

Immunohistological Characterization of Newly Formed Tissues after Regenerative Procedure in Immature Dog Teeth

Nozomu Yamauchi, DDS, MS,* Hideaki Nagaoka, DDS, PhD,[†] Shizuko Yamauchi, DDS, MS,*
Fabricio B. Teixeira, DDS, MS, PhD,[‡] Patricia Miguez, DDS, MS, PhD,[§]
and Mitsuo Yamauchi, DDS, PhD[†]

Abstract

Introduction: In a previous report, we showed that 2 types of mineralized tissues were formed in the canal spaces of dogs after tissue engineering treatments of immature teeth with apical periodontitis: (1) dentin-associated mineralized tissue (DAMT) and (2) bony islands (BIs). The objective of this study was to characterize these mineralized tissues. **Methods:** The maturation and organization of collagen matrices in DAMT, BIs, and the interface between DAMT and the dentin wall were characterized using a histochemical method with picosirius red staining under polarized light microscopy. In addition, the distribution of 2 noncollagenous proteins (ie, dentin sialoprotein and bone sialoprotein) in these tissues was investigated by immunohistochemical methods with specific antibodies. **Results:** The results showed that DAMT is distinct from dentin, bone, or BIs. Although it resembled cementum to an extent showing similar immunoreactivity to the noncollagenous proteins, the organization and maturation of collagen matrix was significantly different from cementum. BIs resembled a bone matrix in terms of morphology, collagen organization, and immunoreactivity. **Conclusions:** The results indicate that DAMT and BIs formed in the canal space are distinct from each other, one exhibiting a unique mineralized tissue and the other a bone-like tissue. (*J Endod* 2011;37:1636–1641)

Key Words

Collagen scaffold, regenerative endodontics, root canal, tissue engineering

The treatment of immature teeth with necrotic pulps and apical periodontitis using regenerative endodontic procedures has recently gained much attention because of its successful outcomes in animal- as well as patient-based studies (1–4). The advantages of regenerative endodontic procedures over apexification procedures are that the former allows possible continued root thickening and lengthening (ie, maturation) and induces vital tissue. With continued research and improvement in regenerative endodontic procedures, these may become the standard of care rather than apexification-type procedures and may have benefits even in mature infected teeth (5).

Recently, Thibodeau et al (1) conducted a study in which bleeding was induced with addition of a collagen solution in canals of necrotic pulps with apical periodontitis. They found that induced bleeding improved the generation of newly formed mineralized tissues within the canal space (1). Using the hematoxylin and eosin–stained sections from this study, Wang et al (6) then further characterized the tissues formed in the intracanal space and identified the cementum-like, bone-like, and periodontal ligament-like tissues. Independent of this study, we have performed a study to improve the vital tissue formation in the canal space by incorporating a cross-linked collagen scaffold and by partially demineralizing the dentin wall. The results showed that the former significantly increased the mineralized tissue formation, and the latter helped adherence between dentin and the mineralized tissue formed along the dentin wall (7).

The organic matrix of bone and teeth (except tooth enamel) consists of primarily fibrillar type I collagen (~90%) and a number of non-collagenous proteins (~10%). In these mineralized tissues, collagen functions as a 3-dimensional, stable template for minerals to deposit and grow in an orderly fashion (8, 9), and a number of noncollagenous proteins may play roles in the initiation and/or inhibition of mineralization. A group of specific noncollagenous proteins called the SIBLING family (Small Integrin-Binding Ligand N-linked Glycoproteins) include dentin sialophosphoprotein (DSPP), osteopontin, bone sialoprotein (BSP), and others (10). Although they have been recently identified in nonmineralized tissues such as kidney and salivary glands (11, 12), these SIBLINGs are mostly found in bones and teeth and are likely associated with the mineralization process. Of these, DSPP is one of the most widely used markers to identify dentin matrix (13, 14). After secretion, DSPP is cleaved into dentin sialoprotein (DSP) and dentin phosphophoryn. Recently, the protease, which is responsible for this cleavage, has been identified to be 3 isoforms of bone morphogenic protein 1 (15). Although osteoblasts have been shown to express DSPP, the level of expression is significantly lower than that of odontoblasts

From the *Department of Endodontics and [†]NC Oral Health Institute, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; [‡]Department of Endodontics, University of Texas Health Science Center at San Antonio, San Antonio, Texas; and [§]Department of Periodontics, University of Pennsylvania, Philadelphia, Pennsylvania.

