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Recombinant Amelogenin Protein Induces Apical Closure and Pulp Regeneration in Open-apex, Non-vital Permanent Canine Teeth

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Abstract

Introduction—Recombinant DNA produced amelogenin protein was compared to calcium hydroxide in a study of immature apex closure conducted in 24 young mongrel dogs.

Methods—Root canals of maxillary and mandibular right premolars ($n = 240$) were instrumented and left open for 14 days. Canals were cleansed, irrigated and split equally for treatment with recombinant mouse amelogenin ($n = 120$) or calcium hydroxide ($n = 120$).

Results—After 1, 3, and 6 months, the animals were sacrificed and the treated teeth recovered for histological assessment and immunodetection of protein markers associated with odontogenic cells. After 1 month, amelogenin-treated canals revealed calcified tissue formed at the apical foramen and a pulp chamber containing soft connective tissue and hard tissue; amelogenin-treated canals assessed after 3 and 6 month intervals further included apical tissue functionally attached to bone by a periodontal ligament. In contrast, calcified apical tissue was poorly formed in the calcium hydroxide group and soft connective tissue within the pulp chamber was not observed.

Conclusions—The findings from this experimental strategy suggest recombinant amelogenin protein can signal cells to enhance apex formation in non-vital immature teeth and promote soft connective tissue regeneration.

Keywords

Amelogenin; apex formation; calcium hydroxide; pulp regeneration; regeneration

Introduction

Dental caries and trauma are among the most prevalent challenges to tooth preservation as these insults can render the dental pulp tissue non-vital. If death of the pulp tissue occurs prior to complete root apex formation and apical closure, normal root development ceases (1). Apexification is one method used with young, necrotic succedaneous teeth to promote continued apical development of an incompletely formed root and/or to induce a calcified barrier in a root with an open apex (2). The most commonly advocated medicament for apexification is calcium hydroxide, although recently the material known as *mineral trioxide aggregate* (MTA) (3) and a biomimetic approach with porcine-derived enamel matrix derivatives (EMD) (4) have been used with success. Nonetheless, apexification is not always successful (5). For these reasons, dental medicine practitioners have searched for more efficient biological methods to repair or regenerate these tissues, including regeneration of the pulp, rather than being forced to rely only on the introduction of a foreign material (6–12). Improved treatment methods that are regenerative rather than reparative, including tissue engineering-based therapies, are no longer part of an anticipated future, but rather are becoming clinical realities. Regenerative endodontic therapies can be defined as biologically based procedures designed to replace damaged odontogenic cells and their bioceramic composite tissues (12). These procedures offer biological solutions to biological problems (2, 12, 13). One appealing approach is to harness strategies that mimic the biological pathways serving to guide tissue and organ development, thereby inducing tissue regeneration (6, 8, 12, 14–20).

The study of recombinant amelogenin protein has permitted structure-function insight into the self-assembly of a multimeric protein structure known as a *nanosphere*, which represents the conformation of amelogenin associated with its function during enamel biomineralization (21–24). Moreover, it is now understood that amelogenin proteins can also provide signals to cells that regulate gene expression and modify the inflammatory response, as well as advance the repair mechanisms of dentinoblasts (20, 25–30). Of recent interest is the 59-amino acid long amelogenin protein isoform that has been shown to alter the fate of human and mouse stem cells towards osteogenic lineages (31) by activating the Wnt signaling pathway (32). Molecules with related biological activity were first described by Amar and colleagues (33, 34), who showed that a small polypeptide recovered from rat incisor dentine induced chondrogenesis that eventually progressed to form bone when ectopically transplanted into neonatal rat muscle. This polypeptide was eventually shown to be an amelogenin splicing isoform, and was suspected to be involved in the epithelial-mesenchymal interaction in neonatal teeth required for dentinogenesis (35–37).

Here, we describe experiments designed to determine whether full-length mouse amelogenin protein produced by recombinant DNA techniques retains bio-signaling capacity to induce healing in the periapical tissues of immature, necrotic teeth with open apices in a dog model. During histologic assessment of apical closure, a novel and unexpected observation was made. We observed that the pulp chamber of teeth treated with recombinant amelogenin protein was filled with soft connective tissue that expressed proteins associated with cells found in pulp-like tissue. Hard tissue of complex organization similar to dentine associated mineralized tissue was also observed (38).

Materials and Methods

Expression and purification of recombinant poly-(histidine)-tagged proteins

Recombinant amelogenin proteins were prepared with the expression vector pQE30 (QIAGEN Inc., Valencia, CA) and purified with a poly-(histidine) amino terminus, as previously described (23, 25). Recombinant poly-histidine-tagged murine 180 amino acid long amelogenin (rp(H) M180) is identical to the authentic mouse full-length amelogenin except for the inclusion of an amino-terminal peptide sequence (RGSHHHHHHGS) used for affinity purification of the bacterially produced protein. The entire DNA fragment within the pQE30 vector was subjected to nucleotide sequence determination to ensure that no errors were generated during the DNA amplification procedures and that the construct was correctly engineered. Recombinant proteins were prepared and purified using nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices (Qiagen, Valencia, CA).

