

## **Curriculum Vitae**

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### **Education and Degrees**

- DDS degree: 1997-2002, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran
- MS degree (specialty in Endodontics) 2007, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran
- MS degree in Biomedical Science 2015, School of Dentistry, University of Maryland Baltimore, Baltimore, MD, USA
- Diplomat, Iranian Board of Endodontics, 2007
- 2012-present: Endodontic Resident, School of Dentistry, University of Maryland, Baltimore, Maryland, USA

### **Professional and Teaching Positions**

- 2005-2007: Clinical instructor in Department of Endodontics, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran
- 2007-2011: Private practice limited to Endodontics
- 2007-2010: Part time Clinical Assistant Professor, Department of Endodontics, School of Dentistry, Rafsanjan University of Medical Sciences, Kerman, Iran
- 2010- present: Assistant Professor and Researcher in Iranian Center for Endodontic Research (ICER), Shahid Beheshti University of Medical Sciences, Tehran, Iran

## Membership in Scientific Societies and Committees

- Active member of American Association of Endodontists (AAE)
- Resident member of the Regenerative Endodontic Committee of the AAE
- Active member of Iranian Association of Endodontists (IAE)

## Grants

American Association of Endodontics Foundation (AAEF)

Project title: Evaluation of the effect of residual bacteria on pulp regeneration

Amount: \$14,480

## Bibliography

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- Evaluation of the effect of blood contamination on the compressive strength of MTA modified with hydration accelerators  
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### **Domestic Publications**

- The effect of carvacrol on Enterococcus faecalis as an intra-canal medicament:  
Invitro study  
Sharifian, Bolhari B, **Nosrat A**, Aligholi M  
Journal of Tehran Dental School 2009; 22(1): 35-40

### **Publications (books)**

- “Cements in contemporary dentistry” (in Farsi)
- “Decision making in treatment planning- Endodontics” (translated to Farsi)
- “Decision making in treatment planning- periodontics” (translated to Farsi)
- “Decision making in treatment planning- prothodontics” (translated to Farsi)
- “Decision making in treatment planning- oral surgery and orthodontics”  
(translated to Farsi)

### **Referee**

- Scientific reviewer of Iranian Endodontic Journal since 2011
- Scientific reviewer of International Journal of Oral Sciences since 2011

- Scientific reviewer of Journal of International Society of Preventive and Community Dentistry since 2015

## **Lectures and Presentations**

### **Poster presentations**

- Isolation, Characterization and Differentiation of Dental Pulp Stem Cells in Ferret  
Homayounfar N, Verma P, **Nosrat A**, Romberg E, Fouad AF  
IADR annual meeting, March 2015, Boston, MA
- Susceptibility of Common Endodontic Pathogens to Common and Potential Intracanal Antibiotics for Regenerative Endodontics  
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### **Table clinic**

- Dental pulp regeneration: problems with current protocols and future directions.  
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AAE annual meeting, April 2013; Hawaii, USA

### **Oral presentations**

- Contribution of new technologies to the understanding of complex root canal anatomy and effective endodontic treatment. AAE annual meeting, May 2014; Washington DC, USA
- Regenerative endodontic treatments (revascularization) for necrotic immature teeth: a review and report of technical modifications. 16<sup>th</sup> scientific congress of Asian Pacific Endodontic Confederation (APEC), April 2011; Shiraz, Iran.
- Apexogenesis treatment with Calcium Enriched Mixture cement. 13<sup>th</sup> annual congress of IAE, September 2010; Mashad, Iran.
- Considerations of periradicular surgery in elderly with real-time live periradicular surgery. 11<sup>th</sup> annual congress of IAE, summer 2008, Tehran, Iran.
- Lecture on different techniques in periapical surgery with real-time live periapical surgery. 10<sup>th</sup> annual congress of IAE, summer 2007, Tehran, Iran.

## **Workshops**

- Biological bases and recent techniques of open apex management in immature teeth. Continuing education for general dentists; summer 2008, 2009, 2010 and winter 2011; Tehran, Iran.
- A 3-days workshop on biologic bases of rotary instrumentation in root canal treatment for general dentists. Summer 2008; Tehran, Iran.
- New material and methods in root canal obturation. Continuing education for general dentists; winter 2008; Tehran, Iran.
- Recent changes in rotary instrumentation techniques in introduction of new rotary instruments. Continuing education for general dentists; winter 2007 and 2008; Tehran, Iran.

## **Honors/Rankings**

- Rank **40** in the Iran Medical Universities National Entrance Exam out of **1,059,800** applicants from all country, 1997.
- Rank **2** in the National Dental Basic Science Exam among second year dental students from all dental schools, 1999.
- Rank **2** in National Endodontic Board Exam in Iran, 2007.
- Performance in National Board Dental Exam (NBDE) part-1 in the USA: Score **97/100** (top 3% in competition with all participants in the USA in 2011).

## **Abstract**

**Title:** The Effect of Residual Bacteria on Dental Pulp Regeneration

Ali Nosrat, Master of Science, 2015

Thesis directed by: Ashraf F. Fouad BDS, DDS, MS

Tissue regeneration requires an interaction of stem cells and growth factors in a bioactive scaffold. This study utilized the ferret canine as an in situ animal model to investigate a clinically applicable tissue engineering approach for dentin-pulp regeneration. On the other hand, ideal root canal disinfection for dental pulp regeneration is a challenge. There is no study available to address the effect of residual bacteria on the outcome of dental pulp regeneration in previously infected root canals. Therefore, the aim of this study was two-fold: 1) To determine histologically, the efficacy of delivering stem cells within a bioactive scaffold directly into the root canal space compared with the traditional revascularization method and 2) to determine the effect of residual bacteria on the histological and radiographic outcomes of dental pulp regeneration procedures.

Periapical lesions were induced in 24 canine teeth of 6 ferrets. Dental pulp stem cells were isolated, characterized, encapsulated in a hydrogel scaffold, and injected in half of the experimental teeth. The other half was treated using the traditional endodontic protocol with a blood clot scaffold. After an evaluation period of 3 months, the animals were sacrificed and block sections were processed for radiographic, histological and histo-bacteriological analyses. Sections were evaluated for the presence/absence of an odontoblast layer, dentin associated mineralized tissue (DAMT), bony islands, intra-canal

and periapical inflammation, and bacteria. Data were analyzed using Fisher exact test and one-way analysis of variance (ANOVA) with the  $p \leq 0.05$ .

Results of the study showed that there was no significant difference between the traditional and the tissue engineering groups in terms of presence and amount of DAMT and bony islands ( $p > 0.05$ ). Presence of residual bacteria was associated with lack of radiographic growth ( $p < 0.0001$ ) and with presence of intra-canal and periapical inflammation ( $p < 0.05$ ). There was significantly higher amounts of DAMT formed in teeth with no residual bacteria compared to teeth with bacteria ( $p < 0.0001$ ). The results of this study showed that residual bacteria play a critical role in the outcome of regenerative endodontic treatments.

The Effect of Residual Bacteria on Dental Pulp Regeneration

by

Ali Nosrat

Thesis submitted to the Faculty of the Graduate School of the University of

Maryland, Baltimore in partial fulfillment

of the requirements for the degree of

Master of Science

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## **Introduction**

The dental pulp frequently succumbs to irreversible disease caused by caries, trauma, congenital abnormalities or consequences of previous dental procedures. Millions of such teeth are saved each year by root canal therapy. While this procedure is highly successful in many situations, teeth with immature apices present some specific challenges. Root canal instrumentation, disinfection and sealing are more technically difficult to perform in these circumstances. Furthermore, depending on the degree of root maturation, the thin root canal walls can render the tooth weak and susceptible to fracture (1).

The literature has shown that the MTA barrier technique has a reasonable degree of success (2). The MTA barrier technique has been shown to be successful (3), however it does not reinforce the thin root walls and the tooth could still potentially fracture in the future.

A more ideal treatment approach is to remove the diseased or necrotic pulp tissues and replace them with regenerated healthy pulp tissues that would continue normal dentinogenesis. In recent years, there has been strong interest in developing such biologically based treatment modalities (4, 5). In immature teeth, this can allow for continued normal physiologic root development. It is generally recognized that tissue regeneration requires an interaction of stem cells and growth factors in a bioactive scaffold, referred to as the tissue engineering triad (6). Current clinical procedures appear to provide components of these elements, sufficient to provide clinically acceptable results. Several reports have demonstrated control of the infectious process, radiographic thickening of canal walls and continued root development following pulpal necrosis in

teeth with immature apices (4, 5). However, recent studies on animal models revealed that tissues formed inside the root canal space after regenerative treatment is not pulp tissue (7-9). These tissues are a mixture of a connective tissue similar to a periodontal ligament and bone-like/ or cementum-like mineralized tissues. Similar to the outcome of animal studies, histological reports from human teeth extracted after regenerative treatments show that although there is vital tissue formed inside the root canal, no pulp tissue forms after traditional regenerative treatment (10-12). In addition, it is not clear if a vital dental pulp is necessary for apical maturation of the root (13), and there are reports showing absence of vital tissues in the root canal space after regenerative treatments (13, 14). The problems that would arise from the lack of vital pulp regeneration include that the technique is not clinically predictable, the resultant mineralized tissue is not strong enough to support masticatory forces and that if endodontic treatment is necessary, the root canal environment would not be conducive to traditional techniques.

In addition, current clinical protocols for regenerative treatments appear to address only the scaffold and growth factor components, and provide for a root canal milieu that is conducive to tissue revitalization. They rely on the presence of stem cells in the apical papilla. In the presence of a periapical lesion, it is difficult to predict whether the apical tissue has a sufficient number of stem cells that can migrate into the pulp space to allow for pulp regeneration.

The other critical aspect that can interfere with the outcome of treatments is the adequacy and quality of root canal disinfection. When regenerative procedures of the dental pulp are being considered following an infection, the traditional level of root canal disinfection may not be sufficient. In most clinical endodontic cases, following chemo-mechanical

disinfection of the root canal space, a well-condensed root filling is placed. Thus, if residual bacteria are left behind, their numbers are thought to be minimal, and the root filling is thought to minimize their access to sustain the periapical lesion, thus creating an environment conducive to healing in most cases. In contrast, if regeneration of the pulp is contemplated, it is likely that the disinfection procedures may need to be performed to a higher level of efficacy than is necessary in clinical endodontics (15). The lack of filling in the canal as the regenerative tissue is developing may be conducive to bacterial proliferation (15). A recent case report shows that regenerative endodontic treatments can fail due to persistent infection (16). The quality of disinfection and the effect of residual bacteria on the outcome of regenerative treatments have never been reported. The proposed study will mainly focus on these two aspects of the regenerative endodontic treatment.

### **Review of the Literature**

The ferret model had been utilized to induce periapical lesions and perform endodontic procedures previously (17). The advantage of using ferrets as experimental animal models is that they have more similar root canal anatomy of the canine to humans than rabbits and rats, with less cost and ethical concerns than cats, dogs or primates (18). Torabinejad et al (19) have recently described this model in terms of age changes and milestones in development. Their findings suggest that this model is suitable for experiments in regenerative endodontics. The permanent maxillary and mandibular cuspids of male ferrets erupt between 51-61 days after birth. Initial signs of closure of the apical foramen are observed at 90-110 days. Complete apical closure and thickening of the root canal walls is seen at 133 days. The most appropriate time to conduct

experiments on ferret cuspids with immature apices is when the animals are between the ages of 70-90 days (19).

In order to predictably regenerate the dentin-pulp complex, sound tissue engineering concepts must be employed. Tissue engineering models used include the tooth slice/scaffold (20), the 6-7mm long human tooth root fragment (21) and in vivo whole pulp regeneration animal studies (22), each of which represents a step closer to the clinical situation, both in terms of simulating the biological response as well as simulating the root canal anatomy. The incorporation of stem cells derived from dental pulp with bioactive factors into a hydrogel scaffold was shown to promote cell proliferation, differentiation and angiogenesis (23). Irrigation with ethylenediaminetetraacetic acid (EDTA) can release bioactive growth factors that are sequestered into the dentin matrix (24), which may aid in the proliferation of regenerated pulp tissue. Conditioning the root canal dentin with EDTA also enhances stem cell attachment and differentiation into odontoblasts (25).

### **Animal studies with no tissue engineering**

Thibodeau et al (26) conducted an animal study using a dog model on the traditional protocol of regenerative endodontic treatments. Histological evidences of hard tissue deposition on the root canal walls (43.9%), apical closure (54.9%), and formation of vital tissue in root canal space (29.3%) were demonstrated (26). They also evaluated the effect of a blood clot and a soluble collagen scaffold on the outcome. Histological outcome of the treatment was not different in the presence or absence of blood clot inside the root

canal space. In addition, presence of a soluble collagen scaffold did not improve the results.

Wang et al (8) further analyzed the specimens from the previous study. Histological evaluation of the tissues produced inside the root canal after regenerative endodontic treatment of the dogs' immature necrotic teeth revealed three types of tissues: cementum-like tissue which was responsible for increases in root length and thickness, bone-like tissue and periodontal ligament (PDL) like tissue inside the canal space (8). There was only one case, with a partially survived pulp tissue, in which the presence of odontoblasts lining was seen. Formation of cemental bridges in different levels inside the canal was also demonstrated which might be related to the hard tissue induction potential of MTA (8).