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Address requests for reprints to Dr Mitsuo Yamauchi, NC Oral Health Institute, School of Dentistry, University of North Carolina at Chapel Hill, Campus Box 7455, Chapel Hill, NC 27599. E-mail address: mistuo_yamauchi@dentistry.unc.edu
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(16). BSP is one of the major noncollagenous proteins in bone matrix (17), and it is relatively low in dentin.

In this study, in an attempt to further characterize the newly formed mineralized tissues in and around the canal space we observed (7), the maturation and organization of collagen matrices and relative distribution of 2 SIBLING members (DSP and BSP) in those tissues were assessed by specific histochemical and immunohistochemical analyses, respectively.

Materials and Methods

The specimens were obtained from our preceding study (7). For the experimental details, different treatment groups (ie, bleeding only, bleeding + EDTA, bleeding + scaffold, and bleeding + EDTA + scaffold) and their effects on mineralized tissue formation refer to this article. All animal procedures followed a protocol approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Histological Processing

After removal of all soft tissue and excess hard tissue from the specimens, they were fixed and decalcified as described previously (7). For general morphologic observations, sections were stained with hematoxylin and eosin and observed under light microscopy. Picrosirius red (PSR) staining combined with or without polarized light microscopy has been used to assess the organization and maturation of collagen matrix (18, 19). With this staining, thicker, tightly packed, mature collagen matrix stains orange to red, whereas thin, immature collagen stains green to yellow. In addition, the directionality of collagen fibers can be evaluated. The sections were stained with 0.1% sirius red in saturated picric acid (Electron Microscopy Sciences, Hatfield, PA) and observed under polarized light microscopy as previously reported (20). The Olympus BX40 microscope (Olympus, Center Valley, PA) was used with $\times 1.25$ –40 magnification.

To determine the relative distribution of DSP and BSP, immunohistochemical analyses were performed. The serial sections were subjected to the antigen retrieval with proteinase K (30 $\mu\text{g}/\text{mL}$) (Thermo Scientific, Asheville, NC) for 10 minutes at room temperature, and the sections were incubated with 0.3% H_2O_2 in methanol for 30 minutes. The sections were then incubated with anti BSP and DSP (1:200 dilution for each) antibodies (10 $\mu\text{g}/\text{mL}$) (LF-84 and LF-151, respectively, kindly provided by Dr Larry Fisher at the National Institute of Dental and Craniofacial Research). To confirm the specificity of those antibodies, the sections were also incubated with a normal rabbit

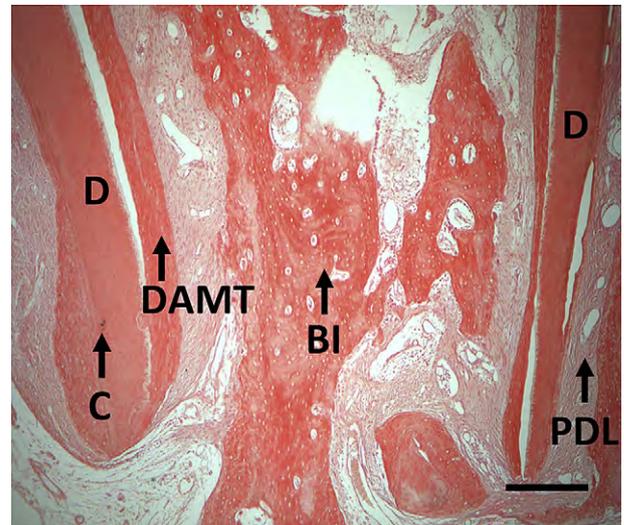


Figure 1. A histologic image of immature dog tooth after the regenerative procedure. The section was stained with picrosirius red. BI, bony island; C, cementum; D, dentin; DAMT, dentin-associated mineralized tissue; PDL, periodontal ligament. For the experimental details, see Yamauchi et al (7). Scale bar = 500 μm .

serum for BSP and DSP immunostaining as negative controls. For detailed information about these antibodies such as antigen sources and specificities, see <http://csdb.nidcr.nih.gov/antisera.htm>. The sections were washed with phosphate-buffered saline several times and further incubated with rabbit or mouse biotinylated immunoglobulin G for 30 minutes and subsequently with avidin and biotinylated horseradish peroxidase for 30 minutes. After several washes with phosphate-buffered saline, the sections were incubated with 3, 3'-diaminobenzidine substrate (DAB; Vector Laboratories Inc, Burlingame, CA) to visualize the immunoreactivity. The sections were observed under light microscopy (Olympus BX40 microscope) ($\times 1.25$ –40 magnification).

