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# Revascularization and tissue regeneration of an empty root canal space is enhanced by a direct blood supply and stem cells

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**Abstract** – *Background*: Regenerative endodontics is an innovative treatment concept aiming to regenerate pulp, dentin and root structures. In the diseased or necrotic tooth, the limitation in vascular supply renders successful tissue regeneration/generation in a whole tooth challenging. The aim of this study is to evaluate the ability of vascularized tissue to develop within a pulpless tooth using tissue engineering techniques. *Material and methods*: A pulpless tooth chamber, filled with collagen I gel containing isolated rat dental pulp cells (DPC) and angiogenic growth factors, was placed into a hole created in the femoral cortex or into its own tooth socket, respectively. The gross, histological and biochemical characteristics of the de novo tissue were evaluated at 4 and 8 weeks post-transplantation. *Results*: Tooth revascularization and tissue generation was observed only in the femur group, confirming the important role of vascular supply in tissue regeneration. The addition of cells and growth factors significantly promoted connective tissue production in the tooth chamber. *Conclusion:* Successful revascularization and tissue regeneration in this model demonstrate the importance of a direct vascular supply and the advantages of a stem cell approach.

Regenerative endodontic procedures can be defined as biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as the pulp-dentin complex for clinical use (1). The objectives of regenerative endodontic procedures are to regenerate pulp-like tissue, ideally, the pulp-dentin complex; damaged coronal dentin, such as following a carious exposure; and resorbed root, cervical or apical dentin (1). Current treatment modalities with synthetic materials offer high levels of clinical success for many conditions. An ideal therapy aims to regenerate the pulp tissue to its healthy state and thereby revitalize the dental pulp. Regenerative procedures are an exciting alternative as the normal pulp homeostasis will be retained.

Stem cell biology has become an area of importance to understand tissue regeneration. In general, stem cells are defined by their capacity for self-renewal and the ability to give rise to transamplifying daughter cells capable of differentiation along various linages. To date, many types of human dental stem cells have been isolated and characterized: (i) dental pulp stem cells (DPSCs) (2), (ii) stem cells from exfoliated deciduous teeth (SHED), (iii) stem cells from apical papilla (SCAP)(3), and (iv) periodontal ligament stem cells (PDLSCs) (4). Among them, all except SHED are from permanent teeth. These mesenchymal stem cells (MSCs) of dental origin vary in their differentiation capacity. DPSCs and SHED are from the pulp, while SCAP is from the pulp precursor tissue (3). Following expansion, these cells can differentiate into odontoblast-like cells and produce dentin-like tissue both *in vitro* and *in vivo* (2, 3). Under specific *in vitro* conditions, DPSCs and SHED have also been shown to be able to differentiate into neuronal and adipogenic cells (5). Remarkable results have been reported in several *in vivo* and *in vitro* studies using DPSC with different types of scaffolds and signalling molecules, confirming the usefulness of this stem cell in tissue engineering (6–12).The role of adult stem cells and scaffold material in this process is becoming an area of great interest. Therefore, the application of these stem cells might be crucial to generate new dental tissue, including dental pulp.

Vascular supply plays a major role in cell survival and tissue generation (13). Regenerating dental pulp tissue by implanting cells in 3D constructs will not succeed if the cells lack nutrients; therefore, encouraging angiogenesis is essential. Recently, a combination of three angiogenic growth factors, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF2) and platelet-derived growth factor (PDGF), was found to synergistically promote angiogenesis and tissue production in a tissue engineering model (14). Therefore, these factors may improve cell survival and tissue generation in the root canal space. Furthermore, enlargement of the apical foramen not only promotes vascularization but can also maintain initial cell viability via nutrient diffusion. Case reports proved this concept when seemingly necrotic immature premolar teeth showed continuing root development after root canal disinfection and formation of a blood clot within the canal space (15, 16). An animal study using dog teeth has partially confirmed this finding (17).

We hypothesize that a direct vascular supply plays a pivotal role in tissue regeneration in an empty root canal. The aim of this study is to evaluate the ability of vascularized tissue to develop within a pulpless rat incisor, to promote dental pulp cell (DPC) survival and to ultimately regenerate dental pulp tissue.

## Materials and methods

All experiments were performed with the prior approval of the St. Vincent's Hospital Animal Ethics Committee, under National Health and Medical Research Council (Australia) guidelines. Male inbred Sprague Dawley rats (Australia) weighing between 220 and 280 g were used for all experiments. Rats were housed in pairs and fed standard rat chow and water *ad libitum*.

