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Decalcification-free technique on the analysis of dental pulp tissue: histological and immunohistochemical analysis

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ABSTRACT

Background: Histological techniques are essential for the microscopic study and investigation of the human dental pulp. The aim of this study was to evaluate the impact of decalcification-free technique by examining dental pulp morphology by histological staining with haematoxylin and eosin and immunohistochemistry.

Materials and methods: The sample consisted of 30 healthy third molars extracted for orthodontic indication, the pulp tissue was obtained by removing the mineralized tissues, separating the enamel and dentine and by marking with a flexible diamond disc on the coronal surface and longitudinal axis of the root. These guides made it possible to separate the fragments and obtain the pulp tissue for fixation and staining with H&E and subsequent immunohistochemistry with CD34 and S-100 antibodies.

Results: The technique showed preservation of pulp morphology with adequate preservation of microscopic structures. No alterations in tissue viability were observed. The staining allowed an accurate assessment of vascular and nervous components by means of CD34 and S-100 markers, respectively.

Conclusions: This technique allows preservation of pulp tissue, maintaining viable tissue for histological analysis and immunohistochemistry tests, as well as reducing sample processing time.

Keywords: CD34, histology, dental pulp, immunohistochemistry, S-100

INTRODUCTION

The dental pulp is a connective tissue consisting of collagen I and III fibres embedded in ground substance composed of chondroitin sulphate, hyaluronate, proteoglycans and interstitial fluid [8]. It is surrounded by highly mineralized tissues; enamel is composed of 95% carbonate hydroxyapatite, 4% water and 1% organic matter [1], dentine is composed of 70% hydroxyapatite crystals, 10% water and 20% organic matter [18]. The pulp is connected to the dentine by odontoblasts, as their cytoplasmic extension penetrates the dentine through the dentinal tubules. This anatomical relationship constitutes a functional unit called the dentin-pulp complex [11].

Microscopically, it is divided into four zones: the layer adjacent to the predentine is the odontoblastic layer; it contains odontoblasts arranged in parallel layers, followed by the cell-free zone called Weil's zone, which is composed of blood capillaries and unmyelinated nerve fibres [13]. This is followed by the cell-rich zone, which contains a high number of fibroblasts, macrophages, dendritic cells and undifferentiated mesenchymal cells and abundant capillary plexus; finally, the pulp centre is observed, consisting of connective tissue, blood vessels, nerve fibres and fibroblasts [16, 24].

Histological techniques allow microscopic observation of tissues, visualising their cellular and morphological characteristics, and recognising structural changes and pathology [21]. The process starts by obtaining a sample from extracted teeth or pulp tissue [6, 7]. This tissue is preserved using a fixative solution such as formaldehyde, picric acid, osmium tetroxide, ethanol, methanol, among others, avoiding degradation but maintaining the cellular architecture and details; this step is vital as the quality of histological sections, staining or molecular biology processes depends on it [3, 20].

For optimal fixation, the pulp tissue should remain in fixative solution at a volume ratio of 1:10 to 1:20 for 24 to 48 hours at room temperature [21]. Subsequently, decalcification is performed; Ricucci et al. [17], Shetty et al. [19], and Couve et al. [2], among others, perform their histological technique with decalcification, using formic acid or chelators for 4 weeks to 6

months; this process must be done with care as the acid can damage the tissue and compromise molecular or immunohistochemical tests. Subsequently, the tissue is dehydrated with alcohol and embedded in paraffin, facilitating sectioning; however, other resin-based materials such as methyl methacrylate and glycol methacrylate are available and are selected mainly depending on the objective of the study [12]. The tissue is carefully oriented in the cassette by determining the plane of section of the sample and embedded in paraffin blocks and sectioned on 3- to 5-micron slides. Tissue sections are low contrast and therefore require stains that react chemically with tissue structures. The routine stain is haematoxylin and eosin (H&E) established by Wissowzky [22] in 1875. Haematoxylin stains cell nuclei purplish blue and eosin stains the extracellular matrix and cytoplasm, including collagen fibres and muscle fibres, pink [4, 25].

Another high specificity staining technique is immunohistochemistry (IHC), which consists of specific antigen-antibody reaction, IHC antibodies mainly bind to enzymes or fluorescent dyes (which express colour for identification) and use specific antibodies to identify different cell lines such as blood vessels, lymphocytes, nerve cells, cancer cells, etc. [14]. Finally, these tissues, stained and mounted on slides, are observed under the microscope for description.

