Pulp Regeneration in Different Prospective

A Thesis

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by

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Dedication

I would like to dedicate my Master Thesis to my beloved family, to the best father in the world Mr. Abdulaziz Khayat, to the best women I ever met my mother Mrs. Awatif Sounbl and to my supportive sisters Sarah, Eman and Roa'a Khayat

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Table of Content

Chapter 1 Literature Review

Recent Advances in Pulpal Regeneration, Literature Review

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Abstract

Apexification, or root end closure, is an option that has been used for many years to develop calcification at the apex in the hope for future root development. However, pulp regeneration therapies are now considered to be a promising alternative to apical closure, as they allow for continued root development and re-establishment of dentin-pulp like tissue. Several clinical studies on pulp regeneration have been published with contradictory outcomes, using a variety of techniques and methods based on clinical challenges. However, some of the histological studies from pulpal regeneration revealed the formation of cementum-bone like tissue, which might interfere with future root canal treatments, if needed. Similarly, in vitro and in vivo studies were able to examine numerous biocompatible, degradable scaffolds combined with stem cells obtained from a variety of sources, to participate in future dentin-pulp complex formation. The ability to devise methods to reliably engineer vascularized dental pulp and dentin tissue formation would encourage dentists to participate in applying this biotechnology as an alternative option for root canal treatment.

This literature review is aimed to increase dentists' awareness of the future incorporation of stem cells into clinical dental therapies, in order to promote the healing and regeneration of damaged tissues, and in turn providing significant benefit to patients.

Keywords: Pulp regeneration, revascularization, dentin-pulp complex, HUVECs, hDPSCs, SCAP and scaffolds

Introduction

Emergence of the field of dental tissue engineering has changed the approach of conventional root canal treatment for both immature and mature permanent teeth. Various clinical studies have showed the ability to revascularization-regenerate tissues in the pulpal space of open apex teeth as well as closed apex teeth[1–4].

Apexification, one of the options used to treat necrotic immature permanent teeth, is defined as "a method of inducing a calcified barrier in a root with an open apex or continued apical development of an incompletely formed root on teeth with necrotic pulp"[5]. What makes apexification a challenging procedure is the negative outcomes associate with treating teeth as thin dentinal walls that are prone to fracture, and difficulties associated with sufficient removal of bacteria from the dentinal walls, and reduced quality of the apical seal[6]. However, apexification does not allow for tooth root development to continue, despite the use of Calcium Hydroxide $Ca(OH)_2$ or Mineral Trioxide Aggregate (MTA) in the treatment[6,7] (Figure 1).

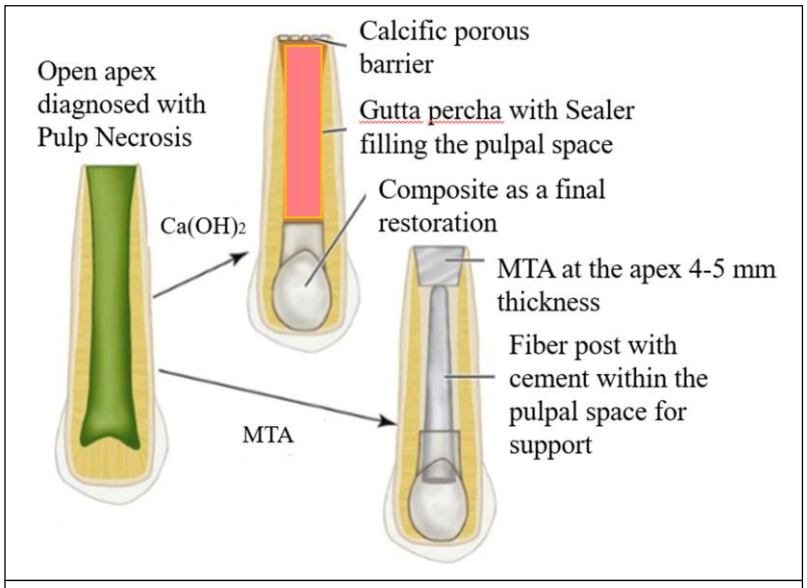

Figure 1: Apexification to Induce Apical Closure without continue of Root Development -Acquired from Hargreaves et al 2010-

Regeneration or revascularization approaches aimed to overcome these disadvantages, by allowing for continued root development, maintenance of apical closure, thickening of the dentinal walls, and regeneration of pulpal tissues within the pulpal space, will in turn provide optimal physiological function[8]. Despite these potential advantages, several challenges were noticed in treated patients, including the presence of an open apex, calcification within root canal space that could interfere with future root canal treatment, and insignificant root development as compared to the adjacent[9,10] (Figure 2).

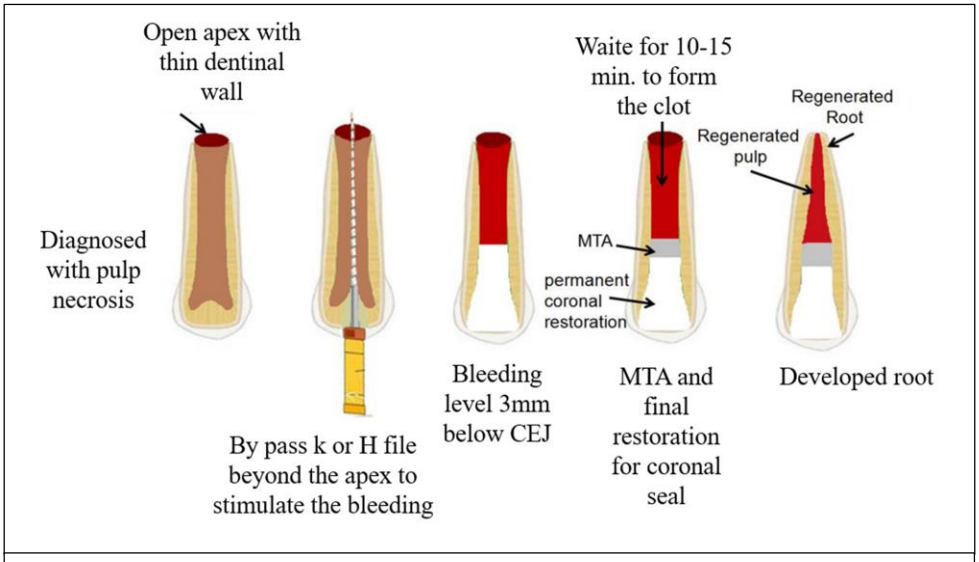

Figure 2: REP Aimed to Induce Root Development of Immature Open Tooth -Bansal et al 2014-

Incorporating biomedical science approaches into the dental clinic will improve our understanding of the reasons behind obstacles encountered in dental pulp regeneration, and of how we can overcome them. The use of tooth slices or tooth root segments to hold the main components of tissue engineering - stem cells (SCs), growth factors (GF) and the scaffolds - will provide a better understanding of how to generate functional, vascularized dentin-pulp like tissue[11]. To date, a variety of SCs including human Dental Pulp Stem Cells (hDPSCs), Human Umbilical Vein Endothelial Cells (HUVECs), and Stem Cells from Human Exfoliated Deciduous Teeth (SHED), combined with scaffolds as PuraMatrix, Collagen and Poly-L-Lactic Acid (PLLA), have demonstrated the ability to regenerate dentin-pulp like structure[12–14].

The aim of this literature review is to highlight the importance of merging the technology of tissue regeneration with clinical dentistry, in order to provide alternative treatment options to those currently used in the dental clinic.

Regenerative Endodontic Procedure Approaches in the Dental Clinic

The concept of pulpal regeneration or re-vascularization has relied on induction of bleeding from the apical area (PA) in order to provide a reservoir for MSCs from the circulating blood[15]. Most of the successful regenerative endodontic procedures (REP) were performed on open apex teeth[1,16–18]. However, two case reports were able to achieve pulpal revascularization in closed apex teeth[4, 19]. In one, pulpal revascularization was achieved in a twenty year old (YO) young adult whose tooth root development had already arrested[20]. In the other, pulp revascularization was achieved in a 39 YO female patient with lesion resolution and bone healing around the root without continued root development[21]. A variety of explanations can

be proposed for tooth root development in immature open apex teeth, including the diversity of stem cells present in the apical part of the tooth, including stem cells at the apical papilla, periodontal ligament stem cells, bone marrow stem cells[17,22,23]. Another possibility is contribution by vital pulp cells remaining in the apical portion of the tooth, which can proliferate and differentiate into tubular dentin producing odontoblasts that can contribute to tooth root length and dentinal wall thickness[17,24]. Hertwig Epithelial Root Sheath cells (HERS) can also help resolve periapical infection, and contribute to repair of damage to tissues in the apical portion of the tooth[25, 26].

Mechanical instrumentation is a mandatory step in regular root canal treatment to achieve sufficient cleaning and shaping of the pulpal space with the aid of irrigation[27]. Protocols for revascularization can vary, with some clinicians preferring to rely on only chemical irrigation and triple paste to eliminate micro-organisms, thus avoiding mechanical instrumentation that may cause damage to the odontoblasts and thin dentinal walls[28–33]. Others proceed with minimal instrumentation when thickness of dentinal walls is sufficient, or root canal treatment was previously initiated, or if pulpal extirpation was mandatory, as in the case of an avulsed tooth[4,9,19,34–37]. Many studies did not report whether they used instrumentation or not, however an assumption was made that instrumentation was not used if not mentioned[1,2,17,18,21,38–41].