An animal study on a dog model by da Silva et al. (27) revealed that the generated tissue inside the root canal space after regenerative endodontic treatment was basically an ingrowth of periodontal connective tissue instead of pulpal connective tissue. The study compared the effect of using triple antibiotic paste with negative pressure irrigation (known as EndoVac) on the histological outcome of the treatment. The group with EndoVac showed less inflammation in newly formed tissues.

### **Animal studies with tissue engineering in non-infected models**

Zhu et al (28) performed a study on the outcome of regenerative endodontic treatments in non-infected dog's teeth. They enlarged the apical foramen of all teeth to size # 80. They examined the effect of the addition of platelet-rich plasma (PRP) and dental pulp stem cells (DPSCs) to the treatment protocol. The study showed that new vital tissues can be

regenerated in permanent canine teeth after pulpectomy and enlargement of the apical foramen. However, the histological outcomes were not different between the blood clot group and the tissue engineering group. Addition of PRP or DPSCs or both did not enhance the outcome of treatment. Cementum-like and periodontal ligament-like tissues along the internal root canal walls were typical structures in most cases. The same group further analyzed these specimens using immunohistochemical techniques to determine the exact type of tissue formed in the root canal space and on the dentinal walls (29). The tissues were examined for the expression of periostin to detect periodontal ligament tissue, nestin and dentin sialoprotein for odontoblasts, and bone sialoprotein and osteocalcin for bone tissues. Samples were also stained for tartrate-resistant acid phosphatase (TRAP) as a marker for osteoclastic lineages. The study confirmed that the tissues formed in dog root canals after regenerative endodontic procedures were not pulp tissues but mainly periodontal tissues.

Wang et al (30) examined the effect of canine DPSCs on the Histological outcome of the pulp regeneration in non-infected dog's teeth. Autologous DPSCs were obtained from extracted first molars and expanded ex vivo to obtain a larger number of cells. After they were transplanted into the pulpless root canal with Gelfoam as the scaffold, DPSCs were capable of generating pulp-like tissues containing blood vessels and dentin-like tissue. Thickening of the root canal wall was also observed. The study showed the feasibility of using stem cell-mediated tissue engineering to achieve pulp regeneration in immature teeth.

Torabinejad et al (7) conducted an animal study on ferrets to examine the effect of PRP on pulp regeneration in ferrets. This was accomplished in an ideal condition (i.e. no

previous infection). The study compared the blood clot and PRP as different scaffolds in 21 canine teeth. Histological evaluation of the outcome showed that almost all of the experimental teeth had presence of intracanal bone-like tissue. No evidence of dentinal wall thickening or apical narrowing was noted in the experimental teeth. The conclusion was that in the ferret model, the use of either PRP or blood clots during regenerative endodontics leads to the formation of intracanal bonelike tissue without continued root maturation.

### **Animal studies with tissue engineering in infected models**

Yamauchi et al (31) designed and evaluated a tissue engineering protocol including use of an insoluble collagen sponge as a scaffold, and 17% ethylenediaminetetraacetic acid (EDTA) as a demineralizing agent which could expose the dentine matrix and possibly promote differentiation of mesenchymal cells and formation of mineralized tissues. Outcomes revealed that the use of cross-linked collagen significantly increased formation of mineralized tissues, and use of 17% EDTA significantly increased attachment of newly formed mineralized tissues to the dentinal canal walls. Two forms of hard tissue were detected: 1) dentine-associated mineralized tissues (DAMT) which were adhered to or detached from the dentinal walls, and were devoid of vasculature and cells; and 2) bony islands (BI) which were in the inner lumen independent of the dentinal walls and contained many embedded blood vessels, cells, and bone marrow-like tissues. In a separate study, further histological assessments and immunohistochemical analyses were performed on DAMT and BI by the same group (9). Outcomes showed that DAMT was clearly different from dentin and bone, and to some extent, from cementum. Although, the lack of vasculature and immunostaining patterns in the DAMT showed resemblance

to cementum, the organization and maturation of collagen fibers were significantly different from cementum. Immunoreactivity of dentine sialoprotein (DSP) and bone sialoprotein (BSP) in the BI was similar to alveolar bone. No odontoblastic cell layer, dentine-like structure, and pulp-like tissue were detected (9).

Zhu et al (32) evaluated the addition of dental pulp cells, PRP or a combination of dental pulp cells and PRP in infected immature dogs' teeth. A group with blood clot formation inside the root canal space served as the control. Radiographic analyses demonstrated no significant difference between the experimental groups in periradicular bone healing, while those groups that used DPCs produced a significantly greater root thickening. The histologic evaluation showed that the groups with PRP formed more tissue in the canals. The groups with DPCs had substantially more mineralized tissue formation in the canal than those without DPCs, especially in the apical third. In the DPCs + PRP group, bone-like tissue grew into the canal space from the periapical tissue. Although the study showed enhanced outcomes in terms of the amount of hard tissues formed following tissue engineering, none of the specimens showed formation of pulp tissue or dentin-like structures.

Tawfik et al (33) examined the difference in the radiographic and histological outcomes between these strategies: MTA apical plug, traditional method of regenerative treatments (blood clot), and regenerative treatment done with the addition of fibroblast growth factor (FGF) plus a gelatin scaffold in previously infected immature dogs' teeth. The radiographic evaluation revealed that in the absence of revascularization, the length and thickness of the root canals did not change over time. The injectable scaffold and FGF were no more effective than a revascularization procedure in promoting tooth

development following root canal revascularization. Histological examination of the specimens showed that the tissues formed in the root canals were not pulp tissue and resembled periodontal tissues.

Yoo et al (34) examined a novel method of tissue engineering for regeneration of pulp tissue in infected dogs' teeth. They used a conditioned medium prepared from pre-ameloblasts of murine apical bud cells as a source for signaling molecules. The conditioned medium was prepared by culturing mouse apical bud cells in the presence of FGF. Then the conditioned medium was added to the blood clot formed in dogs' immature pulpless teeth to regenerate the pulp tissue. Results showed that use of this conditioned medium resulted in formation of a pulp-like tissue plus significantly higher formation of hard tissues inside the root canal space. However, the hard tissue had an atubular structure with cellular components imitating cementum rather dentin (34).

## **Outcomes of clinical studies**

Bose et al (35) conducted a retrospective study on the radiographic outcome of regenerative endodontic treatment compared with MTA apexification and non-surgical root canal treatment. They showed that regenerative endodontic treatment using TAP as an intracanal dressing, produced significantly greater increases in root length and root wall thickness.

A retrospective study by Jeeruphan et al (5) on clinical and radiographic outcomes of regenerative endodontic treatment comparing MTA apexification and calcium hydroxide apexification showed a greater increase in root length and root wall thickness of the teeth treated with regenerative procedures. In addition, their study demonstrated a higher survival rate for teeth treated with regenerative procedures (100%) compared to MTA apexification (95%; not significant) and calcium hydroxide apexification (77.2%; significant).

Nagy et al (36) conducted the first clinical trial in this area of investigation. They compared the outcome of traditional regenerative endodontic treatment, using blood clot, with a tissue engineering strategy using a gelatinous scaffold coated with FGF. The MTA apical plug group served as a traditional control. After an 18-month follow-up, most of the cases in the study showed radiographic evidence of periapical healing. Experimental groups (regenerative treatment and tissue engineering group) showed a progressive increase in root length and width and a decrease in apical diameter. There were no significant differences between these two groups regarding the quality of root development.

Kahler et al (37) ran a prospective cohort study on 16 immature teeth with necrotic pulps treated with regenerative endodontic treatment. They used a blood clot as a scaffold. The study period was 18 months. Assessments at review included an evaluation of clinical signs and symptoms, periapical status, the presence of further root maturation, and pulp sensibility testing. Qualitative assessment showed 90.3% resolution of the periapical radiolucency. Apical closure was assessed as complete in 19.4% of cases. Quantitative assessment showed change in root length varying from -2.7% to 25.3% and change for root dentin thickness of -1.9% to 72.6%. They concluded that the outcome of regenerative endodontic treatments might not be predictable in the short term. In addition, they found the discoloration was the greatest negative outcome of the treatment.

Alobaid et al (38) conducted a retrospective cohort study on the radiographic and clinical outcome of 31 immature necrotic teeth treated either with MTA apexification or regenerative endodontic treatment (revascularization). The average follow up time was 17 months. The majority of treated teeth survived throughout the study period, with 30 of 31 (97%) teeth surviving (18/19 revascularization and 12/12 apexification). Most cases were also clinically successful, with 27 of 31 (87%) meeting the criteria for success (15/19 revascularization and 12/12 apexification; nonsignificant difference). A greater incidence of adverse events was observed in the revascularization group (8/19 vs 1/12 in apexification). Adverse events included discoloration, crown fracture, reinfection (i.e. failure due to persistent endodontic infection). They concluded that although more revascularization cases than apexification cases showed an increase in radiographic root area and width, the effect was neither statistically nor clinically significant.

Saoud et al (39) did a prospective cohort study on the clinical and radiographic outcome of 20 teeth treated with a traditional regenerative endodontic treatment protocol. The period of study was 12 months. All 20 treated teeth survived during the 12-month follow-up period, and all 20 also met the clinical criteria for success at 12 months. As a group, the treated teeth showed a statistically significant increase in radiographic root width and length and a decrease in apical diameter, although the changes in many cases were quite small. The within-case percent change in apical diameter after three months was 16% and had increased to 79% by 12 months, with 55% (11/20) showing complete apical closure. The within-case percent change in root length averaged less than 1% at three months and increased to 5% at 12 months. The within-case percent change in root thickness averaged 3% at three months and 21% at 12 months. This study showed that the most predictable and consistent outcome of regenerative endodontic treatment is complete apical closure.

## **Specific Aims of the Study**

1- To determine, using histology, the efficacy of delivering stem cells within a bioactive scaffold directly into the root canal space compared with traditional revascularization procedures on regeneration of the dentin-pulp complex in an animal model.

2- A) To determine if there is an association between presence of residual bacteria in the root canal system and: 1) histological and 2) radiographic outcome of regenerative treatment.

B) To determine if there is a difference in the amount of newly formed tissue between teeth with residual bacteria and teeth with no residual bacteria.

## **Research hypothesis related to aim-1 (Traditional revascularization vs tissue engineering)**

In immature teeth with pulpal necrosis and in the absence of infection, a histologically-superior treatment outcome (i.e. higher amounts of dentin associated mineralized tissue (DAMT) and bone) of regenerative endodontics will be achieved by directly delivering dental pulp stem cells encapsulated in a hydrogel scaffold, as compared to using a blood clot alone.

**Research hypothesis related to aim-2 (Association between bacteria and outcome)**

In immature teeth with pulpal necrosis, regardless of the regenerative treatment protocol, presence of residual bacteria in the root canal system is associated with the formation of less mineralized tissue, presence of inflammation and lack of radiographic root development.

## **Materials and Methods**

### **Research Design**

This study was conducted at the University of Maryland School of Medicine Animal Research facility and the University of Maryland Dental School. Nine descended 70-day old male ferrets were used for the study. The sample size was determined by power analysis (GPower software). The animals were purchased from an approved, licensed vendor (Marshall BioResources, North Rose, NY; infous@marshallbio.com). All nine ferrets were derived from the same colony of animals. Upon arrival, the animals were acclimated for 7 days before being used in the study. There were two experimental groups in the study: Group 1- Traditional Revascularization and Group 2- Tissue Engineered Construct.

The nine animals were utilized as follows:

Donor animal (1)- stem cell donor, all teeth were utilized to harvest pulp tissues, followed by euthanasia.

Control animals (2)- All 4 canines per ferret were utilized for a total of 8 canine teeth. These 8 control teeth were randomly derived from the 2 control ferrets, each of which contributed 2 canine teeth as positive controls and 2 canine teeth as negative controls.

Experimental animals (6)- All 4 canines were utilized per ferret for a total of 24 canine teeth. Of these 6 ferrets, 3 ferrets were randomly allocated to receive treatment configuration A, and 3 ferrets to receive treatment configuration B, as shown in the tables 1 and 2 below. The teeth in all groups were split randomly to allow for even distribution

between maxillary and mandibular teeth so that if there were differences in tooth dimensions or rate of growth between maxillary and mandibular teeth, they would not affect the analyses. Periapical lesions were induced in the experimental groups and the positive controls as described in previous studies (17). Tissue engineered constructs were prepared in syringes by encapsulating the previously isolated ferret dental pulp stem cells into a hydrogel scaffold.

