Histologic Evaluation of Regenerative Endodontic Procedures with the Use of Chitosan Scaffolds in Immature Dog Teeth with Apical Periodontitis

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Abstract

Introduction: The aim of this study was to evaluate histologically the newly formed tissues after regenerative endodontic procedures (REPs) in dogs using either a blood clot (BC) or 2 different formulations of a chitosan hydrogel as scaffolds. **Methods:** Apical periodontitis was induced by inoculating immature teeth with oral plaque in 4 beagle dogs. Teeth (n = 96) were divided into 2 control (n = 20) and 4 test groups (n = 76) according to the treatment: apexification and REPs with BC, sodium hyaluronate:chitosan (HA:CS) scaffolds, or pectin:chitosan (P:CS) scaffolds. All root canals were disinfected with 2.5% sodium hypochlorite and a triple antibiotic paste intracanal medicament before evoked bleeding, clot formation, or scaffold placement. Thirteen weeks after treatment, the animals were sacrificed and the jaw blocks harvested for histologic processing, histomorphometric analysis, and statistical analysis. **Results:** The lumens of the root canals were completely filled with mineral trioxide aggregate with evidence of a mineralized apical bridge between the root canal walls in 83% of the samples in the apexification group. Vital vascularized tissue was found in the REP groups; apical closure happened in 66.7% of these treatments, and root growth was detected more often as an increase in thickness (85.6%) than in length (45.6%). The greatest amount of mineralized tissue inside the canal was observed in the BC group, with statistical significance compared with the HA:CS and P:CS groups (P < .05). Further histologic evaluation revealed the presence of apical papilla. **Conclusions:** The addition of chitosan scaffolds to blood in regenerative procedures in dogs did not improve the formation of new mineralized tissues along the root canal walls or the histologic evidence of the regeneration of a pulp-dentin complex. *(J Endod 2017;43:1279–1287)*

Key Words

Apexification, apical papilla, chitosan scaffolds, immature permanent tooth, regenerative endodontics procedures, root canal, tissue engineering, tissue regeneration

Significance

We performed a histologic assessment of the regenerative potential of 2 chitosan-based scaffolds compared with blood clot in immature dog teeth with pulp necrosis and apical periodontitis. This study demonstrated the continued survival and differentiation potential of the apical papilla after infection.

During the development of permanent teeth, the occurrence of caries, trauma, or anatomic alterations is quite common and can jeopardize the pulp tissue and impair the pulp-dentin complex physiology and as a consequence normal root development. Premature loss of a functional pulp in immature teeth leads to the arrest of root dentin formation, resulting in a thin and functionally compromised canal wall (1). These anatomic and functional conditions are associated with greater predisposition to treatment failures and fractures and decreased tooth survival (2–4).

Regenerative endodontic procedures (REPs) have emerged such as revascularization and revitalization of pulp tissue in immature necrotic teeth with apical periodontitis (AP) to allow the reinforcement of root canal walls and sometimes the continuation of their development, thus opening new therapeutic possibilities in this field (5–7). The underlying strategies to promote growth of new tissues in pulp canal space are based on 4 fundamental assumptions:

1. Effective endodontic disinfection/antisepsis
2. Recruitment of undifferentiated mesenchymal stem cells (MSCs) from the apical region
3. Creation of a scaffold that allows growth of new tissue
4. Appropriate coronal sealing to prevent reinfection (8, 9)

There have been substantial advances in disinfection techniques that combine the balance between the antimicrobial effect and biocompatibility with MSCs. Also, it has been shown that intracanal bleeding evoked from the apical tissues brings substantial numbers of MSCs into the canal system (10). Although a blood clot (BC) has been traditionally used as a scaffold, it has several limitations that include undefined composition,

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presence of immune cells, unknown breakdown kinetics, and its generation requires traumatizing the apical tissues. Other alternatives have been successfully used in the clinical setting including the use of platelet-rich plasma (11, 12), platelet-rich fibrin (13), and soluble collagen (14). Their clinical use allowed resolution of signs and symptoms of disease leading to the healing of AP and, in certain cases, appreciable radiographic evidence of root development.

The 3 key components for tissue engineering are stem cells, growth factors, and scaffolds. There are ongoing research efforts to develop scaffolds for tissue regeneration using either natural or synthetic materials (15). These alternative scaffolds have been evaluated in animal models of regenerative endodontics, including soluble collagen (16), platelet-rich plasma (17), and absorbable gelatin sponge (18). In these models, they failed to improve the histologic outcome, and, so far, only insoluble cross-linked collagen sponges were associated with better results (19, 20). To overcome some limitations presented by those materials, including a fast resorption rate and a lack of mechanical support for the coronal seal, we sought to test 2 new lyophilized 3-dimensional scaffolds based on chitosan that have been previously evaluated *in vitro* for their biocompatibility (21, 22).

This *in vivo* study assessed histologically the regenerative potential of 2 chitosan-based different scaffolds (hyaluronic acid:chitosan [HA:CS] and pectin:chitosan [P:CS]) compared with the use of an autologous BC in immature dog teeth with pulp necrosis and AP.

Material and Methods

Animals

The study protocol was approved by the Animal Welfare Committee of the Direção-Geral de Veterinária of Portugal (no. 0420/2011) and complied with the International Guiding Principles for Biomedical Research Involving Animals (Geneva, 1985).

Based on previous studies (16, 23), 4 male beagle dogs, aged approximately 6 months, had 4 one and 10 two-rooted premolars involved in the study protocol. All experimental teeth were block randomized, and 76 roots were assigned to the test group and 20 to the control groups. All groups were present in each dog and distributed in alternate quadrants in each animal (24).