Formation of the dentin-pulp complex and laying down of new odontoblasts is the ultimate goal for both endodontists and dental tissue engineers. Several undesirable outcomes were documented by clinicians that could affect the outcomes of REP, including insufficient root development associated with a blunt end, the persistence of open apex, and the formation of severe calcification in the pulpal space[9,16,21]. Due to these unwanted results, and the need to

better understand the etiology of the newly formed tissues, histological analyses were conducted on extracted human teeth previously treated for revascularization[42–46].

Hard tissue formation was found within the regenerated pulpal space, including dystrophic calcification, cellular and acellular cementum-like material, bonelike tissue, osteoblast-like cells, and Epithelial Rest of Malassez (ERM)[44–46]. However, no hard tissue should naturally be found within the pulpal space. In contrast, one study showed the formation of tissue resembling natural pulp tissue, except there was no nerve-like tissue present. The formation of spindle shape of fibroblast or mesenchymal cells dominated the entire pulpal space, and Stro-1 was used to confirm the stem cell origin of the MSCs[43]. Nerve fibers and neurons were detected in one study, as confirmed using Protein Gene Product (PGP) 5.9 to detect neural fibers[45]. Vascularity was not a challenge in these studies[44–46].

1. Blood Clot (BC) Approach

Stimulation of bleeding from the apex is the most commonly used revascularization technique, based on the creation of a natural blood-derived scaffold[1,16,17,30,32,34,36,40]. The value of the BC approach includes rapid formation, within10-15 minutes, and delivery of growth factors to the pulpal space, including Platelet-Derived Growth Factor (PDGF), specifically PDGF, which promotes angiogenesis due to its ability to induce chemotactic and mitogenicity on the MSCs, which in turn leads to a better and more rapid regenerative process[17,20]. Vascular Endothelial Growth Factor (VEGF), which is a robust angiogenesis and vasculogenesis factor that is important in tertiary dentin formation, also induces the proliferation of pulp cells and neovascularization[17,47]. In addition, PDGF and tissue growth factor were found to encourage the differentiation, growth, and maturation of fibroblasts, odontoblasts, and cementoblasts[17].

2. Platelet Rich Plasma (PRP)

Platelet-Rich Plasma (PRP) is an autologous scaffold which has some proprieties to form a better scaffold in compare to BC[34,39]. Jadhav et al., 2012 indicate how the preparation of PRP easy in the dental setting, in the same time PRP able to maintain a three dimension of fibrin matrix to entrap the growth factor which is already rich of it[34,48,49]. PDGF, transforming growth factor (TGF-β), insulin-like GF, vascular endothelial GF, epidermal GF, and epithelial cell GF are examples of GF available in PRP[34]. The ability of PRP to induce healing and regeneration of tissues in the pulpal space is due to the high number of platelets, which in turn has a high number of growth factors that aid in stem cells proliferation[34]. It's been noticed that PRP was able to form vital tissues in a short time as five and half months in premolar diagnosed with necrotic pulp and symptomatic apical periodontitis[39]. Although, PRP is a good alternative for BC due to the difficulty to stimulate the bleeding from the periapical area as reported in several cases [2,3,39,41], some disadvantages have been noticed such as withdrawing blood from children arms, and the high cost of the treatment due to the need to special equipment in the dental clinic as the centrifuge and medication as an anticoagulant to prepare the PRP[39].

Challenges for REP in the Dental Clinic

1. Bleeding Stimulation and Local Anesthesia (LA)

The AAE recommends the usage of LA without vasoconstrictor (VC), especially in the second visit of REP, in order to stimulate bleeding from the PA area of the tooth[50]. This due to the fact that epinephrine reduces blood flow to the injectable site[51]. However, the absence of VC in LA does not necessarily provide a desirable level of bleeding in mandibular first molars, as documented by Norsat et al, perhaps related to the curvature of the molar tooth root[3]. Blood transfer from one canal to another can be used in multi-rooted teeth to overcome any bleeding

deficiency[3]. Positive correlation between bleeding stimulation and root development was noticed after using LA with VC (2% of lidocaine with epinephrine 1:100 000)[4]. In contrast, Torabenjad et al 2011, used PRP for successful REP, which does not require bleeding in the canal space[39].

2. Disinfection

Triple Antibiotic Paste (TAP) is used in REP case to achieve proper disinfection against polymicrobial infection in the pulpal space[52]. Single antibiotics are unable to achieve the desired outcome[52,53]. TAP, composed of Metronidazole, Ciprofloxacin, and Minocycline, has been used to disinfect the pulpal space at the 1st appointment (*Table 1*)[1–3,18,21,30,34,38,39,41]. TAP has been found to reduce the bacterial population in the pulpal space by 30% after irrigation with 10 ml of 1.25% of NaOCL[52]. The viability of fibroblasts was demonstrated using Metronidazole and ciprofloxacin, as confirmed by cytotoxicity testing[54]. Minocycline been noticed to cause dentin discoloration which affect the esthetic, it can be replaced by with Cefaclor and Fosfomycin [2,16,18].

Table 1: The Most Common Used Antibiotic Protocol in Various Clinical Studies:

Author	Year	Dressing Material used for REP
Iwaya, Shin-Ichi et al	2001	2nd to 5th weekly visit: antimicrobial agents (Metronidazole & Ciprofloxacin)
Banchs, Francisco et al	2004	TAP (8 mm in the canal space)
Thibodeau, Blayne et al	2007	TAP
Shah, Naseem et al	2008	Cotton pellet with formocresol was placed in the pulpal chamber
Reynolds, K et al	2008	TAP (to the level of CEJ)
Ding, Rui Yu et al	2009	TAP
Thomson, A et al	2010	TAP (filled to the level just below the CEJ)
Kim, Jong-Hyun et al	2010	TAP
Petrino, Joseph et al	2010	TAP

Cebreli, Zafer et al	2011	$Ca(OH)_2$ Powder (Merck, Darmstadt, Germany) mixed with sterile water 1:3 ratios -thick homogenous paste (places in the coronal 3rd)
Iwaya, Shin-Ichi et al	2011	5th visit: $Ca(OH)_2$ compound (Calcipex, Nippon Shika Yakuhin, Shimonoseki, Japan: calcium hydroxide 24%, Barrium Sulphate 24%, DH2O and others 52%), placed in the upper part of the canal 6th visit: this paste placed in contact with the soft tissue $Ca(OH)_2$ (Vitapex, Neo Dental Chemical Products, Tokyo, Japan: $Ca(OH)_2$ 30%, Iodoform 40.4%, Silicone Oil 22.4%, Inert 6.9%)
Norsat, Ali et al	2011	TAP (3 mm shorter than the estimated WL)
Torabinejad, Mohammed et al	2011	TAP
Chen, M.Y et al	2011	$Ca(OH)_2$ (Henry Schein, Melville, NY) mixed with saline and carried into the canal with files into coronal half of the canal
Norsat, Ali et al	2012	TAP
Jadhav, Ganesh et al	2012	TAP
Paryani, Khimiya et al	2013	$Ca(OH)_2$ paste (Dental therapeutic AB, Necka, Sweden)
		Dusted with Ciprofloxacin powder and carried down to the apex with hand plugger, the canal was coated with powder by using hand file
Soares, Adriana de et al.,	2013	1:1 of $Ca(OH)_2$ (Biodinamica, Ibipora, Brazil) and 2% chlorhexidine gel in a creamy consistency (Endogel) was inserted into the cervical and middle thirds of the root canal with a lentulo spiral (Dentsply Maillefer).
Saoud, Tarek M	2015	Metapaste (calcium hydroxide with barium sulfate; Meta Biomed, Korea).
Wang, Yu et al	2015	TAP
Priya, Harini et al,	2016	Approximately ¼ of a tablet of metronidazole 400 mg (Flagyl; Nicholas Piramal India Ltd, Thane, India) and the contents of a 100-mg capsule of minocycline (Divaine; Cipla Ltd, Mumbai, India) were mixed with saline to a watery slurry consistency and inserted into the root canal with a syringe. The needle was kept just at the entrance of the canal, and the antibiotic mix was gently injected into the canal up to the level of the cementoenamel junction
Zhujiang, Annie et al,	2016	$Ca(OH)_2$ (Dental Therapeutics AB, Nacka, Sweden)

Saoud, Tarek M et al	2016	Metapaste (calcium hydroxide; Meta Biomed Co, Ltd, Chungbuk, Korea)

Double Antibiotic (DAB) could be a mixture of Metronidazole and the Ciprofloxacin or Metronidazole and Minocyclin[28,37]. Iwaya et 2001, applied a mixture of Metronidazole and the Ciprofloxacin in the coronal portion of the tooth to remove the coronal bacteria and allow the remaining vital tissue to repopulate the pulpal space[2] (*Table 1)*. Priya et al. 2016, used another mixture of Metronidazole and Minocycline, to manage internal root resorption that may be caused by loss of the odontoblastic layer or by the infection in the regenerated tissue (*Table 1*)[36]. Paryani et al. suggested that the use of the combination of antibiotics might not be necessary for mature teeth, since chemo-mechanical instrumentation obtained, this could be due to the complexity of root morphology in mature teeth in compare to the immature one[4]. As far as we know, there is no clear information about the exact concentration of ciprofloxacin that not harmful for cell migration, however, 1 mg/ml was suggested to promote 100% survival of SCAP[4].