**Table 1: Treatment configurations**

Treatment Configuration A (3 ferrets):

	Right Side	Left Side
Maxillary Canines	Traditional Revascularization (Group 1)	Tissue Engineered Construct (Group 2)
Mandibular Canines	Traditional Revascularization (Group 1)	Tissue Engineered Construct (Group 2)

Treatment Configuration B (3 ferrets):

	Right Side	Left Side
Maxillary Canines	Tissue Engineered Construct (Group 2)	Traditional Revascularization (Group 1)
Mandibular Canines	Tissue Engineered Construct (Group 2)	Traditional Revascularization (Group 1)

Therefore, each of these 6 ferrets contributed 2 canine teeth to each of the 2 experimental groups, and in total each group had 12 teeth. Power analysis showed that with an N of 12 in each experimental group, power for the independent variable was 81% at a significance level of 0.05 and effect size of .60. The effect size of .60 was determined from a previous study (35), which was a retrospective analysis of radiographic outcomes

of regenerative endodontics in immature teeth with pulp necrosis. Therefore, an N of 12 is sufficient when studying increase in the width of the root canal walls as an indicator of root development following pulp regeneration.

**Table 2: Distribution of teeth in each group**

Type of Treatment	
Traditional Revascularization	Tissue Engineered Construct
Group 1- 12 teeth	Group 2- 12 teeth

	Positive Controls	Negative Controls
Number of Control teeth	4 teeth	4 teeth

All procedures were conducted under aseptic protocol with the use of sterile materials and equipment. A veterinarian or veterinary technician was present for each procedure. The weight of each ferret was recorded before anaesthesia and repeated before each procedure. The initial weight of a 70 day old ferret is approximately 800 gms (40). After a fasting period of 4 hours, the animals were sedated with Ketamine HCl (Ketaject, Phoenix Pharmaceutical, Inc. St. Joseph, Mo) 30-40 mg/kg IM and Xylazine (AnaSed, Lloyd Laboratories Inc. Shenandoah, IA) 2-3 mg/kg IM. The animals were intubated with a size 2.5 to 3 endotracheal tube, and then maintained under 1 to 4% isoflurane gas (Phoenix Pharmaceuticals Inc, St. Joseph, MO). Animals were given IV fluids as needed (Lactate Ringers solution). The animals were maintained in surgical plane of anaesthesia throughout the procedure. An analgesic (Buprenorphine, 0.01-0.03 mg/kg IM every 12

hours or Carprofen 3 mg/kg SC every 24 hours) was given immediately prior to the procedure and continued until the following day to manage any post-operative pain.

## **Controls**

Positive Controls: Periapical lesions were induced but no treatment was rendered.

Positive controls served as reference sections for progression of the disease when untreated and for baseline measurement of root length and thickness. After administration of 2% lidocaine without epinephrine (Dentsply International, York, PA, USA), endodontic access was made by removing 2-3 mm from the incisal edge of each tooth to expose the pulp with a sterile taper fissure bur in a high-speed dental handpiece. After pulp exposure, the orifice was enlarged to facilitate access to the entire canal. Pulp extirpation was done with broaches (# 40) placed to a length of approximately 10mm as measured from a radiograph in a previous study (41). The canal was irrigated with saline and left exposed to the oral cavity for one week. After one week, under the same conditions of general anaesthesia, a cotton pellet was placed in the access and then sealed with Cavit to promote the growth of anaerobes. Lesion development was allowed to occur for 3 weeks. During this period, an analgesic (Buprenorphine 0.01-0.03 mg/kg IM BID or Carprofen 3mg/kg SC QID) was given to manage any pain or soreness.

Immediately before further procedures were performed, the animals received an oral dose of 41.6 nmol/g of body weight of pharmaceutical grade tetracycline hydrochloride to label any new calcified tissue formed after treatment. To give periodic labeling, the tetracycline administrations were repeated every 3 weeks throughout the observation period.

Negative Controls: No lesions were induced for negative controls. They defined the normal growth in terms of increase in root length, dentin thickness and apical closure. They also served as reference sections for the histological appearance of ferret tooth structures.

## **Experimental Groups**

Lesion induction was performed as described previously for positive controls.

Tetracycline doses were also given as described previously to label new mineralized tissue.

## **Phase 1: Disinfection**

Each of the 6 animals was again subjected to general anaesthesia and 2% lidocaine without epinephrine was administered at the treatment sites. The canine teeth were isolated with a rubber dam, #212 clamps and Oraseal (Ultradent Inc., South Jordan, UT, USA). The operative field was disinfected with 2.5% sodium hypochlorite and the Cavit was removed. The teeth were minimally instrumented with endodontic files to avoid damage to the thin root canal walls. Irrigation was done with sodium hypochlorite diluted to a 1% concentration. Max-i-probe needles (25 gauge) with side vents were used for irrigation. Approximately 3ml of irrigant was used per tooth. The canals were then dried and filled with Triple Antibiotic Paste (equal weights of ciprofloxacin, metronidazole and minocycline ground into powder form and mixed with sterile saline). The antibiotic paste

was delivered into all root canals with a Centrix syringe to the level of the cementoenamel junction. The access of all root canals was sealed with Cavit.

### **Phase 2: Pulp Revascularization (Group 1)**

One week later, under the same conditions of general anesthesia, the teeth were isolated and the field disinfected, as described before, then the temporary restoration was removed. Irrigation was performed with 5ml of 17% EDTA. Using EDTA can release bioactive growth factors that are sequestered into the dentin matrix (24) which may aid in the proliferation of regenerated pulp tissue. Conditioning the root canal dentin with EDTA also enhances stem cell attachment and differentiation into odontoblasts (25). The canals were then dried with paper points.

Teeth in group 1 were treated with a traditional revascularization procedure. Hemorrhage was induced by gentle agitation of files in the periapical area (1-2mm past the apex). Coagulation of the blood clot was controlled below the CEJ. In group 2, the same procedure was followed, except that the blood clot step was replaced with the tissue engineering construct, as described below. A layer of 2-3 mm of ProRoot MTA was then placed over the blood clot to fill the access cavity 3mm from the cavosurface margin and a layer of Fuji II glass ionomer material (GC America, Alsip, IL) was placed over it to give an effective and durable seal.

## **Pulp Regeneration (Group 2)**

### **Tissue Engineered Construct preparation and delivery**

Three canine teeth extracted from the donor ferret and used to prepare the tissue engineered construct. The teeth were sectioned and pulp and apical papilla tissues carefully separated out under magnification. The tissues were processed to isolate stem cells and expanded in culture by a biopharmaceutical company that is affiliated with the University of Maryland Stem Cell Consortium (Paragon Bioservices, Baltimore, MD, USA). Clinically provided ferret dental pulp tissues were minced, digested (Collagenase), strained and seeded into culture flasks with alpha-MEM supplemented with FBS and basic-FGF. Non-adherent cells were removed and media exchanged on the first day post seeding and cultures expanded (often as focal colonies). Over the course of 2-3 weeks, the adherent cells were passaged 3-5 times with trypsin, then harvested and cryogenically frozen at a concentration of  $1 \times 10^6$  cells/ml. Sufficient material was grown for injection, reagent qualification, characterization and testing. Cells were tested for post-thaw viability and mycoplasma contamination.

As a scaffold, the oxidized alginate-fibrin hydrogel microbeads developed by Dr. Xu's group were used to encapsulate the stem cells (42). These microbeads degrade inside the root canal to release the cells and initiate cell migration and tissue ingrowth. In brief, a 1.2% (by mass) sodium alginate solution was prepared by dissolving oxidized alginate (UP LVG, 64% guluronic acid, MW = 75,000~220,000 g/mol, ProNova, Oslo, Norway) in saline (155 mmol/L NaCl). Fibrinogen from bovine plasma (Sigma) was added at a

concentration of 0.1% to the alginate solution and incubated at 37 °C for 2 hrs to yield a mixed alginate-fibrinogen solution. Stem cells were added to the alginate-fibrinogen solution at a density of 1 million cells/mL of alginate. The alginate-cell solution was loaded into a syringe which was connected to a bead-generating device (Var J1, Nisco, Zurich, Switzerland). Nitrogen gas was fed to the gas inlet and a pressure of 8 psi was established to form a coaxial air flow to break up the alginate – fibrinogen droplets. The droplets were fed into a well of 100 mmol/L calcium chloride solution plus 125 NIH units of thrombin (Sigma) and cross-linked to form microbeads by calcium chloride, while the reaction between fibrinogen and thrombin produced fibrin. The cell-scaffold matrix was then transported to the animal facility under sterile conditions and immediately used in the experiment. The volume of the root canal of a ferret canine is approximately 32µl, as calculated from the radiographs in a previous study (41).

Slight apical hemorrhage was induced by gentle agitation of files in the periapical area and the cell-scaffold matrix was placed into the root canal space using a lentulospiral or a periodontal probe such that it mixed with the blood and filled the canal up just below the level of the cementoenamel junction. A layer of 2-3mm of ProRoot MTA was then placed to fill the access cavity 3mm from the cavosurface margin and covered with Fuji II as described previously.

An observation period of 3 months was used. The animals were weighed every 3 days. Each animal was observed for food consumption, appetite, general appearance, stool consistency, urination, respiration rate and character and mentation. Any clinical signs of morbidity were reported to the veterinary staff. At intervals of 3 weeks, the animals received oral doses of Tetracycline. After 3 months, the ferrets were deeply anaesthetized

with 5% isofluorane gas and intramuscular injections of 10 mg/kg of Ketamine HCl and 2 mg/kg Xylazine. The animals were weighed. The thoracic cavity was exposed, the thoracic aorta and vena cava were cross-clamped and the left ventricle of the heart was cannulated. After venting of the right auricular appendage the left ventricle was perfused with heparinized saline (Heparin Sodium AAP Pharmaceuticals, Schaumburg, IL) until the perfusate ran clear followed by 120-180 mL of 10% buffered formalin (J.T. Baker Inc. Phillipsburg, NJ). After perfusion was complete, block sections of the upper and lower jaws were dissected and placed in 10% buffered formalin for 2 weeks.

### **Radiographic evaluations**

The blocks were imaged using digital periapical radiographs. All radiographs were taken with a custom jig to ensure that the blocks were in the same orientation and to minimize distortion of the images. The software used to capture the images (MiPACS) was calibrated to ensure that the measurements were made accurately. The images were analyzed for canal wall thickness, root length, apical closure, and periapical lesion size.

Results of radiographic evaluations were analyzed by Dr. Prashant Verma as his research thesis project. The results of this part were briefly as below:

There was no significant difference in root length ( $p=0.75$ ), mid-root canal diameter ( $p=0.15$ ), apical canal diameter ( $p=0.56$ ), periapical lesion presence ( $p>0.05$ ) and number of teeth showing growth versus no growth ( $p>0.05$ ) between the two experimental groups. Six out of twenty two experimental teeth showed radiographic root maturation. There was a significant association between periapical lesion presence and lack of root maturation ( $p<0.05$ ) (for more details please refer to Dr. Verma's thesis manuscript).

Since the results of the radiographic analyses showed a great possibility for presence of residual infection in our specimens it was decided to include a histobacteriological analysis to the project. As it was mentioned under the “aims” of the study, the purpose was to evaluate the presence/absence of residual bacteria and the association between residual bacteria and outcome of the treatment.

### **Histological processing and evaluation**

Included are 30 block sections of the ferrets’ canines from the previous phase of the study. The block sections were decalcified in 7% Formic acid (Leica Biosystems, Buffalo Grove, IL) buffered to 7.4 at room temperature for 7 days. Total decalcification of the specimens was confirmed by randomly taken radiographs. The samples were trimmed with a blade to reduce the size of blocks and reduce the number of slices per sample. The samples were rinsed under running tap water for 5 hours followed by dehydration with ascending concentrations of alcohol. The specimens were embedded in tissue prep paraffin (Fischer Scientific, Fair Lawn, NJ) using standard paraffin processing on a Tissue-Tek VIP 5 (Sakura Finetek USA Inc., Torrance, CA). Serial sections of each sample were cut at a thickness of five micrometers. Six sections cut through the apical foramen (total of 180) showing the entire canal space were selected. 3 sections per specimen were stained with hematoxylin and eosin (HE) (total of 90), another 3 sections were stained with Brown and Bren (BB) (total of 90) to identify the residual bacteria in the root canal system.

All slides were evaluated blindly to determine and describe the type of tissue formed inside the root canal space, the type of hard tissue formed on the dentinal walls, and to determine the presence of residual bacteria in the root canal system.

### **Statistical analyses**

Four Positive and four negative controls were used to show the normal root development and the absence of root development.

### **Analyses related to hypothesis-1**

The histologic sections were examined to determine the presence or absence of an odontoblast layer, bony islands within the root canal, dentin associated mineralized tissue (DAMT) on the root canal walls, intra-canal inflammation, and periapical inflammation. The sections were scored as yes/no for presence of each of these characteristics, and the data were analyzed using a one-tail Fisher exact test at a  $p \leq 0.05$ . Fisher exact test was used because the assumptions for Chi-square were not met (an expected value of 5 in each cell). The area of DAMT and bony islands were measured in all slides (3 for each tooth) using image J software (version 1.41; National Institutes of Health, Bethesda, MD). The mean DAMT and bony islands were calculated for each tooth. The amount of DAMT and bony islands were compared between two experimental groups using one way analysis of variance (ANOVA).