Tooth Preparation

For the first 3 interventions, dogs were anesthetized with an intravenous administration of 0.2 mg/kg diazepam (Labesfal, Campo de Besteiros, Portugal) and 2 mg/kg propofol (Propofol Lipuro 2%; B Braun Medical, Queluz de Baixo, Portugal) and maintained with inhalation of O_2 and 1%–2% isoflurane (Isoflo; Esteve Farma, Lisboa, Portugal). All animals received a single dose of meloxicam 0.2 mg/kg (Meloxidyl; Ceva Santé Animale, Libourne, France) postoperatively. For radiographic follow-up, dogs were sedated with an intramuscular administration of 0.2 mg/kg butorphanol (Dolorex; MSD Animal Health, Porto Salvo, Portugal) and 0.005 mg/kg dexmedetomidine (Dexdomitor; Orion Pharma, Espoo, Finland).

Before any interventions, teeth were radiographed using custom bite registration and paralleling devices (XCP-DS Fit; Dentsply Rinn, York, PA). At the first intervention, teeth had coronal access performed with a bur mounted in a high-speed handpiece and pulp tissue mechanically disrupted with a 30 K-file. Supragingival plaque was collected from the gingival sulcus, mixed with sterilized water (B Braun Medical), placed into the access cavity, and temporarily sealed with Cavit (ESPE 3M, Seefeld, Germany). After 3 weeks, the development of AP was confirmed radiographically. The second intervention was performed under rubber dam isolation, and disinfection of the operative field was achieved with 10% iodopovidone solution (Betadine; Meda Pharma, Lisboa, Portugal). All teeth in the test groups were reaccessed, irrigated with 10 mL 2.5% sodium hypochlorite, and disinfected with calcium hydroxide in the apexification group or filled with triple antibiotic paste (Coimbra Hospital and University Centre, Coimbra, Portugal) composed of ciprofloxacin, metronidazole, and minocycline (20 mg of each antibiotic per mL) before REP protocols. The paste was applied until the cementoenamel junction using a 27-G needle calibrated 2 mm shorter than the working length. A sterile cotton pellet was placed on top of the medication, and teeth were temporarily sealed with Cavit.

Two weeks later, intracanal medication was removed with copious irrigation using 3 mL 2.5% sodium hypochlorite (only on the pulp chamber) followed by a final flush with 10 mL saline solution. Then, canals were dried with paper points and treated according to the following test groups.

1. Apexification (19 roots): mineral trioxide aggregate (MTA) (White ProRoot MTA; Dentsply Tulsa, Johnson City, TN) was mixed according to the manufacturer and inserted into the canal using a suitable-sized carrier (Map System, Dentsply Tulsa). MTA was packed apically using a suitable plugger (Buchanan Hand Plugger #2; SybronEndo, Orange, CA) to fill the root canal 2–3 mm apical to the cementoenamel junction. A sterile wet cotton pellet was then placed in the pulp chamber and coronal access restored with GIC (Ketac Fil, ESPE 3M).

Groups of REP Procedures

2. BC (19 roots): bleeding into the canal was evoked by gentle overinstrumentation with a sterile size 30 K-file leading to filling of the canal space until the level of the cementoenamel junction and waiting for the formation of a BC. After clot stabilization, the coronal portion of the root canal was double sealed with 2–3 mm white MTA and GIC.
3. HA:CS scaffold (19 roots): preparation of sodium HA:CS scaffolds was performed based on a HA:CS of 2:1 mass ratio according to Coimbra et al (21). Induction of bleeding was performed as in the BC group; then, the lyophilized hydrogel of HA:CS was inserted into the canal using a suitable-sized plugger and double sealed.
4. P:CS scaffold (19 roots): preparation of P:CS polyelectrolyte complex scaffolds was performed based on a P:CS of 2:1 mass ratio according to Coimbra et al (22). Induction of bleeding was performed as described in the BC group; then, the lyophilized hydrogel of P:CS was inserted into the canals using a suitable-sized plugger and double sealed.

Control Groups

5. Negative control pulpal (4 roots): pulps were mechanically disrupted and exposed to oral microbiota. At the second intervention, teeth were extracted for histologic analysis of the pulp content.
6. Negative control periapical (4 roots): same protocol performed as in the negative control pulpal group; however, at the second intervention, teeth were restored with GIC and maintained until the end of the study.
7. Positive control (12 roots): normal teeth of the same type as in the previous groups without any intervention included for histologic reference of the physiologic root development.

Histologic Analysis

At 13 weeks post-treatment, animals were euthanized by anesthetic overdose (pentobarbital at 30 mg/kg intravenously; Butler Company, Columbus, OH) followed by bilateral perfusion with 10% phosphate-buffered formalin. Mandibular and maxillary blocks were dissected

from the cuspid to the first molar, post fixed with 10% phosphate-buffered formalin, and decalcified with Morse's solution for 8 weeks. Coronal restorations were removed, the specimens were trimmed and embedded in paraffin wax, and 6-μm serial sections along the mesiodistal plane performed parallel to the long axis of the canal were stained with hematoxylin-eosin and Masson trichrome. Each individual root was histologically examined as an independent sample unit with a light microscope (Eclipse E600; Nikon, Tokyo, Japan). One observer examined all specimens blinded to group allocation.

The intracanal and periapical tissues present in each group were described according to the histopathological parameters and rated in conformity with scores defined in Figure 1*A*. For the REP groups, the newly formed mineralized tissue areas inside the canal, residual scaffold, and total area of canal space were determined using Bioquant 2012 software (Image Analysis Corporation, Nashville, TN). The percentage of mineralized tissue formation and residual scaffold were calculated as follows: (mineralized tissue area + residual scaffold)/total area of canal space × 100 (Fig. 1*B*).

Statistical Analysis

SPSS 17.0 (IBM Corporation, NY) was used to compare the histologic scores using the nonparametric Kruskal-Wallis test followed by the Mann-Whitney *U* test post hoc. $P < .05$ was considered statistically significant.

Results

The experimental procedures did not cause any change in behavior or eating habits of the dogs. During the follow-up, 1 mesial root from a second lower premolar from the apexification group was lost because of a vertical crown-to-root fracture although the distal root was not excluded from the analysis because the coronal access restoration kept the canal sealed.