Calcium Hydroxide $Ca(OH)_2$ is most frequently used as intra-canal medicament between the visits for root canal treatment or in apexification[55]. $Ca(OH)_2$ in combination with other compounds showed promising results in vitro, which require further investigation to prove their ability as a clinical significant way to treat *Enterococcus faecalis*[55–58]. Long term application of $Ca(OH)_2$ into the canal space may result in cervical tooth fracture due to a reduction in the organic content of dentin matrix[59]. Despite its effect against the *E. faecalis*, $Ca(OH)_2$ can cause periapical (PA) coagulation necrosis, which makes revascularization a challenging procedure due to reduced bleeding from PA area[34]. Also, $Ca(OH)_2$ has the potential to destroy remaining vital tissue that can contribute to pulp formation[54]. Iwaya et al.,

2011, used $Ca(OH)_2$ in the form of Calcipex in the coronal portion instead of the full length of the canal, and found that vital tissue was formed 10 mm from the apical part of the canal [33]. Ultracal, a different form of $Ca(OH)_2$, did not affect the survival of SCAP under a variety of concentrations [60]. Nevertheless, high concentrations of TAP, and DAP can terminate the viability of SCAP, while low concentrations of $Ca(OH)_2$ as 0.1 and 0.01 mg promoted SCAP survival[60].

3. Smear Layer and Irrigation

Smear layer that can form above the dentinal walls after instrumentation may interfere with DPSs attachment, causing failure of REP[61]. The smear layer is composed of organic and inorganic substances, fragments of odontoblasts, necrotic material and microorganisms[62,63]. Differences of opinion exist with respect to removing or maintaining the smear layer in the dentinal walls after preparation. Maintaining the smear layer will encourage bacterial proliferation and possibility of marginal leakage. On the other hand, others avoid removal of the smear layer due to the possibility of blocking the dentinal tubule, which in turn will challenge bacterial penetration[64–70]. In Ring et al., partial removal of the smear layer and 50% of exposed dentinal tubules would allow cell attachment, which demonstrated that the presence or absence of the smear layer was not a major factor in cell attachment in REP[61]. Perhaps more important is the proper selection of irrigation for REP, based on bactericidal/bacteriostatic effects on microorganisms and the ability to maintain the survival and proliferation of host stem cells[48].

Sodium Hypochlorite (NaOCl) alone is not sufficient to provide efficient disinfection for REP[54]. However, NaOCl has bactericidal effects, and is capable of dissolving organic tissues[71–76]. Adverse effects on cell survival were detected when using NaOCl, concentrations

of 0.5-1.5% showed a reduction of cell survival by 37%, while the 6% NaOCl reduced the amount of existed SCAP to (5,600 ± 5,500) cells[77].

Chlorhexidine (CHX) is another bacteriostatic/bactericidal agent that cannot dissolve the soft tissues as compared to NaOCl[78,79]. A 2% solution of CHX resulted in cytotoxicity to stem cells, and the complete absence of viable cells in the organotype root model[48]. The investigators believed this was caused by occupying the CHX particles in between the binding sites of stem cells, such as SCAP, and the extracellular matrix of dentin[48]. Several clinical studies included CHX in their irrigation protocol and demonstrated radiographically the complete root development and resolution of the PA lesion in the absence of pathological signs and symptoms[1,3].

The main goal of using 17% EDTA in REP is to maintain the differentiation of DPSCs into dentin producing odontoblast-like cells[80,81]. EDTA enhanced cell adhesion due to its ability to remove smear layer which in turn exposed the collagen fibers in the dentin, and enhanced cell proliferation through the influence of EDTA-soluble morphogens released from the dentin surface, which can aid in DPSC differentiation[80–83]. Furthermore, it has been shown that increased release of growth factors from dentin of up to 2.2 fold can be achieved after using EDTA[77], and also TGF-β, which is important for tertiary dentine formation[82] . Fibroblast growth factor 2 (FGF2) and VEGF have also been detected[84]. Up to 88.66% of viable cells were detected when irrigation with 17% EDTA only[48]. The combination of NaOCl followed by EDTA showed 74.35% of cell viability, and this protocol has been used in multiple clinical cases[4,19,20,36,48].

MTAD is a combination of 3% Doxycycline (a broad spectrum AB), 4.25% citric acid (a demineralizing agent), and a detergent. 0.5% polysorbate 80 [85]. Irrigation of tooth root

segments with MTAD followed by 6% NaOCl and final flush with MTAD, resulted in less cytotoxicity to SHED in compare to irrigation with NaOCl alone, or irrigation with 6% NaOCl and 17% of EDTA followed by a final flush with 6% NaOCl[61]. Also, MTAD has a strong antibacterial reaction against pernicious bacteria such as *E. faecalis*, which is often present in the PA of teeth that have had previous root canal treatment[85]. Both, MTAD and EDTA have similar effect on preventing bacterial leakage as shown by Ghodusi et al. 2007[67].

AquatineEC is another endodontic irrigation that uses hypochlorous acid (HOCl) as an active component, and provides antimicrobial action against a broad range of microorganisms [86,87]. RS irrigation with AquatineEC followed by 17% of EDTA for fifteen seconds followed by final flush with AquatineEC resulted in less cytotoxicity in compare to irrigation with 6% NaOCl alone, or with 6% NaOCl followed by 17% of EDTA and final flush with 6% NaOCl[61]. At the same time, Aquatine*EC* demonstrated increased cell attachment to the surface in comparison to the previously mentioned groups[61].

4. Mineral Tri-Oxide Aggregates vs. Calcium Enriched Matrix

MTA used to serve multi purposes such as in direct pulp capping[88], repair of perforation[89], apexification[90] and revascularization[1,2,4,9,18–21,29–32,35,36,38–41]. However, Calcium Enriched Matrix Cement (CEM) has shown great potential to prevent discoloration, while at the same time showing biocompatibility with the surrounding tissue, lower cost as compared to MTA, and better sealing of the margin[91,92].

MTA – White vs. Grey. White and grey MTA is composed of fine hydrophilic particles that exhibit low solubility, antibacterial activity, superior marginal sealing and is alkaline in nature[93,94].

White MTA (WMTA) has been preferred over the gray MTA (GMTA), due to its ability to enhance the cell proliferation one to five days after exposure to one hr. or 24 hrs. of setting in plain α-MEM[95]. In addition, early short term migration of SCAP was observed at six hrs., as well as osteoinductive properties[95]. However, tooth discoloration from WMTA still a challenge for esthetic purpose [2,9]. Direct contact of hDPSC with GMTA resulted in increased secretion of VEGF [96]. The expression of Osteocalcin (OCN) and dentin sialoprotein (DSP) was elevated in hDPSCs in direct contact with GMTA[96].

CEM has been used instead of MTA for revascularization in order to reduce discoloration observed with MTA [3]. It is composed of calcium hydroxide, calcium phosphate, calcium silicate, calcium sulfate, calcium carbonate, calcium chloride, and calcium oxide [97]. CEM has several advantages in that it is less expensive than MTA, exhibits the ability to form hydroxyapatite, requires shorter setting time and generates less biofilm formation [94]. DPSCs were able to adhere to and spread equally in both MTA and CEM, and both materials allowed for DPSC proliferation, differentiation and exhibited potential osteoinductive properties [98,99].

5. Size of the Apical Foramen

The size of apical foramina also could play a role in revascularization. As Kling et al. suggested, for successful revascularization, the width of the orifice needs to be 1 mm in meso-distal (MD) direction in avulsed permanent teeth after replantation [100], while Paryani et al., 2013 suggested greater than 1 mm in diameter [4]. Since apical orifice is the spot with blood circulation, vascularity, and allows migration of the SCAP to the pulpal space, which in turn promote tooth root development.

6. Root Development and Apical Closure

Radiographic analyses in follow-up sessions revealed several outcomes, depending on a variety of treatments. When clinicians avoided instrumentation, continued root development and apex closure was observed [28–33]. The exception was in molar tooth, the D root exhibited complete development in compare to the M which has a thickening of dentin[3]. When instrumentation of the canal space is performed, either resolved PA lesion and continued root development[9,20,34,36,101], or no significant root development and open apex was observed[4,9]. In addition, slight replacement resorption was also obvious in one case[37]. Finally when the use of instrumentation is not specified, multiple findings were observed, including closure of the apex, thickening of the dentinal walls or fully developed root[1,18,38–40], increased root length without other signs of development[17,2,41], unchanged root length or width[41], and finally open apex was found in one case[21].

Tissue Engineering Approach

Tissue Engineering (TE) is an inter- and multi-disciplinary field whose goal is to develop biological substitutes for the repair, restoration, or regeneration of tissue function[102]. TE approaches employ three basic components - cells, scaffolds and bioactive agents – to create tissues and organs similar to the native human tissues and organs.

Adult Stem Cells

Stem cells are considered to be the first element in tissue engineering[11], as they have several properties that encourage their use in the repair of hard and soft tissues in the oral cavity, including jaw bone, teeth, dental pulp, periodontium including the gum and the periodontal ligaments, and the muscles that enclose the oral cavity. Dental stem cells are relatively new cell populations that have been isolated from various dental tissues [103].

