounds and encloses it, hence this tissue may be helpful on the study of time of death [3]. Pulp cavity extends along from the crown to the apex by the root of the tooth, where blood vessels, lymphatic and nerves of pulp come in and out of the tooth, thus creating a communication channel

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between pulp and the tissues around it. Histologically, the types of cells present on the pulp are fibroblasts, odontoblasts and undifferentiated mesenchymal cells, together with other cells from the immune system such as macrophages, lymphocytes, etc. that are needed for the maintenance and defense of the tissue [4].

The fibrous matrix is composed by collagen fibers of type I and II which are present in disaggregated and randomly dispersed form, with greater density around blood vessels and nerves. Collagen type I is believe to be produce by odontoblast and by dentin itself. Collagen type II is most likely produced by fibroblasts of the pulp due to its increase in frequency with the age of the tooth. The oldest pulps contain more of the packaged and diffuse types of collagen. The fundamental substance is the environment that surrounds both cells and fibers of the pulp, rich in proteoglycans, glycoproteins and large amounts of water [4].

The great number of undifferentiated mesenchymal cells (as perivascular cells) in the pulp eases the recruitment of new differentiating cells to replace other when are lost, specifically odontoblasts. Odontoblasts are located on the external region of the pulp, next to the dentin component. These cells are responsible for dentin secretion and formation of dentinal tubes on the crown and root of the tooth [5].

The estimation of PMI has a great importance and implication on the criminal and forensic investigation. After the death of an individual occur different process of degradation and putrefaction of the body, parameters which have been used widely for the determination of PMI, such as *mortis algor*,*livor mortis*, *rigor mortis*, DNA degradation [6], ARN degradation [7], postmortem biomarkers [8–11], bacterial succession and forensic entomology [12,13], among others that can provide an estimate PMI. It has been described that the potential use of molecular markers like ARN degradation as function of time as an indicator for PMI. Recently, Bishop & Cols publication, they studied the characterization of the degradation of RNA as a function of time on porcine dental pulp, however the usefulness of this approximations is limited to 6 days [7,8].

Nevertheless, up until now there exists few studies that can establish PMI from the study of the integral degradation of dental pulp.

The studies found on which dental pulp is used to estimate PMI are few and they have limited time intervals where they do not overcome 7 days postmortem. Caballín, Gawande, Mehendiratta and Vavpotic studied histological variations that occur on post mortem pulp, making longitudinal and transversal conventional sections of the demineralized tooth, which allows observing the morphology of dental pulp and dentin. The use of conventional section of the tooth implies the complete destruction of the tooth and limits the analysis to a short PMI [14–17]. The aim of this study is to describe the potential use of dental pulp modifications to estimate PMI, using a new methodology [18] that will allow obtainment of pulp tissue in integral conditions that may be used for histological analysis in time intervals that have never been studied before, from 24 h to 6 months.

2. Material and method

2.1. Study design

In this pilot study collection, manipulation and analyses on human tissues and organs were performed within the framework of the Bioethics committee of the Dental School from Universidad de los Andes. An informed consent was especially designed for the research and was signed by every individual who donated a dental piece for the study and by the principal investigator. Dental samples were donated by patients who had extraction of third molars for orthodontic reasons, at Dental Integral Center Cedin Ltd, Paine, Chile. The ages of the individuals, from both genders, were between 20 and 40 years old.

This experimental design considered the day of extraction as equivalent to the time of death, given that at that moment the neurovascular bundle, which gives vitality to the tooth, is sectioned on the apex initiating a process of anoxia and cellular lysis.

2.2. Postmortem intervals for the study of the samples

The sample size was calculated arbitrary because it is the first study of this type.

This study used 24 samples that were grouped in 4 experimental groups:

1. Experimental group 1: 6 dental pulp 24 h from extraction.
2. Experimental group 2: 6 dental pulp 1 month from extraction.
3. Experimental group 3: 6 dental pulp 3 months from extraction.
4. Experimental group 4: 6 dental pulp 6 months from extraction.

All dental specimens were manipulated and storage under laboratory conditions between 20–25 °C, 1 at. and 40% environmental humidity.