Control Groups

All teeth from the positive control group showed mature roots, closed apices, and a normal dentin-pulp complex at the end of the follow-up period. Conversely, in the negative pulp control group, we noticed an intact layer of predentin, but the entire canal space was filled with necrotic tissue with some remnants of collagen fibers. Many resorption lacunae were also present in the apical cementum. Furthermore, in the negative periapical control group, there were evident severe inflammatory periapical lesions as well as resorption areas in the cementum and dentin.

Apexification Group

The histologic findings showed the resolution of periapical lesions in 94% of the cases (Fig. 2*A*, *Ba*, *Bb*, *C*, *E*, and *F*). The lumen of the root canal was completely filled with MTA as well as the induction of a mineralized apical barrier formed mainly by cellular cementum in close contact with MTA (Fig. 2*A* and *C*–*F*). This newly mineralized tissue surrounded by the periodontal ligament formed a bridge between root canal walls in 83% (33% complete and 50% incomplete) of the samples (Fig. 1*A*). All samples without an apical barrier of mineralized tissues were associated with MTA overfilling/overextension, which occurred in 44% of the cases (8/18 roots). Moreover, a large number of the examined cases (34%) showed a considerable periodontal cellular cementum deposition in the apical outer portion of the root (Fig. 2*D* and *G*) when compared with the adjacent dentin.

It was also noted that besides the resolution of the periapical lesions and formation of an apical cementum bridge, the presence of radiopaque tissues (Fig. 2*Bb*) juxtaposed to the apical barrier composed by a bulk structure (* in Fig. 2*E* and 2*F*) of dentin (Fig. 2*H* and 2*I*) and cementum with apical delta was contributing to histologic root length augmentation in 50% of the samples.

BC

The histologic findings showed resolution of periapical lesions in 79% of the cases. The majority of samples showed an increase in root thickness and length (74%) in which 42% showed complete apical closure (Fig. 3*A* and *B* and 4*Aa* and *Ab*) and 32% partial closure.

A bridge of cementum and dentin was frequently observed immediately below the MTA (* in Fig. 3*A* and 3*B*) in close relationship with a highly vascularized connective tissue. Also, the majority of histologic samples showed a layer of mineralized tissue formed in apposition to the dentinal inner walls (*black arrowhead* in Fig. 3*A* and *B*). This newly formed tissue was identified as intracanal cellular cementum (IC) based on its histologic characteristics (Figs. 3*F* and *G* and 4*D*) and the continuation with periodontal cementum (Fig. 4*C* and *D*). Indeed, a perfect link, continuity, and resemblance between intra- and extracanal cellular cementum could be observed (Fig. 4*D*). This IC shows the bulkiest amount near the apex and becomes thinner toward the coronal end (Figs. 3*F* and *G* and 4*D*). Surprisingly, areas exhibiting a normal odontoblast layer were found in 21% of the samples (4/19) (Fig. 3*A*, *B*, and *E*).

It was also noted that the presence of several areas of trabecular bone tissue with a woven bone appearance scattered in the lumen of the canal space. However, it was also possible to recognize in some samples a perfect continuity between the intracanal bone (IB) and the alveolar bone in the periapical area (*blue arrowhead* in Figs. 3*G* and 4*G*). A layer of dense connective tissue similar to the periodontal ligament often attached to these 2 mineralized tissues was also observed (*black arrow* in Fig. 3*G*). Some epithelial cells with a histologic structure resembling the epithelial rests of Malassez were detected (Fig. 3*H*) and subsequently confirmed immunohistochemically by means of an antikeratin antibody (Fig. 3*I*).

HA:CS Scaffold

The histologic findings showed resolution of periapical lesions in 47% of the cases besides an increase in root thickness and length (79%) in which 21% had perfect apical closure and 42% partial apical closure (Fig. 4*Ba* and *Bb*). In this group, large areas of the canal space were still occupied by rests of the scaffold that were often surrounded by cellular cementum (Figs. 3*C* and 4*E* and 4*H*). When the matrix maintained its structure, a bridge of cellular cementum was consistently observed. Also, this layer of cellular cementum was found lining the dispersed matrix particles and both of them were in continuity with the newly formed IC in apposition to dentinal walls. In addition to the thickening root canal walls, newly formed cellular cementum also extended apically, producing a vertical increase in the root length (Fig. 3*C*).

The newly highly vascularized connective tissue was occupying a great area of the root canal space, with a moderate inflammatory infiltrate (Fig. 3*C*) seen in 42% of cases. Also, the presence of several areas of trabecular bone tissue with a woven bone (IB) appearance (Fig. 4*E*) under a remodeling process scattered in the lumen of the canal space was detected.

In 47% of the samples, a thickening of the periodontal ligament was seen suggestive of inflammation (Fig. 3*C*). Pathologic root resorptions were detected on both sides of the dentinal walls where cementum was replacing the lost dentin.

P:CS Scaffold

The histologic findings revealed resolution of periapical lesions in 58% of the cases (Fig. 4*Ca* and *Cb*). An increase in root thickness and