Animal Procedures

An Institutional Review Board charged with the safety and protection of vertebrate animals reviewed and approved the described animal studies conducted at Pharos University. The animals were housed in the vivarium for 1 week under veterinarian observation before intervention for the purpose of assessing their health. A total of 240 root canals from 120 mandibular and maxillary premolars in 24 healthy mongrel dogs at approximately 6 months of age were used. Animals were anaesthetized using sodium pentobarbital intravenous injection (30 mg/kg body weight). Pre-operative radiographs were exposed to confirm the presence of open apices in the right mandibular second, third, and fourth premolars, as well as the right maxillary second and third premolars. Some 120 canals out of the 144 mandibular canals (2 canals in each of 3 premolars in 24 dogs) were filled with recombinant amelogenin. In contrast, 24 mandibular canals were filled with calcium hydroxide, as were the 96 canals in the maxillary premolars (2 canals $\times$ 2 premolars $\times$ 24 dogs) to provide 120 canals. After obtaining endodontic access, the length of each canal was determined and the pulp tissue was removed with K-files. Hedstrom files were introduced to the radiographic apex and used to completely remove all pulp tissue remnants. The same operator performed all of the procedures, aided by a support team, over the course of several days.

The canals were irrigated with distilled water and after achieving hemostasis, a cotton pellet was placed in the entrance of each canal. The teeth were left without a coronal restoration for 14 days to become contaminated with oral microbes (39). After 14 days, the canals were cleansed to within 1 mm of the radiographic apices using larger files and gentle filing movements with 1% sodium hypochlorite. Each canal was dried with sterile paper points, and the teeth were sealed with a temporary filling (Orafil G, Colostol, Fermin, India). At the next visit, 7 days after closure of the canals, teeth that showed signs of infection, such as mobility, odor, purulent exudate, or draining sinus, were excluded in the study (40) as reported in Table 1. The canals meeting these criteria were filled with one of the two experimental materials, either recombinant amelogenin protein (rp (H) M180) or calcium hydroxide (Ca(OH)₂), as described below (Table 2).

Approximately 1 ml of propylene glycol alginate vehicle (PGA) was mixed with 30 mg recombinant amelogenin protein powder rp (H) M180 pre-weighed using an aseptic technique and allowed to rest for 15 minutes before use. Once mixed, the amelogenin material was used within 2–3 hours. The suspension of rp (H) M180 + PGA material was delivered into the canals with a special syringe as previously described (41). This procedure was repeated until all of the 120 canals in the mandibular arches of 20 dogs were treated with rp (H) M180. Intermediate restorative material (IRM) was then carefully placed over the root canal dressing and the access cavity was restored with amalgam.

Prefabricated syringes containing calcium hydroxide paste were used to fill the 96 canals of the second and third premolars in the right maxillary arches. In four dogs, the mandibular right premolar canals (24 canals) were filled with calcium hydroxide to provide a total of 120 canals for the calcium hydroxide treatment group. Intermediate restorative material (IRM) was then carefully placed over the root canal dressing and the access cavity was restored with amalgam.

After each surgery, the animals received Voltaren (Novartis Pharma Egypt, under license from Novartis Pharma, Switzerland), an intravenously delivered painkiller, at 25mg/kg. Tetracycline (Tetracid; Cid Co, Egypt) was administered intramuscularly the first day and thereafter mixed with food for 7 additional days at a dose of 15 mg/kg. The dogs were placed on a soft diet during the postoperative period to reduce the possibility of local trauma to the surgical site.

After 1, 3 or 6 months postoperatively, the animals were euthanized by intravenous overdose injection of thiopental sodium. The teeth and surrounding bone were removed as a block with a water-cooled diamond disc (40). Untreated teeth from the left quadrants were randomly harvested as controls.

Histology and Immunodetection

The treated teeth were recovered at 1, 3, and 6 month time points after treatment in order to obtain approximately 80 canals at each elapsed time interval. In some instances, samples were lost due to technical limitations, and these losses are shown in accompanying tables for each corresponding elapsed time interval (Table 1). Samples were demineralized and standard histologic procedures were used to prepare tissue sections of 3–6 µm thickness that were either stained using hematoxylin and eosin or trichrome stain or used for immunodetection. The histologic samples of the treated canals were examined and scored, as previously described (42) by a blinded observer using the following scoring criteria for apical closure:

Score 0: Complete apical calcified tissue extending from one to the other root canal wall

Score 1: Incomplete apical calcified tissue interrupted by fibrous or inflammatory tissue

Score 2: No new calcified tissue in the apical end of the root

Pulp soft-tissue regeneration was scored as follows:

Score 0: Pulp tissue inside apical portion of root canal and pulp chamber

Score 1: presence of pulp tissue inside apical portion of root canal only

Score 2: Absence of pulp tissue

Immunodetection of dentine sialophosphoprotein (DSPP) calcitonin gene-related peptide (CGRP), Wnt1a and peripherin (Per) were performed. The primary antibody corresponding to the DSPP antigen was obtained from Santa Cruz Biotech Europe (Cambridge, UK), SC. 73632, and used at a 1:100 dilution. This commercially available polyclonal antibody detects both DSP and DPP, proteins expressed in bone. Antibody for the calcitonin gene-related peptide CGRP antigen was obtained from AbCam (Cambridge, UK), AB139264, and was used at 1:50 dilution. Antibody for the Wnt1a antigen was obtained from AbCam (Cambridge, UK) AB91191 and was used at 1:50 dilution. Antibody for peripherin was obtained from AbCam (Cambridge, UK), AB39374, and was used at a 1:50 dilution. All steps were performed following the handling procedures and titers recommended by the supplier. Conjugated secondary antibodies were obtained from Thermo Fisher Scientific (Fremont, CA) and used at a dilution of 1:2000. Control sections were treated in the same manner but without the use of the primary antibody. Sections were imaged using a Nikon Eclipse 80i microscope (Tokyo, Japan) with BX 51 digital camera (Tokyo, Japan) and digital images were recorded.