2.3. Obtaining dental pulp

According to the method described previously for the authors [18]. Each dental sample extracted was kept on a 5 ml polypropylene sterile tube (Micro biologic Rubilator, S.L., Barcelona) until completion of 24 h, 1 month, 3 months or 6 months of PMI period, respectively. A digital radiography was taken for each sample in order to accurately plan access to the pulp cavity. The X-ray equipment used was Sirona, Heliodent, and Charlotte, NC 28273, USA. The tooth must be positioned sideways, in other words in its mesial or distal surface for the radiography to show an image on a facial-lingual orientation. The analysis of the distances on the images was made using the software SIDEXIS (Sirona®). Samples were washed on a saline solution buffered at pH 7.4 by phosphate (Phosphate Buffered Saline, PBS 1x, Hyclone) with successive washes on a volume of 10 ml during 1 min and vortexed (Mrc®).

For external rehydration the samples were immersed in buffered external hydration solution (SRE) at pH 7.4 for 16 h at 37 °C. In a vertical flow hood dental samples were placed on petri dish and perforation was performed on the occlusal surface (upper part of the crown) until communication with pulp chamber using turbine with diamond circular burs and micro-motor with carbide circular burs of 1–2 mm of diameter. Also a lateral perforation was made to gain access to the root canal on the apical third of the root using turbine with diamond circular burs, in accordance with the distances measured before. Once perforation was done, communication with pulp chamber and root canal was corroborated using number 20 K type endodontic files. Perforated samples were immersed in sterile internal hydration solution (SRI) for 48 h at 37 °C and 5% CO_2 in an incubator (modelo MCO 17 AC Sanyo®). Once the tissue is hydrated the pulp is removed with Automated Endodontic Files (1.5/25 mm SAF®) and deposited on petri dish to be fixed in formalin buffered at 10% (Sigma). Each tube was labeled with a number for its posterior histological processing.

2.4. Histology

Standard procedures of dehydration and embedding with histologic paraffin were performed. The orientation of the tissue for inclusion was made so that the major axis of the sample was parallel to the cut area of the paraffin block. The cuts were made on a minot-type rotary microtome (Sakura accut Cut) with a thickness

of 3 μm and were mounted on their corresponding slide. Hematoxylin Eosin (HE) (Sigma) and Masson Trichrome (TM) stains (Merck KGaA) were realized following standard staining protocols. Macroscopic images were acquired with a Canon SX500 IS camera.

2.5. Optical microscopy

Samples were observed with magnification 40× and 100× with the optical microscope Motic Digital BA310 equipped with a built-in 3.0 mega pixel digital CMOS camera that allowed the capture of images processed through the program Micrometrics® SE Premium (Version 3.0).

2.6. Qualitative analysis

Qualitative analysis included the identification of morphologic structures of pulp in sections stained on both protocols. Two blind operators evaluated the histological sections of all the samples.

2.7. Quantitative analysis

Quantitative analysis included the quantification of nucleus and collagen fibers with the software Image J (NIH). Two blind operators measured all the samples.

2.8. Statistics

Averages and standard error were calculated for number of nucleus on the stained samples with HE with magnification 40×. Averages and standard error for the percentage of collagen fibers present on the complete area were calculated from the stained image with TM with magnification 40×. A chart was design on Excel with the data analyzed. Anova-one way and paired T-Test were applied with software Graph Pad Prism 7 to calculate significant differences with IC 95%.

4. Results

4.1. Retrieval of pulp tissue

Using the new technology described on the methodology, it was possible to obtain intact dental pulp tissue from all the teeth with

Fig. 2. Dental pulp recovery. Free floating complete dental tissue at PMI of 24 h.

Fig. 1. Dental pulp recovery. Complete dental pulp tissue obtained with the SAF file system at PMI of 24 h.

PMI analyzed (Fig. 1). It was a homogeneous tissue of white color with pink areas, of soft texture, with a tubular form characteristic of pulp tissue where the mayor axis was 50 mm and 10 mm diameter. This sample was enough for the histology study (Fig. 2).