1. Human Dental Pulp Stem Cells (hDPSC)

hDPSCs can easily be obtained from freshly extracted 3rd molar [104]. The main characteristic of hDPSCs is their ability to maintain high proliferation, to form colonies, to exhibit self-renewal, and the ability to form odontoblast-like tissues, pulp-like tissues, reparative dentin and bone-like tissue [105]. *In vitro* studies showed the ability of hDPSCs to differentiate into odontoblasts capable of forming mineralized nodules and polarized cell bodies [106–108].

2. Stem Cells from Apical Papilla (SCAP)

Immature permanent teeth are a good source of SCAP [109]. During the early stages of tooth development, the apical papilla has an important role in allowing continued formation of radicular dentin and dental pulp, which are needed for tooth root growth and development. SCAP, SHED, and DPSCs can differentiate into different population of cells including osteoblasts, and odontoblasts [110]. Vascularization and regeneration of dentin can be generated from SCAP, as shown in *in vivo* studies [110,111].

3. Periodontal Ligaments Stem Cells (PDLSC)

The periodontal ligament (PDL) is present in between the tooth and the alveolar bone, where its function is to provide repair, hemostasis, and nutrition to the tooth [104]. PDLSCs exhibit the ability to form cementum-PDL-like structures, including collagen type I expressing PDL-like tissues that also contain Sharpey's fibers to attach to the tooth cementum [104]. PDLSCs share with the hDPSCs and SHED the ability to form colonies, and they exhibit higher proliferation as compared to hDPSCs [104].

4. Stem Cells from Human Exfoliated Deciduous Teeth (SHED)

SHED, which can be obtained from extracted primary teeth, have similar properties as hDPSCs, except that SHED exhibit higher proliferation rates and greater colony forming unit capability [104]. SHED have been demonstrated to form fibroblast-like cells, odontoblast-like cells and dentin-like structures [104].

5. Dental Follicle Precursor/Stem Cells (DFSC)

DFSCs can be isolated from the dental sac that surrounds the unerupted tooth [104]. DFSC showed the ability to differentiate into PDL-like structures, and bone-like structures [112].

Bioactive Agents - Growth factors (GF)

GF are defined as "peptide molecules that transmit signals to control cell behavior and activity, by interacting with specific receptors located on the surfaces of cells "[113]. Several functions are mediated by GF including cell differentiation, prolifration, and stimulation of matrix secretion [109,114].

1. Platelet-Derived Growth Factor (PDGF)

Angiogenesis and cell proliferation can be induced by applying PDGF, which normally is released by platelets [115,116]. PDGF can stimulate the proliferation of hDPSCs and promote angiogenesis of the injured pulp [117,118].

2. Transforming Growth Factor β (TGF β)

Various cellular activities are regulated by TGF β including synthesis of the extracellular matrix, cellular proliferation and production in dental pulp tissue in in vitro cell culture [119]. TGF β also showed the ability to promote differentiation of odontoblast in the pulpal tissue [120].

3. Bone Morphogenic Protien (BMP)

BMP is another growth factor that belongs to TGF β family, which has roles in cellular differentiation, apoptosis and proliferation[121]. BMP2 has the ability to stimulate odontoblast differentiation in the pulp, as well as in *in vitro* and *in vivo* studies [122,123]. BMP2 and BMP4 have also been used in pulp capping to induce dentine formation [124,125].

4. Vascular Endothelial Growth Factor (VEGF)

VEGF is an important factor in angiogenisis, and is a known as heparin-binding protien that attracts endothelial cells [126]. Neovascularization, endothelial cell proliferation and enhanced survival of these cells are functions of VEGF [127]. VEGF also can enhance the differentiation of hDPSCs into endothelial cells [128].

5. Fibroblast Growth Factor2 (FGF2).

Cell proliferation and differentiation are two processes regulated by FGF2 during tooth morphogenesis [129]. Furthermore, FGF2, PDGF and VEGF have the ability to induce new blood vessel formation within the dental pulp [117,130–132]. The combination of FGF2 and TGF β1 are capable of differentiating dental pulp cells into odontoblast-like cells, however, FGF2 alone can induce hDPSC proliferation without inducing their differentiation [120]. Moreover, several studies showed the FGF2 induced the formation of reparative dentin at dental pulp exposure site [133–135].

6. Insulin-Like Growth Factor (IGF)

Somatomedin C or IGF-1, is capable of inducing the odontoblast growth and differentiation in serum-free media [136]. Additionally, the proliferating cell nuclear antigen (PCNA) and IGF-1R have been detected in mature teeth as compare to immature teeth, implying roles for IGF-1 in tooth mineralization [137].

7. Nerve Gorwth Factor (NGF)

Neutropins, another name for NGF, participate in tooth morphogenesis and innervation as studied in rats [138]. In addition, NGF induces the differentiation of immortalized dental papilla into odontoblasts, suggesting roles in dental formation[139].

Scaffolds

Scaffolds can be used to promote a 3 dimensional (3D) growth environment for stem cells present within the pulpal space [48]. The main goal of incorporating scaffolds in the regeneration process is to facilitate cell migration, attachment and proliferation, and to allow cells to be organized to best achieve the final structure and function of the targeted tissue [140]. Furthermore, since there is host tissue interaction, scaffolds are required to be biocompatible materials that encourage vascularity, and tissue infiltration. Also, scaffolds should be able to be remodeled and degraded, in order to eventually be replaced by regenerated tissue [140]. In general, four main categories of scaffolds have been used in the field of pulp regeneration: natural and synthetic polymers, hydrogels and bioceramics [140].

1. Natural Polymers

Natural Polymers can be fabricated from environmental materials including plant, sea algae, plant cell walls, crustaceans, and crops [140]. Natural polymers exhibit advantages in that they can be formed from a variety of sources, exhibit complex and unique structures, are known for their intrinsic biodegradability, can be used for the temporal release of growth factors, and are favorable for tissue remodeling [140]. In contrast, challenges include the possibility of pathogen transmission, unwanted immune response, difficulties in processing and sterilizing,

and unique properties based on their origin [140]. Chitosan, starch, dextran, cellulose, hyaluronic acid, and polysaccharide extracellular matrix (ECM) are all examples of natural polymers [140].

2. Synthetic Polymers

Synthetic polymers overcome certain disadvantages of natural polymers in that they exhibit minimal antigen-antibody reaction, can easily be fabricated into desired shapes, can be tuned to exhibit desired mechanical properties, are inexpensive and easy to fabricate in large numbers, can be degraded, and provide the option of adding functional groups to attract cells and/or to bind growth factors [140]. The main drawback of synthetic scaffolds is an accumulation of acids from degradation products [140]. Polylactic acid (PLA), polyether ester, polyglycolic acid (PGA), polyethylene glycol (PEG), and copolymer of PLA and PGA are examples of widely used synthetic polymers for TE [140].

3. Hydrogels

Hydrogels provide additional features including high compatibility with host tissues, similar viscoelastic properties as native host tissues, high tissue like water content, easy applications for injectability, allow for uniform cell encapsulation, can be photocross-linked either via chemical or physical (heat) methods, are capable of transferring nutrients and waste products, and finally can be tailored to include GFs or bifunctional molecules [140]. The noticeable weakness associate with hydrogels is that they exhibit limited mechanical stiffness, making them unsuitable for certain applications such for bone grafts[140]. Gelatin methacrylate (GelMA), self-assembling peptide hydrogel, and multidomain peptide hydrogel are examples of hydrogel scaffolds [141–143].

4. Bioceramic Scaffolds

Bioceramic scaffolds can be used to generate hard tissues such as bone, and are known for their biocompatibility with host tissues, osteoconductive nature, excellent bone-bonding, and are biodegradable with wide degradation range [140]. Bioceramic scaffolds can be prone to fracture and are predisposed to fatigue. Humid conditions decrease the mechanical strength of bioceramic scaffolds[140]. Hydroxyapatite, bioactive glasses, calcium phosphate and biphasic calcium phosphate (HA/TCP) are examples of biocermic scaffolds [140].

In vitro and in vivo studies

Many studies have shown the capacity of hDPSCs, HUVECs and SCAP to generate pulp-like structures, blood vessel formation, and reparative dentine to replace the damaged dentin-pulp complex [13,14,110,142,144–151]. Furthermore, in vivo studies aimed to incorporate DPSCs and collagen scaffolds with the dentin matrix protein-1 (DMP-1) at root perforation, found that the scaffold had been degraded, and that various cells were detected at the defect site, including fibroblasts, and blood vessels. However, Von Kossa stain did not reveal mineralization at 6 week time point [152].

Various scaffolds have been used in conjunction with stem cells, including PuraMatrix (peptide hydrogel), collagen scaffold, nanofibrous gelatin/silica bioactive glass (NF-gelatin/SBG) hybrid, flouroapetite crystal coated with poly caprolactone nanofibers, and poly L lactic acid (PLLA) [13,14,110,142,144–151]. None of these scaffolds interfere with cell differentiation, providing the clinician and bioengineers the opportunity to select scaffolds that serve their best interests.