### Dental pulp cell preparation

DPC containing a subpopulation of DPSC were obtained from the incisor teeth of neonatal Sprague Dawley rats using a protocol adapted from Gronthos (5). Under sterile conditions, the incisors were extracted, and the dental pulp tissue was then removed. Tissues were evenly sliced into 1–2 mm sections, then incubated with PBS containing 3 mg $ml^{-1}$ Collagenase I and 4 mg $ml^{-1}$ Dispase II (Roche, Mannheim, Germany) in a shaking water bath (at 37°C) for 45 min. The isolated cells were then centrifuged (1500 rpm) at room temperature for 5 min, washed in PBS, and resuspended in Alpha MEM (Gibco, Grand Island, NY, USA) supplemented with 20% FCS, 100 $\mu$mol $l^{-1}$ L-ascorbic acid, 2 mM L-glutamine, 100 units$^{-1}$$ML^{-1}$ Penicillin/Streptomycin and 1% sodium pyruvate (Sigma, St. Louis, MO, USA) (Complete Media). Cells were incubated in complete medium at 37°C/5% $CO_2$, with regular media changes every 3–5 days. The cultures were monitored regularly using a light microscope. Cells at passage 3–4 were used in the experiments. On the day of surgery, prior to implantation, the DPC were labelled with Cell Tracker CM-DiI (Molecular Probes, Eugene, OR, USA). A stock solution of the dye was prepared at a concentration of 1 $\mu g$ $\mu l^{-1}$ using dimethyl sulfoxide (DMSO). Cells were detached from the tissue culture flasks, counted and resuspended in sterile phosphate-buffered saline (PBS). Dye was added to the cell suspension with final DMSO concentration $< 0.1\%$ and incubated for 5 min at 37°C. The cells were then transferred onto ice for a further 15 min then washed three times with PBS (centrifuged at 1200 rpm for 5 min each). Uniformity of cell labelling was confirmed by placing a drop of the cell suspension onto a histology slide, cover-slipping and viewing under a fluorescent microscope (Axioskop 2; Zeiss, Göttingen, Germany).

### Scaffold and growth factor preparation

Gelatin microspheres were prepared at the University of Melbourne Department of Chemical and Biomolecular Engineering, as previously described (18). Fibroblast growth factor-2 (FGF-2) (Peprotech Inc, Rocky Hill, NJ, USA), VEGF (R&D Systems, Minneapolis, MN, USA) and PDGF (R&D Systems) at 100 ng $ml^{-1}$ each were conjugated to the gelatin microspheres and incubated at 4°C overnight. On the day of surgery, 700 $\mu$l of chilled purified, pepsin-solubilized bovine dermal collagen I (Vitrogen; Cohesion Technologies, Inc., Palo Alto, CA, USA) was mixed with 100 $\mu$l of 10× PBS and the pH adjusted to 7.4 using 200 $\mu$l $NaHCO_3$. The growth factor-conjugated gelatin beads were added at a concentration of 2 mg beads per 100 $\mu$l collagen, and single cell suspensions of DiI-labelled DPCs were suspended in the neutralized collagen I at $2 \times 10^6$ cells per 200 $\mu$l of collagen.

### Experimental groups

All surgical procedures were performed under general anaesthesia (intraperitoneal injection of 75 mg $kg^{-1}$ ketamine, 10 mg $kg^{-1}$ xylazine final 0.2 ml per 100 g rat). Two models were used focusing on the important role of the vascular supply from underlying bony tissue. The first model consisted of allogenic transplantation of a rat tooth chamber into the femur. This model was adopted to access the rich vascular supply from the bone marrow space, with minimal blood clot formation around the implant. The second model was the orthotopic replantation of a rat incisor into its socket after apical resection. This model aims to mimic the clinical situation more closely, while excluding the regenerative potential of stem cells situated in the apical area of the pulp.

