A Regenerative Endodontic Approach in Mature Ferret Teeth Using Rodent Preameloblast-conditioned Medium

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Abstract. *Background: This study evaluated the effectiveness of a regenerative endodontic approach to regenerate the pulp tissue in mature teeth of ferret. The presence of odontoblast-like cells in the newly-formed tissue of teeth treated with or without preameloblast-conditioned medium was evaluated based on morphological criteria. Materials and Methods: Twenty-four canines from six ferrets were treated. The pulp was removed, and the apical foramen was enlarged. After inducing the formation of a blood clot, a collagen sponge with or without preameloblast-conditioned medium was placed underneath the cementoenamel junction. The samples were analyzed at the eighth week of follow-up. Results: Vascularized connective tissue was observed in 50% of teeth, without differences between groups. The tissue occupied the apical third of the root canals. Odontoblast-like cells were not observed in any group. Conclusion: Revitalization of mature teeth is possible, at least in the apical third of the root canal. Further experimental research is needed to produce more reliable outcomes.*

Preclinical and clinical research aimed to regenerate the pulp tissue necrotic teeth has substantially increased during the past decade (1). The main objective of regenerative endodontics is to replace damaged structures, including dentin and root structures, as well as cells of the pulp–dentin complex (2).

This therapy has been studied mainly for treating immature necrotic teeth, as an attempt to complete the development of the fragile dentin walls and revitalize the tooth.

In clinical practice, most cases of pulp necrosis are found in mature teeth. However, studies oriented to regenerate or repair pulp tissue of mature teeth are today scarce, most of them published in recent years (3-7). Currently, the most reliable option for the treatment of necrotic mature teeth is still root canal treatment. However, endodontically treated teeth are susceptible to reinfection and fracture, and the failure rate is close to 25% at 3-5 years (8). Moreover, endodontically treated teeth will remain devitalized throughout the patient's lifetime and therefore defenseless to new caries lesions, as the absence of pulp implies the lack of tooth defense mechanisms (9, 10). Therefore, teeth revitalization offers several advantages over conventional root canal treatment, such as the recovery of the natural immune system and a structurally more resistant tooth (8, 11, 12).

There are two main limitations to treating permanent mature teeth with a regenerative endodontic approach. One is the small diameter of the apical foramen (13), which acts as a physical barrier hindering new tissue ingrowth into the root canal; and the second is the absence of an apical papilla. Immature permanent teeth present an apical papilla, which is a reservoir for mesenchymal stem cells (14). Stem cells from the apical papilla (SCAPs) have odontoblast-like differentiation potential, and produce dentin *in vitro* and *in vivo*, being responsible for root maturation (15). However, a previous study showed that even though the apical papilla is no longer present in mature teeth, an evoked-bleeding technique in these teeth is able to deliver mesenchymal stem cells from the periapical tissues (16). These cells also express mesenchymal stem cell markers, similarly to SCAPs (16).

Although these mesenchymal stem cells have odontogenic potential (15, 17), once in the root canal, they seem to

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Table I. *Summary of histological findings found in the intracanal and periapical tissues two months after treatment.*

Parameter	Control group (n=11), n (%)	Experimental group (n=11), n (%)
Presence of newly formed iCT	4 (36.36%)	7 (63.63%)
Apical third	4 (100%)	7 (100%)
Medium third	0 (0%)	0 (0%)
Coronal third	0 (0%)	0 (0%)
Presence of inflammatory infiltrate in the iCT (percentage of teeth with iCT)	2 (50%)	4 (57.14%)
Presence of inflammatory infiltrate in the periapical tissues	6 (54.54%)	7 (63.63%)
Debris from biomechanical preparation	9 (81.81%)	6 (54.54%)
Apical cementum remodeling	8 (72.72%)	9 (81.81%)
Dentin resorption	0 (0%)	1 (9.4%)
Presence of intracanal hard tissue (cementum-like tissue)	2 (18.8%)	2 (18.8%)
Presence of Sharpey's fibers in the intracanal space	2 (18.8%)	1 (9.4%)

iCT: Intracanal connective tissue.

differentiate into cementoblast-like cells and osteoblast-like cells, both in mature and immature teeth (18). This might suggest that these cells do not receive the correct signals necessary to promote odontoblast-like differentiation.