4.2. Histology

The studied samples at 24 h PMI (Fig. 3) showed a connective tissue with homogeneous structure, with abundant basophilic nuclei with an average of 602 ± 214 (Fig. 7) and with different cell morphology characteristic to cell types that can be found on a lax connective tissue as dental pulp, such as mesenchymal stem cells, fibroblasts, endothelial and immune system cells. Also homogenous chromatin and in some samples the lumen of blood vessels or lymphatic with intact endothelial cells that had a well-defined figure of the nuclei (Fig. 3A, B and Table 1).

Collagen fibers in the tissue represented an average percentage of 21% ± 15 from total area of the sample (Fig. 8) and they formed a thin homogeneous network distributed throughout the connective tissue (Fig. 3B, Table 1).

At 1-month PMI samples showed connective tissue with a partial loss of homogeneity with a slight vacuolation on some areas (Fig. 4A and B, Table 1). The average of nuclei was 379 ± 142 (Fig. 7) distributed in the center of the sample and some grouped on the periphery of the tissue (Fig. 4A, B, Table 1). Nuclei preserved intact morphology. It was found neither blood nor lymphatic vessels in the samples. The analysis of variance (one way ANOVA) showed that F value was 11,38 and the p value was 0,0002. The t-test showed statistically *significant difference* between groups of PMI at 24 h and 6 months p = 0,0012 also at PMI 3 and 6 months p = 0,0095.

Collagen fibers in the samples represented an average percentage of 36% ± 20 from total area of the sample (Fig. 8). The fibers were mostly located in the center of connective tissue and it also showed a loss of fine lattice structure and the appearance thickened collagen fibers forming large, well-defined bundles (Fig. 4B Table 1).

The samples of 3 months PMI showed disaggregated areas in the extracellular matrix and vacoulation of other areas in the connective tissue (Fig. 5A and B, Table 1). Nuclei were in average 294 ± 154 (Fig. 7). It was revealed as a loss of nuclei morphology, and they were grouped and located in the periphery of the tissue (Fig. 5A). There was no evidence of blood or lymphatic vessels. The percentage of collagen fibers was 40% ± 15, from total area of the sample (Fig. 8). The fibers were organized in large groups of well-