Statistics

The Chi-Square test was used to compare the outcomes for the study groups. A P value $< .05$ was chosen as the criterion to reject the null hypothesis. The statistical package used for this study was IBM SPSS v22.

Results

Amelogenin-Treated Canal Cohort

At 1 month post-treatment, the canals treated with recombinant amelogenin consistently revealed the formation of dentin and/or the presence of dentin deltas (Figs. 1A and 2A and B, Table 3). These cellular responses produce a histological appearance consistent with apical closure with regenerated fibers of the periodontal ligament. The walls adjacent to the root apices appear thickened due to the formation of secondary dentin (Fig. 2A). In 82% of the recovered samples (Table 3), delicate soft tissue was located in the radicular pulp chamber with the microanatomy of pulp-like tissue without polarized odontoblasts. The soft connective tissue varied in volume, from a modest amount of tissue occupying only the apical portion of the root canal, to greater amounts of soft tissue extending into the coronal area of the chamber (Fig. 1A). Also, observed among the dentin deltas were vascular elements (Fig. 2A). Moderate to intense immune-reactive staining for the protein dentin sialophosphoprotein (DSPP) could be identified in the newly formed dentin at the root apex, as well as among the dentin deltas (Fig. 4A and B). The presence of DSPP antigen was recognized immunologically in the soft connective tissue within the pulp chamber and in the tissue of the regenerated root apex, while no DSPP was detected in control sections (Fig. 4C). Cells within the regenerated soft tissue expressed peripherin, a neuro-filament protein identified with a peripherin-specific antibody (Fig. 4G and H) compared to controls (Fig.

4J). Cells found in the regenerated soft tissue within the pulp chamber and canals also expressed Wnt1a protein, identified by immunodetection with a Wnt1a-specific antibody (Fig. 4J and K) that was absent from control sections (Fig. 4L).

At 3 months post-treatment, 77% of the amelogenin-treated teeth revealed root closure, as evidenced by micro-anatomic analysis of the apical area, which showed a continuity of cells and a dentin-like bioceramic layer (Fig. 2C and D, Table 4). The appearance of an attachment apparatus was observed histologically in samples from this time interval. Approximately 85% of the pulp cavities of the recombinant amelogenin-treated canals now contained soft connective tissue that approximated the microanatomy of pulp-like tissue but without polarized odontoblasts. The appearance of this soft tissue was composed of fine fibrous elements containing spindle-shaped cells with thin-walled vascular elements (Fig. 2C and D).

At 6 months post-treatment with amelogenin, 65% of the teeth showed histologic evidence of closure of the apical area achieved by a thickened layer of cellular dentin (Fig. 2E and G). The dentin walls appeared thickened with newly formed dentine (Fig. 2F and H). The pulp chambers of 87% of the amelogenin-treated teeth from this time interval now showed delicate soft connective tissue, containing cellular, fibrous and vascular elements (Fig. 2E–J). Cells within the soft tissue showed expression of CGRP protein (Fig. 4D and E), whereas the control section lacked immunodetection of the CGRP antigen (Fig. 4F).

Calcium Hydroxide Canal Cohort

In contrast to the outcomes reported for amelogenin treatment, the calcium hydroxide treatment group was more variable in its histoanatomic outcomes over the post-treatment time intervals. At 1 month post-treatment with calcium hydroxide, 88% of the treated canals revealed root apices that were either wholly open or showed signs of incomplete closure (Table 3), with 2% of the samples showing histologic evidence of complete apical bridging (Figs. 1B and 3A and E, Table 1). Apical to the mineralized tissue, granulation tissue was observed (Fig. 3A, D, and E).

Those canals treated with calcium hydroxide showed signs of persistent inflammation, evidenced by the granulation tissue and the persistence of thin radicular walls, along with extensive bone resorption at the apical portion of the root (Fig. 1B; Fig. 3A and E). In some samples, a thin layer of granulation tissue bridged the opened apex (Fig. 1B; Fig. 3A, D, and E). Cells resembling Hertwig's epithelial root sheath cells (HERS) were identified based on their anatomical position and micro-anatomic appearance at the root apex in canals treated with calcium hydroxide at 1 month post-treatment (Fig. 3A and D).

For canals treated with calcium hydroxide at the 3 month interval, granulation tissue surrounding the nearly closed root apex predominated, with only minimal amounts of bone or PDL tissue were observed histologically (Fig. 3B, Table 4).

At 6 months post-treatment with calcium hydroxide, 2 histologic patterns were recognized. Approximately 22% of the canals showed complete apical closure by a continuous layer of dentin with thickened lateral walls along with granulation tissue, with mineralized tissue

present inside the apical portion of the root canal (Fig. 3C, and Table 5). The other pattern was of a thin, but histologically incomplete apical bridge with evidence of root resorption (Fig. 3F). Thickening of the lateral portions of the root were only sporadically observed. In approximately 19 samples (47.5%), incomplete islands of bone and some disoriented PDL fibers were observed, with no attachments to the tooth apical portion.