### **Analyses related to hypothesis-2**

The presence of bacteria in the root canal system was determined in slides stained with B&B method. The association between presence of residual bacteria in the root canal

system and absence of radiographic growth was determined using one-tail Fisher exact test. The association between intra-canal and periapical inflammation with residual bacteria was determined using one-tail Fisher Exact test. The significance level was set at  $p \leq 0.05$ . The amount of DAMT formed in teeth with residual bacteria was compared with teeth with no residual bacteria using one way ANOVA.

Statistical analyses were performed using online tools (Vassarstat.com) for Fisher exact tests and SPSS (Version 21, IBM, Armonk, NY) for one way ANOVA.

## **Results**

### **Animal welfare**

During the 4-weeks period of lesion induction three animals developed swellings and stopped gaining weight. One of the experimental animals lost a mandibular canine during this period due to formation of an acute abscess. This canine was designed to be in the “tissue engineering” group. Therefore, the veterinarian gave clindamycin to all animals for one week. After the disinfection, none of the experimental animals developed swelling and they continuously gained weight. One of the control animals developed swelling twice on a maxillary canine which was controlled by giving clindamycin, each time for one week. The weighing data shows that this animal stopped gaining weight when the swelling appeared. However, the ferret never lost weight. Therefore, the animal was retained in the study.

One specimen in the “traditional revascularization” group was lost during the experiment due to breakage of coronal seal. Therefore the total sample size was reduced to 30 (4 negative controls, 4 positive controls, 11 traditional revascularization, and 11 tissue engineering).

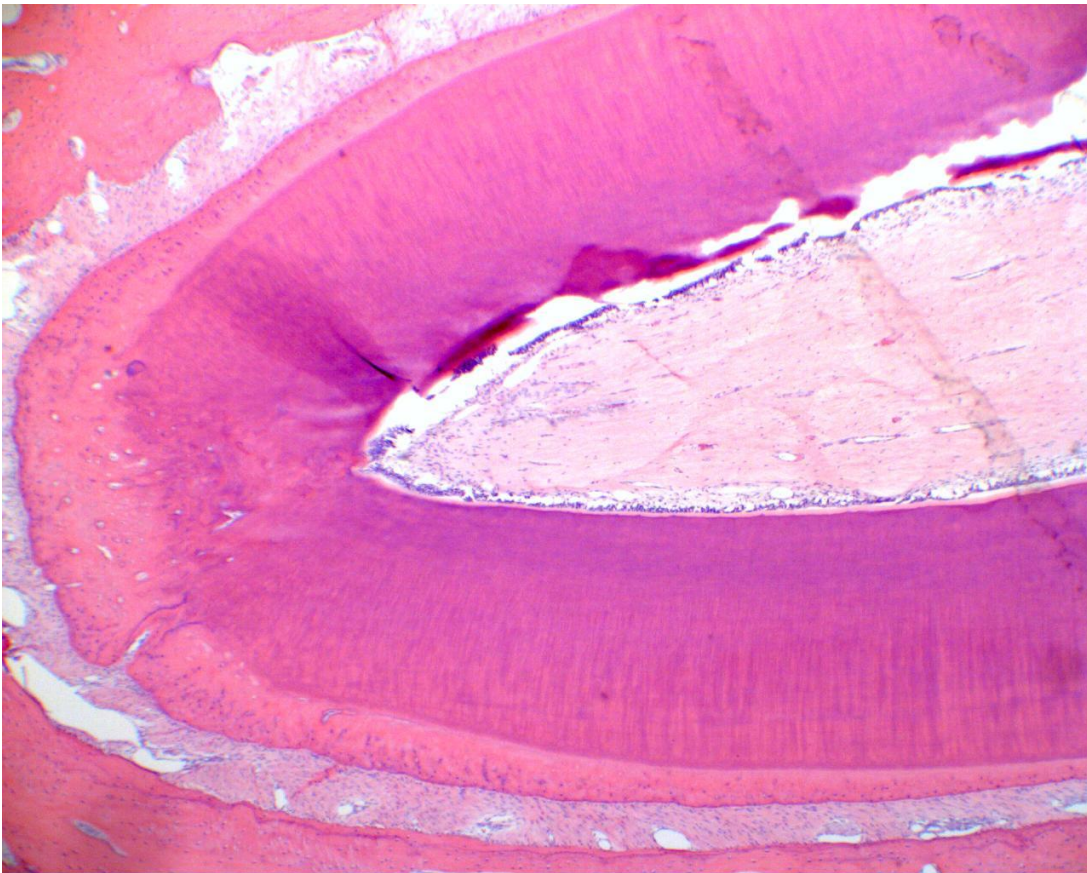
### **Results of histological and histo-bacteriological evaluations**

#### **Control teeth**

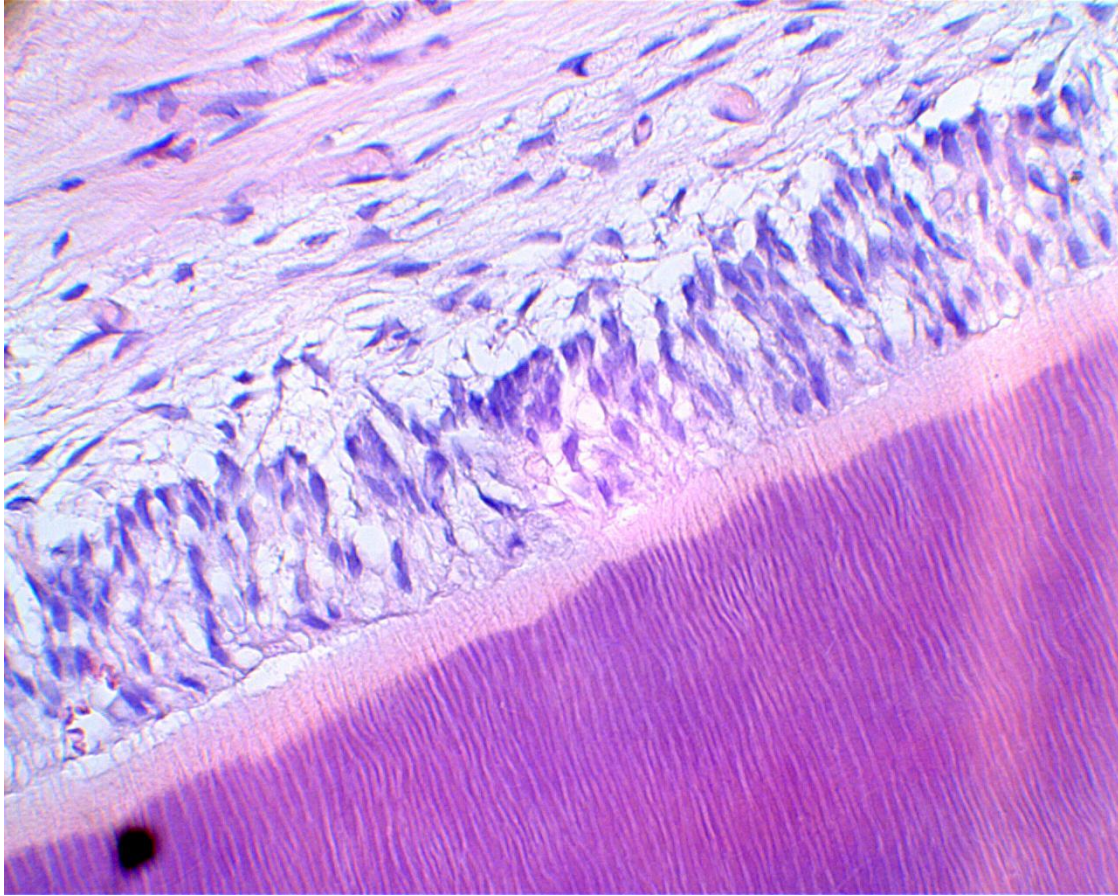
H&E staining of the control negative group (n=4) showed presence of odontoblast layer, normal pulp and normal periapical tissues, and absence of intra-canal/periapical inflammation. The cementum on the root surface was continuous, thicker and cellular on

the apex, thinner and acellular on the root surface (Figures 1 and 2). B&B staining of the control negative group showed no bacterial contamination.

**Figure 1: H&E image of a negative control tooth showing presence of odontoblast layer, normal pulp and normal periapical tissues, and absence of intra-canal/periapical inflammation, thick and cellular cementum layer on the apex, and a thin and acellular cementum on the root surface**

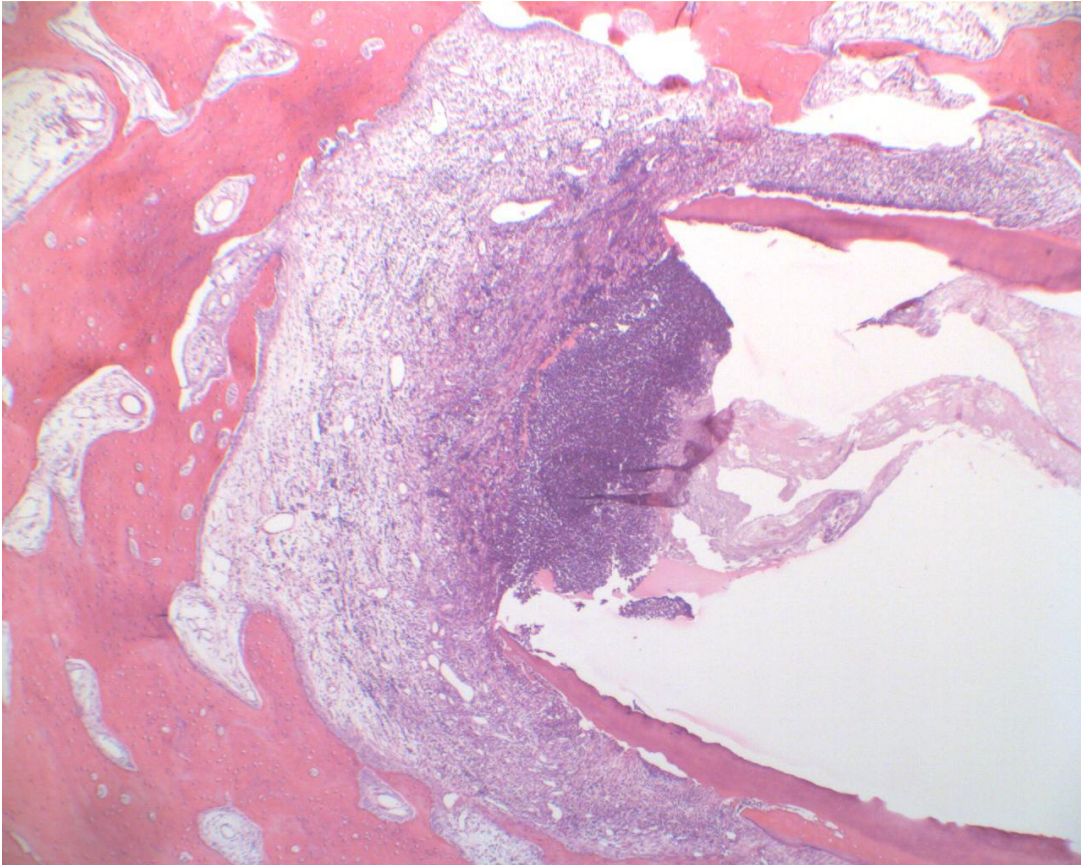


**Figure 2: High magnification ( $\times 400$ ) of the pulp-dentin complex (odontoblast layer) from the same specimen showed in Figure 1.**

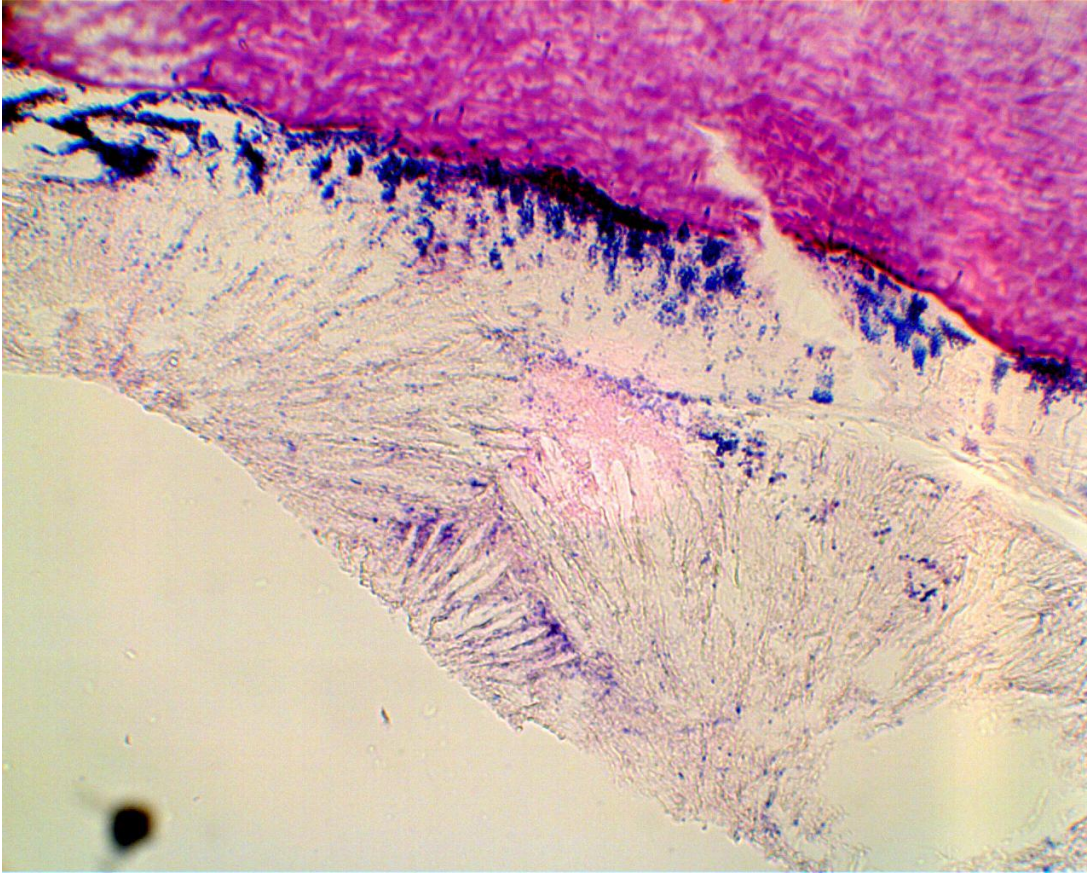


H&E staining of the control positive group (n=4) showed absence of tissue in the root canal space, immature apices, thin dentinal walls with resorptive lacunas, periapical and periradicular inflammation, and resorption of the cementum (Figure 3). B&B staining of the control positive group showed heavy intra-canal and intra-tubular bacterial infection (Figure 4).

