Histological observations of pulpal replacement tissue in immature dog teeth after revascularization of infected pulps

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Abstract – Background and aim: Many studies have examined the nature of tissue formed in the canals of immature necrotic teeth, following revascularization in animals and humans. While speculations have been made that regeneration of the pulp tissue might take place in the canal, the tissue has been found to be cementum-like, bone-like, and periodontal ligament-like. The purpose of this study was to histologically examine the tissue in the root canals in immature dog teeth that had been artificially infected and then revascularized. Methods: Two 4- to 5-month-old mongrel dogs with immature teeth were used in the study. In one dog, four maxillary and four mandibular anterior teeth, and in another dog, four maxillary and five mandibular anterior teeth were used in the experiment. Pulp infection was artificially induced in the immature teeth. Revascularization was performed on all teeth by disinfecting the root canals with sodium hypochlorite irrigation and triple antibiotic intracanal dressing, completed with induction of intracanal bleeding, and sealed with an MTA plug. The access cavity was restored with silver amalgam. The animals were sacrificed 3 months after revascularization procedures. The revascularized teeth and surrounding periodontal tissues were removed and prepared for histological examination. Results: Besides cementum-like, bone-like, and periodontal ligament-like tissues formed in the canals, residual remaining pulp tissue was observed in two revascularized teeth. In four teeth, ingrowth of alveolar bone into the canals was seen; presence of bone in the root canals has the potential for ankylosis. Conclusions: Within the limitation of this study, it can be concluded that residual pulp tissue can remain in the canals after revascularization procedures of immature teeth with artificially induced pulp infection. This can lead to the misinterpretation that true pulp regeneration has occurred. Ingrowth of apical bone into the root canals undergoing revascularization can interfere with normal tooth eruption if ankylosis occurs.

Revascularization is defined as ‘restoration of the blood circulation of an organ or area, achieved by unblocking obstructed or disrupted blood vessels or by surgically implanting replacements’ in Stedman’s Medical Dictionary (1). However, revascularization is often misused as regeneration in endodontic therapy. The term ‘revascularization’ is used in this report to describe the neoangiogenesis and development of replacement tissue that take place in the clinical treatment described. Clinically, revascularization procedures in human immature permanent teeth with a necrotic pulp and/or an apical periodontitis can result in resolution of apical periodontitis, thickening of the canal walls, continued root maturation, as well as restoration of tooth vitality, as seen in numerous published case reports and case series (2). Similar radiographic findings were also observed in animal studies (3–6). As such, it was hypothesized that revascularization procedures of immature permanent teeth with a necrotic pulp and/or an apical periodontitis in humans could promote pulp tissue regeneration through recruitment and differentiation of stem/progenitor cells from the apical papilla (7). However, several histological studies in animals have found that the tissues formed in the canals of revascularized teeth were cementum-like, bone-like, and periodontal ligament-like rather than pulp-like tissue (3–6). Subsequently, similar tissues were also observed histologically in the canals of human immature permanent teeth with a necrotic pulp and/or an apical periodontitis after revascularization procedures (8–10).

A recent study demonstrated that vital pulp tissue could remain in the canals up to 60 days after artificially induced apical periodontitis in an animal model (11). Therefore, residual vital pulp tissue might remain in the canals after revascularization procedures of
immature teeth with artificially induced apical periodontitis. Consequently, this surviving residual pulp tissue could be misinterpreted as regeneration of the pulp tissue. The purpose of this study was to histologically examine the tissue in the root canals in immature dog teeth that had been artificially infected and then revascularize.

Materials and methods
Two 4- to 5-month-old healthy male mongrel dogs with immature teeth, weighing 5–6 kg, were used in this study. Preoperative periapical radiographs were taken of all experimental teeth to confirm the incomplete maturation of the roots, with an open apex and large pulp cavity (Fig. 1a,c). In one dog, four maxillary and four mandibular anterior teeth, and in another dog, four maxillary and five mandibular anterior teeth were treated with revascularization procedures. All teeth were free of caries and periodontal disease. The dogs were kept in an animal facility under constant observation throughout the experiment period to ensure their health. This study was approved by the Institutional Review Board of Alexandria University, Egypt, in accordance with the International Guidelines for Animal Care and Use.