A

Filling of pulp canal space with vital tissues	Groups				Total cases (%)
	Apexification	BC	HA:CS	P:CS	
Absent (0)	18 (100%)	1 (5.3%)	6 (31.6%)	2 (10.5%)	27 (36.0%)
Light < 50%(1)	0 (0%)	4 (21.1%)	12 (63.2%)	10 (52.6%)	26 (34.7%)
Partial >50% (2)	0 (0%)	4 (21.1%)	1 (5.3%)	4 (21.1%)	9 (12.0%)
Complete (3)	0 (0%)	10 (52.6%)	0 (0%)	3 (15.8%)	13 (17.3%)
p< 0.05 a=SS for Apexification vs BC, HA:CS and P:CS b= SS for BC vs HA:CS and BC vs P:CS c=SS for HA:CS vs P:CS	a	a, b	a, b, c	a, b, c	
Vascularization					
Absent (0)	18 (88,9%)	1 (5.3%)	6 (31.6%)	5 (26.3%)	28 (37.3%)
Present with hypermedia (1)	0 (0%)	3 (15.8%)	11 (57.9%)	5 (26.3%)	20 (26.7%)
Present with normal (2)	0 (0%)	15 (78.9%)	2 (10.5%)	9 (47.4%)	27 (36.0%)
p< 0.05 a=SS for Apexification vs BC, HA:CS and P:CS b= SS for BC vs HA:CS and BC vs P:CS	a	a, b	a, b	a, b	
Mineralized tissues in the root canal					
Absent (0)	18 (88.9%)	2 (10.5%)	9 (47.4%)	4 (21,1%)	31 (41.3%)
Present (1)	0 (0%)	17 (89.5%)	10 (52.6%)	15 (78.9%)	44 (58.7%)
p<0.05 a=SS for Apexification vs BC, HA:CS and P:CS b=SS for BC vs HA:CS	a	a, b	a, b	a	
Mineralized tissues formed on root canal wall *					
Absent (0)	6 (33.3%)	0 (0%)	4 (21.1%)	3 (15.8%)	13 (17.3%)
Present with increased the thickness of the root wall (1)	3 (16.7%)	7(36.8%)	7 (36.8%)	9 (47.4%)	26 (34.7%)
Present with increased the length of the root (2)	6 (33.3%)	1 (5.3%)	0 (0%)	0 (0%)	7 (9.3%)
Present with increased the length and thickness of the root (3)	3 (16.7%)	11 (57.9%)	8 (42.1%)	7 (36.8%)	29 (38.7%)
p=0.270 *(outside wall of the 1/3 apical portion of root canal)					
Apical closed histologic					
Absent (0)	3 (16.7%)	6 (31.6%)	7 (36.8%)	6 (31.6%)	22 (29.3%)
Partial (1)	9 (50%)	4 (21.1%)	7 (36.8%)	7 (36.8%)	27 (36%)
Complete (2)	6 (33.3%)	9 (47.4%)	5 (26.3%)	6 (31.6%)	26 (34.7%)
p=0.681					

SS – Statistically Significant

B

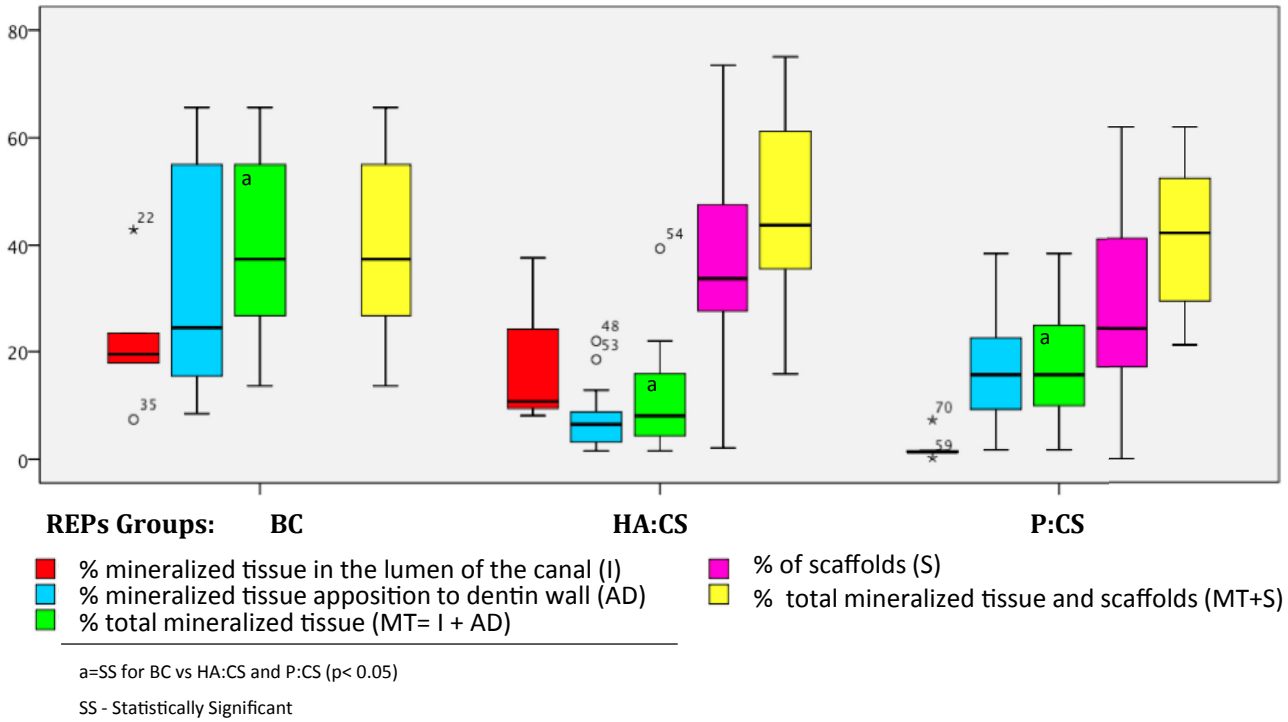

Figure 1. (*A*) Histologic parameters and rated in conformity with scores defined for this experimental study. (*B*) Histomorphometric analysis performed for the REP groups (BC, HA:CS, and P:CS) with quantifications of newly mineralized tissue and residual scaffolds present on the root canal.

length was present in 84%; 32% had perfect apical closure and 36% partial closure. There was a presence of residual fragments of scaffold often sparsely surrounded by mineralized tissue (Fig. 3*D*). The root canal space was mainly occupied by the matrix but also by large amounts of highly vascularized connective tissue (Figs. 3*D* and 4*F*) without signs of inflammation. IC was observed in contact with radicular dentin (84% of the cases) with greater deposition noted in the root apical third (Figs. 3*D* and 4*F*). Interestingly, in 1 sample, dentin could be observed in the middle third, allowing an increase in thickness of the root canal wall, and established a connection with the newly formed IC in the apical