The aim of this study was to evaluate the impact of the decalcification-free technique by examining the morphology of dental pulp by histological staining with haematoxylin and eosin and immunohistochemistry from orthodontically extracted third molars.

MATERIALS AND METHODS

Pulp tissue was obtained from 30 freshly extracted third molars with fully formed roots, free of caries, periodontal disease, and restorations. All molars were extracted for orthodontic purposes from donor patients from dental practices and all subjects signed their informed consent authorising the donation of the teeth. The research project was reviewed and approved by the Ethics Committee of the Universidad Autónoma del Estado de México (2021/P11).

Immediately after extraction, the teeth were rinsed and cleaned with saline and transferred to the laboratory in a hermetic plastic jar with 10% buffered formalin, and pulp tissue was obtained on the same day of extraction. This process was carried out by making a mark with a flexible diamond disc (Plexoflex Fine Grain, DFS-Diamon, Riedenburg, Germany) 0.17 mm thick and 22 mm in diameter using abundant irrigation; the first mark was made in the coronal area below the amelocementary line (Fig. 1A), this was done around the crown to divide it from

the root with a spatula (Fig. 1B); with this cut the coronal pulp tissue in the root zone was exposed and for its removal a Glick 2 endodontic excavator was carefully introduced at the level of the pulp chamber attached to the dentine to carefully separate the soft tissue (Fig. 1C), when separated from the dentine with the same endodontic excavator a single intention cut was made on the floor of the pulp chamber to make a clean cut between the pulp of the chamber and the root pulp.

The second mark was made longitudinally to the midline of the root from vestibular to lingual or palatal without communicating side to side with the disc; then with a spatula it was carefully separated by dividing it into two parts (Fig. 1). The root tissue was carefully extracted with the dental explorer #5 following the root anatomy (Fig.1E); both tissues were kept in 10% buffered formalin fixation to be stained to distinguish whether the root tissue was mesial, distal or palatal. This process was started by blotting the formalin from the tissue with absorbent paper and using a fine brush, red dye (MARK-IT TM, Red Dye, Thermo Fisher Scientific, MI, USA) was applied for mesial tissue and green dye (MARK-IT TM, Green Dye, Thermo Fisher Scientific, MI, USA) for distal or palatal tissue. Excess dye was removed with absorbent paper to place 5% acetic acid on the pulp tissue, which served to fix the dye to the tissue. The stained tissue was placed on filter paper and in the embedding cassette and fixed in 10% buffered formalin for 24 hours at room temperature: 1:10 volume ratio.

After this time, the pulp tissue was dehydrated in alcohol and placed in the paraffin embedding cassette. The paraffin blocks were cut into 5 μm sections on a manual microtome (CUT 4062, Slee Medical GmbH, Nieder-Olm, Germany) and mounted on slides with H&E staining.

Immunohistochemistry was performed with the streptavidin-biotin system and signal amplification with tyramide (ImmunoMax method). Paraffin blocks were cut on a manual microtome (CUT 4062, Slee Medical GmbH, Nieder-Olm, Germany) into 3-μm sections and mounted on loaded slides (Surgipath® X-tra®, Leica Microsystems, IL, USA). Specimens were deparaffinised in an oven at 60°C for 30 min and the tissue was hydrated in a descending train of alcohols. Antigen retrieval was performed in pressure cooker with buffered citrate solution pH 6 (10× Citrate Buffer, Diagnostic Biosystems, CA, USA) for CD34 and EDTA pH 9 (10× Tris-EDTA Buffer, Diagnostic Biosystems, CA, USA) for S-100 for 40 min. To block endogenous peroxidase, 3% hydrogen peroxide (Tissue Primer, Diagnostic Biosystems, CA, USA) was used

for 5 min. Primary antibodies were incubated for 45 min (Tab. 1). The slices were incubated with secondary antibody (PolyVue Plus Mouse/Rabbit HPR Label, Diagnostic Biosystems, CA, USA) and streptavidin peroxidase complex for 20 min, the chromogenic reaction was with diaminobenzidine (DAB) and finally the slices were counterstained with Harris haematoxylin.