**Figure 3: H&E staining of the control positive specimen. Please note presence of necrotic tissues inside the root canal space, presence of intense intracanal and periapical inflammation and several resorptive lacunae in the root canal space and on the external root surface.**



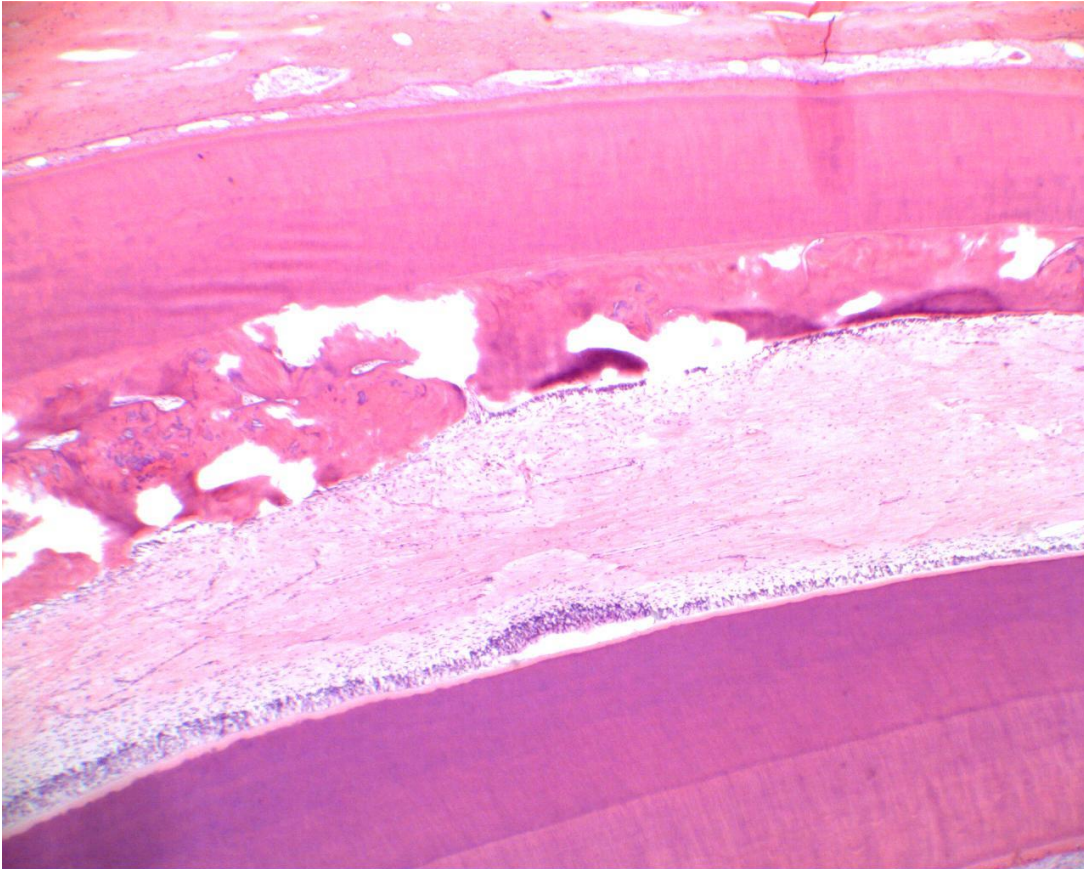
**Figure 4: B&B staining ( $\times 400$ ) of the same specimen (control positive) shown in figure 3 showing presence of heavy intra-canal and intra-tubular bacterial infection.**



### **Experimental teeth**

Experimental teeth showed a wide range of tissue formation from “no tissue” to partial presence of odontoblast layer and normal pulp in cases with radiographic growth. The specimens with radiographic growth (n=6), Three cases (all from revascularization group) showed areas of presence of original odontoblast layer (comparable to control negative group) within a normal pulp; or presence of a pulp-like tissue without odontoblast layer on one of the walls (Figure 5).

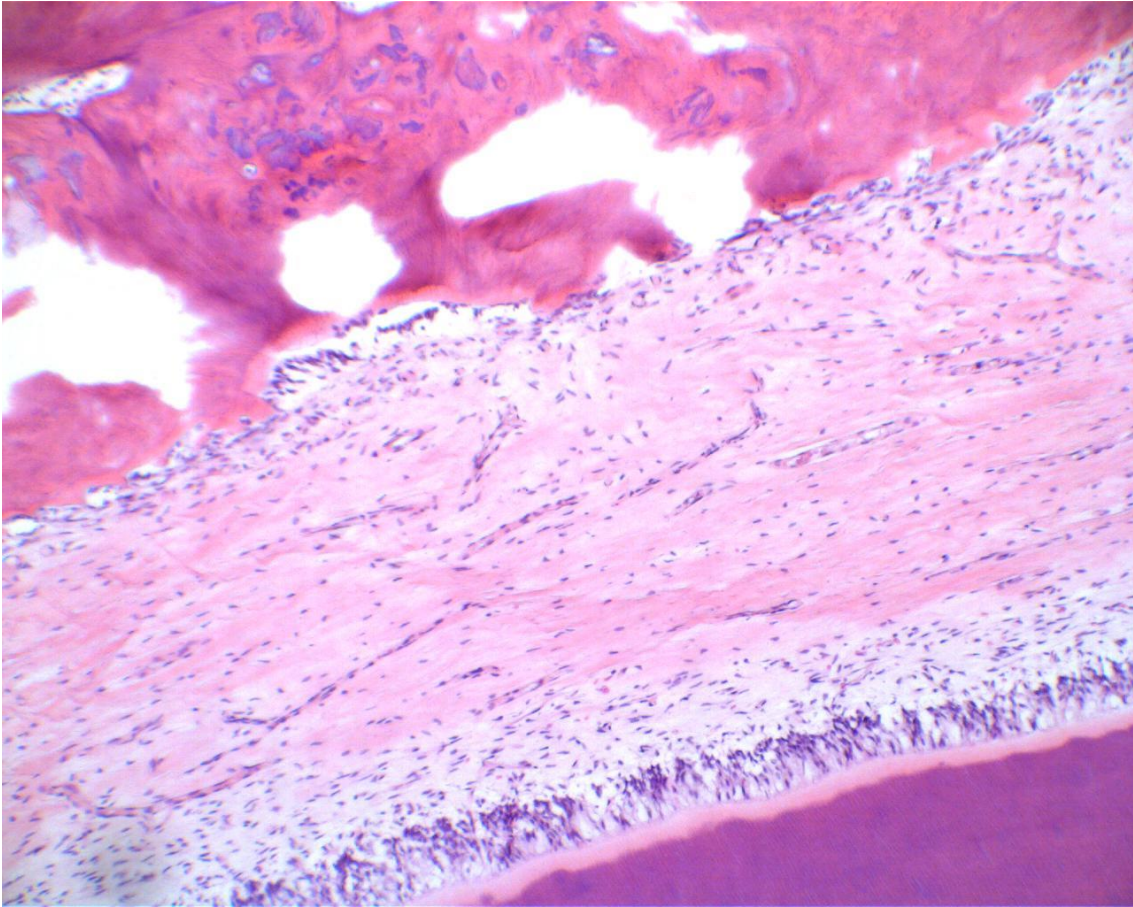
**Figure 5: H&E staining of a case with radiographic growth showing presence of original pulp tissue on one side and presence of newly formed hard tissue on the other side.**



The hard tissue produced on the dentinal walls was normal dentin in those areas showed presence of original odontoblasts; or irregular dentin/bone-like (osteodentin) structure in those areas showing absence of original odontoblasts (Figure 6).

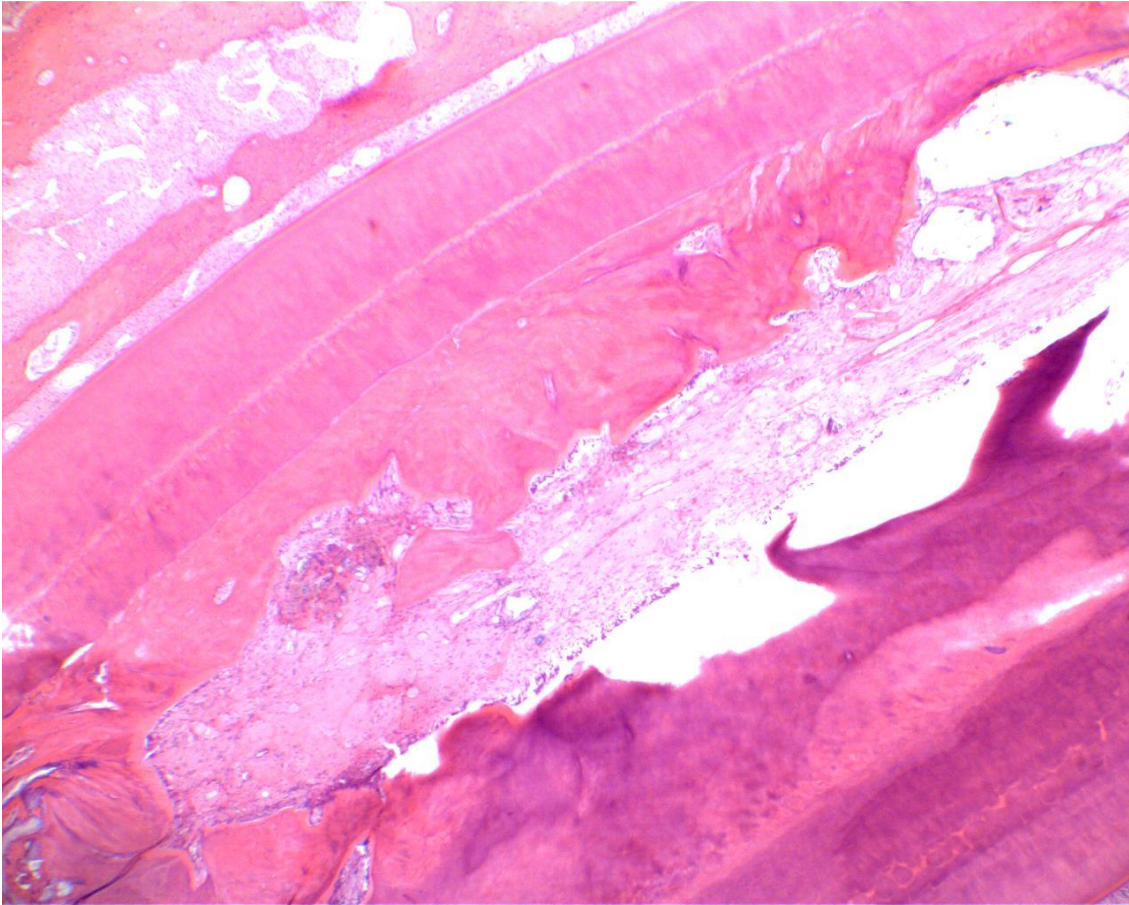
**Figure 6: Higher magnification ( $\times 100$ ) of the same specimen shown in figure 5.**

**Please note the presence of odontoblast layer on one side and presence of osteodentin on the other side of the dentinal walls.**