*Group 1: Allogenic implantation of tooth chamber into the femur*

A 5-mm cylindrical tooth chamber was prepared using an extracted rat incisor tooth. The pulp was removed, and then the root canal was mechanically prepared with hand endodontic instruments. After the root canal was cleaned, one end of the tooth chamber was sealed with light-cured composite resin (Dentsply, Caulk, Milford, DE, USA) following the manufacturer's protocol (Fig. 1a). The tooth chamber was ultrasonicated in 17% EDTA for 3 min followed by a $dH_2O$ wash for

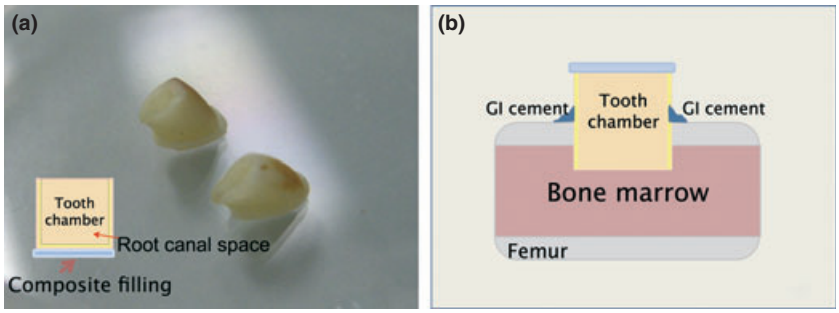


*Fig. 1.* Tooth chamber. (a) A schematic to illustrate the design of tooth chamber with composite resin cover, shown also in photo. (b) Schematic diagram shows how the tooth chamber was placed. Tooth chamber, filled with the mixture of dental pulp stem cell, collagen I and growth factors, was placed directly into the bone marrow space through the hole created and then held in place using glass ionomer cement.

15 min and sterilized using 25 kGy gamma irradiation. On the day of surgery, the rat leg was shaved and the skin surface decontaminated using alcoholic chlorhexidine solution. A longitudinal incision was performed along the thigh to expose the femur. A hole was created through the femoral cortex using a small tungsten carbide dental bur. The tooth chamber was filled with collagen I alone ($n = 2$) or the mixture of DPC, collagen I and growth factors ($n = 6$), and then placed vertically into the prepared hole. Glass ionomer cement (Fuji IX GP®; GC Co., Tokyo, Japan) was placed around the tooth and bone to fix the tooth in position (Fig. 1b). The skin wound was closed. Rats were monitored regularly and sacrificed at 4 and 8 week.

*Group 2: Autoreplantation of rat tooth after apical removal*

The oral cavity and the outer tooth surface were decontaminated with alcohol chlorhexidine solution. Under microscope magnification, one incisor tooth was extracted. Then, 2 mm of the apical part of the tooth was removed. Pulp tissue was removed using a sterile barbed broach, and the root canal space was then filled with sterile distilled water for 15 min to eliminate remaining odontoblasts by osmotic shock. For the experimental group, a volume of 2 $\mu$l per chamber of the mixture of cells, scaffold and growth factors, was added into the tooth r ($n = 9$). In a control group, the canals were left empty ($n = 1$) or the pulp was retained ($n = 3$) except for the apical 2 mm, which was also resected in this group. The tooth was replanted back into its socket and sutured using 6-0 nylon. The other upper incisor tooth was kept as a control. Lower incisors were ground off using a dental bur to prevent contact of the upper and lower teeth. Soft food and water containing analgesic were provided up to 1 week, and tissues were harvested after 4 and 8 weeks.

#### Histological assessment

At sacrifice, intravenous perfusion was performed using 4% paraformaldehyde. Femurs and maxillas were removed and soaked in fixative solution overnight. Tissues were decalcified using 10% EDTA for 1 month before histological processing. Histological sections (5 $\mu$m thick) were mounted onto 3-aminopropyltriethoxy-silane (Menzel-Glaser, Braunschweig, Germany) coated slides and routine haematoxylin and eosin staining performed for morphological analysis. To assess DPC survival fluorescent histochemistry using DAPI was applied. DAPI (Molecular Probes, Eugene, OR, USA) at a concentration of 1:1000 was added to slides for 5 min at room temperature then washed with PBS and distilled water. Tissue sections were monitored under a fluorescent microscope (Axioskop 2; Zeiss) with a 100-W HBO light source with a rhodamine bandpass filter set with an excitation filter wavelength of 546 nm and emission filter wavelength of 590 nm. The sections were assessed morphologically and histomorphometrically by one blinded observer. Special attention was devoted to location of connective tissue formation, percentage of new connective tissue formation area/total chamber area (%, Osirix for MacOS X), cell number (cells per 100 $\mu m^2$), location of bone and vascular formation and percentage of vessel area/total chamber area (%). Statistical analysis was performed using student's *t-test*. $P < 0.05$ was considered significant.