Induction of pulp infection and necrosis
General anesthesia with intravenous injection of 3% sodium thiopental (Pentobarbital, Butler Co., Columbus, OH, USA) in the dose of 30 mg/kg body weight was administered to the animals. The experimental teeth were accessed through the lingual surface into the pulp chamber using #2 carbide round bur mounted on a high-speed handpiece. The canal length was estimated from the preoperative radiograph. The pulp tissue was extirpated using Hedström files. The tooth was left open to the oral environment for microbial contamination of the root canal for 7 days, and then, the access cavity was sealed with a cotton pellet and Intermediate Restorative Material (IRM; Dentsply DeTrey, Konstanz, Germany) for 4 weeks. Because clinical and/or radiographic periapical radiolucency associated with an immature tooth could be due to apical periodontitis or a developing apical papilla, the primary intention of

Fig. 1. (a, c) Preoperative radiographs of maxillary and mandibular experimental anterior teeth to show incompletely developed roots. (b, d) Postoperative radiographs of maxillary and mandibular experimental anterior teeth 3 months after revascularization to demonstrate maturation of the roots and increased thickening of the canal walls.

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this study was to induce pulp infection and necrosis of immature teeth in dogs for revascularization procedures.

Revascularization procedures

First treatment
Anesthesia was administered as previously described. The experimental teeth and surrounding gingival tissue were first disinfected with 5% tincture of iodine (Amriya Pharm. Ind., Alexandria, Egypt) and then isolated with a rubber dam. The teeth and surrounding rubber dam were disinfected with 35% hydrogen peroxide, followed by 5% tincture of iodine. The IRM and cotton pellet were removed from the access cavity of all experimental teeth using #2 carbide round bur mounted on a slow-speed handpiece. The canals were carefully irrigated with copious amounts of 2.5% sodium hypochlorite, followed by flushing with sterile saline solution. The canals were dried with sterile paper points. A sterile precurved cotton pellet was placed over the access cavity of the experimental teeth using a lentulo spiral in a slow-speed handpiece. The access cavities of all experimental teeth were closed with a sterile cotton pellet and IRM.

Second treatment
Three weeks after the first treatment, if no signs, such as swelling or sinus tract, and intracanal exudate were present, a second treatment was performed. Again, general anesthesia was administered to the animals. The experimental teeth were isolated and thoroughly disinfected as described previously. The IRM and cotton pellet were removed from the access cavities. The canals were irrigated with copious amounts of 2.5% sodium hypochlorite and sterile saline solution to remove the antibiotic paste in the canals. The canals were dried with sterile paper points. A sterile precured #25 stainless steel K-file was introduced into the canals through the open apex into the periapical tissues to provoke bleeding into the canals to the level of cemento-enamel junction (CEJ). A sterile cotton pellet was placed over the blood for approximately 10 min to achieve partial coagulation and then removed. Mineral trioxide aggregate (Pro-Root MTA; Dentsply Tulsa Dental Specialties, Tulsa, OK, USA) was mixed with sterile saline solution and placed over the semi-coagulated blood clot with a MAP System carrier (Roydent, Johnson City, TN, USA) to achieve a 3–4 mm thick plug and gently condensed with pluggers into the canal to the level of the CEJ. The access cavity was closed with silver amalgam.

Postoperative periapical radiographs were taken of all experimental teeth 3 months after completion of revascularization procedures (Fig. 1b,d). The animals were sacrificed by an overdose of sodium thiopental. Two types of mineralized tissue were formed beneath the MTA plugs in nine revascularized teeth. One type of the mineralized tissue was similar to bone, lacking tissues were carefully dissected out and removed as a block and processed for histological examination.

Tissue processing for histological examination
The specimens were immediately fixed in 10% neutral-buffered formalin solution for 10 days, washed under running tap water over night, and then decalcified in 5% trichloroacetic acid. Complete decalcification was confirmed when the specimen could be easily pierced with a sharp instrument. The decalcified specimens were washed under running tap water overnight, dehydrated in ascending concentrations of ethanol, and cleared in xylene. The specimens were embedded in paraffin and serially sectioned at 5 μm. The sections were mounted on slides, deparaffinized, and hydrated through descending concentrations of ethanol. The sections were stained either with Harris hematoxylin and eosin (H&E) for cellular identification or Gomori’s trichrome for collagen identification.

Criteria of histological findings
The following criteria were used to characterize the nature of hard and soft tissues formed in the canals:

Mineralized tissue beneath the MTA plug, on the canal walls, and at the root apex
Dentin—mineralized tissue containing tubular structures without cell inclusions.
Cementum—mineralized tissue with cementocytes housed in the lacunae.
Bone—mineralized tissue with osteocytes housed in the lacunae and having osteons or bone marrow.