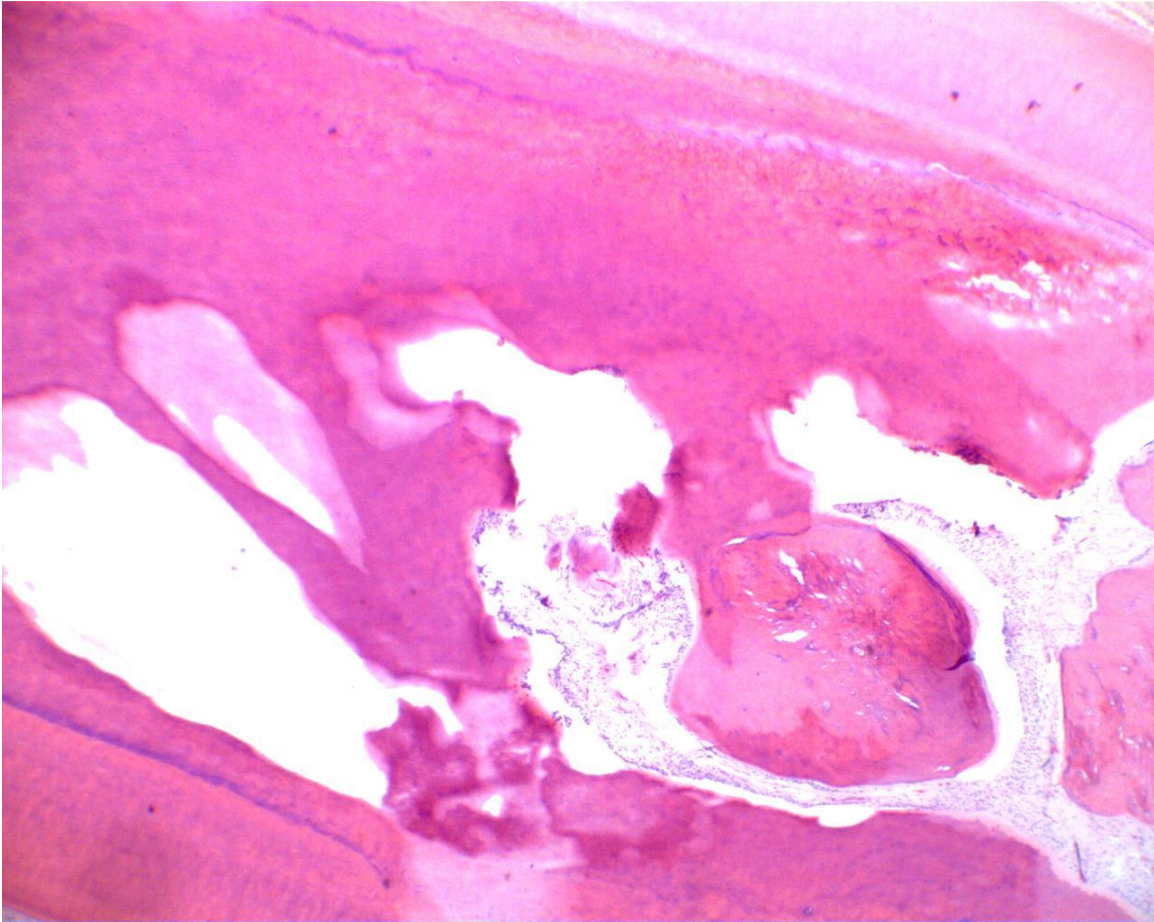


Three cases (Two from tissue engineering group and one from revascularization group) show presence of osteodentin admixed with a loose connective tissue (Figures 7 and 8).

**Figure 7: H&E staining of another case with radiographic growth. Two cases show that the root canal space is filled with osteodentin type of tissue admixed with loose connective tissue.**

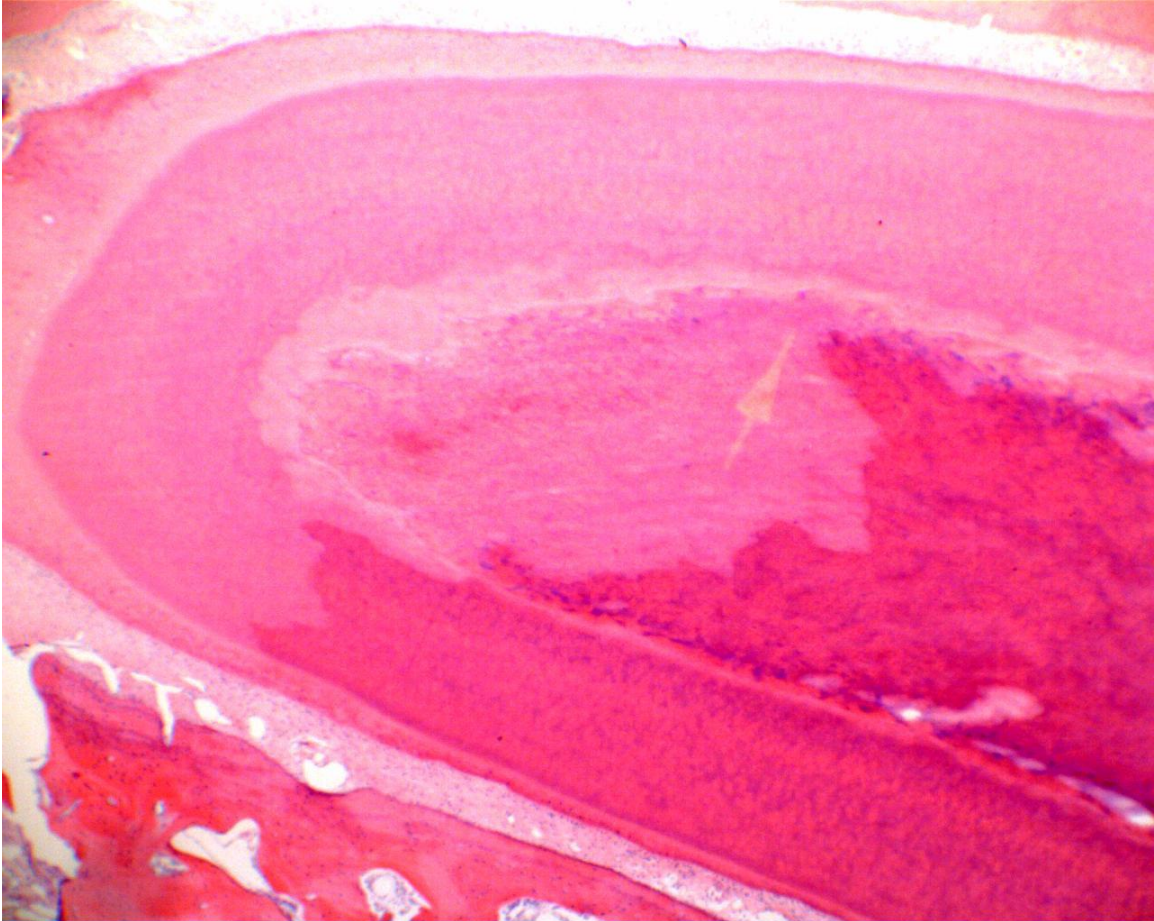


**Figure 8: H&E staining of a tooth with radiographic growth showing the same pattern as what was shown in figure 7. (i.e. formation of steodentin admixed with loose connective tissue).**



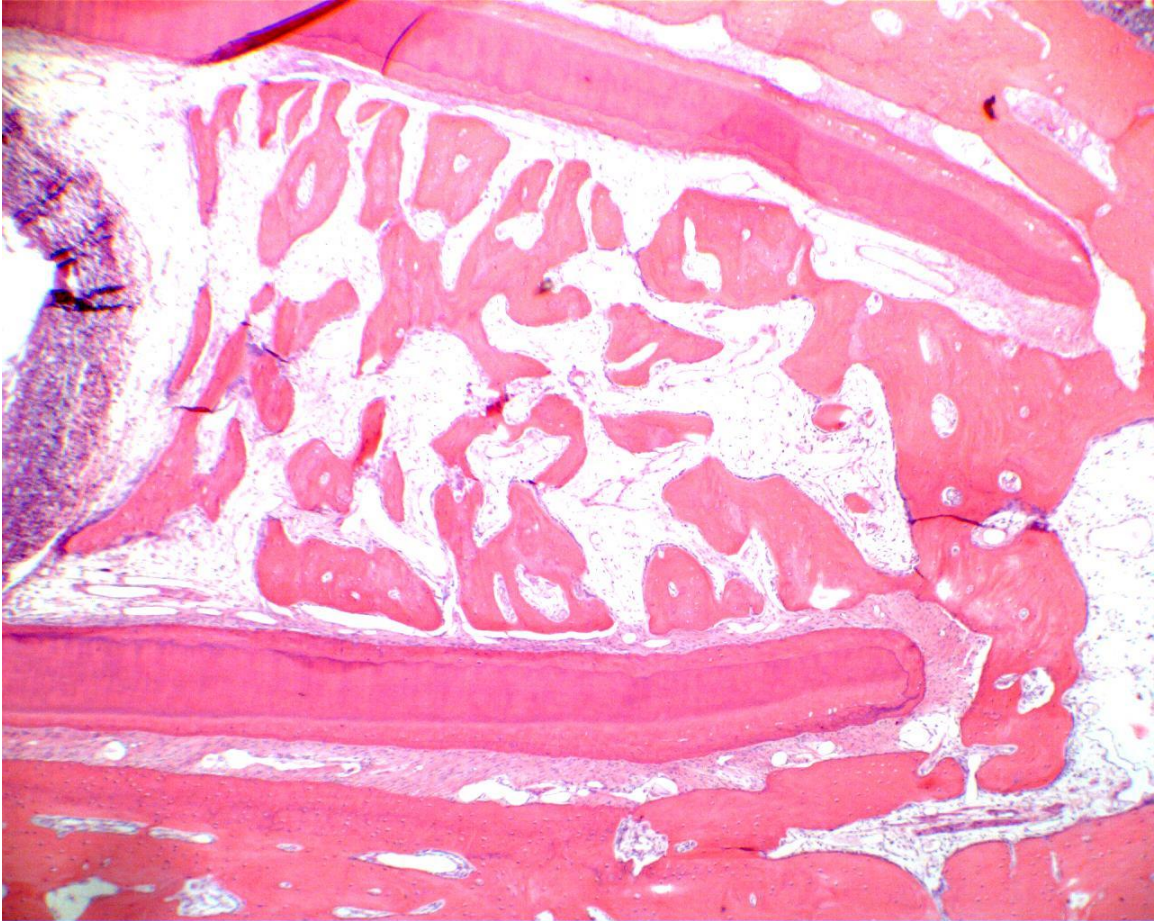
One case of them showed complete calcification at the apical third of the canal (Figure 9).

**Figure 9: Same specimen shown in figure 8. This case shows complete calcification of the root canal space in the apical third.**



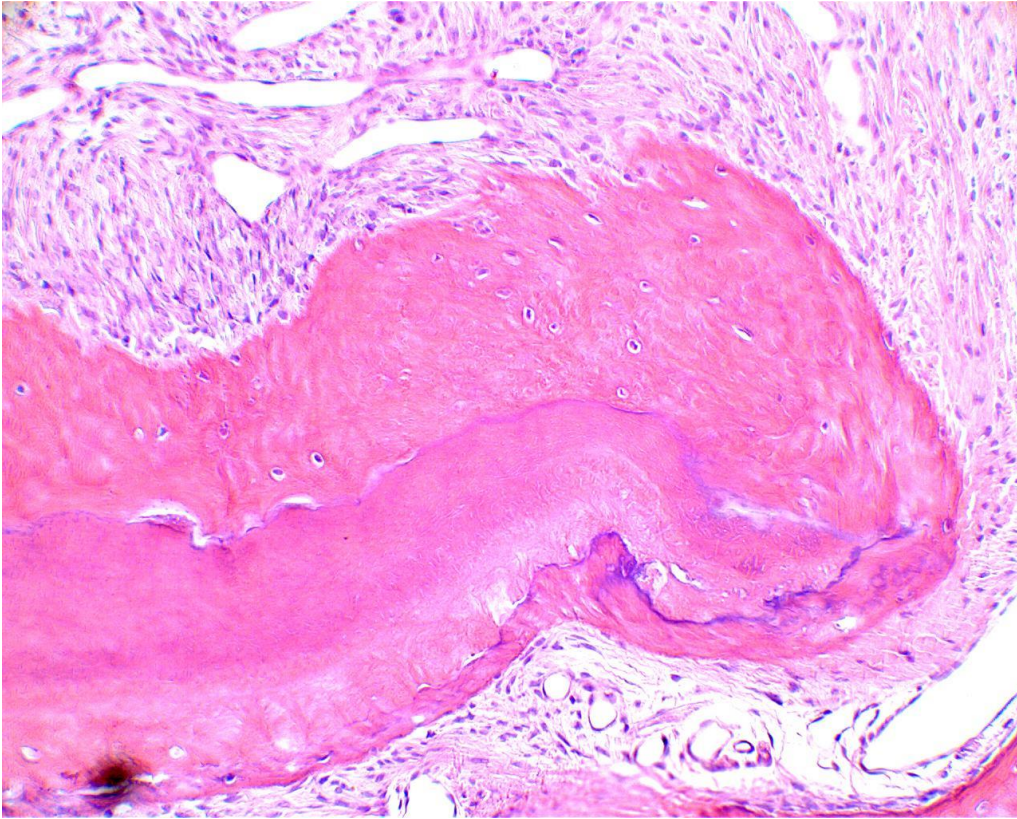
H&E staining of the specimens with no radiographic growth showed presence of a newly formed tissue in the apical 1-2 mm of the most of the specimens (some specimens showed no tissue formation). This intra-canal newly formed tissue is composed of bony islands with connective tissue formed in between, or only a loose connective tissue (Figure 10).