Results

General Observations of Dentin-associated Mineralized Tissue and Bony Islands

As shown in the preceding report (7), at 3 and a half months after the treatment, 2 types of newly formed mineralized tissues were

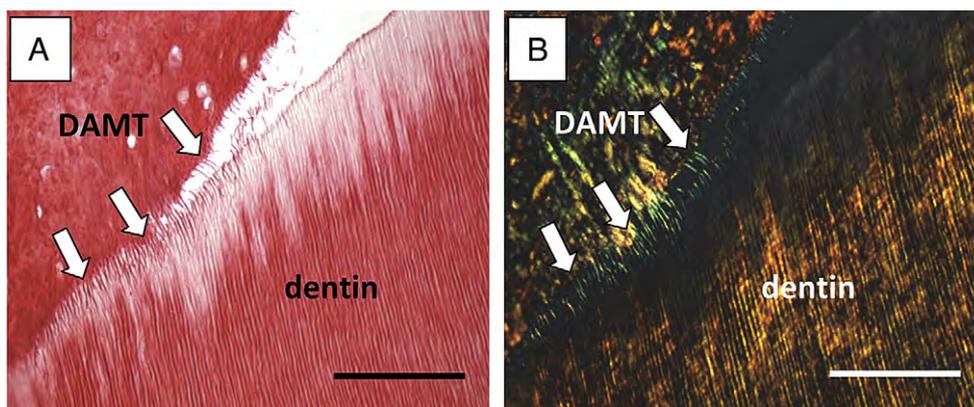


Figure 2. PSR-stained images of the interface between dentin-associated mineralized tissue (DAMT) and dentin (A) without and (B) with polarized light. The arrows indicate hair-like projections from the DAMT interlocking with the partially demineralized dentin. Scale bar = 100 μm .