Tissue regeneration without scaffolds is also possible. Syed-Picard et al. 2014, successfully used DPSC cell sheets to create 3D cylinders, to induce odontoblast differentiation as confirmed by the expression of DSP, and also the presence of CD31 expressing blood vessels [148]. Microtissue spheroids are another method that exhibited successful incorporation of DPSCs and HUVECs without a scaffold, where cell feeding was depending on agarose in the 3D petri dish [151]. Syed-Picard et al. 2014, the DPSCs demonstrated the ability to support blood vessel formation in co-cultured HUVEC and DPSCs[151]. However, HUVECS alone did not survive for more than 12 hours., suggesting that VEGF produced by DPSCs is required for HUVECs survival [151]. Table 2 summarizes some of the *in vivo* studies that participated in formation of pulp-dentin like [13,110,142,144,146–148,151,153].

Table 2: Summery of *in vitro* and *in vivo* studies

Author	**Year**	**Stem Cells**	**Scaffold**	**Main Results**
M.M. et al.,	2008	SHED alone or with human dermal microvascular endothelial cells (HDMEC)	PLLA-based scaffold	* after 14–28 days, Dentin-pulp like structure was formed * (IHC) DSP Suggested odontoblasts-like cells differentiation, this was much stronger in the combination of cells rather than SHED seeded scaffold * Transmission electron microscope revealed similar morphology to odontoblast in predentin area * SHED Proved the ability to differentiate to blood vessels by using LacZ-positive cells

George T et al.,	2010	SCAP & DPSCs	Poly-D-L-lactide/glycolide	* Vascularized Pulp-like tissue within the entire pulpal space * Dentin like structure was formed (tertiary dentin), and formation of odontoblast like structure, DSP, BSP, Alkaline Phosphatase and CD105 are used to confirm odontoblasts expression
F. F. Demarco et al.,	2010	DPSCs	Poly-L-lactic acid (PLLA)	DPSCs are capable to form pulp-like structure, expression of DMP-1, DSPP, MEMPE are expressed in the newly formed tissue
K. M. Galler et al.,	2012	DPSCs	Self-Assembling Peptide Hydrogel, incorporation with GF as (TGFb1), (FGF2), & (VEGF)	* Vascularized Connective tissue similar to dental pulp was formed within cell seeded scaffold with GF * Odontoblasts like was detected in predentin area and expressed by using DSP
Tiejun Qu et al.,	2013	DPSCs	Nano-Structured Gelatin/Bioactive Glass Hybrid	Nano-Structured Gelatin/Bioactive Glass Hybrid promote differentiation of DPSCs and biomineralization, this result confirmed by using ALP
F.N. Syed-Picard et al.,	2014	DPSCs	Scaffold free	Cellular, fibrous, and vascularized scaffold free tissue formed within pulpal space. In the same time odontoblast like structure was noticed by using DSP
W. L. Dissanayaka et al.,	2015	DPSCs & HUVECs	scaffold free prevascularized micro tissue spheroid	a successful scaffold free pulp like tissue formation, furthermore, vasculature and HUVECs survival were supported and maintained in presence of DPSCs
W. L. Dissanayaka et al.,	2014	DPSCs & HUVECs	Injectable Peptide Hydrogel (PuraMatrix)	PuraMatrix promote survival of cells as well as migration. Furthermore, it maintains vasculature formation in absence of GF. DPSCs participate in network formation of VEGF and paly important role in angiogenesis. The newly formed tissue has pulp like structure

W. L. Dissanayaka et al.,	2014	DPSCs & HUVECs	scaffold free prevascularized micro tissue spheroid	this model was successfully able to form pulp-like structure with vasculature within RS. Odontoblast like structure was noticed and incorporation of host vasculature with the construct was noticed. However, origin of vasculature was recognized by human mitochondria and it proved the originality of the formed vasculature to the used HUVECs

Challenges in Tissue Engineering

Age of the Donor

Stem cell isolation from individuals of different ages appeared to affect cell division, as cell doubling times were longer in people greater than or equal to 20 years as compared to the younger age group [154]. Furthermore, there was a positive relationship between the developmental stage of DMSCs and the doubling time [154].

Optimization of Scaffolds for Pulp Regneration

Selecting the proper scaffold composition and the properties requires careful attention from the clinician in order to generate the target tissues. Additionally, most scaffolds need to be prepared in the lab before reaching the dental chair, which in turn indicates the necessity to include bioengineers and technicians into the dental team, to prepare cell lines, achieve proper cell density, and to devise methods to easily prepare and apply the mixture to the defect site.

Future Prospective

Most of the published *in vitro* and *in vivo* studies mentioned in this review focused on the successful formation of the dentin-pulp complex. However, closer attention must be paid to the regeneration of nerve-like structure within the tooth pulp. In addition, gene therapies

should be incorporated to promote pulp tissue survival and formation of the odontoblast layer. Likewise, subcutaneous root segment/slice implant models are not suitable for evaluating pathogenic responses in the periapical area, which require the need to use large animal models, such as the canine model. To the best of our knowledge at this time, the proper mix of growth factors, cells and scaffolds must be evaluated to achieve reliable pulp regeneration.

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Chapter 2 *in vitro* and *in vivo* Study

GelMA Encapsulated DPSCs and HUVECs for Dental Pulp Regeneration

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Abstract

Pulpal revascularization is commonly used in the dental clinic to obtain apical closure of immature permanent teeth with thin dentinal walls. Although sometimes successful, stimulating bleeding from the periapical area of the tooth can be challenging, and in turn may deleteriously affect tooth root maturation. Our objective here was to define reliable methods to regenerate pulp-like tissues in tooth root segments (RS) injected with human dental pulp stem cells (hDPSCs) and human umbilical vein endothelial cells (HUVECs) encapsulated in 5% gelatin methacrylate (GelMA) hydrogel. RS injected with acellular GelMA alone and empty RS were used as controls. Combined hDPSCs and HUVECs (1:1) were encapsulated in 5% GelMA and injected into root segments (RS) of 6 mm length and 2-3 mm orifice wide. White mineral trioxide aggregate (WMTA) was used to seal one of the orifices while the other was left open. Samples were cultured in-vitro in osteogenic media for 13 days, and subsequently implanted subcutaneously in nude rats for 4 and 8 weeks. At least five sample replicates were used for each experimental and control groups. Analyses of harvested samples found that pulp-like tissues formed in hDPSC/HUVEC encapsulated GelMA filled RS, and that host cell infiltration was observed in the acellular GelMA and empty RS groups. Mesenchymal stem cells and endothelial cells were identified using immunofluorescent IF for Vimentin and CD31, and hDPSCs and HUVECs were distinguished from host cells using IF for anti-rh-mitochondrial antibody. Our results showed that GelMA supported hDPSC/HUVEC proliferation and organization, and also demonstrated the ability to attract and provide attachment for host cells. GelMA hydrogels promoted the establishment of host vasculature within the segments, and extracellular matrix (ECM) deposition. Together, these

results identify GelMA hydrogel as a promising alternative therapy for clinically relevant pulpal revascularization.

Key words: Apexification, pulp vascularization, hydrogel scaffold, pulp like structure, & endodontic treatment.

Introduction

The goal of successful pulpal revascularization of an injured or infected tooth is to facilitate continued root development, prevent possible fracture of thin dentinal walls, to achieve apical closure development similar to that of adjacent teeth, to prevent tooth and supporting bone loss, and to avoid the financial burden of dental implant placement [10]. Regardless of these good intentions, undesirable outcomes can occur after clinical revascularization procedures, including arrested tooth root development, incomplete closure of the tooth apex, and calcification within the pulpal space that could impede future root canal treatment [9]. Induced bleeding at the tooth apex is a procedure commonly used to activate the proliferation and migration of stem cells from the apical papilla (SCAP) into the pulpal space, and to release growth factors such as platelet-derived growth factor (PDGF), which participate in angiogenesis [17,20]. But unfortunately, bleeding deficiency can occur, leading to possible arrested tooth root development [2,38,41].

Several *in vivo* studies attempted to regenerate the dentin-pulp complex by incorporating cells, such as human dental pulp stem cells (hDPSCs), human umbilical vein endothelial cells (HUVECs), SCAP – with scaffolds such as PuraMatrix, nanofibrous gelatin/silica bioactive glass (NF-gelatin/SBG) hybrid, collagen scaffold, and poly L lactic acid (PLLA) and flouroapetite crystal coated with poly caprolactone [13,14,110,142,144–151]. Others demonstrated the ability to regenerate pulp like tissue using scaffold-free approaches including cell sheet technology, and using DSC aggregates formed on agarose 3D petri dishes [147,148].

Here we propose the use of gelatin methacrylate (GelMA) hydrogels as a scaffold material for pulp regeneration. Recently, we have established a 3D biomimetic tooth bud model using photopolymerizable GelMA hydrogel formulas, designed to facilitate dental epithelial and

dental mesenchymal cell interactions leading to ameloblast and odontoblast differentiation, respectively, and the formation of bioengineered teeth of predictable size and shape [155–157]. GelMA hydrogels exhibit interesting properties that make it an attractive material for a variety of tissue engineering applications including [158,159]: i) it is largely composed of denatured collagen and relatively inexpensive; ii) it retains many of collagen's natural properties including arginine-glycine-aspartic (RGD) adhesive domains and matrix metalloproteinases (MMP) sensitive sites which are known to enhance cell binding and cell-mediated matrix degradation; iii) the physical properties of GelMA hydrogels can be tuned by varying GelMA and/or photoinitiator (PI) concentrations; and iv) GelMA is suitable for cell encapsulation at 37°C, and promotes cell viability and proliferation. We have identified GelMA formulas suitable for bioengineered tooth development based on the elastic moduli of natural tooth bud derived enamel organ and pulp organ tissues. And in addition to dental cells, we have incorporated HUVECs in these bioengineered 3D tooth bud constructs, to promote neovascular formation in bioengineered tooth tissues, and to facilitate *in vivo* engraftment with host vasculature [155,156].