**Figure 10: A figure representative of formation of bony islands in the root canal space and DAMT on the dentinal walls. This case is from tissue engineering group.**



Also, presence of DAMT, which was continuation of the cementum from external root surface on the root canal walls, was evident in most of the specimens. In some specimens there are cellular components in the DAMT (similar to cellular cementum). DAMT doesn't show a tubular structure (Figure 11).

**Figure 11: High magnification illustration of DAMT which doesn't show a tubular structure. It also shows presence of cellular components similar to cementum. This case is from tissue engineering group.**



The rest of the root canal spaces were either empty or full of necrotic cells with inflammatory cells.

The images taken from control negative specimens (normal root development) under fluorescent microscope revealed absence of tetracycline marks. This can be due to long term storage of the specimens or the decalcification methods. Therefore, we lost our labeling for newly formed mineralized tissues. As an alternative we used calcio-traumatic lines formed between DAMT and dentin to determine the area of newly formed

mineralized tissues and to measure it. All the new tissues formed in experimental teeth with radiographic growth were assumed as DAMT.

**Results of analyses related to aim-1 (Traditional revascularization vs tissue engineering)**

Twenty seven percent of the specimens in the traditional revascularization group showed formation of bony islands versus 55% in the tissue engineering group (p=0.19, Table 3).

**Table 3: Distribution of bony islands in experimental groups**

Group	Bony islands	
	Yes	No
Traditional revascularization	3 (27%)	8 (73%)
Tissue engineering	6 (55%)	5 (45%)

Fisher Exact Test, p=0.19

In both groups, 91% of the specimens showed formation of DAMT (p=0.76, Table 4).

**Table 4: Distribution of DAMT in experimental groups**

Group	DAMT	
	Yes	No
Traditional revascularization	10 (91%)	1 (9%)
Tissue engineering	10 (91%)	1 (9%)

Fisher Exact Test, p=0.76

The comparison of the area of DAMT and bony islands between the traditional revascularization and tissue engineering groups showed no statistical difference (p>0.05, Tables 5 and 6).

**Table 5: Comparison of the area of DAMT between experimental groups**

Groups	n	Mean ± SD	F	p value
Traditional revascularization	11	4.199 ± 5.331	.061	.81
Tissue engineering	11	5.136 ± 11.358		

**Table 6: Comparison of the area of bony islands between experimental groups**

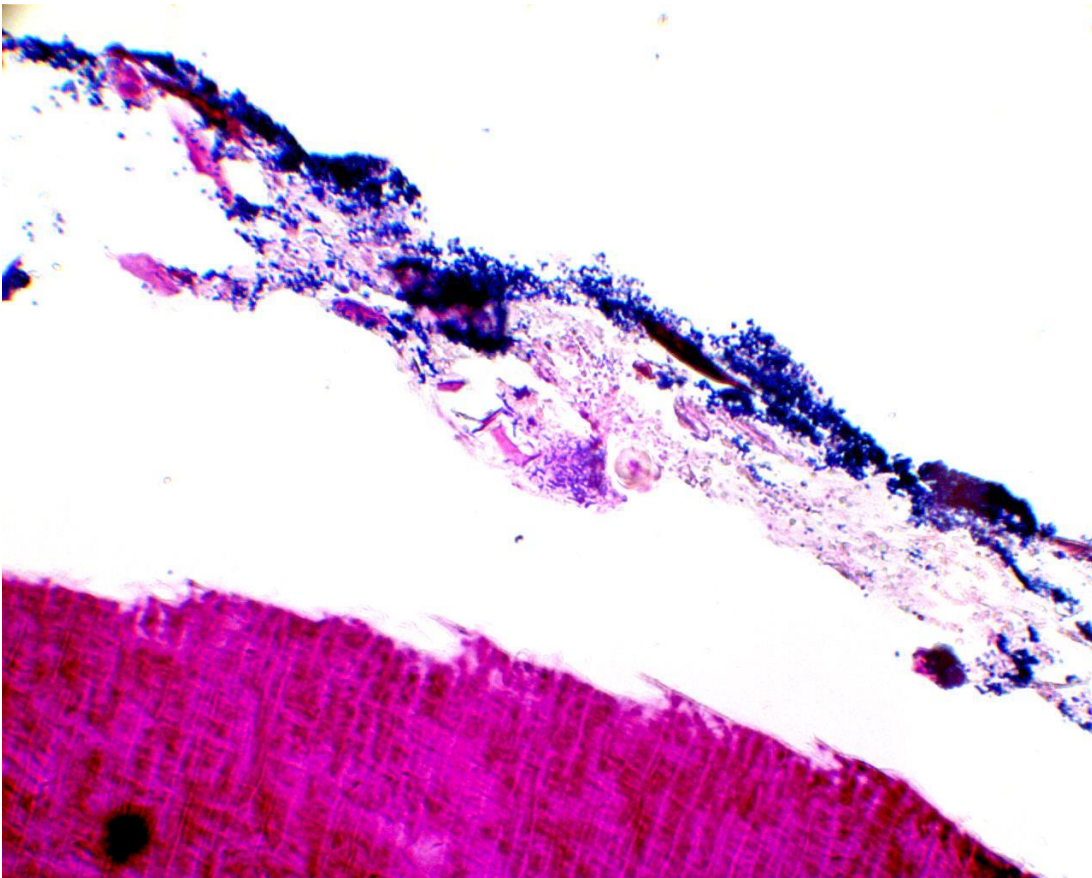
Groups	n	Mean ± SD	F	p value
Traditional revascularization	11	0.098 ± 0.302	3.242	.087
Tissue engineering	11	0.626 ± 0.922		

**Results of analyses related to aim-2 (Association between bacteria and outcome)**

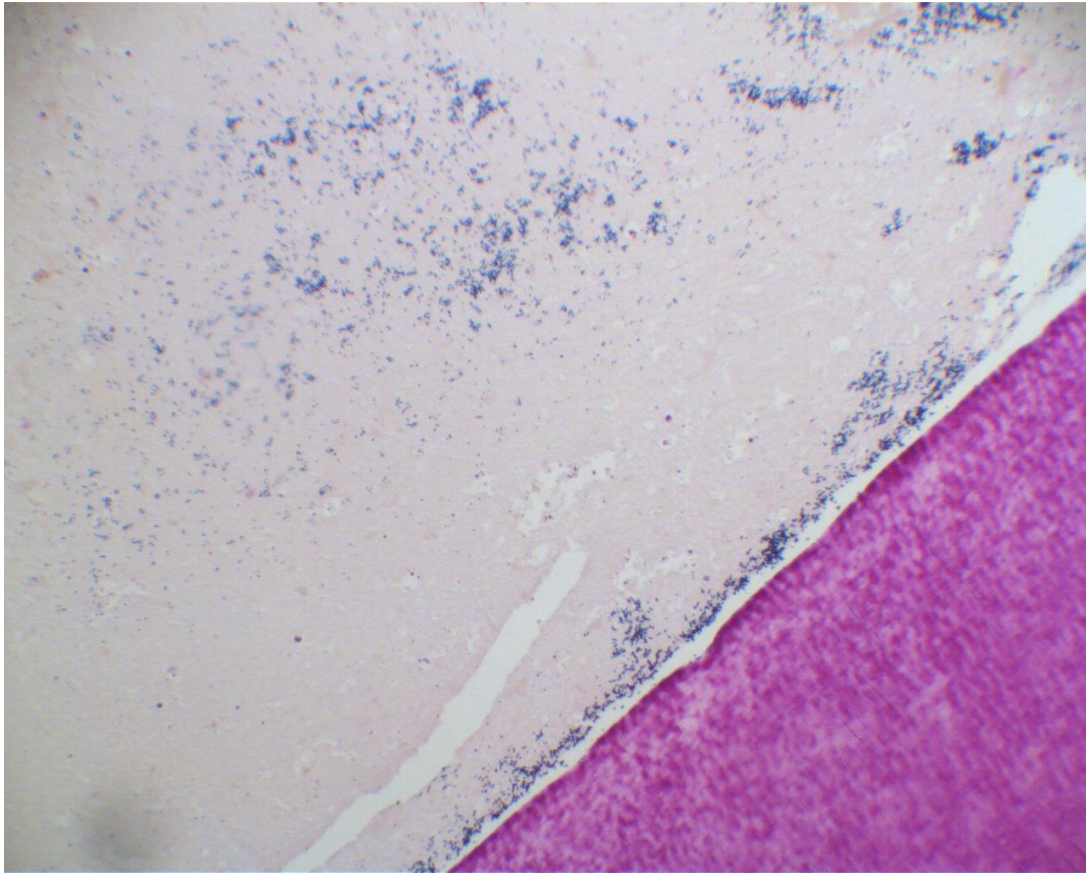
Sixty eight percent of the experimental teeth (15 of 22) showed presence of residual bacteria in Brown and Bren (B&B) slides. All cases with no radiographic growth showed presence of residual bacteria except one. Bacteria were found in the form of thick biofilms (Figure 12) or planktonic colonies in the coronal third of the root canals where the root canal space was empty or occupied with necrotic tissues (Figure 13). Three cases showed presence of sparse bacteria which were detectable under ×1000 magnification (Figure 14).

There was no tissue regeneration adjacent to the bacteria. The root canal space was empty (Figure 12) or filled with necrotic tissues (Figures 13 and 14) where the bacteria were located.

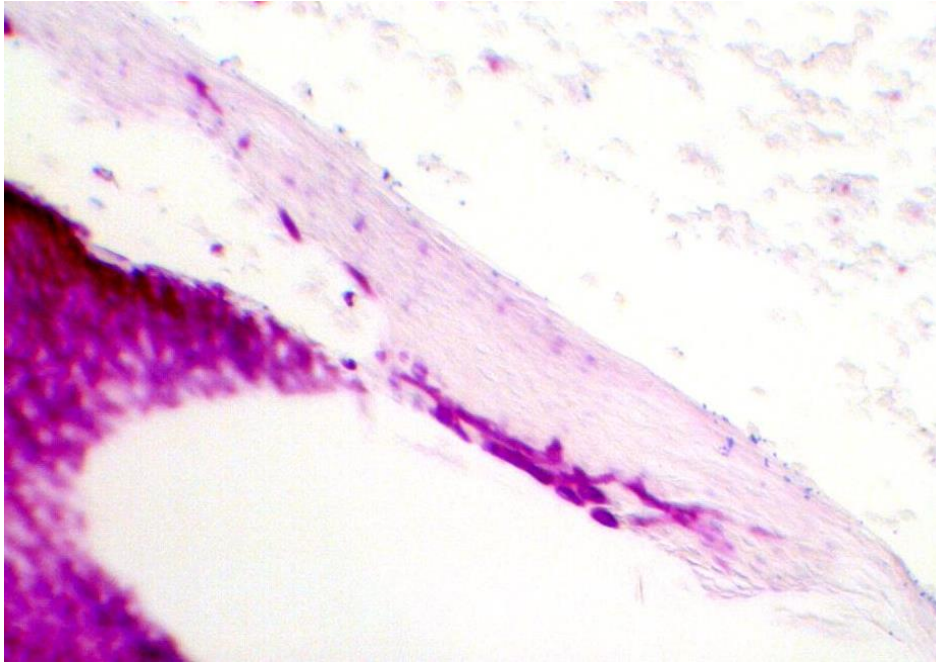
**Figure 12: A bacterial biofilm in the coronal third of an experimental tooth from tissue engineering group. (B&B staining; ×400)**



**Figure 13: Presence of planktonic colonies in the coronal third of the root canal of one of the experimental teeth from revascularization group (B&B staining; ×400).**



**Figure 14: Three cases showed presence of sparse residual bacteria (B&B staining, ×1000).**



#### **Association between bacteria and lack of growth**

Out of 16 teeth with no radiographic growth, 15 (94%) showed presence of residual bacteria. Analysis of this data showed a significant association between residual bacteria and radiographic growth ( $p=0.00009$ , Table 7). When residual bacteria are present, there is no radiographic growth, and the reverse.

**Table 7: Distribution of residual bacteria based on presence/absence of radiographic growth**

Growth	Bacteria	
	Yes	No
Radiographic growth	0 ( 0%)	6 (100%)
No Radiographic growth	15 (94%)	1 ( 6%)

Fisher Exact Test,  $p=0.00009$

### Association between bacteria and inflammation

Since inflammation can be an indicator of presence of bacteria, we looked at the presence of intra-canal and periapical inflammation in teeth with or without residual bacteria. Intra-canal inflammation was found in 15 of the 22 experimental teeth (68%). There was a significant association between residual bacteria and intra-canal inflammation ( $p=0.01$ , Table 8). When bacteria is present, intra-canal inflammation is present and the reverse.