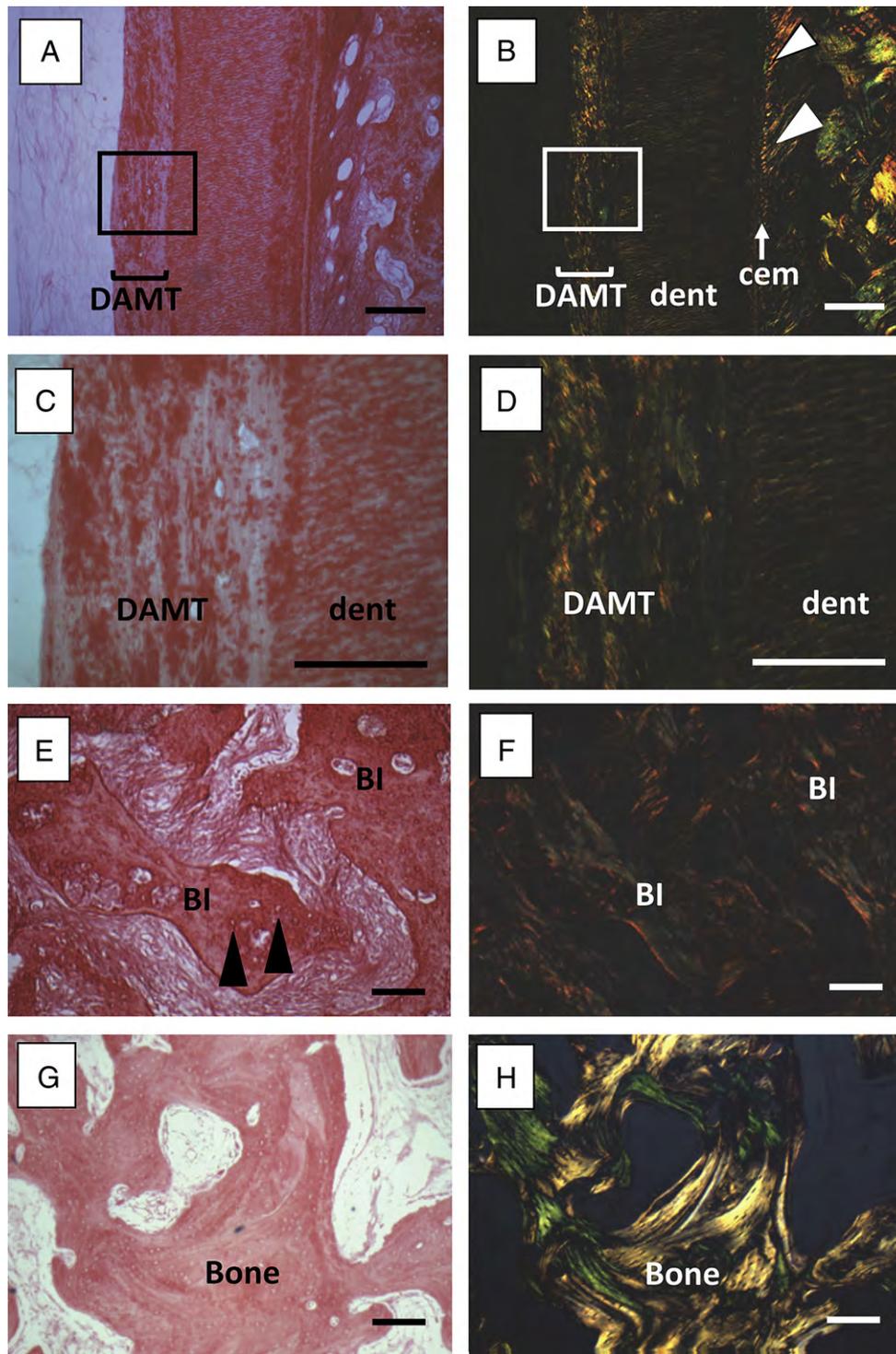


Figure 3. PSR-staining patterns of dentin-associated mineralized tissue (DAMT), bony island (BI), and surrounding tissues without (A, C, E, and G) and with polarized light (B, D, F, and H), respectively. Images of C and D are higher magnifications of the boxed areas shown in A and B, respectively. Bone, periapical compact bone; cem, cementum; dent, dentin. *White arrowheads* indicate periodontal ligament and *black arrowheads* indicate lacunae present in BI. Scale bar = 100 μ m.

constantly observed in the canal space: 1 adhering to/detached from the inner dentin wall (dentin-associated mineralized tissue [DAMT]) and another forming bony islands in the inner lumen independent of the dentin wall (bony island [BI]) (Fig. 1). There was no significant difference in the histochemical and immunohistochemical staining pattern of DAMT and BI among the treatment groups. Thus, the following images were all taken from the bleeding + EDTA + scaffold group, except

Figure 2 in which the hair-like projections were most clearly shown in the bleeding + EDTA group.

PSR Staining

In most of the specimens, DAMT formed in the canal space was significantly thicker than the cementum counterpart (Figs. 1 and 3A and B). When stained with PSR, DAMT appeared as a nonlamellar,

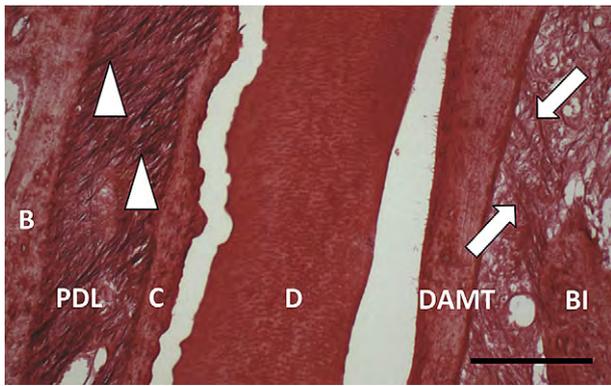


Figure 4. PSR-stained image showing bone (B), bony island (BI), cementum (C), dentin (D), dentin-associated mineralized tissue (DAMT), and periodontal ligament (PDL). There is a cleavage plane showing cementum separated with some attached dentin and DAMT separated from dentin. The PDL (*arrowheads*) is well organized and tightly packed, connecting cementum and bone. The *arrows* show the sparse PDL-like connective tissue loosely connecting DAMT and BI. Scale bar = 200 μm .

