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

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Abstract

Introduction: Recombinant DNA–produced amelogenin protein was compared with 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 histologic 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 nonvital immature teeth and promote soft connective tissue regeneration. *(J Endod 2016;42:402–412)*

Key Words

Amelogenin, apex formation, calcium hydroxide, pulp regeneration, regeneration

Dental caries and trauma are among the most prevalent challenges to tooth preservation because these insults can render the dental pulp tissue nonvital. If death of the pulp tissue occurs before 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 ($Ca[OH]_2$) although recently the material known as mineral trioxide aggregate (3) and a biomimetic approach with porcine-derived enamel matrix derivatives (EMDs) (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 toward osteogenic lineages (31) by activating the Wnt signaling pathway (32). Molecules with related biological activity were first described by Amar et al (33, 34), who showed that a small polypeptide recovered from rat incisor dentin 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).

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Here, we describe experiments designed to determine whether full-length mouse amelogenin protein produced by recombinant DNA techniques retains biosignaling 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 pulplike tissue. Hard tissue of complex organization similar to dentin-associated mineralized tissue was also observed (38).

Materials and Methods

Expression and Purification of Recombinant Polyhistidine-tagged Proteins

Recombinant amelogenin proteins were prepared with the expression vector pQE30 (QIAGEN Inc, Valencia, CA) and purified with a polyhistidine amino terminus as previously described (23, 25). Recombinant polyhistidine-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 metal-affinity chromatography matrices (QIAGEN Inc).

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, Alexandria, Egypt. 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 anesthetized using sodium pentobarbital intravenous injection (30 mg/kg body weight). Preoperative 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 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 $Ca(OH)_2$, 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. Hedström 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 1 of the 2 experimental materials, either recombinant amelogenin protein (rp[H] M180) or $Ca(OH)_2$, as described later (Table 2).

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	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

Approximately 1 mL propylene glycol alginate vehicle was mixed with 30 mg recombinant amelogenin protein powder (rp[H] M180) preweighed using an aseptic technique and allowed to rest for 15 minutes before use. Once mixed, the amelogenin material was used within 2 to 3 hours. The suspension of rp(H) M180 + propylene glycol alginate 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 was then carefully placed over the root canal dressing, and the access cavity was restored with amalgam.

Prefabricated syringes containing $Ca(OH)_2$ paste were used to fill the 96 canals of the second and third premolars in the right maxillary arches. In 4 dogs, the mandibular right premolar canals (24 canals) were filled with $Ca(OH)_2$ to provide a total of 120 canals for the $Ca(OH)_2$ treatment group. Intermediate restorative material 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, Basel, Switzerland), an intravenously delivered painkiller, at 25 mg/kg. Tetracycline (Tetracid; Cid Co, Giza, 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 to obtain approximately 80 canals at each elapsed time interval. In some instances, samples were lost because of 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- to 6-μm thickness that were either stained using hematoxylin-eosin or trichrome stain or used for immunodetection. The histologic samples of the treated canals were examined and scored