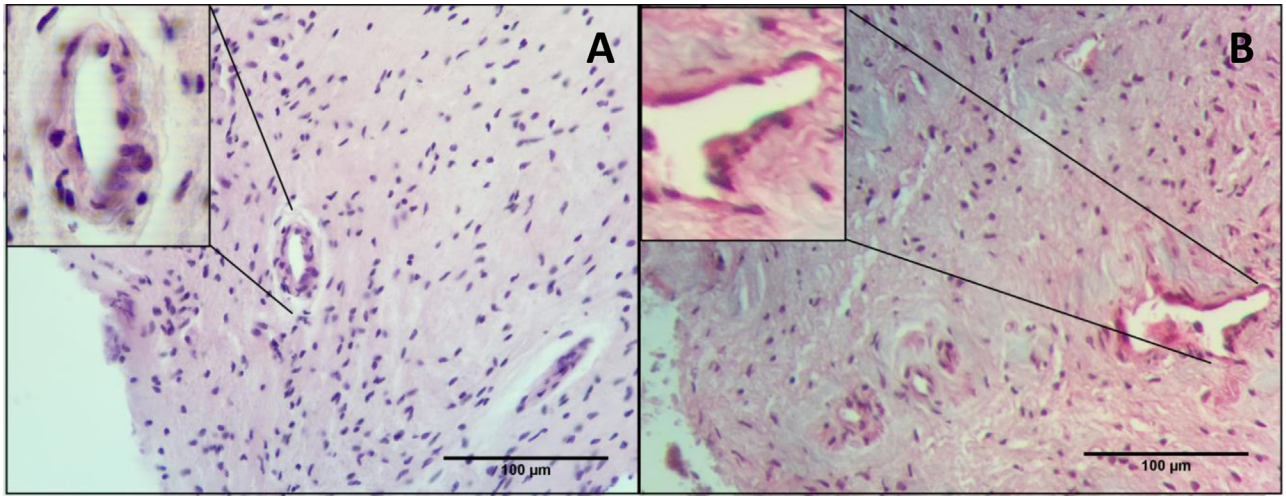

Fig. 3. Recovered dental pulp tissue at PMI of 24 h stained with HE and Masson dyes. (A) Representative haematoxylin and eosin-stained sections of dental pulp tissue of N = 6 samples. It shows homogeneous stromal component and a large amount of nuclei with different morphologies due to the cells types that compose the cell-rich zone of dental pulp. Presence of blood vessels of different size, Scale bars = 100 µm, magnification 40×. Solid line inset shows the blood vessel in a transversal section, lumen surrounded by endothelial cells nuclei, magnification 100×. (B) Histological preparations of dental pulp with Masson stain of N = 6 samples. It shows light blue collagen fibers with a wavy shape, distributed randomly and homogeneously on the tissue. The elongated irregular lumen surrounded by endothelial cell nuclei is compatible with a lymphatic vessel lumen, Scale bars = 100 µm, magnification 40×. Inset of figure shows a lymphatic vessel in a cross section with collagen fibres present, magnification of 100×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

defined bundles, without losing the proportion between the collagen fibers and the extracellular matrix (Fig. 5B, Table 1).

At 6 months PMI, the samples showed a totally disaggregated structure of the tissue (Fig. 5A, Table 1) with an average of nuclei of 9 ± 11 (Fig. 7). There is a raise in the percentage of collagen fibers, $68\% \pm 24$ from total area of the sample, and a considerable loss of extracellular matrix (Fig. 5B, Table 1). The analysis of variance (one way ANOVA) showed that the p value was 0, 0071. The t-test showed significative differences between groups of PMI 24 h and 6 months p = 0,0055 also PMI 3 and 6 months p = 0,0403 (Fig. 6).

5. Discussion

Estimating post mortem interval remains as one of the most complex unsolved problems in forensic pathology. The accuracy and applicability of the procedures depends on the characteristics and circumstances of death and how long is the postmortem interval. There are few references that describes the utility of teeth or dental tissues in the estimation of PMI. Most of them focus on structural, biochemical, and morphological analyses of dentin-pulp complex in samples with early PMI. In 2003 Boy et al. [19]

Table 1
Summary of the histological transformations of the human dental pulp at PMI of 24 h, 1 month, 3 months and 6 months.

PMI	Stain Hematoxilin and Eosin	Stain Masson's Trichrome
24 h	• Big amount of nuclei with homogeneous distribution in the conjunctive tissue • Circular, ovoid, and extended shape nuclei that suggest different cell types: macrophages, fibroblasts • Well conserved endothelial cells of blood vessels • Homogeneous distribution of extracellular matrix without vacuolization	• Lax conjunctive tissue • Great amount of nucleated cells on homogeneous distribution over the tissue • Higher proportion of fundamental substance on collagen fibres • Big amount of nuclei with nuclear morphology well conserved • Blood and lymphatic vessels. • Thin and wavy collagen fibres, without a defined pattern distributed homogenous in the tissue
1 month	• Nuclei redistribution from the centre to the periphery of the tissue • Nuclear morphology well conserved • Absence of blood and lymphatic vessels • Partial loss of homogeneity of extracellular matrix. Areas with vacuolization	• Nuclear morphology well conserved • Dense wavy collagen fibres. Partial loss of homogeneity of the extracellular matrix
3 months	• Nuclei in the periphery of the tissue • Not defined nuclei morphology, with irregular chromatin • Extracellular matrix with disaggregated areas and vacuolization areas	• Nuclei in the periphery of the tissue • Absence of blood and lymphatic vessels • Collagen fibres with homogeneous distribution • Vacuolization of conjunctive tissue
6 months	• Fibrous appearance of the tissue • Low nuclei density • Random distribution of nuclei • Disaggregated extracellular matrix and with total loss of homogeneity	• Low cellular density • Abundant collagen fibres • Disaggregated extracellular matrix