irregular, and often patchy matrix (Fig. 3A and C). Under polarized light, the tissue generally showed a green to light yellow color (Fig. 3B and D), indicating an immature/thinner collagen matrices. In comparison to other tissues such as cementum, dentin, and periodontal ligament (PDL, indicated by *white arrowheads* in Fig. 3B), DAMT lacked the uniformity and continuity of color exhibiting patches of green, yellow, and, in some areas, orange color and significantly varied in size and direction (Fig. 3D). This suggests that the collagen fibers in DAMT are not packed in an orderly fashion, the directionality is poor, and the maturity is not consistent. The cementum layer (indicated by a *white arrow* in Fig. 3B) appeared as a tightly packed collagen matrix with a relatively uniform color (ie, green to light orange), with insertion of PDL fibers that run obliquely exhibiting a yellow to orange color. Dentin showed a yellow to orange color indicative of more mature collagen fibers and had clear directionality likely along the dentinal tubules (Figs. 2B and 3B and D). The BI was formed in the canal space varying in size and generally appeared as woven bone-like structures lacking osteon (Fig. 3E). Many lacunae, like alveolar bone, were observed in BIs (Fig. 1 and *black arrowheads* in Fig. 3E). When observed under polarized light (Fig. 3F), BIs showed green to orange color with some directionality, and most of them lacked a clear lamellar structure. For comparison, PSR-stained periapical compact bone is shown without and with polarized light in Figure 3G and H, respectively. The typical lamellar structure is obvious in this section (Fig. 3H).

As noted in our previous report (7), the projections/hair-like structures were often seen in the partially demineralized sections (bleeding + EDTA + EDTA + scaffold groups) (Fig. 2A). These characteristic structures indicated by white arrows, most likely collagen fibers, extended from the DAMT into the demineralized dentin surface connecting the 2 mineralized tissues. When observed under polarized light, they showed a green color indicative of immature collagen fibers (*white arrows* in Fig. 2B).

In some sections, there were cleavage planes between cementum and dentin and between DAMT and dentin (Fig. 4). The PDL present between cementum and alveolar bone showed a clear direction and tightly attached to the 2 mineralized tissues (*white arrowheads* in Fig. 3B and Fig. 4). The soft connective tissue present between the DAMT and the BI appeared to be less organized and loosely packed (Fig. 4). In some sections, the continuous strands connecting DAMT and BI were observed (*white arrows* in Fig. 4).

Immunohistochemistry

Immunohistochemical analyses were performed for DSP and BSP in areas showing dentin, DAMT, cementum, BI, and bone, with the respective negative controls. Positive immunoreactivity for DSP was obvious throughout the thickness of dentin matrix, but it was particularly intense in the areas near the canal space (Fig. 5A). In DAMT, a layer with appreciable immunoreactivity was observed near the areas that were originally attached to dentin, and overall moderate immunoreactivity was seen in this tissue. No significant immunoreactivity was found in cementum except at the edge of the tissue, which could be an edge effect. For BSP, immunoreactivity was seen in alveolar bone matrix but not in DAMT, dentin, or cementum (Fig. 5C). Periodontal ligament revealed significant immunoreactivity for BSP. For BI, no significant immunoreactivity for DSP was found (Fig. 5E), but positive immunostaining for BSP was apparent in this tissue (Fig. 5G), which is similar to that of alveolar bones (Fig. 5C). No immunoreactivity was seen in all of the respective negative controls (Fig. 5B, D, F, and H).

Discussion

It has been reported that in a dog model, after regenerative endodontic treatment, newly mineralized tissues were formed (1). However, the nature of these mineralized tissues is poorly understood. Thus, the current study was undertaken to characterize the 2 types of mineralized tissues, DAMT and BI, formed in the canal space (7) and to compare them with other mineralized tissues.