Statistical Evaluation

This study was based on an initial sample size of 240 canals harvested and observed over 3 time intervals of 1, 3, and 6 months. There were some canals excluded from the study by selection criteria and others that were lost during processing, and these values are shown in Table 1. The summative outcome of this study are described in Table 1 and B, in which root apex closure was observed in 40.8% of the 240 canals. In the group treated with recombinant amelogenin, 70.9% revealed complete apex closure whereas only 10.8% showed complete closure with calcium hydroxide treatment. Incomplete closure was recorded for 29.6% of the recovered canals, including 18.3% of those treated with recombinant amelogenin and 40.8% treated with calcium hydroxide. Of the total canal samples, 20.4% revealed no root closure. In the recombinant amelogenin treatment group only 8.3% revealed no root closure, compared with 32.5% of the calcium hydroxide treatment group. Comparison among the apex closure groups with respect to treatment modality revealed the outcomes were significantly different, with a P value of $<.001$.

An unexpected finding in this study was the observation of soft connective tissue within the pulp chambers and canals which contained cells expressing immunodetected marker proteins associated with native pulp cells that were found to occur only with the use of recombinant amelogenin, not with calcium hydroxide, at a significance level of $p < 0.001$. In total, 42.5% of the samples soft connective tissue within the pulp cavity, with 100% of these samples occurring with recombinant mouse amelogenin, while 0% of the canals treated with calcium hydroxide showed soft connective tissue.

Discussion

Amelogenin proteins are commonly associated with their role as structural guides to the organization of hydroxyapatite crystallites during formation of enamel bioceramic tissue. During this process, they serve to guide enamel's unique material properties of hardness and fracture toughness (43). Alternative splicing of the primary RNA transcript produces amelogenin protein isoforms identified to be involved during the development of an integrated system of dental tissues and bioceramics composing the mammalian tooth organ (44). Roles for amelogenin protein during induction of dentin formation (33, 45) and cementum formation (25, 26, 28) have also been reported. Moreover, previous studies have identified that enamel matrix proteins including amelogenin and ameloblastin play a significant role in accelerating and improving periodontal tissue repair and regeneration in primates (17). Finally, the small 59-amino-acid amelogenin isoform known as leucine-rich amelogenin peptide (LRAP) was recently shown to induce a shift in the determination of stem cell phenotypes through its activation of the powerful Wnt signaling pathway (31, 32, 46–48). Together, these and several other studies (4, 20, 30) suggesting amelogenin could be

a useful molecule to signal cells to participate in repair and regeneration of dentin and cementum prompted our efforts to test the competency of recombinant DNA produced full-length mouse 180-amino-acid amelogenin to participate in repair of the root apex.

In recent years, it has been recognized that tissue repair and regeneration can be influenced by cell-to-cell and cell-to-extracellular matrix (ECM) interactions (49). Other investigators point to the capacity of the ECM to provide clues through their integration of signaling molecules and that their ECM protein(s) can accentuate or attenuate their signaling activity (49–53). Post-natal wounds, including irreversible types of damage such as pulpal necrosis and apical periodontitis, most often heal by repair or by a combination of repair and limited regeneration because of the very modest regenerative potential of the relevant somatic cells at the site (54). Therefore in this study, like others before us, we sought to augment the natural potential of these cells by providing biological signals for the restoration of the physiological, structural and mechanical integrity of the diseased pulp and dentin/cementum complex of the root apex.

At the time of the inception of this project there were no meta-analysis studies available to better inform the design of this study. Our experimental strategy intended to examine the capacity of recombinant amelogenin protein to complete the developmental program for radicular apex formation using a young dog model of open, non-vital root canals. Histomorphometric examination of the treated teeth after 1, 3, and 6 months was used to compare outcomes obtained using amelogenin with outcomes obtained using one of the common chemical signals for apex formation, calcium hydroxide. Our experimental strategy was purposely intended to provide an unfavorable environment for the treatment modalities to exert their potential impact on apex completion. This design included pulp extirpation and canal cleansing, followed by permitting the canals to become contaminated with the oral flora before cleansing and shaping them for the treatment phase.

Meticulous and ordinal scaled observations of the histological presentation of the canals at the 1, 3, and 6 month post-operative assessments revealed the development of dentin hard tissue at the radicular apex in canals treated with recombinant amelogenin and to a lesser extent in canals treated with calcium hydroxide.

We note that in this study design we could not document the duration that the recombinant amelogenin remained in the treated canals, but such a measurement will be pursued in subsequent studies. The study design also failed to identify which cell type responded to the amelogenin signal(s). It should be noted that many cell types, including periodontal ligament fibroblasts and other mesenchyme-derived cells, actively uptake enamel matrix proteins (55). It is not yet clear whether amelogenin uptake is required for the cell signaling (37, 56, 57), although recent data on a tethered amelogenin isoform revealed cellular uptake is not required (47). It is clear from the work of multiple investigators, however, that enamel matrix proteins have the capacity to induce the regeneration of cementum, alveolar bone and the periodontal ligament in a variety of animal models, including humans (17, 58). Investigators have also shown that the mouse 59-amino-acid amelogenin isoform known as *leucine-rich amelogenin peptide* (LRAP) induces bone formation in human and mouse stem cells by activating the Wnt signaling cascade (31, 32, 48, 59). Prior investigations have

shown that enamel matrix proteins can be successfully used as a pulpotomy agent in vital primary teeth (60). In this study of non-vital teeth, increased angiogenesis and dentine islands were observed over a period as short as 1 month, with dentine bridging the full width of the apical pulp chamber by 6 months.