During tooth embryogenesis, the inner enamel epithelium and the underlying mesenchyme participate in a mutual interaction by secreting growth factors that induce their differentiation into ameloblasts and odontoblasts, respectively (19). Previous studies have identified 71 factors in the conditioned medium (CM) from preameloblasts (20). These molecules participate in cellular transduction, cell growth, transcription and metabolism and may act as attractants of bioactive signaling molecules required to induce differentiation of mesenchymal stem cells into odontoblast-like cells. An *in vitro* study has shown that preameloblast-CM induces odontoblast-like differentiation of mesenchymal stem cells (20). Moreover, an *in vivo* study in immature teeth claimed that newly formed dentin-like and pulp-like tissues were more frequently observed in CM-treated teeth (21).

Taking into account these data, our objectives were the following: Firstly, to evaluate the effectiveness of a regenerative endodontic approach to regenerating the pulp tissue of mature teeth of ferrets after pulpectomy; secondly, to evaluate the presence of odontoblast-like cells in the newly-formed tissue of teeth treated with and without preameloblast-CM; and thirdly, to analyze the suitability of the ferret as an animal model for regenerative endodontics.

Materials and Methods

This study was approved by the Ethical Committee for Animal Experimentation of the University of Barcelona, Spain (no. 519116). All animal experiments were conducted using the EU Directive 2010/63/EU for animal experiments and the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society.

Preameloblast-CM. Apical bud cells (ABCs) from Sprague–Dawley rats were isolated and cultured as previously described (22). The handling of ABCs was performed inside a laminar flow hood with sterile instruments. Briefly, 20 apical bud tissues of lower incisors of 4-day postnatal rats were collected. The ABCs were enzymatically isolated with type I collagenase (0.62 mg/ml; Sigma, St. Louis, MO, USA) for 30 min, and collected by centrifugation. Cells were cultured in 10 mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carlsbad, CA, USA) with 10% inactivated fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin) and 2.5 mg/ml ascorbic acid, under 5% carbon dioxide at 37˚C, until confluence. After differential repeated trypsinization (0.25% trypsin/EDTA), cells were seeded onto 6-well plates in keratinocyte-free medium (Gibco BRL) with 0.01 ng/ml epidermal growth factor and 25 μm/ml of bovine pituitary extract until confluence (Invitrogen Corp., Carlsbad, CA, USA). Cells were then washed twice with PBS and cultured with DMEM without FBS. After 24 hours, supernatants were collected and filtered using sterile 0.22-μm diameter pore size membranes, aliquoted and stored at −20˚C.

Ferret model. Six 4- to 6-month-old male and female ferrets (*Mustela putorius furo*) were commercially acquired (Isoquimen, Sant Feliu de Codines, Catalunya, Spain) and used in this study. Periapical radiography of the teeth showed complete root development. The four canine teeth from each ferret were treated and randomly assigned to the control (n=12) or the experimental group (n=12); randomization was performed with a coin. In order to study the normal histology of pulp tissue, four untreated molars were also analyzed (negative controls). Ferrets were deprived of food for 12 h before surgical intervention. Dental surgeries were performed at the Biotherium of the University of Barcelona (UEA de Bellvitge B-9900046), in the morning of 6 working days, under general anesthesia (25 mg/kg ketamine and 2 mg/kg xylazine) and analgesia (1 mg/kg meloxicam). The clinical protocol was as follows: Teeth crowns were disinfected with 2% chlorhexidine. Treatment was carried out under local anesthesia (Mepivacaine HCl 3%; Septodont, Lancaster, PA, USA) and endodontic access was performed using a high-speed hand piece under constant irrigation with saline. Pulp was removed with a #15 sterile stainless-steel endodontic hand file (Dentsply Maillefer, Ballaigues, Switzerland) and working length was determined