Based on both PSR staining pattern and immunohistochemical analysis, DAMT was clearly different from dentin and bone (Figs. 2 and 3). Although to an extent it resembles cementum (eg, lack of vasculature and immunostaining pattern), the organization and maturation of collagen matrix appeared to be significantly different from cementum. Because collagen defines the space for the mineral deposition and growth, the results indicate that the pattern of mineralization in DAMT is less uniform compared with other mineralized tissues including cementum. The investigation on collagen at the ultrastructural level (ie, diameter and shape of the fibrils) and biochemical level (ie, collagen types and post-translational chemistries [21]) may provide further insights into the similarity and difference between DAMT and cementum. The nature of minerals in DAMT also needs to be investigated to further characterize this tissue. The BI resembles bone in the following aspects: (1) it is vascularized, (2) there are lacunae housing cells resembling osteocytes, and (3) there were some bone marrow-like structures (7). The PSR staining pattern was similar to a woven bone-like structure. Possibly, with time, a more mature, lamellar structure could be formed. The pattern of immunoreactivity of BIs for DSP and BSP was also similar to alveolar bone but distinct from dentin, cementum, and DAMT.

The origin of the cells responsible for the formation of DAMT and BI is not clear at this point. It has been recently shown that mesenchymal stem cells are delivered into the canal space after induced bleeding and that they may be derived from the local tissue adjacent to the apex (22). The apical papilla identified in various species and the stem cells in it (23–27) could be a potential source of cells to form DAMT and BI although the presence of such cells has not been shown in a dog model. It has been reported that these cells have the potential to differentiate into odontoblasts forming new roots (23–26). In the current study, however, there was no odontoblastic cell layer, dentin-like structure, or new pulp-like tissue observed in any of the specimens examined. It is likely that rigorous instrumentation followed by infection of root canal spaces destroyed not only pulp tissue including the odontoblasts and predentin layer but also the apical papilla even if it was present.