To our knowledge, ours is the first study to investigate the use of GelMA encapsulated hDPSCs and HUVECs for clinically relevant applications for pulpal regeneration.

Materials and Methods

Human Teeth, Cell Isolation and Expansion

Human teeth that had been extracted for clinically relevant reasons were collected from the Tufts University School of Dental Medicine and the Back Bay Oral Maxillofacial clinic in Boston, MA. hDPSCs were isolated from dental pulp obtained from wisdom teeth as previously published [6]. Dental pulp was minced and enzymatically digested using 0.3 mg/mL

collagenase type I and 0.4 mg/mL dispase, and filtered through a 40 mm cell sieve. The resulting single cell suspensions were expanded by culturing in mesenchymal medium [Advanced DMEMF12 (GIBCO), 10 % FBS, 25 ug/mL Ascorbic Acid, 1 % PSA, 1 % Glutamax], respectively, in 5% CO_2 at 37°C. HUVECs (PSC100010, ATCC, Manassas, VA) were pre-cultured in vascular basal media (VBM) (PCS100030, ATCC) with VEGF kit (PCS10004, ATCC) in humidified 5% CO2 at 37°C. Expanded cells were cryopreserved in 10% DMSO in appropriate culture media until use.

hDPSCs and HUVECs that had been cryopreserved at Passage 2 and 5, respectively, were used in this study. Cryopreserved hDPSCs were suspended in 9 ml mesenchymal medium, centrifuged for 5 min. at 1500 rpm, resuspended in 25 mL medium, plated in T175 flasks at expected $10x10^6$ cells/flask, and incubated at 37^0C in an atmosphere of 5% CO_2 for 2 weeks with media changes every 2 days. Osteogenic Media (OM) was prepared using DMEM/F12 supplemented with 1%PSA, 10% FBS, 100 nM Dexamethasone, 10mM beta Glycerol Phosphate, 0.05 mM Ascorbic Acid. Cryopreserved HUVECs were suspended in 9 ml of (VBM), centrifuged for 5 min. at 1500 rpm, resuspended in 25 ml VBM, plated in T175 flasks and incubated at 37^OC in an atmosphere of 5% CO_2 with media changes every 2 days.

Research Design

Three groups of root segments (RS) were examined in this study: 1) hDPSC/HUVEC encapsulated GelMA filled RS (G1); 2) acellular GelMA filled RS (G2); and empty RS (G3). Briefly, replicate samples were cultured for 13 days in osteogenic media (OM) *in vitro.* Five replicates of each group were fixed at this time, and used to characterize cell proliferation and morphology using histological and immunohistochemical (IHC) methods. The remaining RSs were implanted subcutaneously in nude rats, and grown for 4 and 8 weeks (Figure 1).

GelMA Preparation

Lyophilized GelMA was fully dissolved in DMEM/F12 media (w/v), and photo-initiator (Irgacure2959, Sigma, St. Louis, MO) was added to create 5 percent GelMA formulation, denoted as 5% GelMA, which was sterilized by filtration using a 0.22 µm filter, and placed in the dark conditions until use.

Tooth RS Selection Criteria, Disinfection & MTA Placement

Teeth were collected from healthy patients aged 15 to 30 years old, including single and multi-rooted teeth with Type I & V Vertucci root canal configurations. Teeth containing caries, Type II - IV Vertucci root canal configuration, calcified canals, or those with prior root canal treatment were excluded. Tooth RS were prepared using sterilized 330 and fissure burs, from the coronal and middle 3rd of the roots to avoid curvature in the root. RS were 6 mm in length, with 2-3 mm pulpal chamber orifice width, to facilitate injection of cellular and acellular GelMA. The pulpal space lumen was enlarged using Gates Glidden (GG) sizes 1 and 2, and the previously mentioned burs. Next, RSs were prepared using the protocol of Galler KM et al 2011. Briefly, RS were soaked in 0.5 M EDTA for 1 minute, rinsed in Phosphate Buffering Solution (PBS) for 5 minutes, rinsed in 6.15 % Sodium hypochloride (NaOCl) for 10 minutes, and washed three times in sterile PBS. RS were then soaked in 0.5 M EDTA for 10 minutes followed by rinsing three times in PBS. Finally, to test for any microbial growth of these segments, RS were cultured in DMEM/F12 media at 37^{0}C for four days [80]. Finally, White Mineral Trioxide Aggregate (WMTA) from (ProRoot DENTSPLY Tulsa Dental Specialties, Tulsa, OK) was used to create a plug at one side of each RS to mimic the clinical situation, while the other end was left open to allow host cell invasion.

Cell Preparation and GelMA Encapsulation

Confluent flasks of hDPSCs and HUVECs were trypsynized by using 0.25% Trypsin-EDTA (1X) (Gibco Life Technology, City, State) and 0.5% Trypsin-EDTA from Primary Cells (ATCC, Manassas, VA) respectively. Briefly, cells were washed with 10 ml of PBS, treated with trypsin solution for 5 min at 37° C in 5% CO_2, and examined for cell detachment using an inverted microscope Olympus CKX41 (Binocular, Waltham, MA). Next, 12 mL of DMEM/F12 media added to hDPSC flasks, and 12 mL of VCBM was added to HUVEC flasks, each were transferred into 50 conical tubes, and centrifuged at 1500 rpm for 5 min. Cells were resuspended in their respective media, and cell densities were calculated using a Countess Automated Cell Counter (Invitrogen ™, Carlsbad, CA). A total of 6×10^5 hDPSCs and 6×10^5 HUVECs (1:1) were combined into one 50 ml tube, and resuspended in 0.5 mL of filtered 5% GelMA. Approximately, 28 µL of GelMA encapsulating hDPSCs and HUVECs was injected in the RSs and photo-crosslinked via exposure to 9.16 W/cm^2 UV light for 20 seconds using an Omnicure S2000 (Lumen Dynamics Group Inc., Mississauga (ON) Canada).

Subcutaneous Implantation in Nude Rats

Using Tufts University approved IACUC Protocols, 8 female Nude rats aged 4-6 weeks were prepared as follows. Isoflurane 1-4% was administered in sedation chamber then Buprenorphine was administered for pain management before performing the procedure. The fur was shaved at the incision site followed by disinfection with Iodine and Ethanol 75% three times. Eye lubricant was used to prevent eye dryness. Weight was recorded and ear tags were placed. Four incisions were created, two on each side, and fascia was separated from the muscle to form a lateral sac deep enough to hold an individual RS. After implantation, incisions closed

with wound clips. Rats were checked every day for one week, and Buprenorphine was administered once every two days for one week.

Root Segments (RS) Harvest

Replicate samples were harvested at 4 and 8 weeks using TUSDM approved IACUC protocols. Briefly, rats were euthanized by 5% CO_2. Harvested RSs were washed 3 times in PBS and fixed in 10% Formalin overnight. RS were then washed with PBS and decalcified in 10% EDTA at pH 7.00 for 4 months. Decalcification was monitored by taking 5 ml of the 10% EDTA solution and adding a drop of HCL, followed by 1 ml of saturated Ammonium Oxalate. The solution was mixed thoroughly and allowed to sit for 20 min, at which time the solution was monitored for CaPO4 precipitate. Lack of precipitate formation was determined to be complete decalcification.

Histological Analyses

Samples were prepared for cryostat sectioning as follows. Demineralized samples were washed 2 times in PBS for 10 min. and soaked in 40% sucrose solution for one night at -40ºC. Optimal Cutting Temperature (OCT) solution from (Sakura Finetek, Torrance, CA) was added to samples in holders. Vacuum was used to remove all air bubbles, and the OCT was solidified in liquid nitrogen. Samples were placed in plastic wrap to prevent dehydration, and stored at -80ºC.

Cryosectioning

A cryostat (Leica biosystems, Nussloch, Germany), set at -21ºC, was used to section samples at 10 µm or 30 µm intervals, for histological/IF and confocal analyses, respectively. Magic Tape (Cryofilm Type2C, Section-Lab, Hiroshima, Japan) was used to transfer sections

to Superfrost ® Plus Microscope Slides Precleaned (Fisher Scientific, Atlanta, GA). Slides were stored in slide boxes at -20^{0}C until use.

Hematoxylin and Eosin (H&E) Stain

To avoid dislodging samples from the glass slides, a modified H&E protocol was used, starting with dipping the slides in DI H_2O (3X) to replace regular HE staining protocol rehydration step. Slides were stained with Hematoxyline (H) by dipping the slides (3-4X) in H solution, followed by gentle rinsing with DI H_2O. Slides were then dipped into dilute hydrochloric acid (2 ml of Hydrochloric acid in 250 ml of DH_2O) (2-3X), then into ammonia water (2 times). Slides were dipped in DI H_2O (2-3 times) and finally in Eosin (3-4 times). Dehydration was performed by dipping each slide (2-3 times) in an alcohol series: 2X in 95% EtOH, 2X in 100% EtOH, and finally once in Xylene. Samples were covered by cover slipped using Permount (Fisher Scientific, Atlanta, GA).