using a portable digital X-ray device (iRay D4, Dexcowin, Los Angeles, CA, USA). The apical cemental barrier was penetrated with #15 K-file and root canals were prepared 0.5 mm beyond the canal terminus. The root canals were prepared and the apical foramen was enlarged using a #80 K-file (Dentsply Maillefer, Ballaigues, Switzerland) with 2% sodium hypochlorite (23). Root canals were rinsed with 17% EDTA and sterile saline and dried with paper points. A sterile #15 K-file was inserted 2 mm beyond the canal terminus to irritate the periapical tissues and induce bleeding and the formation of a blood clot inside the root canal. A hemostatic absorbable lyophilized collagen sponge (Hemospon; Technew, Rio de Janeiro, RJ, Brazil) was placed over the blood clot. The collagen sponge was cut to a size slightly superior to the coronal part of the root canal, and then submerged in DMEM (control group) or preameloblast-CM (experimental group). Teeth were sealed with grey MTA (CHL Medical Solutions, Milan, Lombardy, Italy), flowable resin composite (CHL Medical Solutions) and amalgam (Bestdent, Montebello, Vicenza, Italy).

After completing the surgical procedures, animals were given supportive analgesics for 3 days (1 mg/kg meloxicam). A special soft diet was not needed. Ferrets were given *ad libitum* access to food and water, kept under a 12-h light/12-h dark cycle with three animals per cage. Eight weeks after surgery, animals were sacrificed using an anesthetic overdose (200 mg/kg pentobarbital).

Jaws and maxillaries including the treated teeth were sectioned and immediately submerged in 10% formalin for 24 hours. Using a diamond burr, a small cavity was created in the crown of the canines to ensure intracanal tissue fixation.

Each mandible/maxilla was scanned in a micro-computed tomographic (CT) device (Quantum FX microCT; PerkinElmer, Waltham, MA, USA). Briefly, the samples were scanned at 90 kV and 160 mA through a 20 mm FOV using an exposure time of 4.5 min. The acquired images were reconstructed into cross-sectional slices using PerkinElmer software (PerkinElmer, Health Sciences) by the Feldkamp method. The reconstructed image stacks of the bone blocks were co-registered using OsiriX DICOM Viewer v. 9.0 software (Pixmeo DARL, Geneva, Switzerland) for three-dimensional visualization.

Histological processing. All samples were decalcified using a solution containing 14.28% EDTA and 5% formic acid for approximately 3 weeks. The samples were then processed with a tissue processor (Shandon Citadel 1000; Thermo Scientific, Waltham, MA, USA), dehydrated through ascending gradations of ethanol in the following manner: one cycle of 70% ethanol (8 h), one cycle of 90% ethanol (8 h), three cycles of 100% ethanol (8, 16 and 14 h), three cycles of xylene (5, 5 and 6 h), and two cycles of paraffin (6 and 16 h).

Samples were sectioned using a Leica Jung RM2045 microtome (Leica, Nussloch, Baden-Württemberg, Germany). Longitudinal sections of 5 μm through the root apical foramen were obtained and placed on poly-L-lysine-coated glass slides. Tissues were stained with hematoxylin and eosin (H-E), and toluidine blue, and evaluated under light microscopy (Olympus CH30; Olympus, Tokyo, Japan). Histological analysis was performed by two histologists blinded to the experimental groups. Histological characteristics of the newly formed tissues were performed using morphological criteria and scored according to the histopathological parameters reported by Gomes-Filho *et al*. (24).

Statistical analysis. The categorical data (presence/absence of cementum-like or dentin-like tissues/inflammatory cells, fibroblast, odontoblast-like cells) were analyzed using a Fischer's exact test, with a significance level of $p<0.05$.

Results

Due to the lack of blood clot formation and to fracture of the file in the root canal, one control and one experimental tooth were excluded, respectively. Thus, 11 teeth (from six ferrets) per group were analyzed. No periapical lesions in any teeth were radiographically observed.

Histological analysis: Control and experimental group. Newly formed intracanal connective tissue (iCT) was observed in 50% of the teeth, without statistical differences between groups (Table I) ($p>0.05$). Intracanal tissue was characterized as non-organized loose connective tissue with a low to moderate number of blood vessels, localized in the central region of the canal (Figures 1 and 2A and B). However, the iCT only occupied the apical third of the root canal. Cells were mostly fibroblasts (Figure 1B, D and F) and odontoblast-like cells were not observed in any group. Root canals without iCT were observed as being mostly empty and with some chronic inflammatory infiltrate and debris from biomechanical instrumentation (Figure 2C and D).