Slides were examined at 10× to 40× with an optical microscope (DM750, Leica Microsystems, IL, USA) equipped with a Leica 1CC50W microscope camera with which a series of photographs were taken. Pulp tissue preservation and morphology were analysed with H&E staining. For immunohistochemistry, CD34 and S-100 were considered positive when brown colour was detected in endothelial cells and nerve tissue.

RESULTS

Slides stained with H&E were analysed; the results showed pulp tissue with adequate fixation and adequate morphological structures, observing in the root zone that the tissue maintained the shape of the root canal (Fig. 2A) and in the pulp chamber the presence of pulp horns (Fig. 2B).

The histoarchitecture showed the odontoblastic zone of the coronal portion was observed in 24% discontinued and 76% of the samples continuously, as well as the Weil zone, as opposed to the cell-rich zone and the pulp centre which were observed in 100% of the samples (Fig. 2C). In the root pulp, the odontoblastic zone and the Weil zone were observed in 14% of the samples, the cell-rich zone in 84% and the pulp centre in all samples. The odontoblastic zone was mainly shown in the coronal pulp and was reduced in the root pulp. As the tissue approached the apex, the cell-rich zone became more evident, as did the pulp centre (Fig. 2D). Cellular details were clearly visualised in appropriate contrast; observing the eosinophilic colour of the collagen fibres, blood vessels, erythrocytes; and the basophilic colour of the pulp tissue cells (Fig. 2E).

The expression of S-100 (Fig. 3A, B) and CD34 (Fig. 3C) antibodies was positive in all samples, showing light brown staining of nerve fibres (S-100) and endothelial cells (CD34) of blood vessels, both distributed longitudinally.

DISCUSSION

The histological study of pulp tissue is challenging as it is surrounded by calcified structures, histological processing from fixation to staining can suffer from difficulties in its development, for example, lack of fixation in the specimens or tearing of the pulp tissue at the time of procurement. The purpose of this study was to evaluate the impact of the decalcification-free technique on dental pulp morphology, using histological staining with haematoxylin and eosin and immunohistochemistry; in addition to providing an updated protocol of the technique, proving useful and practical for future research.

The pulp tissue fixation process is of vital importance to preserve the cellular elements, allowing their microscopic evaluation and the use of histological and immunohistochemical staining. This process allows tissues to preserve their morphology and avoids cell destruction by autolysis or putrefaction due to bacterial contamination, compromising histological processes [15, 20].

The fixation technique used was performed in two phases, starting immediately after extraction when the teeth were placed in 10% buffered formalin for transfer to the laboratory, this allowed the fixative to enter through the root canal and the second fixation was performed immediately after removal of the enamel and dentine; in contrast, Daud et al. [3] studied four different ways of preparing the tooth for pulp tissue fixation: tooth cut longitudinally at the midline, cut from the apical third of the root, cut coronally at the cervical line and fixed intact tooth, were subjected to decalcification with formic acid for 2 weeks, the result showed that the fixative did not penetrate sufficiently through the root canal due to the short fixation time, showing changes in shrinkage, cellular organization, clarity of pulp morphology and H&E staining; Therefore, the result was classified as good but not excellent compared to their technique of extracting the apical third of the root, compromising the histological observation of the apical pulp tissue. In our study, although the teeth were in fixative solution while they were transferred to the laboratory, the main fixation occurred after the removal of the hard tissues, this process allowed a greater penetration of the fixative solution to the tissue, achieving an excellent H&E staining, allowing a perfect observation of the architecture of the dental pulp, both in the coronal zone with its pulp horns and in the root zone, preserving the shape of the canal. It also allowed the cellular elements of the tissue to be adequately stained and the epitopes to be kept intact for molecular tests such as immunohistochemistry.

The technique to obtain the pulp tissue is essential to maintain its integrity, as it is a soft tissue surrounded by highly mineralised tissues; when these tissues are removed, the pulp tissue may suffer structural damage. Keklikoglu and Daud [3, 12] in their studies performed a root section with a carbide bur, making a longitudinal cut to divide the root into two parts, which resulted in tearing and loss of pulp tissue structure; In contrast, we use a fine-grained diamond disc, marking the surface of the tooth without communicating the disc from side to side, and with a spatula exerting light pressure we separate the parts, taking care of the central part of the root and the crown, avoiding tearing the tissue and thus preserving most of the pulp tissue.