Evaluating the expression of distinctive protein markers using specific antibodies assessed the differentiation state of the cells found in the pulp chamber. Dentin sialophosphoprotein (DSPP) is critical for the formation and proper mineralization of the dentin bioceramic. DSPP-derived proteins are expressed and secreted by odontoblasts (61) and are detected by specific antibodies used in this study. One month after treatment, the restored cells comprising the soft connective tissue showed moderate to intense immunoreactivity for the DSPP antigen, indicating that the calcified tissue formed during this interval was most likely dentin or a very closely related hard tissue that incorporates the DSPP protein. However, the recovered hard tissue could include osteogenic or cementogenic lineage outcomes. DSPP is also expressed in bone (62), and more recently, it was shown to be expressed in the pericytes of blood vessels in the dental pulp, which are believed to be able to differentiate into odontoblasts (63). Nerve fibers immunoreactive to antibodies directed against calcitonin gene-related peptide (CGRP) represent small neurons present in the central and peripheral nerves of mammalian dental pulp. CGRP is mainly distributed in the root apex area (64). The restored tissue described here in response to recombinant amelogenin protein treatment shows CGRP immunoreactivity consistent with typical pulpal cells. Peripherin is a specific marker for peripheral small-sized neurons such as those that comprise the majority of neurons in mammalian dental pulp. In this study, peripherin immunoreactivity was identified in the soft connective tissue within the pulp chamber and canals. Peripherin is highly enriched in neuronal derivatives of the neural crest, the embryological tissue from which the dental ectomesenchyme derives (65), and plays a role in neurite elongation during development and axonal regeneration cells (66). Lastly, we showed Wnt1a immunoreactivity is present throughout the restored soft connective tissue found within the pulp chambers, suggesting that Wnt activation is locally available and thus may exercise a role in promoting regeneration. Wnt1a belongs to the Wnt family of signaling molecules active during many developmental and regenerative processes including tooth development (7, 67). With exposure to the amelogenin isoform LRAP, mesenchymal stem cells are induced to express Wnt messenger RNA (48, 59). The restored soft connective tissue observed in this study is largely a loose connective tissue similar in appearance to pulp-like tissue, but one that lacks visible polarized odontoblasts. It is similar to the outcomes described by Yamauchi and colleagues (11) and Zhu and colleagues (68), and to a recent human case report described by Torabinejad and colleagues (10).

Historically and currently, the treatment of choice for teeth with immature roots and infected pulp leading to necrosis is calcium hydroxide ($\text{Ca}(\text{OH})_2$) apexification, which can take from 1 to 2 years to induce apical closure (69). The use of calcium hydroxide has been shown to lead to a weakening of the root structure, often resulting in cervical root fractures (70, 71). However, calcium hydroxide is reported to have desirable antimicrobial activity that is related to the release of hydroxyl ions (72). In the present study, we observed delayed root apex closure by cellular mineralized tissue, the thin lateral walls, and failure to regenerate the attachment apparatus in the previously infected area at 3 months post-treatment with

calcium hydroxide. This likely reflects the picture Sonoyama and colleagues (73) described: the apical papilla cells contribute only to root dentine and not to root cementum formation upon stimulation by appropriate inductive signals from Hertwig's Epithelial Root Sheath (HERS) cells which arise from the enamel organ epithelia forming the enamel crown. HERS cells are capable of signaling stem cells in the apical papilla to differentiate into odontoblasts and produce root dentin (Sonoyama et al. 2007). Following apexification procedures, HERS cells are thought to be more resistant than apical papilla cells to apical periodontitis/abscess formation in immature permanent teeth (70). The high pH of calcium hydroxide is known to be toxic to vital cells and when placed in the apical region, may have a toxic effect on the stem cells of the apical papilla (2). In the calcium hydroxide group, the presence of a cell-free zone might explain the distance often seen between the newly grown dentin bridging the apex and the granulation tissue (Fig. 1B, Fig. 3C, E, and F).

A consistent result in the present study is the absence of prominent inflammatory reaction following the use of the recombinant amelogenin protein (27, 74, 75). One of the amelogenin splicing isoforms, leucine-rich amelogenin peptide (LRAP) induces Wnt10b expression that attenuates the release of tumor necrosis factor alpha and interleukin-8, providing a potent anti-inflammatory effect (48, 76). No root resorption was observed in the amelogenin-treated study groups, an outcome that is consistent with similar studies using enamel matrix derivative (29) (EMD, commercially sold as Emdogain from porcine sources) proteins in which amelogenin is the dominant protein (77). Root resorption was not seen with EMD treatment in those studies, but was a frequent phenomenon in the control groups (18–20, 29, 30, 78).