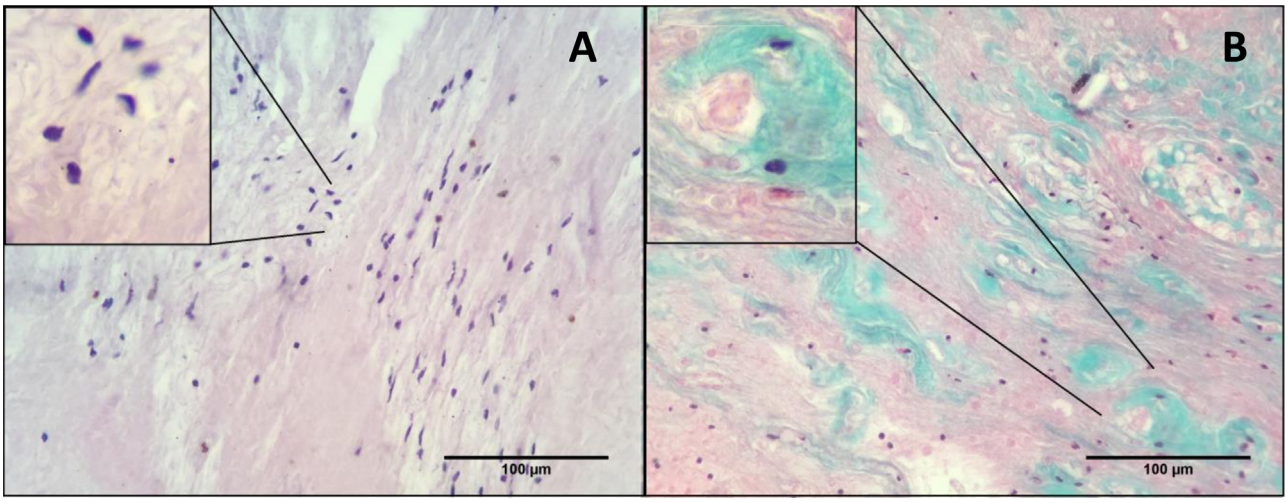

Fig. 4. Recovered dental pulp tissue at PMI of 1 month stained with HE and Masson dyes. (A) Representative haematoxylin and eosin-stained sections of dental pulp tissue of N = 5 samples. It shows a slightly vacuolated stroma with fusiform nuclei clusters. Decrease in number of nucleic. Solid line inset shows nuclear morphology and the vacuolization of the stroma, Scale bars = 100 µm, magnification 40×. (B) Histological preparations of dental pulp with Masson stain of of N = 6 samples. It shows large bunches of light blue collagen fibres with nuclei clusters. Square inset shows a bundle of light blue collagen fibres in a wavy shape with presence of nuclei trapped in between the fibres, magnification 100×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used degradation of dental pulp DNA as PMI marker for determinations up to 144 h (6 days). In 2009 Vavpotic et al. [16] counted the number of odontoblasts in the pulp related to the PMI, he described that after 5 days was not possible to analyze histologically the presence of whole cells. Caballin et al. [17] in 2010 performed the assessment of the histological dental pulp modifications for estimating PMI. Preliminary results showed three stages in the gradual loss of pulp parenchyma and its organization up to the seventh day. In these cases, methods only can be used in estimating early PMI up to one week. The current data represents the first histological study of dental pulp for estimation of PMI up to 6 months. The used methodology adds value to the tooth and pulp content, whereas other researchers, in their attempt to obtain dental tissues for study, have treated the tooth and its components in a very aggressive manner (pulverizing, demineralization, cutting with discs). This methodology improves existing designs incorporating an increase in tooth value and pulp content in order to prevent the destruction of tooth and also provide the best conditions for tooth structure and content to enable a full recovery of pulp content. This is achieved by protecting the tooth and its pulp content, recreating real life conditions, that is, high outside humidity, at 37 °C and with inside humidity in the pulp chamber. The use of low-speed rotary instruments for removing pulp content is an important part of the methodology because it produces less heat and damage than others methods commonly used. Low-speed rotary instruments used are designed for the complete removal of the dental pulp or its remains, allowing morphological analyses, which was totally impossible until now. SAF System (ReDent Nova, Israel) was used for obtaining rehydrated dental pulp content. SAF System is a hollow file in a low speed rotation tool. The design of the file is very sophisticated; as the abrasive net acts as a fishing net that retains