## Results

#### Allogenic implantation of tooth chamber into the femur

At all measured time periods, all tooth chambers showed extensive revascularization. Blood vessel formation was observed in a matrix of fibrous connective tissue. In the group containing only scaffold, new fibrous connective tissue formation was occupied only 24.65% of the total chamber and was found only in the apical 1/3 of the chamber, adjacent to the bone marrow (Fig. 2a,b). Bone also formed inside the connective tissue and was observed to adhere to the tooth structure. In contrast, in the group containing DPCs and growth factors, new connective tissue was significantly observed ($P < 0.05$), which new tissue was occupied at 31.8% and 62.62% of the chamber space for 4- and 8-week group, respectively. In addition, the location of new tissue always presented at middle part of the chamber for the 4-week group, and at middle to coronal part of the chamber for the 8-week group (Figs 2c,d and 3b).

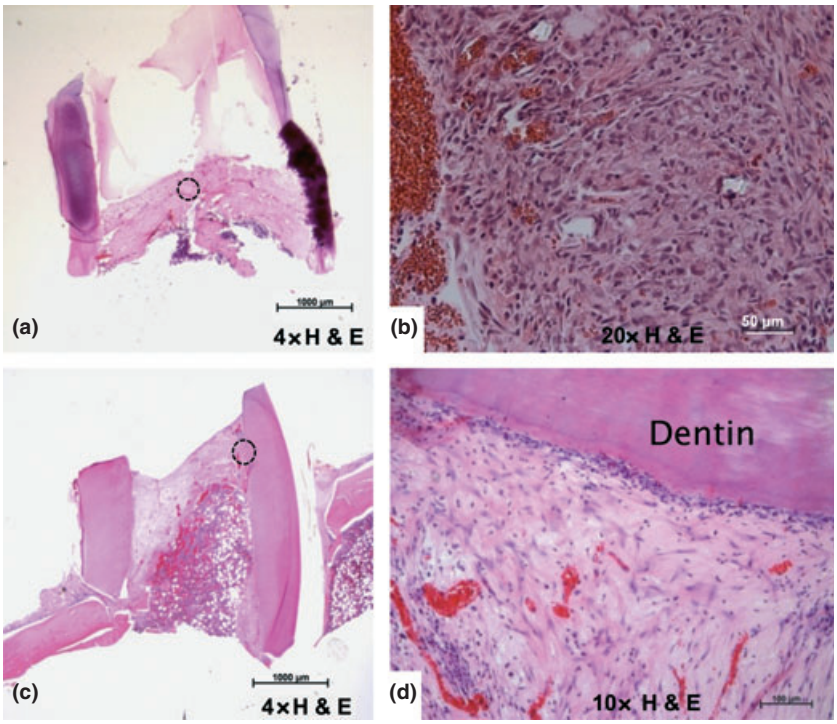


*Fig. 2.* Allogenic implantation of the tooth chamber. After 4 and 8 weeks, samples were harvested and processed. (a, b) In tooth chambers containing only scaffold at 4 weeks, connective tissue was observed at the apical 1/3 of the tooth. (c, d) In chambers containing dental pulp cell, scaffold and growth factors at 8 weeks, highly vascularized connective tissue was observed in the coronal 1/3–2/3 of the tooth chamber. The regions in a and b corresponding to the higher magnification areas in c and d are circled.

In the same experimental group, DPC survival in the chamber was confirmed by double fluorescent staining with DAPI. Positive staining cells generally observed near blood vessels (Fig. 3c). Cells around the edge of the construct were found to be attached to the pulpal surface of dentin (Fig. 3d). An amorphous extracellular matrix was observed associated with the cells (Fig. 3e). Bone also formed inside the chamber which occupied around 7% of the total chamber space. Islands of active bone formation were found underneath the connective tissue and being attached to the dentin in all chambers (Fig. 3f). Bone marrow invasion was always observed at the apical 1/3 of the tooth chamber. In addition, the percentage of area of blood vessel was found more in the groups containing cells and growth factors, especially in the 8-week group ($P < 0.05$). These vessels were found inside the newly formed connective tissue.