Acute inflammatory cells in the intracanal tissue were observed in 50% and 57% of control and experimental groups, respectively (Figure 1C, E and F and Figure 2C), without statistical differences between groups ($p>0.05$). In the experimental group, dentin resorption in the apical third of one tooth was observed (Figure 2D).

Cementum-like tissue was observed in four teeth, both attached to the wall canals as well as free in the iCT (Figure 1A-D).

Scarce inflammatory cells in the periapical area of most teeth of both groups were present (Table I) (Figure 2D). Cementum remodeling at the apical portion of most teeth was also observed (Table I) (Figure 2B).

Normal histology of mature teeth from ferrets (negative control group). The normal histology of mature teeth from the ferrets can be seen in Figure 3. Pulp was observed as an organized, mature and vascularized tissue (Figure 3A and B), with odontoblasts arranged in a pseudostratified palisade (Figure 3B), compatible with a mature although young pulp tissue. Dentin was observed with ordered dentinal tubuli and predentin (Figure 3B). The maturation of the root is evident. Teeth presented a small apical foramen and cellular cementum at the apical area (Figure 3A).

Ferret as an animal model for regenerative endodontics. The mean weight of the animals was 2.8 kg and all animals were healthy prior to and after the surgery. No adverse events were
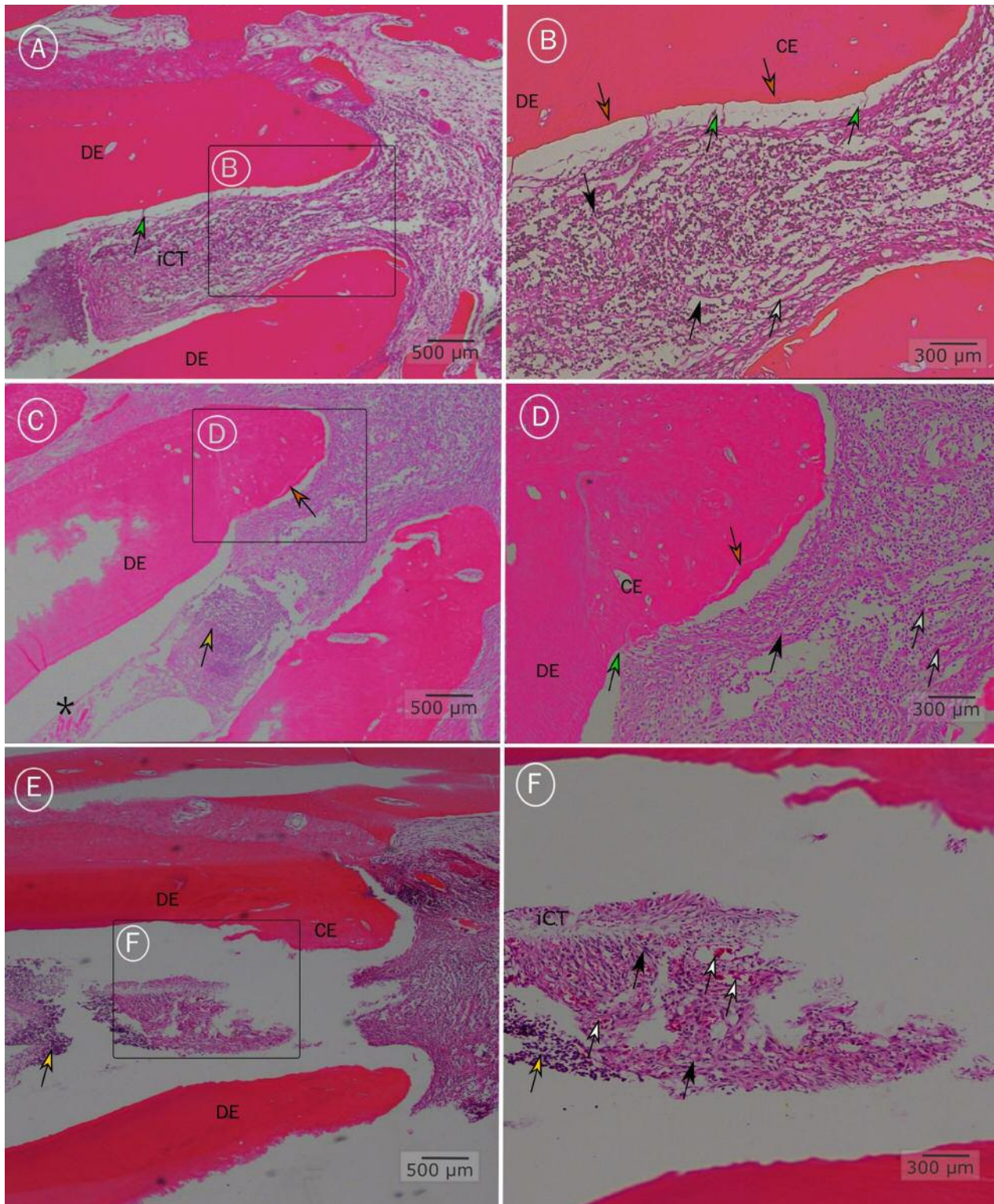
Figure 1. *Histological appearance with hematoxylin-eosin staining (H-E) of mature teeth treated with a regenerative endodontic approach. The intracanal connective tissue (iCT) occupied the apical third of the root canal of control (A, B) and experimental (C-F) teeth. A large number of fibroblasts (black arrows), inflammatory cells (yellow arrows) and a moderate number of blood vessels (white arrows) were observed (A-D). Intracanal Sharpey's fibers (green arrows) (B and D) and cementum-like (CE) tissue attached to the inner walls (orange arrows) were also observed. DE: Dentin. *Debris from biomechanical preparation.*
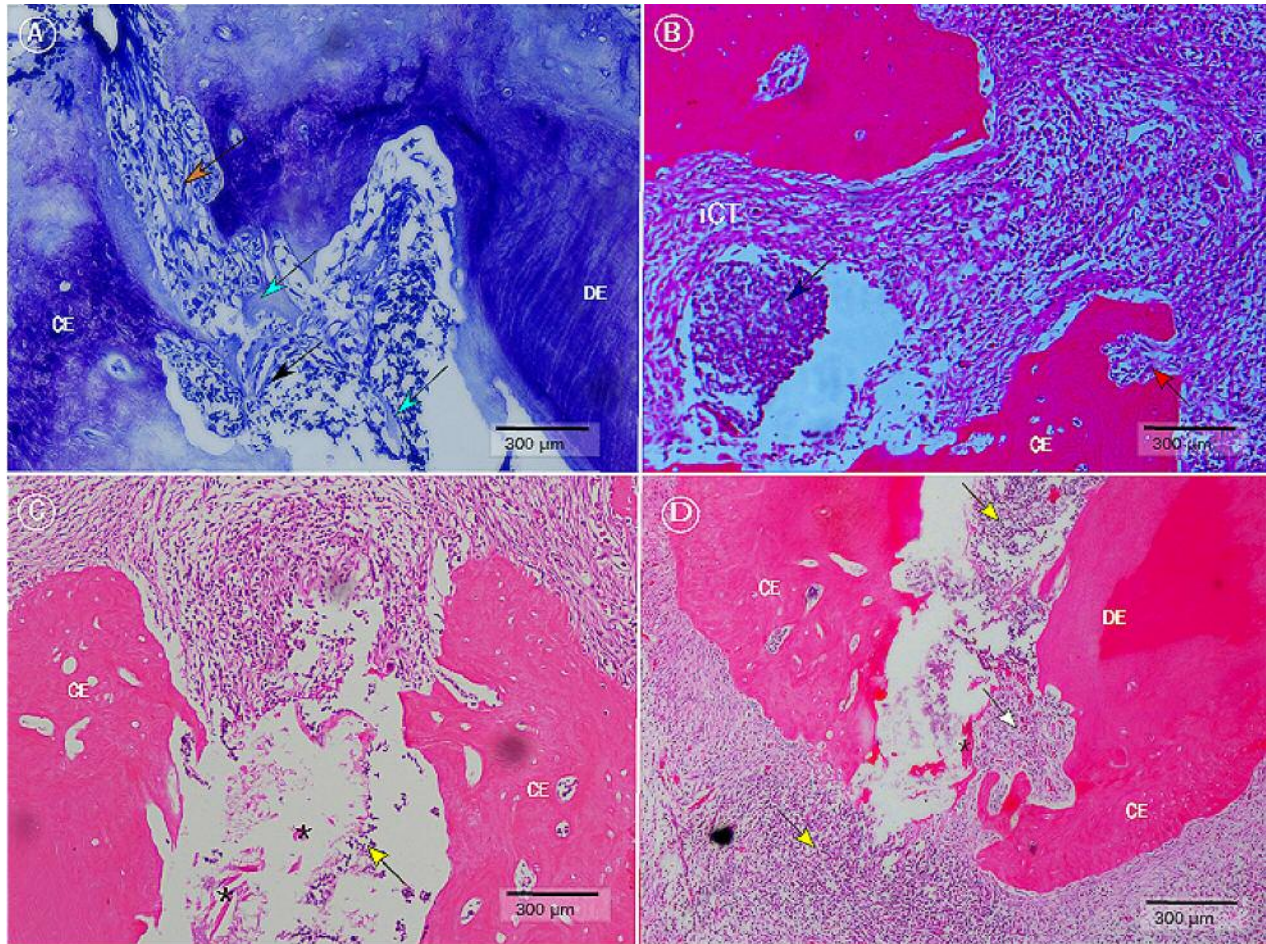
Figure 2. *Histological appearance with toluidine blue (A) and H-E (B-D) staining of mature teeth in the control group treated with the regenerative endodontic approach. The apical delta and accessory canals (brown arrow) were observed (A). A lack of intracanal connective tissue (iCT) was observed in most samples of the control group (C and D). Fibroblasts (black arrows), inflammatory cells (yellow arrows), necrotic tissue (blue arrow) were observed in samples with iCT (A and B). Islets of cementum like tissue (CE) (A) (light blue arrow) were also observed. Cementum (CE) (B) and dentin (DE) (D) resorption were observed in some samples (red arrows) (D), as well as debris from biomechanical preparation (*) (C and D).*