Soft tissue in the canal space
Pulp proper-like tissue—loose connective tissue rich in blood vessels and nerve fibers without odontoblast-like cells, similar to the tissue in the core of the pulp.
Pulp tissue—loose connective tissue rich in blood vessels and nerve fibers and having odontoblast-like cells lining along the predentin.
Periodontal ligament—dense cellular connective tissue with fibers inserted in cementum or bone.

Results
One maxillary left lateral incisor had a crown fracture during the experimental period and was excluded from the study. A total of 16 revascularized teeth were available for histological examination. All experimental teeth showed continued root development, with thickening of the canal walls, and no presence of periapical lesions (Fig. 1b,d).

Mineralized tissue beneath the MTA, on the canal walls, and at the root apex
Two types of mineralized tissue were formed beneath the MTA plugs in nine revascularized teeth. One type of the mineralized tissue was similar to bone, lacking...
the tubular structure observed in dentin (Fig. 2a). Cells resembling elongated fibroblasts or mesenchymal cells, together with collagen fibers, aligned the mineralized tissue on the pulp cavity side (Fig. 2b). Scattered inflammatory cells were present in the loose connective tissue in the canal. Another type of mineralized tissue observed was cellular cementum-like tissue (Fig. 2c). Flattened cementoblasts lined the hard tissue along the pulp cavity side, and cementocytes were housed in the lacunae (Fig. 2d).

Varying thicknesses of cellular cementum-like tissue was deposited on the canal walls in all revascularized teeth (Fig. 3a). A layer of plump cementoblast-like cells lined the cellular cementum-like tissue on the pulp cavity side, and cementoblasts were housed in the lacunae (Fig. 3b). In one case, reparative dentin-like tissue (RD) with a few irregular tubular structures was formed on the local area of the canal dentin wall (Fig. 4). The reparative dentin-like tissue was covered by predentin-like tissue with palisade odontoblast-like cells on the pulp cavity side. There was a concentration of inflammatory cells and scattered mineralized tissue nodules in the loose connective tissue in the canal (Fig. 4). In seven cases, a hard tissue barrier was formed more apically in the canal, rather than directly beneath the MTA plug.

In five cases, the apical foramina appeared to be completely closed by deposition of cellular cementum in a two-dimensional histological picture, yet the canal contained vital tissue (Fig. 5a). In four other cases, apical alveolar bone was seen growing into the canal through the apical foramina (Fig. 5b).

**Soft tissue in the canal space**

Different degrees of ingrowth of soft tissue into the canals from the periapical tissues were observed in all revascularized teeth. In 10 cases, the soft tissue was a loose connective tissue rich in blood vessels without the presence of polarized columnar or flattened odontoblast-like cells, resembling the pulp proper-like tissue (Fig. 6a). Scattered chronic inflammatory cells were present in the loose connective tissue in two cases. In six cases, the connective tissue appeared to be more fibrous and continuous with the apical periodontal ligament (Fig. 5b). No definite nerve fibers were identified in the soft connective tissue.

Remnants of normal pulp tissue were observed on the canal wall in two revascularized teeth (Fig. 6b). Tall columnar odontoblasts lined the predentin. On the opposite side of the canal wall in the same tooth, no normal pulp tissue was present (Fig. 6b).

**Discussion**

When parenchymal cells and supporting extracellular matrix of an organ such as the dental pulp are completely destroyed by infection or injury, *in situ* regeneration of the damaged organ by the living host is unlikely (12, 13), without organ transplantation or the
application of tissue engineering. The histological findings of revascularized teeth in the present study, although limited by the sample size, are similar to those observed in previous animal studies (3–6). The new tissues formed in the canals of revascularized teeth are cementum-like, bone-like, and periodontal ligament-like tissue. Importantly, normal pulp tissue with odontoblasts aligning the predentin was observed on the canal wall in two revascularized teeth in the present study (Fig. 6b). Although the pulps were extirpated and the canals were exposed to oral microorganisms for 7 days and then closed with a cotton pellet and IRM for 4 weeks to induce pulp infection and necrosis in the present study, it is possible that the vital pulp tissues were not completely extirpated and remnants of the pulp tissue survived in the canal after revascularization procedures during the present experimental period. Recently, a study showed that radiolucency in the periapical region cannot be used as a determining factor of total pulp necrosis because vital tissues can be present in the pulp chambers of animal immature teeth 60 days after experimentally induced apical periodontitis (11). Histologically, the presence of vital pulp tissue in the canal, in spite of the presence of periapical abscess in re-implanted immature animal teeth, has also been demonstrated (14). Accordingly, remnants of vital pulp tissue in the canals of revascularized teeth in short-term animal studies can be misinterpreted as regeneration of the pulp tissues, as in the present study. In human immature permanent teeth clinically diagnosed as having a necrotic pulp and an apical periodontitis, the disease process has typically been ongoing for a long period of time, especially in trauma cases. Therefore, the infection is well established. Consequently, the histological features of revascularized teeth in animal models in short-term experiments can be different from those in humans with long-standing apical periodontitis.