#### Autoreplantation of rat tooth after apical removal

Four and eight weeks after the tooth replantation, all rats were in a healthy condition. Replanted teeth showed neither pathological mobility nor macroscopic infection, and periodontal ligament reattachment was confirmed histologically (Fig. 4d). However, external root resorption was reported in all samples (Fig. 4e). No revascularization or tissue regeneration was observed in any group. Necrotic pulp remnants were found in the replanted teeth in which the pulp remained, with inflammatory cell infiltration ($n = 3$) (Fig. 4b). A blood clot was found occluding the apical area (Fig. 4a,c).

*Comparison of new tissue formation*
Statistical analysis demonstrated a significant improvement in tissue formation within the allogenic implantation of tooth chamber into the femur group when compared with the autoreplantation of rat tooth after apical removal at the same level ($P < 0.008$).

*Comparison of angiogenesis*
Statistical analysis demonstrated a significantly higher area of capillaries in the allogenic implantation group when compared with the autoreplantation group ($P < 0.05$).

*Comparison of dental pulp cell survival*
Statistical analysis demonstrated a significant improvement in DPC survival within the allogenic implantation of tooth chamber into the femur group when compared with the autoreplantation of rat tooth after apical removal ($P < 0.05$).

## Discussion

This study demonstrates that revascularization and tissue regeneration can be induced in a tooth chamber connected with direct blood supply. Addition of DPC and growth factors to the tooth chamber did improve tissue and vessel formation. Moreover, survival of the implanted cells could be observed in these allogenic transplanted teeth up to 8 weeks. On the contrary, very limited growth of tissue and vessel was observed in the

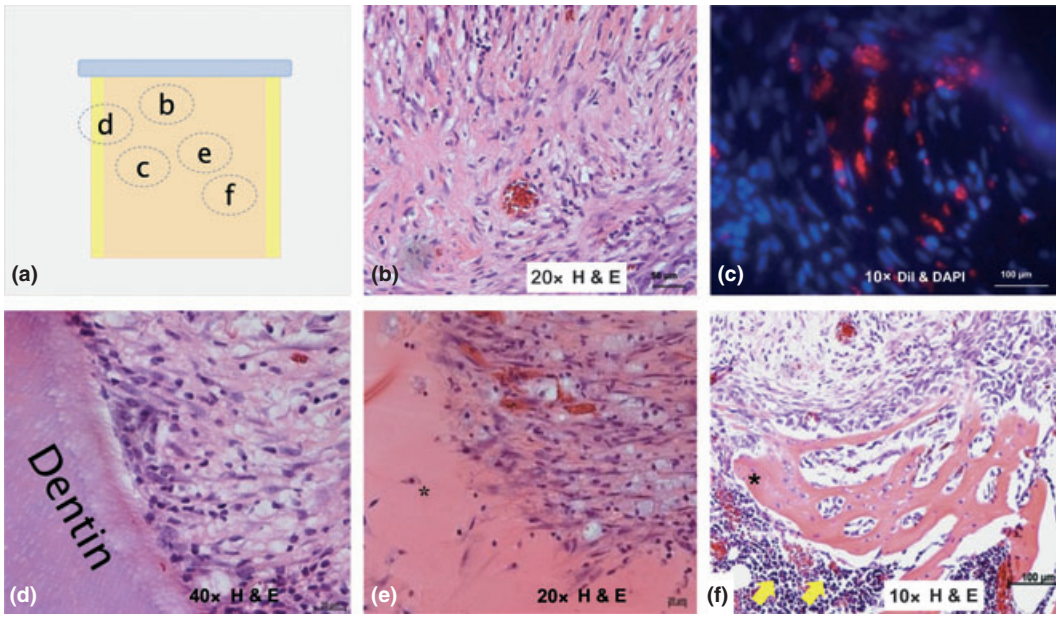


*Fig. 3.* Allogenic implantation of a tooth chamber containing dental pulp cell (DPC), scaffold and growth factors. (a) A schematic shows the location for the followings pictures. (b) Highly vascularized connective tissue was observed in the coronal 1/3–2/3 of the tooth chamber. (c) Surviving DiI- and DAPI-positive DPC were found. (d) Cells and connective tissue were found attaching to the dentin of the tooth chamber. (e) Amorphous matrix with adjacent cells was observed (asterisk). (f) Bone formation was observed in the tooth chamber (asterisk), together with bone marrow invasion (arrows).

autoreplantation teeth even cells and growth factors were added. Implanted cells could not survive in the replanted tooth.