observed. Although isolation of teeth with a rubber dam was not possible, the size of ferret canine teeth was adequate to prepare the canals using wide files (#80), irrigation and placement of restorative materials without major complications (Figure 4A-C). The size of the mouth also allowed intraoral radiographs to be taken. The dimension of incisors, however, was too small to perform endodontic experiments (approximately 1 mm width ×2 mm height) (Figure 4B). Periapical radiographs and clinical examination revealed a pronounced curvature of the canine teeth, especially of mandibular teeth. Thus, instrumentation with flexible files is highly recommended. In the micro-CT analysis, canine teeth presented characteristic apical delta, with several accessory canals (Figure 2A and 4D), which may explain the clinical difficulties encountered in reach the working length.

Discussion

In our study, 50% of teeth showed some degree of vascularized connective tissue at the intracanal space. However, it occupied only the apical third of the canal. Moreover, the neo-formed tissue was disorganized and did not have the characteristic layers of mature pulp.

In this study preameloblast-CM was used to simulate the cellular and molecular conditions occurring during tooth embryogenesis between the inner enamel epithelium and the underlying mesenchyme. These tissues participate in a mutual interaction by secreting growth factors that induce their respective differentiation into ameloblasts and odontoblasts (19). Therefore, we hypothesized that growth factors present in the CM from preameloblasts (20) would
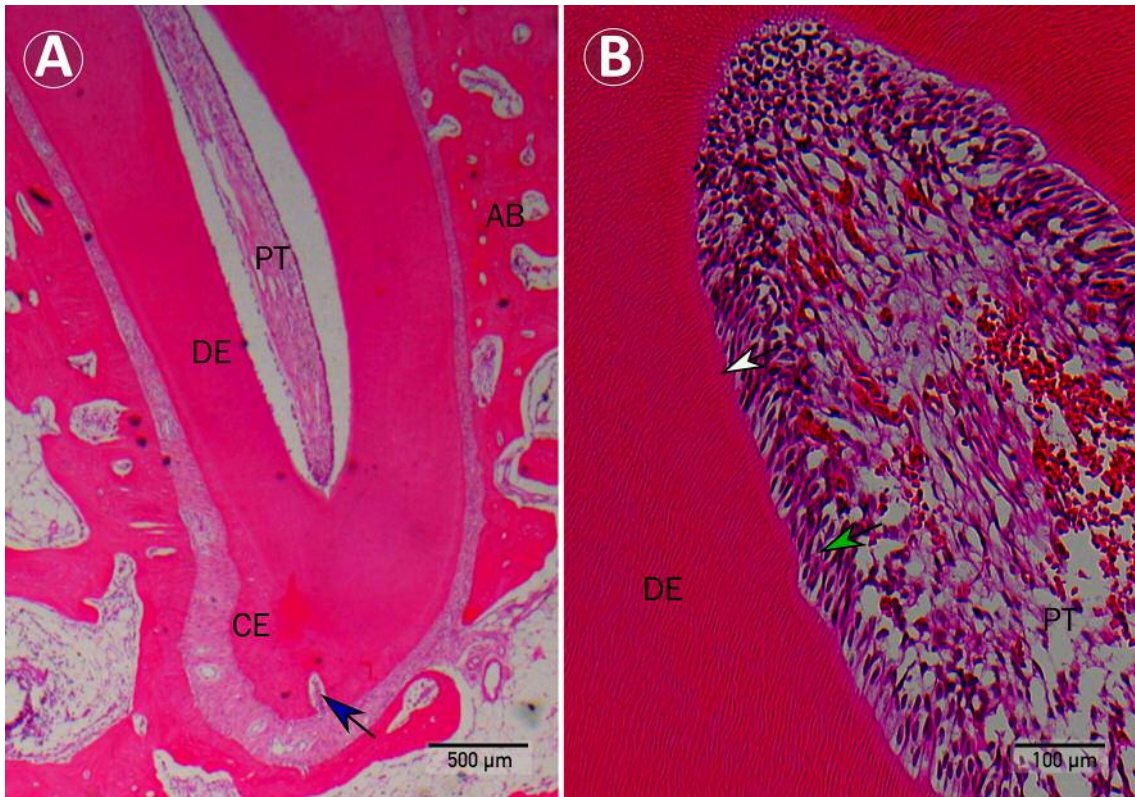
Figure 3. *Normal histology (H-E) of ferret non-treated molars in longitudinal (A) and transversal (B) views. DE: Dentin; CE: cementum; PT: pulp tissue; AB: alveolar bone; blue arrow: apical foramen; green arrow: odontoblasts arranged in a pseudostratified palisade; white arrow: predentin.*
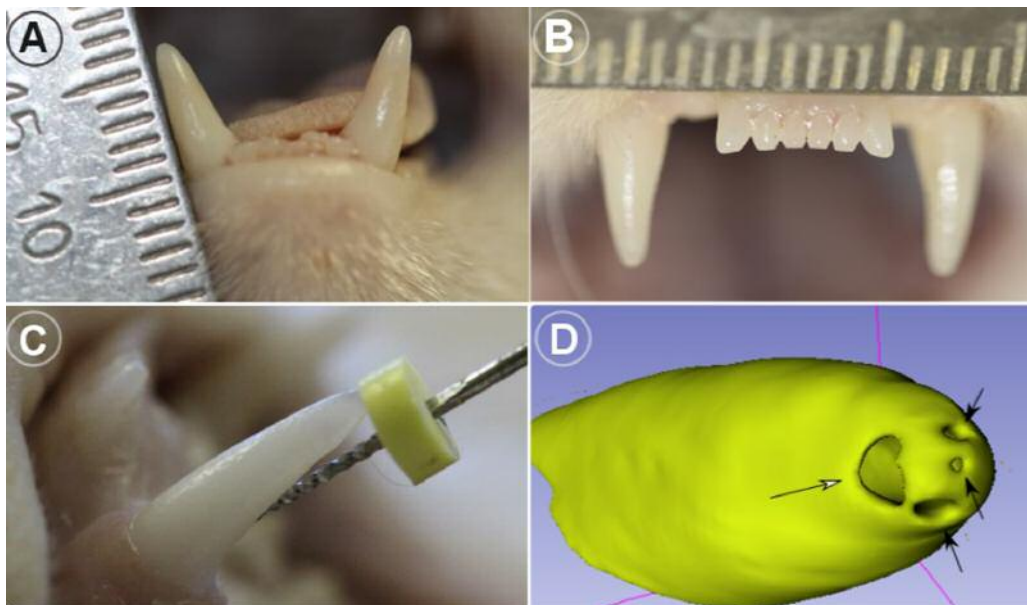