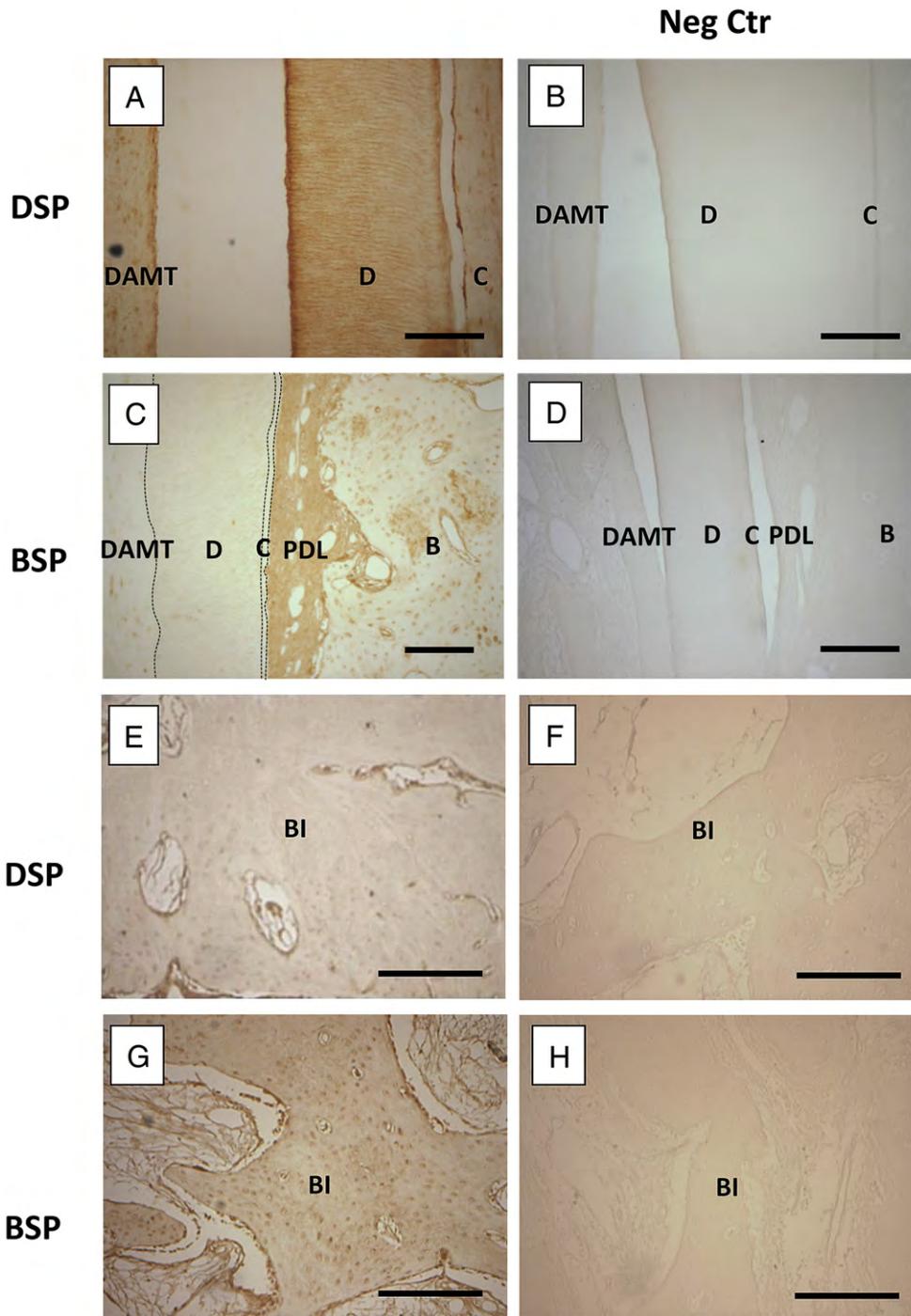


Figure 5. Typical immunohistochemical staining for dentin sialoprotein (DSP) and bone sialoprotein (BSP). B, alveolar bone; BI, bony island; C, cementum; D, dentin; DAMT, dentin-associated mineralized tissue. The respective negative controls using normal rabbit serum are shown on the right of each image. Scale bar = 100 μ m.

Histologically, it appeared that PDL showed in-growth along the dentin walls to create DAMT. Because PDL cells can repopulate damaged or exposed cementum/dentin surfaces and form cementum-like tissues (28), it is likely that the stem cells present in the PDL form DAMT (29). The bone-like BI tissue was likely formed by osteoblast-like cells derived from periapical tissues, such as bone marrow and/or PDL. The damage of apical PDL because of the over-instrumentation could also induce a significant influx of PDL stem cells that may help to form DAMT and BI.

Recently, Wang et al (6) reported the presence of Sharpey's fiber- and PDL-like structures between cementum-like and bone-like tissues in the canal space on Thibodeau's specimens (described earlier). The PDL-like structures were also observed in our study (Fig. 4), but the occurrence was sparse and irregular. When it occurred, it did not appear to connect tightly the 2 mineralized tissues (DAMT and BI) as natural PDL does between cementum and alveolar bone. The nature of this soft connective tissue should be characterized by the presence of PDL markers such as periostin (30, 31) and asporin/periodontal

ligament associated protein (PLAP) (32–34). The apparent lack of connectivity between the DAMT and BI could be related to the lack of mechanical stress in the intracanal space. Possibly, the apposition of DAMT along the dentin wall is necessary to stabilize the tooth structure rather than forming the periodontium-like tissue. It would be interesting to see if DAMT and BI will be eventually fully connected and fuse to close the lumen space or if the soft connective tissue will remain between the 2 mineralized tissues. A long-term study needs to be performed to address this.

Replantation and autotransplantation studies may give us some insight into the biological process of these regenerative treatments. Skoglund and Tronstad (35) showed that in transplanted teeth the odontoblastic layer rarely survived, and over a 180-day course the lumen was filled with mineralized tissue. A recent study showed that in a rat model, in replanted teeth, some cases exhibited revascularization of the pulp with surviving odontoblast-like cells, which were able to form a dentin-like structure on the root wall. These teeth were extracted and replanted immediately; thus, it is possible that the pulp tissue remained vital and revascularization occurred. In the cases in which the pulp tissue and odontoblasts were lost, these teeth were filled with bone-like tissue (36). To regenerate dentin and pulp complex in teeth that were previously infected, further studies need to be done possibly by incorporating some factors into the scaffold that facilitate the differentiation of stem cells to odontoblasts.

In conclusion, the mineralized tissue forming along the inner dentin wall generated by our tissue-engineering approach is a unique mineralized tissue with some resemblance with cementum. The mineralized tissue formed independently in the lumen space resembled a bone matrix.

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The authors deny any conflicts of interest related to this study.

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