The present study focused on revascularization and the survival of DPC using tissue engineering concepts in an empty root canal space. Two different models were used to mimic the clinical situation of tooth situated in bone. A potential problem with replanting an extracted incisor is that the occurrence of periapical blood clot, which may delay healing (19, 20). Therefore, revascularization of the canal space was suspended leading to the death of implanted cells (DPC). On the other hand, all allogenic implants in the femur, with a minimal blood clot between the root segment and bone marrow, showed highly vascularized connective tissue formation within at least part of the tooth segment. We hypothesize that the abundant access to bone marrow space reduces cell stresses and hypoxia and allows improved maintenance of the transplanted DPC population in the allogenic transplant group. As demonstrated in the results, the increased number of capillaries and fibrous connective tissue formation supports this hypothesis. This hypoxic-ischaemic insult to the autoreplanted cells may exacerbate inflammation. Inflammation is necessary to remove cellular debris and plays an essential role in the process of angiogenesis, but disturbed inflammation may concomitantly result in cellular injury.

Implanted DPC s and growth factors also participated in tissue generation. This novel model provides an insight into both the limitations of the development of a direct vascular supply, and also the role of a stem cell approach. Notable results have been reported from case studies encouraging the use of regenerative treatments in endodontics, whereby only root canal disinfection was utilized (15, 16). Three biological possibilities may be involved in this regenerative situation. Firstly, stem cells may survive in the apical area after the infection (3, 21). Several stem cell candidates have been suggested, because many stem cells have been identified in the dental pulp and surrounding tissue including DPSC (2), stem cells from the apical papilla (SCAP) (3) and PDLSCs (4). However, SCAP are favoured to play this role because of their proximity to the periapical tissue and are most likely to survive after infection (3). Secondly, stem cells from other areas may migrate to the dental pulp and promote tissue regeneration. Several types of mesenchymal progenitor cells that can participate in tissue repair have been identified in the circulatory system (22, 23), which may play a role during pulp regeneration. In our study, we eliminated the possibility that SCAP would survive by resecting the apical part of the tooth. No revascularization was observed in the replanted teeth. This may be because SCAP really do play a role in revascularization, or that the blood supply was not sufficient to promote tissue regeneration along the long canal space even when angiogenic factors were added. The tooth chamber connected to the bone marrow space further underpins the importance of a vascular supply in the regenerative process. In this model, the vascular supply was sufficient because a shorter tooth chamber was used (1, 24), and a direct vascular supply developed. Tissue regeneration was observed in all tooth chambers, but the extent varied with the experimental conditions. In the chamber containing only the collagen scaffold, new connective tissue was observed in the root canal, while a greater amount of highly vascularized connective tissue formation was observed in the chambers containing stem cells and