When pulp tissue is obtained directly in vivo from a diseased tooth, it is obtained by accessing the pulp chamber to take the tissue sample with an endodontic excavator and the root pulp tissue with Hedström files, which must be immediately fixed in buffered formalin for 48 hours. This process will keep the tissue in an optimal state for histological processing and staining [15]. For the study of pulp tissue in extracted teeth, our technique allows a good morphological evaluation in H&E staining and immunohistochemistry, as it avoids endodontic access to the pulp chamber, preventing mechanical instruments such as carbide burs from damaging the tissue. Furthermore, in the root zone, it prevents endodontic files from tearing the pulp tissue, preserving the original morphology, maintaining curvatures and bifurcations.

For decades, studies of pulp tissue have been based on decalcification techniques, which have the advantage of removing calcium salts from mineralised tissues while preserving their organic components, thus allowing easy cutting of calcified tissue while maintaining enamel, dentine, and pulp in one histological section. Gupta et al. [9] report that 10% nitric acid decalcifies teeth rapidly in 7 days, 10% formic acid in 10 to 12 days, 8% potassium formate in 18 to 20 days and finally EDTA in 28 to 30 days. These acids, in contact with the tissue for longer than necessary, can damage it, affecting the staining characteristics. On the contrary, the technique without decalcification allows for a shorter processing time of the samples, having them ready in 2 days since after the fixation step they can be processed immediately maintaining the morphological integrity of the pulp tissue with the epitopes intact for immunohistochemical tests since the tissue is not subjected to acidic substances, which agrees with Johansson et al. [10]. It can also be used in studies where bacterial staining is required, such as the modified Brown and Brenn technique, the PAS technique for mucopolysaccharides [15], ELISA or PCR.

With respect to the pulp histoarchitecture, Tjäderhane et al. [23] and and Frank et al. [5] found that the odontoblastic zone is present mainly in the coronal zone and decreases in visibility towards the apical zone due to the decrease in dentinal tubules; therefore, in the root zone, the cell-rich area and the pulp centre are more evident, which coincides with our results.

The weak point of this technique is that in the histological sections the relationship of the dentine with the odontoblastic layer is not observed, as when the hard tissues are removed this relationship disappears. Another point is that the odontoblastic layer is not continuously observed, and this may be due to the removal of tissue from the pulp chamber. However, this technique can be used in research where it is necessary to obtain processed samples in a short period of time; also research where healthy pulp tissue from extracted teeth is required as a control group to compare it with diseased tissue, observing changes in its cellular components, morphology, vascularisation, among others.

CONCLUSIONS

The results obtained in this study demonstrate that the immersion fixation technique and the application of the free decalcification technique allows the preservation of pulp tissue for H&E staining, immunohistochemical techniques or molecular procedures; This makes it possible to reduce sample processing time, evaluate microscopic components, and successfully express immunohistochemical markers. It is suggested as a tool in pulp tissue research for use in future studies.

ARTICLE INFORMATION AND DECLARATIONS

Data availability statement

The authors declare that all data underlying the results are available as part of the article and no additional source data are required.

Ethics statement

The research project was reviewed and approved by the Ethics Committee of the Universidad Autónoma del Estado de México (2021/P11).

Author contributions

Maria Teresa Hernandez-Solis: design, execution, interpretation of the data being published. **Edith Lara-Carrillo:** conception, design, scientific supervision. **Victor Hugo Toral-Rizo:** conception, design, interpretation of the data being published. **Ronell Eduardo Bologna-Molina:** conception, scientific supervision.

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None.

Conflict of interest

The authors declare that there is no conflict of interest.

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Table 1. Monoclonal antibodies used in this study

Antibodies	Isotype	Clone	Dilution	Company
CD34	Mouse Monoclonal Antibody IgG1, kappa	QBEnd/10	1:100	Diagnostic Biosystems
S-100	Mouse Monoclonal IgG2a, Kappa	4C4.9	1:100	Diagnostic Biosystems