Double Immunofluorescent (IF) Histochemical Analyses

Sample slides were warmed to RT for 5 min, rehydrated in DI H_2O for 5 min, re-fixed in 10% formalin for 10 min, and washed twice for 10 min in PBS. Samples were blocked with 5% BSA in a humid chamber for 15 min. Primary antibodies were then added, including mouse α CD31(1:200, ab187377, Abcam, Cambridge, MA) and rabbit α Vimentin (1:25, bs-0756R, Bioss, Woburn, MA), and samples were incubated for 1 hr at RT, followed by washing 10 min 2X in PBS. Secondary antibodies including goat α mouse (Invitrogen, 568, 1:50, West Grove, PA) and goat α Rabbit (Invitrogen, 488, 1:50, West Grove, PA) were applied to the specimen for 1 hr, followed by washing three times in PBS. Finally, 40 µl of VectaShield Hardset mounting media (Vector Laboratories, H-1400, Burlingame, CA) were added, samples were cover slipped and stored at -4^{0}C. Mouse α rh-Mitochondria (1:25, Millipore Sigma,

MAB1273, Temecula, CA) was used to discriminate between HUVEC and host endothelial cells.

Sections were analyzed using a Zeiss Axiophot Imager microscope and digital camera (Zeiss, Germany), and images acquired and processed with AxioVision software (Zeiss, Germany, V.4.9.1), Nikon A1R confocal (Nikon Instruments Inc.) running NIS Elements 4.2. Collagen deposition was performed using polarized light (Pol) microscopy (Zeiss, Germany).

Results

GelMA encapsulated Cells Filled Tooth Root Segments (G1)

A functional vascularized network is a requirement for the long term survival of bioengineered tissues and for proper integration with the recipient host [160]. In natural tissues, blood vessels are composed of a luminal endothelial cell layer, surrounded by a layer of smooth muscle cells. Published reports showed that mesenchymal stem cells (MSCs) and endothelial cells exhibit the ability to self-organize into capillary-like networks after encapsulation in GelMA hydrogel *in vitro* and *in vivo* [110,161]. In this work, the mesenchymal cells isolated from dental pulp (hDPSCs) obtained from wisdom teeth and HUVEC encapsulated in GelMA were used to study pulp regeneration. hDPSCs showed a typical spindle shaped fibroblast morphology, and HUVEC exhibited a typical cobble-stone like morphology with large dark nuclei (Figure 1). Both types of cells retained their characteristic morphologies after achieving one hundred percent confluence. hDPSC and HUVEC were combined and encapsulated in 5% GelMA, and injected into RSs. H&E staining confirmed the presence of cellularized bioengineered pulp-like tissue within GelMA encapsulated hDPSC/HUVEC filled RSs at 13 days *in vitro* culture (Figure 2 A, B). Cellularity appeared to increase over the time in 4 & 8 weeks *in vivo* implanted constructs (Figure 2 E, F and I, J), and eventually occupied the entire

pulpal space not containing MTA. Pol revealed the collagen deposition and organization in derived extracellular matrix (ECM) after 4 and 8 weeks *in vivo* implanted samples (Figure 2 C, G and K). However, a more organized collagen formation was observed within the RSs after 8 weeks than 4 weeks (Figure 2G, K). Collagen fiber alignment within the RS appeared to be oriented perpendicular to the encapsulating host tissue, and parallel to the dentin. Neovascularization was observed in 4 week *in vivo* implanted samples (Figure 2F, G), while 8 week *in vivo* implants showed patent blood vessels containing red blood cells (Figure 2G, K). IF analyses were used to examine neo-vessel formation and organization within hDPSC-HUVEC encapsulated GelMA RSs. IF analyses of VM expressing (green) hDPSCs and CD31 expressing (red) HUVECs revealed network formation in hDPSCs/HUVEC GelMA RSs (Figure 2D, H, L).

Acellular GelMA Filled Tooth Root Segments (G2)

H&E staining showed early degradation of GelMA in 13 days *in vitro* cultured acellular GelMA RSs (Figure 3A, B). In *in vivo* implanted constructs, host cells attached to both the GelMA and to the dentin surface, and cellularity appeared to increase over the time in 4 and 8 weeks implanted RS (Figure 3 D, E and H, I). GelMA scaffolds were detectable at both 4 and 8 weeks *in vivo* times. Red blood cells were evident surrounding the GelMA. Pol revealed the collagen deposition and organization of derived ECM deposited by infiltrating host cells after 4 and 8 week implanted RSs (Figure 3F, J). Double IF using VM and CD31 antibodies was used to demonstrate host MSCs and endothelial cells in *in vivo* periods only due to presence of cells in compare to 13 days *in vitro* which they are missing (Figure 3G, K).

Empty Tooth Root Segments (G3)

H&E staining showed host cellularity and infiltration in both 4 (Figure 4A, B) and 8 weeks (Figure 4E, F) *in vivo* implanted RSs, suggesting that host cell infiltration starts from the open site of the RS. Host ECM appeared to be more mature at 8 weeks as compared to 4 week samples. Pol also revealed more organized collagen formation within the RSs after 8 weeks than 4 weeks (Figure 4C, J). The collagen produced by the host cells in the empty RSs appeared to be more organized than that produced in the acellular GelMA after 4 and 8 weeks. Collagen fiber alignment within the RSs appeared to be oriented perpendicular to the encapsulating host tissue and parallel to the dentin. More organized network formation was observed in GelMA encapsulating hDPSC/HUVEC RS than empty root segments. IF analyses revealed host vascularity and mesenchymal stem cells (Figure 4D, K), which is similar to that of the acellular GelMA group.

Human and Host Cell Contribution to Bioengineered Dental Pulp

In order to evaluate the long-term survival and to discriminate the hDPSC and HUVEC from the host rat tissue, sectioned samples were stained with rh-mitochondria antibodies. Rh-mitochondria stains all human cell types and does not cross react with rat and mouse tissue [162]. IF analyses of rh-mitochondria expressing (green) human cells showed that the GelMA encapsulating hDPSCs and HUVEC injected in RSs survived after 4 weeks in vivo implantation (Figure 5 B), but did not survive after 8 weeks in vivo implantation (Figure 5 C). In contrast and as expected, host cells did not stain positive for the rh-mitochondria antibody for acellular GelMA and empty RSs.

Discussion

Huang et al., 2010 reported DPSCs was capable to regenerate dentin-pulp complex after being encapsulated in a scaffold and transplanted *in vivo* [110]. However, there are two major hurdles that still need to be overcome: i) the identification of a scaffold that mimics the ECM of natural pulp, ii) and securing a good blood supply to ensure the survival of transplanted DPSCs *in vivo* [147]. The goal of this study was to define a more effective, clinically relevant method for pulpal revascularization and regeneration in human using tooth root segments.

In this study, we used human DPSCs in combination with HUVECs, based on the important roles of both cell types for pulpal tissue formation and vascularity, encapsulated in GelMA material. To overcome the challenge of vasculature, applying endothelial cells which in turn incorporate with the host vasculature following transplantation *in vivo* [13]. Moreover, DPSCs promoted early vascular network formation by increase expression of VEGF and facilitating the migration of HUVECs [13]. GelMA is a preferred hydrogel over other types of hydrogel scaffolds, due to distinctive features including the ability to facilitate cell attachment, spreading, proliferation, and interactions with host tissues to create a 3D tissue environment that is highly conducive for tissue engineering [158]. More importantly, its injectable and photo-crosslink properties make GelMA a very attractive material for root canal treatment and pulpal regeneration, further studies are needed to ensure complete photo-cross link for curved and full length root. Thorough photo-crosslinking of GelMA was validated first using an unpublished lab trial, which revealed that the GelMA was efficiently crosslinked through the entire 6 mm segment. This was also evident by the fact that the color of the GelMA scaffold turned from red -pink to colorless (data not shown).

Our group have described the design and characterization of dental and endothelial cell encapsulated GelMA constructs as 3D biomimetic tooth bud models, consisting of biomimetic enamel and pulp organ bilayers [155,157]. We used *in vitro* analyses to identify GelMA formulas that supported dental epithelial and mesenchymal cell attachment, spreading, metabolic activity, and neo-vasculature formation by co-seeded endothelial cells (HUVECs) [156,157]. Therefore, the fabricated 3D tooth bud model consists of a biomimetic enamel organ layer (dental epithelial and HUVEC encapsulated in 3% GelMA) and biomimetic pulp organ (dental mesenchymal and HUVEC encapsulated in 5% GelMA). We have also demonstrated that the selected GelMA formulas supported dental cell differentiation, vascularization, and *in vivo* formation of mineralized tissues of specified size and shape [156,157]. In this study, the biomimetic pulp organ (hDPSC and HUVEC encapsulated in 5% GelMA) was injected in RSs to study pulp regeneration.