Interest in regenerating pulp tissue has grown as our understanding of stem cell biology has improved. Bianco and colleagues (79) postulated that HERS is destroyed by long-standing apical periodontitis/abscess, so the resident periapical mesenchymal stem cells are required to transdifferentiate into HERS to promote continued root development. They concluded that after the fetal period, transdifferentiation of mesenchymal stem cells into epithelial cells does not appear to occur readily. On the other hand, Veis and collaborators (45) argued that amelogenin gene splice products have the ability to interact with immature mesenchymal cells, both in culture and in vivo on implanted tissue and can thereby initiate a change in cell phenotype and differentiation pathway. They further argued that pulp healing and dentin bridging may be stimulated by the application of amelogenin proteins, rather than by chemical components of dental materials (29, 36, 80), a hypothesis supported by the present findings.

Angiogenesis/vasculogenesis and neurogenesis are critical for pulp regeneration. Revascularization during pulpal wound repair and regeneration may be due to angiogenesis (formation of blood vessels by endothelial cells) and vasculogenesis (formation of new blood vessels by angioblasts derived from bone marrow and by endothelial progenitor cells from circulating peripheral blood). The precise mechanism of angiogenesis/vasculogenesis during the pulpal healing process is not known (81, 82). There has been no report on successful angiogenesis/vasculogenesis and pulp regeneration in teeth, although a report of angiogenic activity for EMD, which includes amelogenin protein isoforms, has been reported (83). Cao and colleagues have recently shown the promise of induced mesenchymal

stem cells (12). The amelogenin group in the present study showed restored soft connective tissue that was lined by viable cells with the capacity to form new hard tissue inside the root canal and thereby contribute to the strength of the root structure. The restored soft connective tissue showed numerous regenerated blood vessels and cells expressing markers associated with pulp-like cells. The nascent blood vessels were seen as early as 1 month post-operatively within the vicinity of the newly regenerated dentin-like deltas or as delicate stands in the pulp space near the regenerated root apex. The massive bone destruction that resulted from the infection of the root canal and periapical area was replaced by cellular granulation tissue as early as 1 month post-operatively. The regenerated mineralized tissue that bridged the root apex was cellular and connected to the PDL fibers inserting into the bone to establish the tooth attachment apparatus.

Discussion

Further study of recombinant amelogenin is as it shows potential as an apexification agent. The current study revealed the complete closure of the root apex in addition to the partial regeneration of a soft tissue resembling pulp-like tissue. The restored soft connective tissue showed appropriate architecture and positive expression of proteins expressed by cells of the pulp. An experimental strategy deploying recombinant produced amelogenin does not require cell donation (allogeneic or syngeneic) for restoration of a soft connective tissue. The unique outcomes reported here may be due to the effect of recombinant amelogenin on stem cells in the periapical region. When confirmed by other investigators, and extended from a canine model to humans, this approach could modulate dental treatment and prognosis by providing a biological solution to a biological problem. Based on these studies performed in dogs, recombinant amelogenin protein is a promising material that proves to have the potential for clinical success; it revives the expectations for pulp regeneration, which would improve the long-term prognosis of non-vital teeth in humans.

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Figure 1.

Premolar teeth treated with recombinant amelogenin or calcium hydroxide at 1 month. (A) Sagittal image of dog premolar tooth roots treated with recombinant amelogenin at the end of 1 month showing closure of the root apices with hard tissue consistent with dentin (D) or bone-like (B) material and the presence of a well-organized periodontal ligament (arrows). Restored tissue that resembles canonical pulp (P) tissue is observed, either near the closed apices or extending into the coronal areas. This histologic state was seen in approximately 75% of the specimens. H & E stain. Original magnification, $\times 20$. (B) Sagittal image of dog

premolar tooth roots treated with calcium hydroxide at the end of 1 month, showing open apices and extensive bone resorption at the apical portion of the 2 neighboring roots. The presence of a periapical lesion (PL) is also seen. A thin layer of granulation tissue (GT) is observed to bridge the patent apex. Degeneration of the inter-radicular and inter-dental bone (*arrows*) is observed. This histologic state was seen in approximately 45% of the specimens. RC, root canal. H & E stain. Original magnification, $\times 20$.

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Figure 2.

Premolar teeth at selected time intervals following treatment with recombinant amelogenin.

(A) Greater magnification of apical area shown in boxed area of Figure 1A after 1 month showing regenerated soft connective tissue (P) containing a regenerated blood vessel (V). Apex closure is indicated by the presence of apical deltas formed of dentin (D) along with regenerated apical periodontal ligament fibers (*arrows*). This histologic state was seen in approximately 45% of the specimens. H & E stain. Original magnification, $\times 100$. (B) Apical area of a canal after 1-month showing the widely opened apex with granulation tissue bridging the apex (GT). Delicate soft tissue resembling the pulp tissue (P) is seen in the apical area of the root canal. Newly regenerated bone (B) and regenerated periodontal ligament fibers (*arrow*). This histologic state was seen in approximately 75% of the specimens. H & E stain. Original magnification, $\times 40$. (C) Apical area of premolar tooth after 3 months showing hard tissue consistent with dentin (D) bridging the apical foramen and attached to bone (B) by regenerated periodontal ligament (*arrow*). The regenerated soft