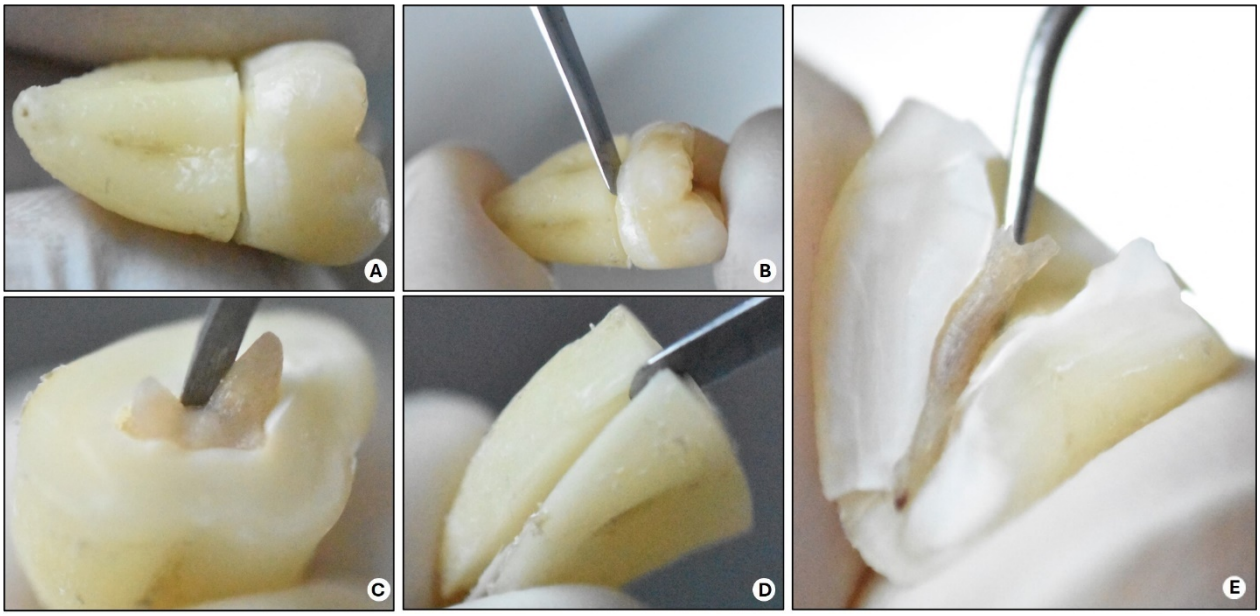

Figure 1. Method of obtaining pulp tissue with the free-decalcification technique. **A.** Coronal marking with a diamond disc at the cemento-enamel junction. **B.** Spatula was used to separate the coronal part of the roots. **C.** Exposure of the coronal pulp tissue removed from the pulp chamber with a endodontic excavator. **D.** Root marking with a diamond disc and separation of the roots to expose the pulp tissue. **E.** Careful removal of the pulp tissue from the root canal with a dental explorer.