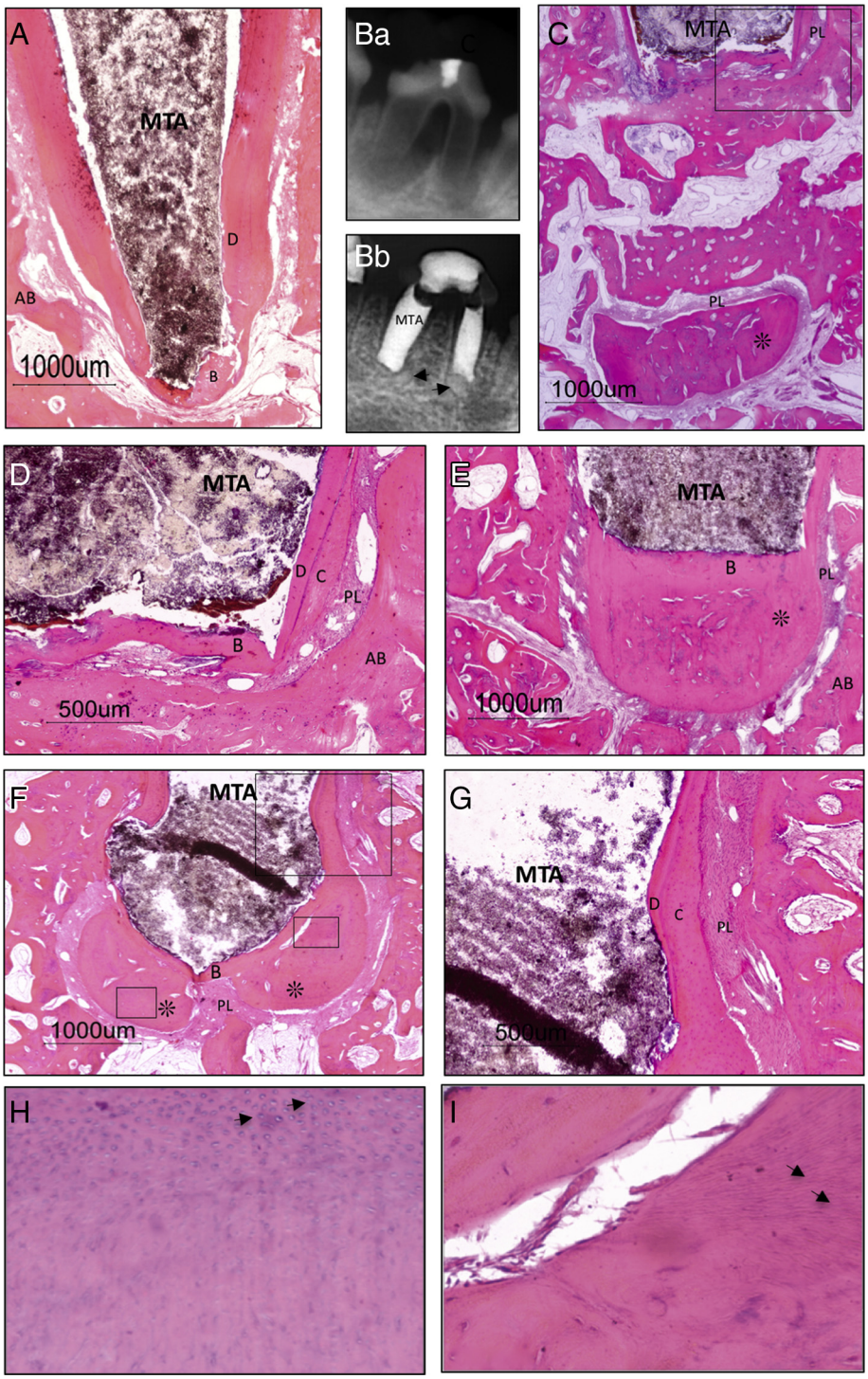

Figure 2. The apexification group. (*A*) A microphotograph showing the lumen of the root canal completely filled with MTA as well as a closed apex by an apical cementum bridge (B). (*Ba*) Radiographic confirmation of AP at 2 weeks after pulp exposure. (*Bb*) Thirteen weeks post-treatment, resolution of periapical lesions and the presence of radiopaque tissues on the apical part of the mesial and distal root (*black arrows*). (*C*) The presence of mineralized structures (*) composed by dentin and cementum surrounded by the periodontal ligament (PL) located at the expected position of the apex and separated from the corresponding root end by trabecular bone tissue seen as an independent root tip detected. (*D*) A high magnification of *C* of an apical barrier (B) adjacent to the MTA formed by cellular cementum and surrounded by an extension of the PL and alveolar bone (AB). (*E* and *F*) Histologic images of those mineralized structures (*) juxtaposed to the apical barriers (B) comprising dentin and cementum with apical delta enclosed by an extension of the PL, contributing to histologic root length augmentation. (*G*) A high magnification of *F* with the presence of a considerable thickening of periodontal cementum (C) when compared with the adjacent dentin (D). (*H* and *I*) A high magnification of *F* to demonstrate tubular dentin (*black arrows*) on these mineralized formations (hematoxylin-eosin).