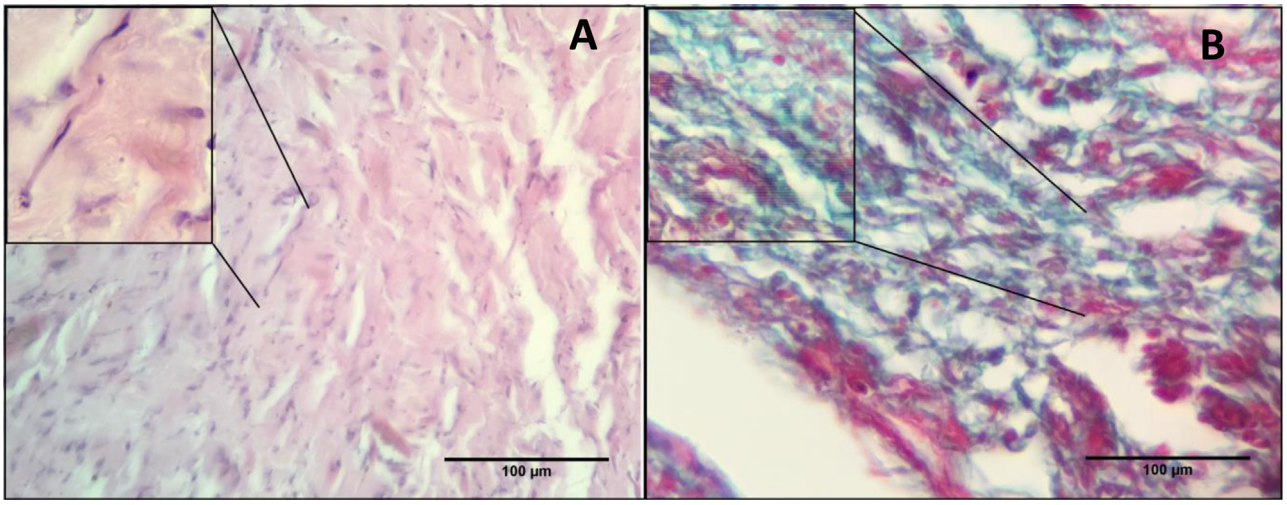

Fig. 5. Recovered dental pulp tissue at PMI of 3 months stained with HE and Masson dyes. (A) Representative haematoxylin and eosin-stained sections of dental pulp tissue of N = 6 samples. Decrease in number of nucleic. Nuclei redistribution to the periphery of the tissue (left side), evident loss and vacuolization of some areas of stroma, Scale bars = 100 µm, magnification 40×. Inset solid line shows the nucleus and cytoplasm of a fusiform cell, magnification 100×. (B) Histological preparations of dental pulp with Masson stain of of N = 6 samples. It shows evident loss and vacuolization of some areas of stroma. A prevailing presence of ligth blue collagen fibres as well defined bundles, presence of cellular cytoplasm (red), Scale bars = 100 µm, magnification 40×. Square inset shows collagen network, magnification 100×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