Figure 4. *A: Canine crown of 6 mm in height. B: Maxillary incisors, dimension 1×2 mm. C: Canal preparation with file #20 after creating the coronal access of a maxillary canine. D: Three-dimensional reconstruction of the apical third of a ferret canine tooth. Three accessory foramens (black arrows) and one enlarged apical foramen (white arrow) can be observed.*

induce the differentiation of mesenchymal stem cells into odontoblast-like cells, as previously reported in *in vitro* studies (20, 21).

Several attempts at treating immature teeth with a regenerative endodontic approach have been made. With few articles reporting success until date (12), these attempts tried to induce differentiation of SCAPs and dental pulp stem cells into odontoblast-like cells *in vivo* (18, 25). In mature teeth, the induction of intracanal bleeding attract mesenchymal stem cells from the periapical tissues (16). The differentiation process of stem cells after revascularization procedures always leads to cementoblastic or osteoblastic lineage formation instead of generating odontoblast-like cells. Hence, this leads to the formation of cementum or bone-like tissues instead of dentin (18, 24-26). In this sense, even with the use of preameloblast-CM, the results of our study confirm those previously published. Only periodontal tissues (cementum-like and Sharpey's fibers) were observed.

The size of the apical foramen seems to be a major concern in pulp repair of mature teeth, since a small apical foramen diameter might be a physical limitation for the ingrowth of new tissue inside the root canal. Although some studies refuted the influence of the apical diameter on clinical outcomes (27), others showed that an apical foramen diameter of <1 mm exhibited a greater increase in root thickness, length, and apical narrowing (28). To date, there is no established standard size of apical foramen required to ensure new tissue ingrowth into the root canal. In the present study, canal preparation was made using a #80 file 0.5 mm beyond canal terminus to ensure the widening of the foramen (13). However, the achieved size might not have been enough to allow new tissue ingrowth, that might explain why only 50% of the teeth presented vital intracanal connective tissue. Therefore, it is necessary to determine the diameter at which the apical foramen should be enlarged in order to arrive at more predictable outcomes when treating mature teeth with a regenerative approach. Moreover, the method for enlargement should also be properly studied since the effect of instrumentation over the apical constriction may also explain the inflammatory response of the periapical tissues, as well as the reaction of the apical cementum, which showed zones of remodeling. In summary it is necessary to establish a standardized clinical protocol for regenerative endodontics in mature teeth, as already exists for immature teeth (23), since the characteristics of mature teeth (a closed apex and the absence of apical papilla) deserve special consideration.

This study confirms the utility of ferrets as a model for regenerative endodontics, as previously proposed (29). Ferrets are easy to handle (2-3 kg), and their mouths and teeth are large enough to perform the clinical procedures for this therapy, although not to perform isolation with a rubber dam. It is also necessary to consider the apical delta and the pronounced curvature of the mandibular canines when planning a treatment.

Conclusion

Revascularization of mature teeth is possible, at least in the apical third of the root canal. However, further research in animal models is needed to create quicker and more reliable regenerative endodontic procedures that can be evaluated in clinical trials.

Conflicts of Interest

The Authors declare that no conflicts of interest exist regarding this study.

Authors' Contributions

CB, AGS and CMC planned the study; CB, AGS and JMA performed the animal experiments; CB and IVG processed the samples and did the histological analysis; CB researched and wrote the article; CMC, IVG and JMA offered scientific advice and proof-read the article. All Authors read and approved the manuscript.

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