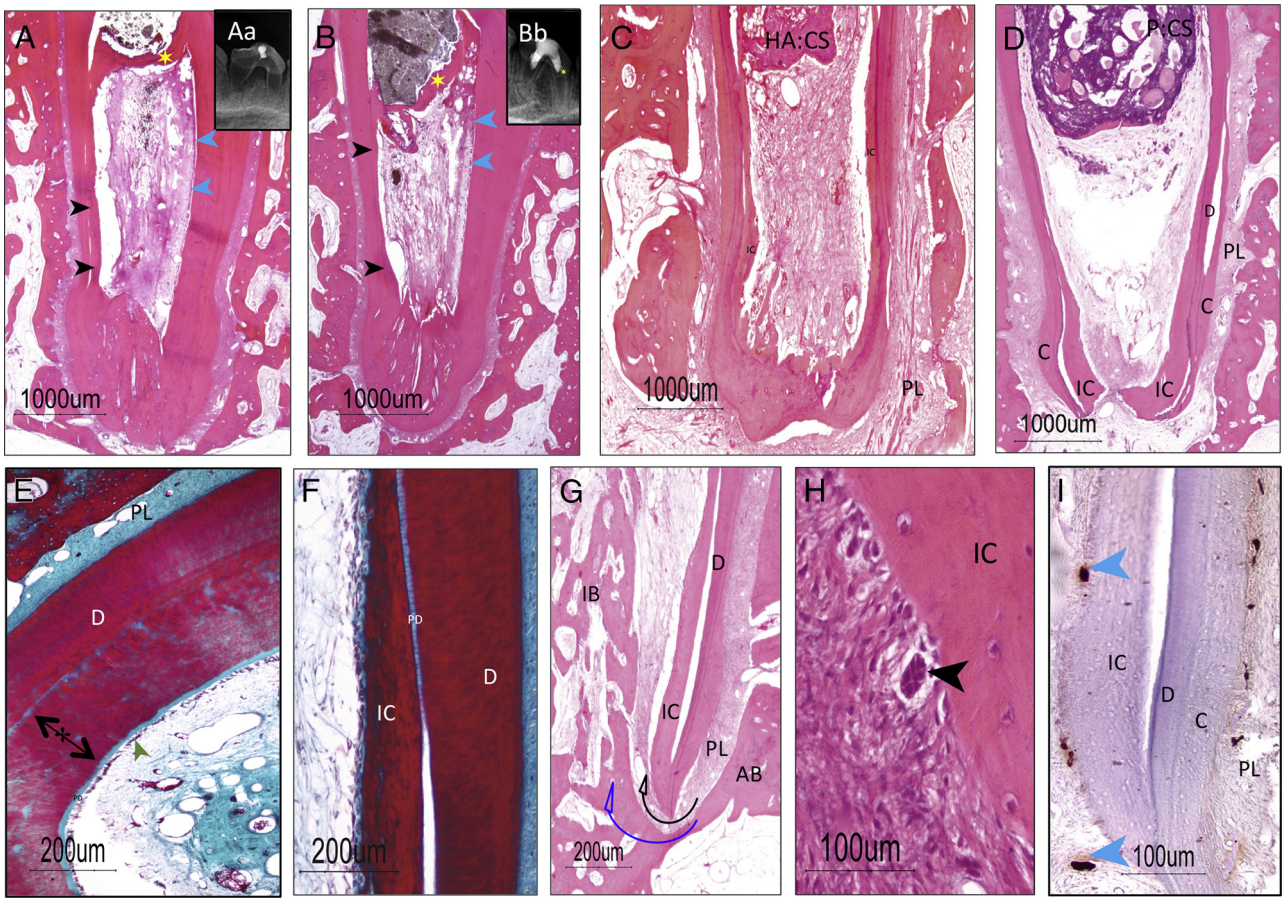

Figure 3. Histologic images from the REP groups. (*A* and *B*) The mesiodistal section of 2 roots of a tooth from the BC group showing a typical pulp tissue exhibiting a normal odontoblastic layer (*blue arrowheads*) on the right side of the canal space. The other half showed the presence of a mineralized bridge (*) below the MTA and highly vascularized connective tissue and IC (*black arrowheads*). Complete apical closure is also present (hematoxylin-eosin). (*Aa*) Initial and (*Bb*) final radiographs of both roots seen on top. (*C*) An image of the HA:CS group; the presence of large areas of scaffold with a presence of a moderate inflammatory infiltrate on pulp space, complete apical closure, and thickening of the root canal wall by new IC and increased periodontal ligament (PL) thickness. (*D*) A P:CS group image with a residual scaffold remnants and areas of intracanal cementum (IC) formed in apposition to dentin (D). (*E*) High magnification of a sample from the BC group showing newly formed dentin (+) along the dentinal wall as well as predentin (D) and an odontoblastic layer (*arrow*) (Masson trichromic stain). (*F*) The presence of a small space separating the IC and the dentin (D) followed by an area of continuity between this space and a conserved region of the predentin (Masson trichromic stain). (*G*) A perfect continuity between the intracanal bone (IB) tissue (*blue arrow*) and the connective tissue (*black arrow*) formed in the canal space, resembling alveolar bone (AB) and PL. (*H*) Epithelial cell rests of Malassez (*black arrowhead*) present in connective tissue, nearby the IC, in the apical portion of canal space (hematoxylin-eosin). (*I*) Positive immunocytochemistry staining (with antikeratin antibodies) of epithelial cell rests of Malassez (*blue arrowheads*) located inside the root canal space and in the PL. C, cementum.

third. The presence of bone tissues or osteoidlike tissue (IB) was noted, but it occurred seldomly and in small confined areas of the root canal (Fig. 4*F*).

Apical Papilla

In all the experimental groups, histologic and radiographic evaluation revealed the presence of apical papilla that had survived the infection and disinfection process and was found detached from the root end in 19% of samples (14/75). This tissue was found mineralized and distinct from the surrounding tissue (Figs. 2*C* and 4*E*, *G–J*, and *L*). It appeared to be composed of cellular cementum and dentin surrounded by the periodontal ligament, containing epithelial rests of Malassez and exhibiting a similar composition to the walls of any ordinary root apex, including the presence of foramina (Figs. 2*F* and 4*J–M*).