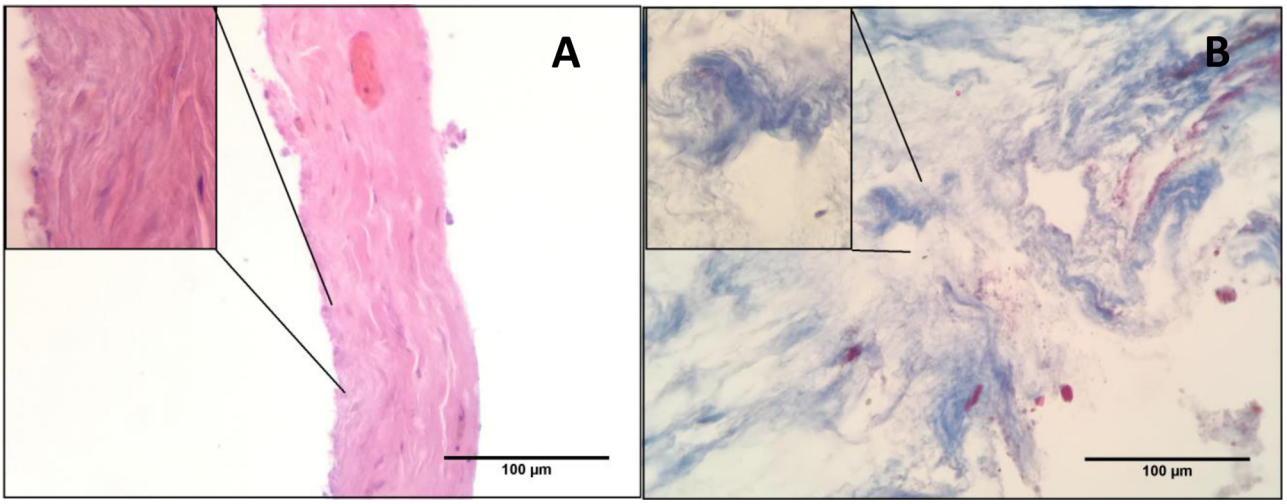

Fig. 6. Recovered dental pulp tissue at PMI of 6 months stained with HE and Masson dyes. (A) Representative haematoxylin and eosin-stained sections of dental pulp tissue of N = 5 samples. Decrease in number of nucleic It shows disaggregation and vacuolization of connective tissue stroma. Scale bars = 100 µm, magnification 40×. Square inset shows cellular nuclei from connective tissue, magnification 100X. (B) Histological preparations of dental pulp with Masson stain of of N = 6 samples. Predominance of collagen fibers as small fibres and large wavy bundles with some cellular remains (red) from the cytoplams, Scale bars = 100µm, magnification 40×. Square inset shows an area of concentration of collagen fibres, magnification 100×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the content without damaging the remains of pulp. In regards to the obtainment of dental pulp tissue, the new technology proposed showed to be efficient in the retrieval of dental pulp at the PMI (24 h, 1 month, 3 months and 6 months), in contrast to other methods used by other investigators [17–20]. The method allowed obtaining pulp tissue in integral conditions to be used for histological analysis. Studies found on which dental pulp was used for estimating PMI at different time intervals did not overcome 7 days post-mortem [14–17]. The studied histological variations that occur on post-mortem pulp, by longitudinal and transversal conventional sections of the demineralized tooth, allowed observing the morphology of dental pulp and dentin. The use of conventional section cuts of the tooth implies the complete destruction of the tooth and the analysis its restricted to a short PMI [14–17]. The present study used the new technology and described the potential use of dental pulp modifications to estimate PMI. The results showed histological cuts that are possible to analyze from 24 h up to 6 months. This methodology is not limited to time, allows extending evaluation interval up to 6 months pos-mortem. The time 6 months PMI had never been studied before. Histological analysis permitted observing morphological features that are characteristic of a lax tissue at the PMI studied. The analysis of the results was qualitative and quantitative. Qualitative analysis showed morphological modifications according to the PMI. First stage at 24 h was considered as control image, because we could recognize cellular nuclei, blood and lymphatic vessels, collagen fibres and fundamental substance. As PMI increases, characteristic morphologic changes were observed for each time interval. We proposed a comparative chart based on the qualitative analysis, which correlates histological patterns at each PMI. In quantitative analysis, possible patterns were quantified regarding PMI, such as total number of nuclei and percentage of collagen fibres in the total area of the sample. It was determined that the number of nuclei showed a significant decrease as the PMI increased, in contrast with the percentage of collagen fibres that showed a significant increase in relation to the PMI. Significant differences were found between 24 h and 6 moths PMI; and between 3 months and 6 months PMI for the two parameters studied (number of nuclei and percentage of collagen fibres). However, there was not a significant difference between groups in comparison to the 1-month interval. These results suggests that in the present experimental conditions, the