In the present study, inflammatory cells were present in the soft connective tissue in the canals in two cases. Similar findings were also observed in other revascularization studies (4, 5, 10, 15). Therefore, revascularized teeth are not necessarily free of inflammation. The inflammation might be due to immune response of the newly formed vital tissue to residual bacteria in the canal after revascularization. The exact cause of inflammation in the revascularized teeth should be investigated.

Two types of mineralized tissues, cellular cementum-like and bone-like tissue, were formed beneath the MTA plugs in revascularized teeth. When MTA was used as a direct pulp capping or pulpotomy agent, reparative dentin-like tissue was formed beneath the MTA (16, 17). It was assumed that MTA might induce pulp progenitor cells to differentiate into odontoblast-like cells and produce reparative dentine-like tissue (16, 17). However, in revascularized teeth, when the pulp is either mostly or completely necrotic, it is unlikely that reparative dentin-like tissue can be formed by the pulp progenitor cells beneath the MTA plug. Mineralized tissue barriers were formed in the middle of the canals in seven cases. This was also observed in previous animal study (4). The cellular mechanism of this type of mineralized tissue barrier formation is not clear.

Similar to other animal revascularization studies (3–6), increased thickening of the canal walls was
observed and appeared to be due to deposition of cellular cementum-like tissue. However, in one case, reparative dentin-like tissue with some irregular tubular structures was formed on the canal wall, which was covered by predentin-like tissue with palisade odontoblasts (Fig. 3). The exact repair process that occurred in that case is not known. Perhaps the progenitor cells from the remaining vital pulp tissue might be able to produce the reparative dentin-like tissue in response to induced pulp infection and revascularization procedures during healing process.

Histologically, the apical foramina appeared to be completely closed by the deposition of cellular cementum-like tissue in five cases. However, as vital tissue was present in the canals of revascularized teeth, the apical foramina could not be completely closed. Based on conventional radiography or cone beam computed tomography, this kind of root apex may be misinterpreted as complete closure of the apical foramen. In four cases, apical alveolar bone was seen growing into the canal spaces through the open apex, indicating possible ankylosis of the revascularized tooth with the apical alveolar bone. This might interfere with the normal eruption of the revascularized immature teeth.

Although nerve fibers were not observed in the newly formed tissue in the canals of revascularized teeth in the present study, it does not imply that nerve fibers were absent. Nerve fibers are not easy to identify in tissue sections stained with hematoxylin and eosin. If neurofilament markers such as 200 N52 or special staining, Bodian’s protagol or silver stain were used, nerve fibers might be detected. The sensory nervous system plays important roles in maintaining tissue vitality, immune defense, wound healing, and angiogenesis (18–20). Many blood vessels were present in the newly formed soft connective tissue in the canals. Blood flow is regulated by neuropeptides released from sensory nerve fibers (17). Usually, blood vessels and nerve fibers run side by side in the tissue. Accordingly, nerve fibers are likely present in the newly formed tissue. The role of sensory nerve fibers and their neuropeptides in revascularization needs to be investigated.

Based on a few animal studies (3–6), the stem/progenitor cells introduced into the canal spaces of immature teeth with necrotic pulp after revascularization procedures appear to be capable of differentiating into cementoblast-like or osteoblast-like cells rather than odontoblasts. These stem/progenitor cells are likely to be derived from the periodontal ligament or the periapical alveolar bone marrow (21, 22) because the newly formed tissues in the canals of revascularized teeth are cementum-like or bone-like tissue. No evidence of regeneration of the pulp tissue was observed in revascularized teeth in the present study.

**Conclusions**
Residual pulp tissue can remain in the canals after revascularization procedures of immature teeth with
artificially induced pulp infection and necrosis in short-term animal experiments, thus leading to misinterpretation of regeneration of the pulp tissue. Revascularized teeth are not necessarily free of inflammation. Ingrowth of apical bone into the root canals undergoing revascularization can interfere with normal tooth eruption if ankylosis occurs.

Conflict of interest statement

The authors deny any conflict of interests related to this study.

References