Discussion

In this study, a comparative histologic and histomorphometric evaluation was performed in the apexification procedure and 3 variations of REPs in dogs. It was found that REPs that used the induction of a BC allowed for more predictable healing and formation of a vascularized tissue that was devoid of inflammation than the other groups that incorporated the use of chitosan-based scaffolds. In general, the tissues formed in the BC and chitosan groups were composed of a highly vascularized loose connective tissue with evidence of ectopic calcification islands and the presence of newly deposited cementumlike tissue along the dentinal walls. Importantly, areas of dentinal formation with odontoblastlike cells could be seen in approximately 21% of the samples in the BC group. Conversely, the apexification group showed a significantly higher rate of apical healing and the formation of a biological seal apical to the biomaterial that was composed of cementum present in all samples showing MTA confined inside the dentinal walls. Also, in 50% of the samples, histologic augmentation of the root was observed, which

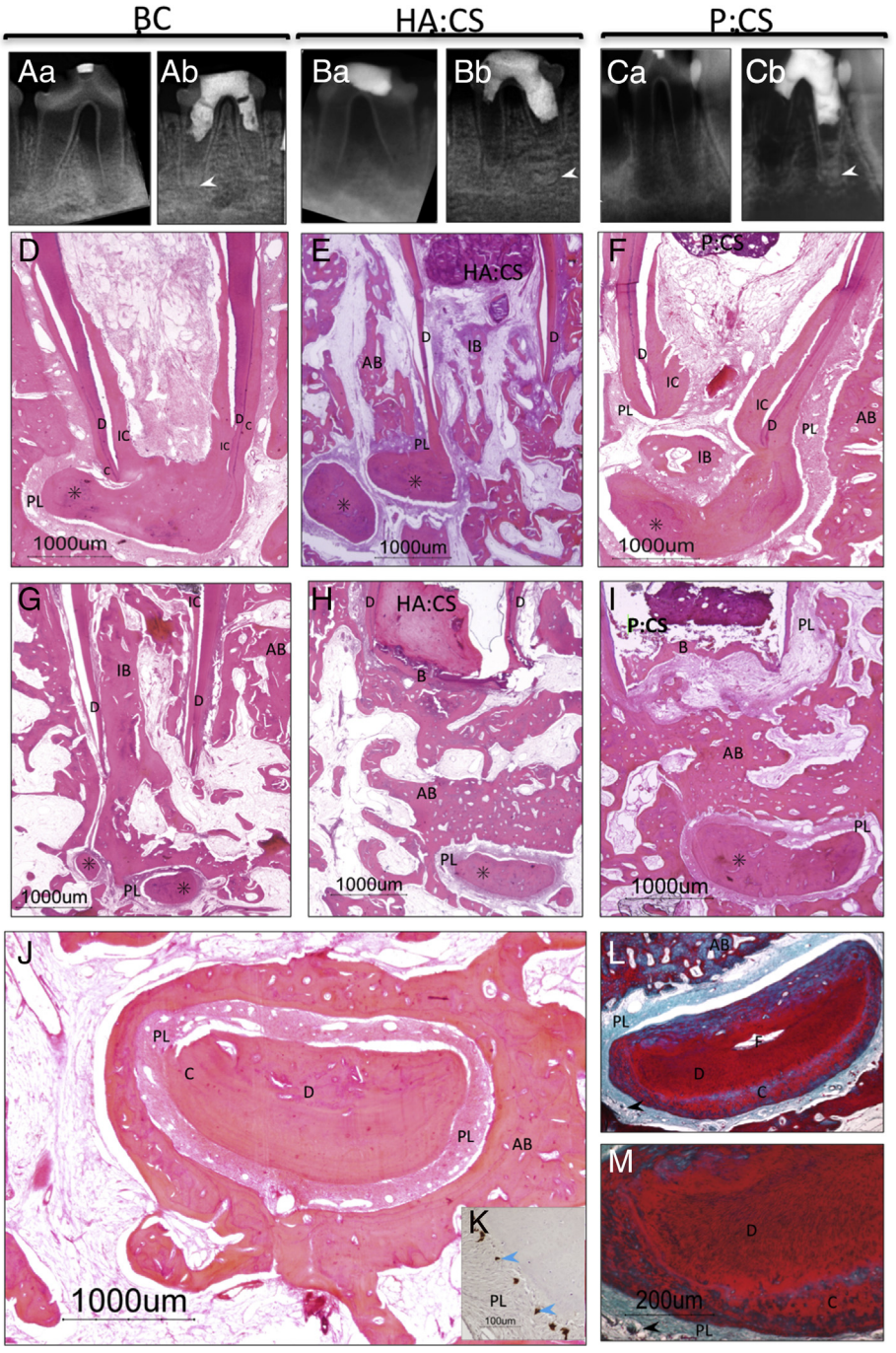

Figure 4. Radiographic and histologic images of REP groups. (*Aa*) Initial and (*Ab*) final radiographs of the BC group, (*Ba* and *Bb*) the HA:CS group, and (*Ca* and *Cb*) the P:CS group. The final radiographic evaluation revealed the presence of radiopaque tissues (*arrows*) apically positioned from the root end. (*D*) An image to elucidate the location and structural components present in the apical papilla (✳) in continuity and in the proximity of canal walls, dentin (D), IC, and periodontal ligament (PL). (*E*) An image of HA:CS in which the same root had 2 mineralized apical papilla (✳), 1 attached to the root wall but moved (spin out) from the natural site and the other detached from the dentin wall. Observed areas of trabecular bone tissue with a woven bone (IB) appearance scattered in the lumen of the canal space. (*F*) Partial detachment of the apical papilla from 1 side of the root dentin wall in the P:CS group. IC with greater deposition in the root apical third and the presence of IB (hematoxylin-eosin). (*G*) The BC group: areas of woven intracanal bone (IB) in continuity with alveolar bone (AB). Apical papilla (✳) (hematoxylin-eosin). (*H* and *I*) Histologic sections showing the presence of mineralized apical papilla (✳) separated from the main root by trabecular bone surrounded by the PL (hematoxylin-eosin). (*J*) Mineralized apical papilla composed of cellular cementum (C) and dentin (D) surrounded by the PL similar to the tip of an ordinary root apex (hematoxylin-eosin). (*K*) Positive immunocytochemistry staining (with antikeratin antibodies) of epithelial cell rests of Malassez (*blue arrowheads*) located in the PL. (*L*) Masson trichrome pointing out the similar structural constitution of these mineralized structures to any ordinary root apex, including the presence of foramina (F) and epithelial cell rests of Malassez (*arrowhead*) on the PL space. (*M*) High magnification of *L* depicting dentinal tubules (D) (Masson trichrome stain).

supports a couple of case reports in humans (25, 26) but is not corroborated by other radiographic quantification studies (3, 4, 14).