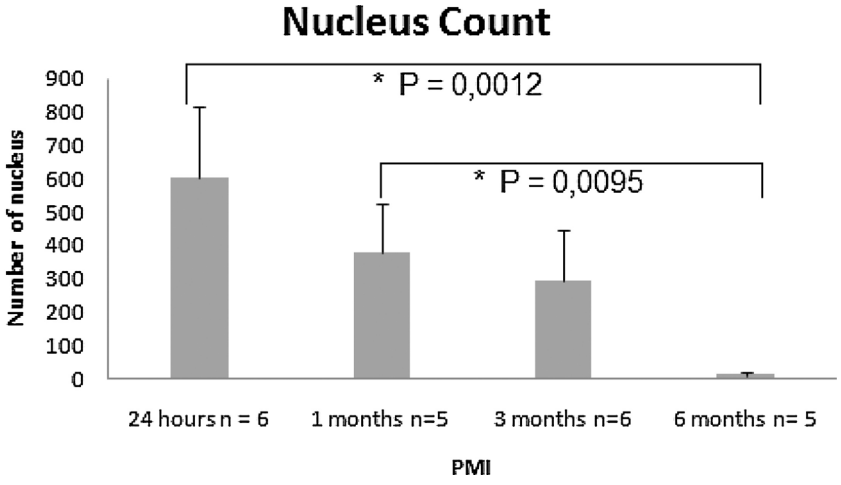

Fig. 7. Number of nucleus stained with HE at different PMI: 24 h, 1, 3 and 6 months of PMI. Statistical analysis shows P value, significative *P < 0,05.

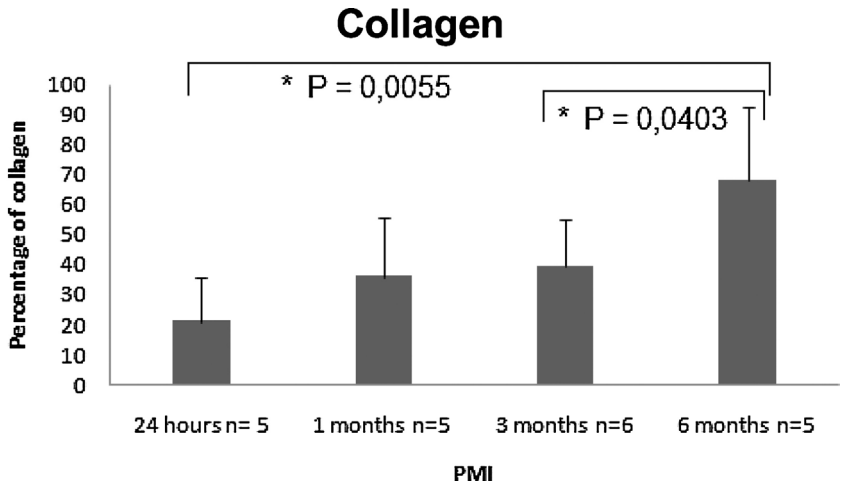

Fig. 8. Percentage of collagen fibres stained with Masson at different PMI: 24 h, 1, 3 and 6 months of PMI. Statistical analysis shows P value, significative *P < 0,05.

parameters analyzed give reliable information for the estimation of PMI at 24 h, 3 and 6 months. It has been hard for the forensic odontology area to propose methods that are easy to apply and feasible to be used in the estimation of PMI of more than one week, because the techniques for the recovery of dental pulp have not been able to produce useful tissue to be analyzed by histology. Furthermore, these techniques are not conservative since they cut or destroy the dental piece completely making it impossible to use for further investigations. Our technology provides an effective solution to both problems. Pulp tissue was treated under physiologic conditions in order to facilitate its retrieval and the histologic management of the sample [18].

6. Conclusion

It is necessary to acknowledge the work realized by other investigators referred previously, which had proposed the study of dental pulp for the first time for the estimation of PMI. We continued this research proposal but with a new methodology that extends the estimation of PMI up to 6 months. The qualitative and quantitative results support the use of this methodology in the estimation of PMI to be applicable to forensic caseworks. Further research should focus on using a higher sample number and in different environment conditions to allow a wider application of the methodology and eventually to help narrowing the PMI timeframe estimations.

Acknowledgments

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