connective tissue (P) shows many vascular elements (V). This histologic state was seen in approximately 70% of the specimens. Trichrome stain. Original magnification, $\times 40$. (D) Greater magnification of apical area shown in (C) showing apical closure achieved by a layer of cells producing a dentin/osteoid-like bioceramic tissue. The pulp space contains delicate regenerated soft tissue and vascular elements (V). A PDL space is observed that contains fibers of the periodontal ligament (*arrow*). H & E stain. Original magnification, $\times 100$. (E) Apical root region of premolar tooth after 6 months with apical closure by a layer of dentin (D). The pulp space shows regenerated soft connective tissue (P) composed by a delicate fibrous connective tissue and cells. The PDL space contains PDL fibers (*arrows*) attached to the regenerated alveolar bone (B). This histologic state was seen in approximately 60% of the specimens. H & E stain. Original magnification, $\times 40$. (F) Greater magnification of the boxed inset from (E), showing dentin (*arrows*) and regenerated soft connective tissue (P), vascular elements (V), and some calcified material (CL). H & E stain. Original magnification, $\times 200$. (G) The apical region of premolar tooth after 6 months showing apical closure by a layer of dentin (D). The pulp space shows delicate regenerated soft connective tissue (P) with vascular elements (V). The PDL space contains PDL fibers (*arrows*) attached to alveolar bone (B). This histologic state was seen in approximately 25% of the specimens. H & E stain. Original magnification, $\times 40$. (H) Greater magnification of box 1 from (G), showing newly formed dentin (*arrow*) and regenerated soft connective tissue (P). H & E stain. Original magnification, $\times 400$. (I) Greater magnification from box 2 from (G), showing cellular dentin (D) and regenerated tissue (P). H & E stain. Original magnification, $\times 200$.

Figure 3.

Premolar teeth at selected intervals following treatment with calcium hydroxide. (A) Apical root region of a premolar tooth after 1 month from box 1, Figure 1B, showing thin apical root dentin and failed apical closure. The root canal (RC) contains scarce amounts of soft tissue. Granulation tissue (GT) surrounds the open apex. This histologic state was seen in approximately 45% of the specimens. Trichrome stain. Original magnification, $\times 40$. (B) Apical and mid-root region of premolar tooth after 3 months of healing showing apical bridging of the open apex by a thin layer of cellular calcified tissue. Granulation tissue (GT) surrounds the closed apex. Bone and periodontal tissue regeneration is not observed in the apical root area. The root canal (RC) is largely devoid of soft tissue. This histologic state was seen in approximately 7% of the specimens. Trichrome stain. Original magnification, $\times 100$. (C) Apical root area of premolar tooth after 6 months showing bridging of the apex by a layer of mineralized tissue (MT). The space of the root canal (RC) contains debris and some calcific material (arrow). Granulation tissue (GT) surrounds the closed root apex. This histologic state was seen in approximately 15% of the specimens. H & E stain. Original magnification, $\times 100$. (D) Greater magnification of box in (A) showing cells resembling Hertwig's Epithelial Root Sheath (arrows) and granulation tissue (GT). Trichrome stain. Original magnification, $\times 400$. (E) Greater magnification of box 2 in (B) showing the absence of an apical closure. A thin layer of granulation tissue (GT) and collagen deposition (arrow) surround the apical root dentin. Trichrome stain. Original magnification, $\times 100$. (F) Apical root area of tooth after 6 months showing bridging of the apex by a thin layer of dentin that also reveals an apparent resorption pit (arrow) on the external root surface. Regenerated bone (B) is seen near the root apex bounded by granulation tissue (GT). The root canal (RC) space is devoid of soft

tissue. This histologic state was seen in approximately 5% of the specimens. H & E stain.
Original magnification, $\times 40$.

Figure 4.

Protein expression by immunodetection in tissue from premolar teeth at selected intervals following treatment with recombinant amelogenin. (A) Apical root area of premolar tooth at 1 month showing bridging of the apical foramen by dentin deltas (D), while granulation tissue (GT) surrounds the regenerated root dentin. The pulp (P) space contains sporadic areas of soft connective tissue resembling the architecture of pulp tissue. This histologic state was seen in approximately 45% of the specimens. H & E stain. Original magnification, $\times 100$. (B) An adjacent tissue section to that shown in (A). The tissue shows apical closure by