**Table 8: Distribution of residual bacteria based on presence/absence of intra-canal inflammation in experimental teeth only**

Inflammation	Bacteria	
	Yes	No
Intra-canal inflammation	13 (87%)	2 (13%)
No intra-canal inflammation	2 (28%)	5 (72%)

Fisher Exact Test,  $p=0.01$

Also, analysis of the data showed a significant association between intra-canal inflammation and radiographic growth ( $p=0.004$ , Table 9). When intra-canal inflammation is present there is no radiographic growth and the reverse.

**Table 9: Distribution of intra-canal inflammation based on presence/absence of radiographic growth**

Growth	Intra-canal inflammation	
	Yes	No
Radiographic growth	1 (17%)	5 (83%)
No radiographic growth	14 (87%)	2 (13%)

Fisher Exact Test,  $p=0.004$

Evaluation of the periapical tissues showed presence of periapical inflammation in 50% (11 of 22) of the experimental teeth. There was a significant association between residual

bacteria and periapical inflammation (p=0.03, Table 10). When residual bacteria is present, periapical inflammation is present and the reverse.

**Table 10: Distribution of residual bacteria based on presence/absence of periapical inflammation**

Inflammation	Bacteria	
	Yes	No
PA inflammation	10 (91%)	1 ( 9%)
No PA inflammation	5 (45%)	6 (55%)

Fisher Exact Test, p=0.03

None of the cases with radiographic growth showed presence of periapical inflammation. Analysis of the data showed a significant association between periapical inflammation and radiographic growth (p=0.006, Table 11). When periapical inflammation is present there is no radiographic growth and the reverse.

**Table 11: Distribution of periapical inflammation based on presence/absence of radiographic growth**

Growth	PA inflammation	
	Yes	No
Radiographic growth	0 ( 0%)	6 (100%)
No radiographic growth	11 (69%)	5 ( 31%)

Fisher Exact Test; p=0.006

There was significantly higher amounts of DAMT formed in teeth with no residual bacteria compared to teeth with bacteria (p<0.0001, Table 12).

**Table 12: Results of one way ANOVA comparing the area of DAMT between teeth with residual bacteria and teeth with no residual bacteria**

Teeth	n	Mean $\pm$ SD	F	p value
No residual bacteria	7	13.827 $\pm$ 10.923	24	<.0001
With residual bacteria	15	0.393 $\pm$ 0.365		

## **Discussion**

In the present study, we evaluated the effects of residual bacteria on the histological and radiographic outcome of pulp regeneration. The outcomes showed poor radiographic and histological results for teeth with residual bacteria. All cases with radiographic growth showed presence of no residual bacteria. The amount of mineralized tissue produced in cases with no residual bacteria was significantly higher than cases with residual bacteria. It has been assumed that successful pulp regeneration needs a higher level of disinfection compared to successful root canal treatment (15). That means that even in the presence of some residual bacteria, root canal treatment can result in the healing of periapical disease. However, the same finding doesn't apply to pulp regeneration treatments. Our study provides some proof for this concept. Adequate root canal disinfection for pulp regeneration is a challenge (43, 44).

The quality of disinfection using full strength triple antibiotic paste has been examined in several studies. Both Hoshino et al (45) and Sato et al (46) conducted in vitro studies in "mature" human teeth extracted due to endodontic infections. They used culture methods to examine the effect of triple antibiotic paste and its ingredients on endodontic bacteria. The results of these studies showed that triple antibiotic paste was not only capable of killing endodontic bacteria inside the root canal space, but also was able to penetrate into the dentinal tubules and sterilize the dentine. On the other hand, in the younger teeth bacteria penetrate through more dentinal tubules and advance deeper in comparison with older ones (47). Windley et al (48) evaluated the effect of NaOCl irrigation followed by triple antibiotic dressing for two weeks on previously infected immature dog teeth. They used a culture technique followed by spiking of the "negative" samples to eliminate the

biases related to unintentional delivery of the antibiotic remnants to the culture media. Their study showed that this protocol of disinfection rendered 70% of the root canals bacteria free. On the other hand, a study by Weiger et al (49) showed that the root canal system may contain viable, but non-culturable, bacteria following use of antibacterial dressing. Therefore, the culturing method used in the aforementioned studies might not be a reliable method to evaluate the effectiveness of triple antibiotic paste. The results of the present study showed that 68% of the experimental teeth still had residual bacteria following one week of triple antibiotic dressing. The period of one week for antibiotic dressing was chosen based on previous clinical studies (50). None of the cases with radiographic growth in this study showed presence of residual bacteria. This is a critical finding which showed that the presence of residual bacteria is a potential threat for the success of regenerative procedures. Also, it showed that bacterial biofilms formed on the dentinal walls might be resistant to antibiotics and become non-culturable after dressing.

Our data showed the association between the presence of residual bacteria and intra-canal and periapical inflammation. We also found an association between both periapical and intra-canal inflammation and the lack of radiographic growth. Periapical inflammation and intra-canal inflammation were reported to be present in previous animal studies on infected immature teeth (8, 27, 32-34, 51). In a similar study on ferrets, Torabinejad et al (51) showed presence of periapical inflammation in 75% of the teeth following treatment. Some authors assumed that this inflammation might be due to the residual antibiotic paste (27) or might be an attempt for healing in the newly formed tissue (8). Our data showed that the inflammation might be due to residual bacteria. None of the previous studies had

conducted a histo-bacterial analysis to explore this critical factor. Inflammation could be an indirect indicator of the presence of residual bacteria.

Torabinejad et al (7) conducted a study on pulp regeneration in uninfected immature ferret canine teeth. They used two different scaffolds: a blood clot and PRP. PRP served as a source of concentrated growth factors as well. The outcome of the study showed formation of bone inside the root canals in all teeth regardless of type of scaffold used. Also, minimal root thickening was observed due to formation of a cementum-like hard tissue on the dentinal walls. The same group ran another study using the same scaffolds on infected immature canine teeth of the ferret (51). They induced periapical lesions before the experiments began. The outcome was the same as their previous study in terms of type of tissue formed in the root canal space (bone and cementum) and none of the teeth showed signs of root development. None of the teeth showed regeneration of the pulp-dentin complex. These findings are consistent with our findings. On the other hand, a few case reports have indicated that root development following regenerative endodontic treatments is due to the deposition of cementum on dentinal walls in human teeth (10-12). Therefore, although we didn't achieve root development in all teeth in our study, the deposition of DAMT in our samples was very similar to previous findings in human teeth.

We used dental pulp stem cells (DPSCs) mixed with a fast-hydrolyzing hydrogel scaffold as a source of stem cells for pulp regeneration. DPSCs have the capability of self-renewal and the capacity of multi-lineage differentiation. They are also capable of differentiating to adipocytes and neural-like cells (52). DPSCs are the precursors of odontoblast-like cells (53). As shown previously, DPSCs are capable of regenerating the pulp-dentin

complex in vivo in a controlled sterile environment (54). Huang et al (21) examined the combination of DPSCs and SCAP mixed with a scaffold inserted into human root slices. Their results showed formation of a pulp-like tissue with a well-established vascularity, a continuous layer of odontoblast-like cells on the dentinal walls, and production of dentin-like tissue along the dentinal walls (21). Nakashima and Iohara (55) showed that mobilized DPSCs of dogs are capable of regenerating pulp tissue in uninfected pulpotomized teeth (55, 56). Iohara et al (57) isolated CD105+ cells (a must-to-be-present marker for mesenchymal stem cells) from dog's pulp tissue (58). Then they regenerated the entire pulp tissue in mature dog teeth using CD105+ cells and stromal cell derived factor-1 (SDF-1). Therefore, DPSCs are considered a potent stem cell source for regeneration of the pulp-dentin complex. In addition studies on dog model have shown that DPSCs obtained from young animals are more capable of regenerating the pulp tissue compared to aged animals (59). In the present study we obtained the DPSCs from a young (80 days old) ferret.

On the other hand, in a side project in our lab, we ran an extensive evaluation on differentiated ferret DPSCs using qRT-PCR methods (unpublished data). We compared the expression of dentin related and bone related genes in differentiated ferret DPSCs with that of human dental pulp cells. We found that ferret DPSCs express bone related genes (osteopontin and alkaline phosphatase) significantly more than human dental pulp cells. These findings showed the differences between species and the possibility of different responses in the same condition. Therefore, the difference between the species can be a critical factor in interpreting the results of animal studies. Based on the data from our unpublished project, production of bone and bone-like tissues in ferret teeth can

be justified and might be species specific. Although the difference in the amount of bony islands formed in the canals of teeth in traditional revascularization group and teeth in the tissue engineering group was not statistically significant (due to low sample size), the p value of 0.08 shows a tendency for production of more bone in the tissue engineering group. The amount of bony islands formed in the tissue engineering group was six times higher (0.626) than the traditional revascularization group (0.098). This finding might be clinically important.

In the present investigation, 1.25% NaOCl was used as an irrigant during instrumentation and 17% EDTA was utilized as a final irrigant before inducing bleeding. Previous studies have shown that NaOCl had detrimental effects on the survival and differentiation of stem cells from apical papilla (SCAP) (60), however, this negative effect might be prevented by using a low concentration of NaOCl (1.5% or less) followed by 17% EDTA irrigation (60). The lower concentration of NaOCl was chosen in the present study because full strength hypochlorite might interfere with stem cell survival (61), might prevent attachment of stem cells to root canal dentin (62) and might denature the proteins of the dentin matrix. These three factors are involved in promoting tissue regeneration (63). Furthermore, 1.25% sodium hypochlorite has been shown to be sufficiently effective in significantly reducing bacterial presence in infected root canals (64). EDTA releases bioactive growth factors that are sequestered in the dentin matrix (24, 65). The use of EDTA as a final irrigant during regenerative endodontic procedures might promote stem cell survival (61). Conditioning the root canal dentin with EDTA also enhances stem cell attachment and differentiation into odontoblasts (25).

The importance of a bacteria-tight coronal seal for successful revascularization is well documented (66). A majority of reported clinical cases have used a double-seal over the blood clot formed inside the canal made up of MTA and a resin bonded restoration (66-68). Like many previous studies (7, 8, 31, 51), we used MTA as the coronal barrier on the blood clot. Also we added a layer of self-setting glass ionomer over the MTA to protect the MTA from chewing forces. MTA is a bioactive material which produces hydroxyapatite crystals when it is in contact with body fluids (69). MTA is biocompatible, promotes cell differentiation, and induces hard tissue production without adverse tissue reactions (70). Sealing ability of MTA makes it a suitable biomaterial for sealing of the blood clot and preventing bacterial leakage overtime (70). In our study, all teeth with radiographic growth showed formation of a hard tissue barrier underneath MTA with no inflammation.

### **Study limitations**

Although this study is the first one to evaluate the effect of residual bacteria on the outcome of regenerative endodontic procedures, it has limitations. The low sample size (n=11) did not provide us sufficient power to show significant differences between groups in terms of formation of mineralized tissues. The amount of bone formation in tissue engineering group was more than 6 times higher than revascularization group.

Although this difference was not statistically significant, it can be clinically significant.

The ferret model itself is not an easy model with which to work. The opening of the access cavity on canine teeth is much smaller than the diameter of the root canal space. This specifically causes problems in removing the pulp tissue from the concave area underneath the cingulum. Two cases with radiographic growth showed remnants of the

pulp tissue. The pulp tissue was remained intact on the cingulum side. A recently published animal study (71) showed that mechanical removal of the pulp tissue and induction of infection in a short period of time might not mimic the true clinical picture of an endodontic infection. This study showed that residual pulp tissue can remain in the canals after regenerative procedures and result in misinterpretation of the outcomes of pulp regeneration experiments (71).

Also, due to the above limitation, access of the irrigants and dressing to the concave area beneath the cingulum might be limited. Thick bacterial biofilms were frequently found in this area.

Another limitation of this study was the lack of commercial antibodies for ferret dentin-specific proteins to run immunohistochemical analyses to determine the type of new tissues that were formed. Therefore, the nature of the dentin-associated mineralized tissue has yet to be determined.

## **Future studies**

The results of our study underscore the importance of an “ideal” disinfection in regenerative endodontic treatment. Our findings show that the current disinfection protocols may not produce an ideal environment for pulp regeneration in the root canal space of previously infected teeth. Future studies should focus on finding more biologically acceptable disinfection protocols which not only eliminate bacteria, but also make the root canal space an inductive environment for pulp regeneration. In addition, basic science studies are needed to obtain a better understanding of the mechanisms of differentiation of multipotent stem cells. This will help clinicians design clinically applicable protocols for true pulp regeneration.

## Conclusions

- Residual bacteria is a main contributor to the failure of pulp regeneration attempts.
- The present protocols for disinfection of the root canal space of infected immature teeth might not be adequate to prepare an environment conducive to pulp regeneration.
- The protocol for pulp regeneration in this study resulted in formation of bone in the root canal space and deposition of a cementum-like tissue or steodentine-like tissue on the dentinal walls (collectively known as “dentin associated mineralized tissue” in this manuscript).
- Addition of dental pulp stem cells encapsulated within a hydrogel scaffold did not enhance the outcome of pulp regeneration in ferrets.

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