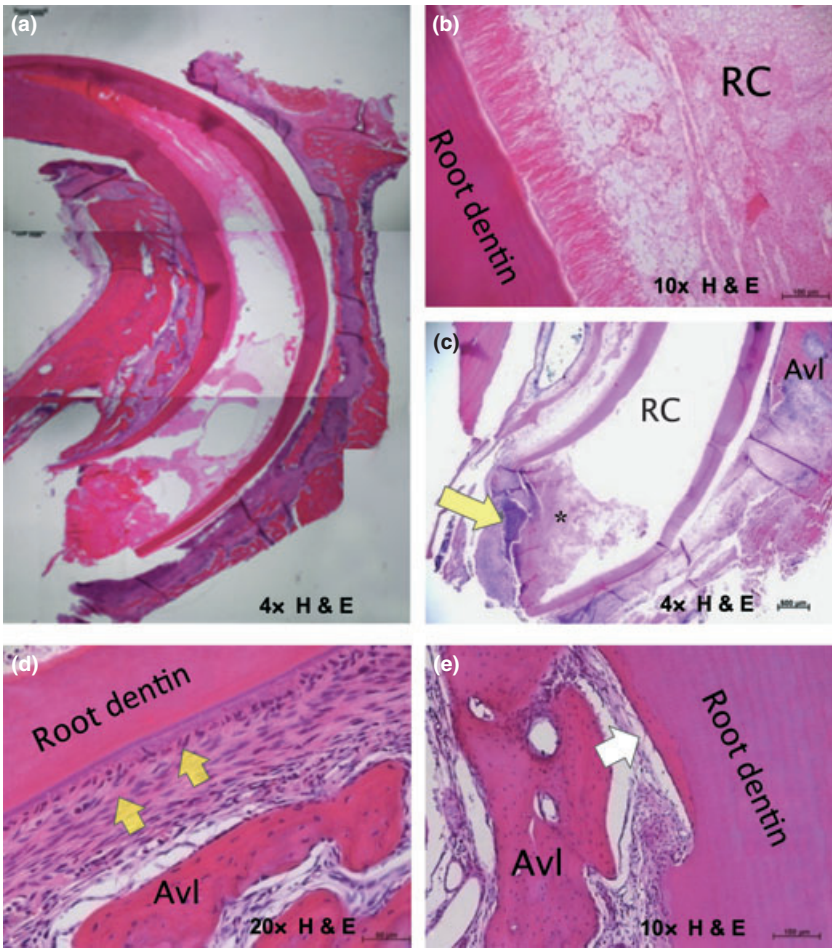


*Fig. 4.* Autoreplantation of the rat tooth. (a) An experimental tooth illustrating a lack of revascularization. (b) Necrotic pulp without inflammatory reaction was observed in a control replanted tooth, with remaining pulp. (c) Blood clot (asterisk) and inflammatory cell infiltration (arrow) were located at the root apex. (d) In all replanted teeth, periodontal reattachment was generally observed (arrows). (e) External replacement root resorption was found in a few areas (arrow). Avl: alveolar bone, RC: root canal.

growth factors, indicating the benefit of these factors. Thirdly, a scaffold created from a blood clot has been proposed to play a role in the cell attachment and signalling, which leads to pulp revascularization (15). An animal study focusing on this alternative treatment recently confirmed the regenerative potential remaining after root canal disinfection using a mixture of antibiotics (17). However, the addition of collagen I and blood clot as scaffolds did not significantly improve revascularization (17). In our study, a blood clot apical to the replanted tooth did not appear to exert any positive effects. Indeed, it appeared to block revascularization. Collagen I was used in this study to support the cells and growth factors in the root canal. Collagen was replaced by vascularized connective tissue containing surviving DPC after 8 weeks implantation, confirming its biodegradability and potential use as a scaffold for pulp cell engineering as collagen I gel has been confirmed to support DPSC proliferation in culture (25). The biocompatibility of this collagen was excellent, because a severe inflammatory reaction was not observed. The manipulation was simple, and the material could be injected into the canal. However, several studies have identified limitations (1, 25), so further development of appropriate injectable scaffolds for regenerative endodontics is required.

Bone formation within the canal space is a common negative finding after immature tooth replantation in humans (17, 24, 26, 27). A similar finding has been reported in revascularized dog teeth following root canal disinfection (17). Two prerequisite factors that seem to play a role in this regard are: (i) preosteoblastic cells, which have the potential to differentiate into bone-forming osteoblasts, either survive or migrate into the canal space. Cells in periodontal tissue and/or resident pulpal mesenchymal cells have been proposed as potential preosteoblasts (28, 29). Moreover, several recent studies reported that DPSC have potential to produce bone (9, 11, 30); (ii) The vascular supply should be sufficient to support the growth of all cells, including bone cells. Because the vascular supply after mature tooth replantation is inadequate, pulp tissue tends to become necrotic without hard tissue formation (31, 32). In our study, bone formation was observed in all tooth chambers connected to the femurs, but not in the incisor replantation model. This confirms the important roles of

the blood supply because revascularization did not occur in the replanted incisors. To date, the mechanisms participating in bone formation after tooth replantation are unclear. A recent study suggests that the osteoclast lineage of progenitor cells forming tissue after tooth injuries plays a role in osteoblast induction (27). From our study, two possibilities regarding the reparative potential of bone marrow leading to hard tissue production in the tooth chamber may be the mesenchymal stem cells (BMSCs) migrating from the bone marrow, and/or the induction of implanted DPSC towards an osteoblast lineage. In any case, bone deposition within the canal space must be regarded as an adverse effect that will interfere with normal root development. Further studies of factors predisposing to (or preventing) bone formation after tooth injuries are needed, because successful inhibition may optimize the regenerative result.

In conclusion, we have shown that vascular and tissue regeneration can be induced in an empty root canal space as long as a blood clot does not impede tissue ingrowth. Direct access to a vascular supply together with addition of cell and growth factor did participate in this generative situation. However, care should be taken as intracanal bone formation is a risk. Therefore, to generate a practical paradigm for dental tissue engineering, various aspects should be explored, for example pathway of dental-related cell differentiation, appropriate cell source and suitable scaffold, which may lead to a development of an alternative treatment using these techniques.

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