Immature permanent tooth roots exhibit remarkable reparative potential. The resolution of periapical lesions and the progression of root development in immature permanent teeth with pulp necrosis after apexification and REP procedures are yet to be well described by several authors (3, 27, 28). These procedures have been generally focused on immature teeth in order to promote continuity of root canal growth, thickening, and apical foramen closure (29), possibly increasing their resistance to fracture and survival (4, 30). Moreover, according to some authors, this approach would also help restore pulp vitality (6, 8). There have been some published cases showing a remarkable radiographic progress in root development after apexogenesis/maturogenesis or REPs (3, 27, 28, 31). There is increasing histologic evidence in the literature from both human and animal studies that the tissues formed after these procedures may not completely recapitulate the once lost dental pulp (12, 16, 20, 23, 32). Although the primary goal of healing AP and continued root development is often achieved in REPs (33), the lack of predictability of histologic evidence of regeneration represents a shortcoming. It is well appreciated that this scientific goal is multifactorial and very challenging. The use of a suitable scaffold that is better defined than the BC has been proposed as one of the components of tissue engineering that needs to be further explored in REPs (15).

Chitosan is a cationic polymer derived from chitin comprised of β-(1–4)-glucosamine and N-acetyl-D-glucosamine monomeric units (34, 35). Chitosan presents different biological properties such as antimicrobial activity; biocompatibility; biodegradability; fungistaticity; hemostatic potential; noncarcinogenicity; remarkable affinity to proteins; and promotion of cell adhesion, proliferation, and differentiation (36). Because of these and other favorable properties, chitosan and its derivatives, alone or in combination with other polymers and biomaterials, have been used to produce a large number of different matrices for tissue engineering applications. Therefore, 2 forms of chitosan scaffolds have been evaluated in this study. Interestingly, the use of these scaffolds was marked by incomplete degradation and the presence of persistent inflammation after REPs. Indeed, it has been reported that there is an initial inflammatory reaction associated with chitosan application to hard and soft tissues that impairs its application as a suitable scaffold for clinical applications and it takes too long for biodegradation of implanted chitosan *in vivo* (37).

Procedures performed with the formation of a BC allowed for complete histologic apical healing in 79% of the cases. This number is significantly lower than the apical healing observed in the apexification group (94%). Nonetheless, this high rate of apical healing along with the presence of a vascularized tissue is an indicator of sufficient microbial disinfection and eradication of infection-related inflammatory reaction. This important finding highlights the now well-reported success of these procedures in promoting the resolution of disease in clinical studies, achieving an important patient-centered outcome (4, 14, 33, 38). It can be concluded that the disinfection protocol used in this study allowed for adequate disinfection of the root canal system and that the persistent inflammatory reaction seen in the chitosan scaffolds may be attributed to the incomplete degradation of these scaffolds.

Although the intracanal tissue formed in the REPs in this study did not fully resemble the native dental pulp, there was the presence of dentin formation with surrounded odontoblastlike cells in 21% of the samples in the BC group. This desirable tissue formation was detected in isolated areas of the root juxtaposed to regions that formed ectopic tissues such as cementum on the dentinal walls and mineralized tissues typical of the periodontium. This dentin formation is not likely because of surviving odontoblasts because the root canal was infected and medicated with triple antibiotic paste for 1 month. Furthermore, all REPs were treated the same way with the exception of the different scaffold used; yet, this finding was only noted in the BC group. To the best of our knowledge, this is the first demonstration, albeit in isolated areas of the canal space, of "true regeneration" as defined by dentin formation by odontoblastlike cells in a previously infected root canal after REPs.

This study also aimed to evaluate the periradicular tissues after apexification and REPs. Homogenous mineralized tissues were found in close proximity and continuity with the cementum bridges of the apical barrier in both the apexification and REP groups. The homogeneity of the tissue and the presence of dentinlike tissue, periodontal ligament, and apical foramina suggest that it consists of the apical papilla, which survived the endodontic infection and its interaction with the Hertwig's Epithelial Root Sheath (HERS) allowed for differentiation to take place once the infection was solved. Importantly, complete detachment of this tissue was noted in some 23% of samples (13 of 57) of groups treated with REPs, suggesting that the mechanical forces applied to evoke bleeding are capable of dislodging the apical papilla from its continuum with the canal space. Their partial or total separation may be because of the fact that apical papilla and HERS are loosely attached to the apex so they can be easily detached by infection, mechanical trauma, iatrogenic factors, or as a result of the treatment protocol. Interestingly, there is clinical evidence of root tip formation separate from the main root after trauma (26, 39). In another study, the apical papilla and its residing cells were also shown to survive advanced endodontic infections in rats (40). Likewise, apical papilla was detected in this study, suggesting that this tissue is resilient and survives adverse conditions.

Collectively, these findings suggest that stem cells of the apical papilla and HERS cells will remain vital in continuity or close to the main root after infection. It is expected that mechanical disruption of the apical tissues was the main reason for their detachment. Thus, a conservative approach should always be considered as a first choice of treatment for immature teeth with necrotic pulp. Vigorous attempts of evoking apical bleeding should be avoided, and the use of chemotactic-based cell homing strategies represents an important future clinical approach.

Conclusion

The addition of the chitosan scaffolds to the blood in REPs did not improve the formation of new mineralized tissues along the root canal walls and histologic evidence of regeneration. Despite the use of scaffolds, REPs allowed continued development of root walls with the presence of bonelike tissue, cementum, and periodontal ligament observed in the canal space. Some samples treated with evoked bleeding and the formation of a BC showed evidence of dentin formation in restricted areas of the root canal. Furthermore, this study showed the continued survival and differentiation potential of the apical papilla after infection.

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