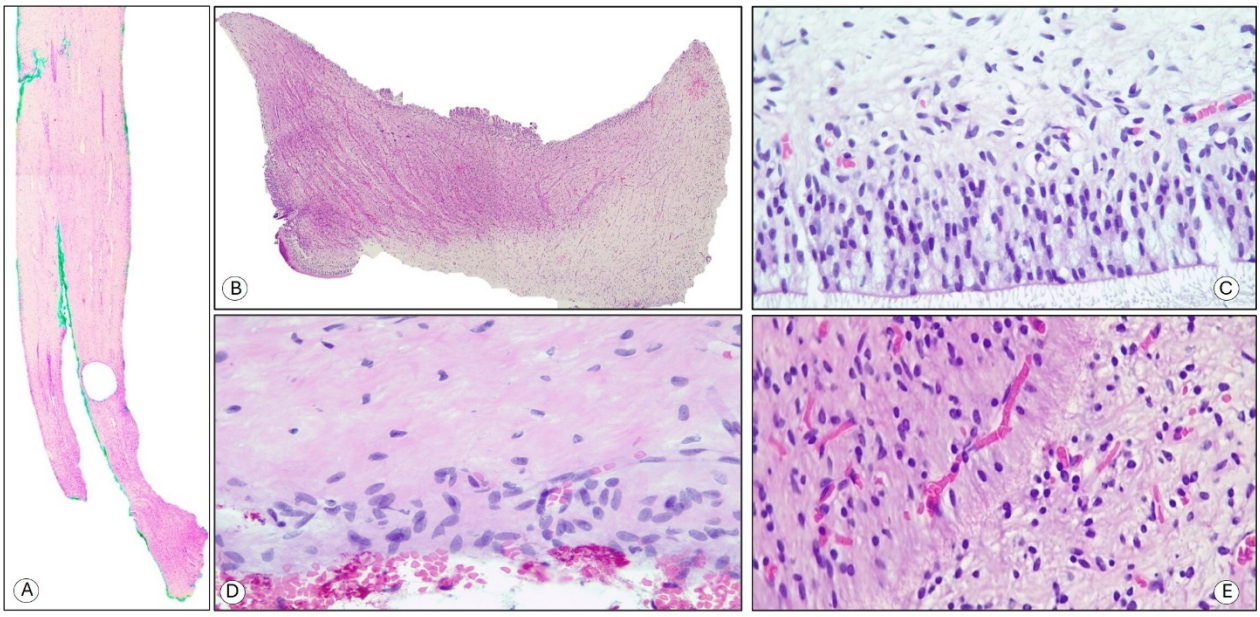

Figure 2. Microphotographs of the pulp tissue morpho-architecture. **A.** Root pulp tissue that maintained the root canal morphology showing the apical bifurcations. **B.** Coronal pulp tissue with intact morphology of the pulp horns (Hematoxylin and Eosin, 10× magnification). **C.** Histological findings in the coronal pulp tissue showing the odontoblastic zone, the cell-rich zone and the pulp center. **D.** In the apical zone of a pulp tissue the cell-rich zone, the pulp center and the absence of the odontoblastic zone are clearly shown (Hematoxylin and Eosin, 40× magnification). E. Integrity of the pulp tissue, showing abundant collagen fibers, numerous fibroblasts and blood vessels (Hematoxylin and Eosin, original magnification 400×).

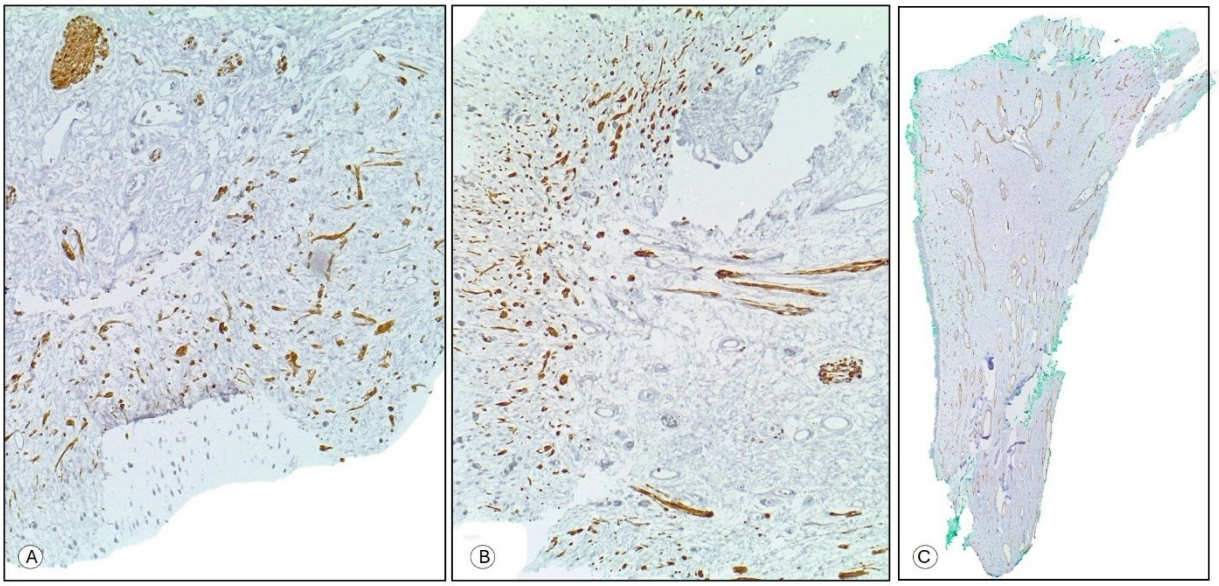

Figure 3. Microphotographs of immunohistochemistry. **A, B.** They show the positive expression of S-100 in the nerve tissue fibers of the coronal and radicular dental pulp. **C.** CD34 positivity in blood vessels of different caliber in the radicular pulp tissue from the cervical to the apical portion. (Immunohistochemistry, original magnification 10×).