dentin deltas (D) that are immunoreactive to a dentin sialophosphoprotein (DSPP) antibody. Immunoreactivity is recognized in the granulation tissue (GT) apical to the regenerated dentin and in cells resident to the soft tissue within the canal (P). Dentin sialophosphoprotein antibody. Original magnification, $\times 100$. (C) An adjacent tissue section to that shown in (A). In this control section lacking primary antibody, no immunoreactivity to the DSPP antigen is observed. Dentin sialophosphoprotein antibody control. Original magnification, $\times 100$. (D) Apical root area of premolar tooth at 6 months showing a thick layer of dentin at the root apex that fails to completely bridge the opened apex. A large vascular element (V) is seen travelling towards the restored soft connective tissue that resembles pulp-like (P) tissue, along with regenerated bone (B). This histologic state was seen in approximately 60% of the specimens. Trichrome stain. Original magnification, $\times 40$. (E) An adjacent tissue section to that shown in (D). Moderate immunodetection of calcitonin gene-related peptide (CGRP) is observed throughout the restored soft tissue that resembles pulp-like tissue (P). Dense immune-reactive fibrous elements are recognized near the mineralized cell layer (*arrow*) and bone (B), representing antibody trapping common to demineralized tissues. Calcitonin gene-related peptide (CGRP) antibody. Original magnification, $\times 40$. (F) An adjacent tissue section to that shown in (D). In this control section lacking primary antibody, no immunoreactivity to the CGRP antigen is observed. Calcitonin gene-related peptide antibody control. Original magnification $\times 40$. (G) Apical and mid-root area of premolar tooth root at 1 month showing bridging of the open apical foramen by dentin deltas (D). Regenerated bone (B) surrounds the regenerated root dentin. The pulp space contains restored soft connective tissue that reaches towards the coronal area (P) of the chamber. This histologic state was seen in approximately 35% of the specimens. H & E stain. Original magnification, $\times 40$. (H) An adjacent tissue section to that shown in (G) that reveals positive immune-reactivity to the peripherin (PER) protein. Moderate to dense immune-reactivity to peripherin is observed in the restored soft tissue (P). Regenerated bone (B) and dentin (D) shows immune-reactivity due to antibody trapping. Peripherin antibody. Original magnification, $\times 40$. (I) An adjacent tissue section to that seen in (G). In this control section lacking primary antibody, no immunoreactivity to the peripherin antigen is observed. Peripherin antibody control. Original magnification, $\times 40$. (J) Apical root area of premolar tooth root treated for 1 month, showing bridging of the open apical foramen by dentin (D). Regenerated bone (B) surrounds the regenerated root dentin. The pulp space contains restored soft tissue (P). This histologic state was seen in approximately 50% of the specimens. H & E stain. Original magnification, $\times 40$. (K) An adjacent tissue section to that shown in (J). Moderate immunoreactive staining for Wnt1a protein is seen in the restored soft tissue (P), especially in the cell layer nearest the mineralized front resembling odontoblasts (od). Regenerated bone (B) shows antibody trapping. Wnt1a antibody. Original magnification, $\times 40$. (L) An adjacent tissue section to that shown in (J). In this control section lacking primary antibody, no immunoreactivity to Wnt1a antigen is observed. Wnt1a antibody control. Original magnification, $\times 40$.

Table 1

Sample Distribution Overview

Variable	Outcome	No.	%
Total number of canals		240	100.0
Time distribution	1 month	80	33.3
	3 months	80	33.3
	6 months	80	33.3
Group distribution	Amelogenin	120	50.0
	Calcium hydroxide (CaOH)	120	50.0
	Sample lost	22	9.2
Root closure	Complete	98	40.8
	Incomplete	71	29.6
	Missing	49	20.4
Pulp regeneration	Yes	102	42.5
	Canal	36	42.5
	Canal + chamber	66	64.8
	None	116	48.3

Table 2**Summative Results**

Canal variable	No. (%)	Treatment		P value
		Amelogenin	CaOH	
Total number of canals	240 (100.0)	120 (50.0)	120 (50.0)	—
Root closure	Complete	85 (70.9)	13 (10.8)	<.001 *
	Incomplete	22 (18.3)	49 (40.8)	
	None	10 (8.3)	39 (32.5)	
Pulp regeneration	Yes	102 (100.0)	0 (0.0)	<.001 *
	No	27 (22.5)	89 (77.5)	

* Significant using Chi-Square test at <0.05 level.

Table 3

Sample Distribution and Statistical Analysis of Variables and Outcomes at 1 Month

Canal variable	No. (%)	Treatment		P value
		Amelogenin	CaOH	
Total number of canals	80 (100.0)	40 (50.0)	40 (50.0)	—
Regeneration	Yes	33 (41.3)	0 (0.0)	<001 *
	Root canal	31 (38.8)	0 (0.0)	
	Root canal + chamber	2 (2.5)	0 (0.0)	
	None	41 (51.3)	33 (82.5)	
Root closure	Complete	29 (36.3)	1 (2.5)	<001 *
	Incomplete	21 (26.3)	9 (22.5)	
	None	24 (30.0)	24 (60.0)	

* Significant using Chi-Square test at the <0.05 level.

Table 4
Sample Distribution and Statistical Analysis of Variables and Outcomes at 3 Months

Variables	No. (%)	Treatment		P value
		Amelogenin	CaOH	
Total number of canals	80 (100.0)	40 (50.0)	40 (50.0)	—
Regeneration	Yes	34 (42.5)	0 (0.0)	<001 *
	Root canal	4 (5.0)	0 (0.0)	
	Root canal + chamber	30 (95.0)	0 (0.0)	
	None	8 (20.0)	32 (80.0)	
Root closure	Complete	31 (77.5)	3 (7.5)	<001 *
	Incomplete	5 (12.5)	21 (52.5)	
	None	3 (7.5)	11 (27.5)	

* Significant using Chi-Square test at the <0.05 level.

Table 5
Sample Distribution and Statistical Analysis of Variables and Outcomes at 6 Months

Variables	No. (%)	Treatment		P value
		Amelogenin	CaOH	
Total number of canals	80 (100.0)	40 (50.0)	40 (50.0)	—
Yes	35 (43.8)	35 (87.5)	0 (0.0)	<001 *
Root canal	1 (1.2)	1 (1.2)	0 (0.0)	
Root canal + chamber	34 (98.8)	34 (98.8)	0 (0.0)	
None	35 (43.8)	11 (27.5)	24 (60.0)	
Complete	35 (43.8)	26 (65.0)	9 (22.5)	<001 *
Incomplete	24 (30.0)	5 (12.5)	19 (47.5)	
None	11 (13.8)	7 (17.5)	11 (27.5)	

* Significant using Chi-Square test at the <0.05 level.