The H&E staining and Pol imaging confirmed that no tissue formation was observed in acellular GelMA RSs after 13 days *in vitro.* (Figure 3). In contrast, GelMA encapsulating hDPSC and HUVEC exhibited cellularized bioengineered pulp-like tissue, and the cellularity increased over the time after 4 & 8 weeks in vivo (Figure 2). The analyses showed that the GelMA scaffolds were largely biodegraded by the cells over time, but was detectable after 8 weeks in acellular GelMA RSs. The results showed close attachment of host tissues to the GelMA scaffold, and also over the inner surface of the dentin in the RS (Figure 2). Additionally, GelMA promoted host tissue infiltration, proliferation, and promoted vascularization of the implant area.

Within each RS, WMTA was used to seal off one end of each RS, to mimic what would be experienced by a treated natural tooth [95]. Remnant MTA is clearly identifiable in some of

the histological sectioned samples, although most of the MTA was dissolved during sample processing. No reparative dentin was observed to form below the MTA as was documented by Huang et al., 2010 [110]. The lack of dentin formation in our study could be due to the relatively short duration of our *in vivo* study (1-2 months) as compared to the Huang et al. study (3-4 months) [110].

As noted, GelMA supported cell proliferation and this result is also consistent with our previous results [155,157]. By polarized light microscopy, the collagen produced by rat host cells in the empty RSs appeared more organized than that produced in the acellular GelMA after 4 and 8 weeks (Fig. 4, Panel J). A possible explanation is that GelMA impedes host cell infiltration. Recent published reports noted host cell infiltration within empty RSs, which had orifice widths of 1-2.25 mm and 1-1.5 mm [110,148]. Since the apex of the root is the only site for vasculature invasion into the tooth, apex needs to be wide enough to enhance cell migration into the pulpal space. Further studies are needed to define the optimal width of the apex for efficient pulpal regeneration.

We performed IF histochemical confocal analyses to examine neo-vessel formation and organization within *in vitro* cultured and in vivo samples. We identified elaborate neo-vascular (Figure 2) and capillary-like network formation in all in vivo groups, after 4 and 8 weeks [163]. However, more organized network formation was observed in GelMA encapsulating hDPSC/HUVEC RS than empty root segments. Importantly, the presence of host red blood cells within the bioengineered vasculature confirmed the ability of GelMA encapsulating hDPSC/HUVEC constructs to form functional vascular networks in vivo, as required to support tooth integration and growth after implantation. The IF analyses of rh-mitochondria expressing human cells showed that the GelMA encapsulating hDPSCs and HUVEC injected

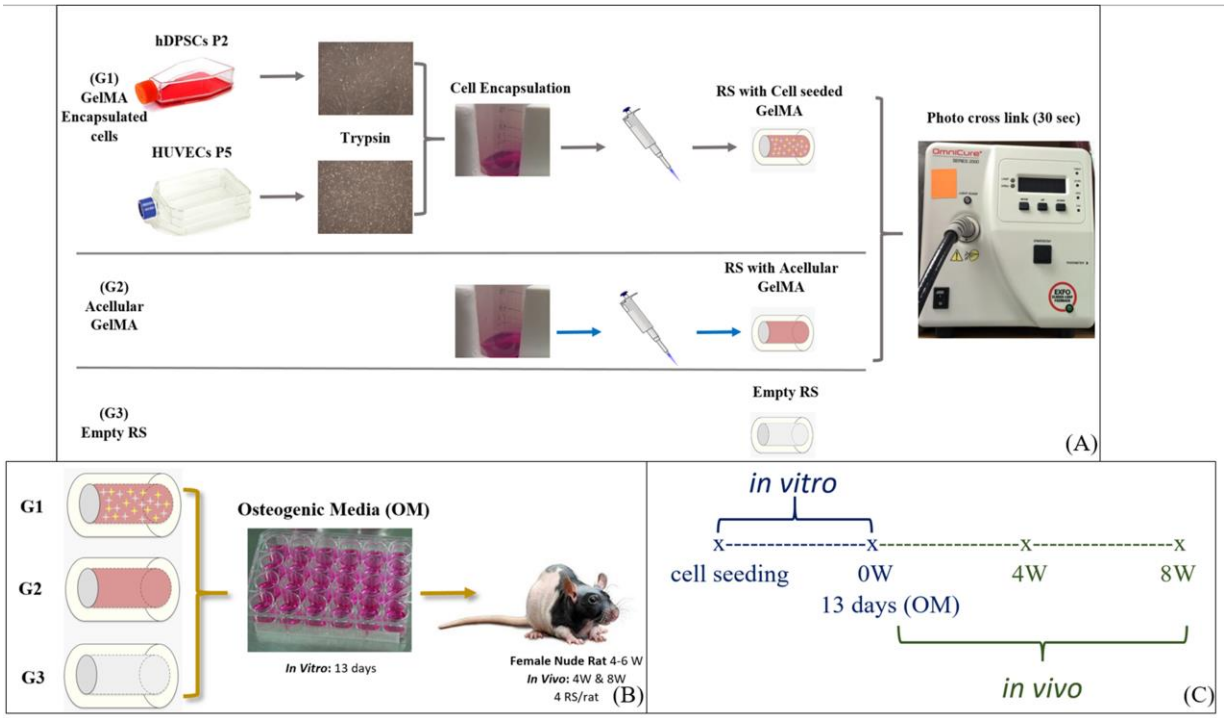
in RSs survived after 4 weeks in vivo implantation, but did not survive after 8 weeks in vivo implantation (Figure 5 C).

Our studies showed that pulp-like tissues formed within pulpal space of GelMA encapsulated hDPSC/HUVEC, acellular GelMA and empty RS. Published reports also demonstrated the formation of pulp-like tissues, including one using DPSCs and self-assembling peptide hydrogel PuraMatrix, along with growth factors FGF, TGF-β1 and VEGF [13,14]. However, a potential drawback of PuraMatrix hydrogel is that it contains animal derived components that could elicit an unwanted immune response, and form acid byproducts during degradation that might be toxic for the cells. To the best of our knowledge, GelMA degradation does not interfere with cell viability or proliferation [164].

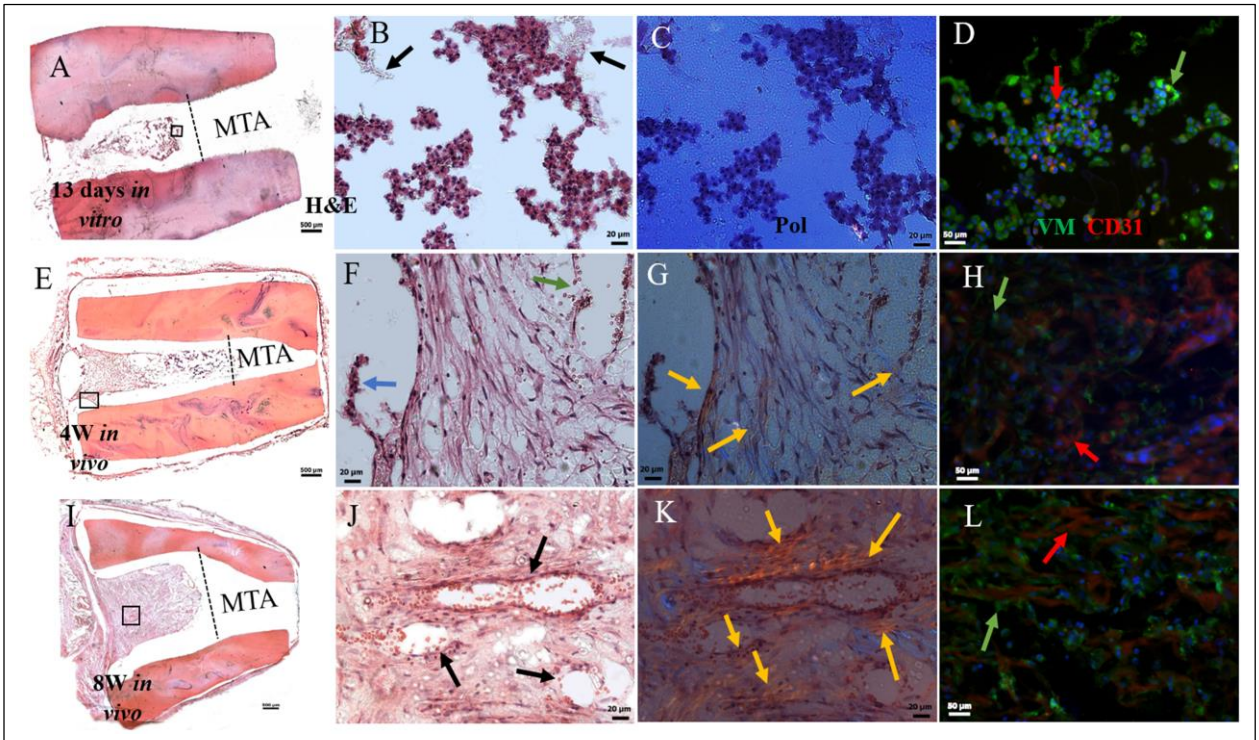
Conclusion & Future Studies

In conclusion, here we show that GelMA hydrogels can be used to support hDPSC proliferation and differentiation, and HUVEC revascularization of bioengineered dental pulp tissues. Furthermore, GelMA was attached to the host tissues and promote increased cellularity and vascularization over the time. Also, hDPSCs-HUVECs were proliferated over the time *in vitro* and *in vivo* and form pulp-like structure. Based in our results, would be a promising solution for bleeding deficiency in pulpal revascularization also it could be used to replace the infected pulpal tissues in pulpotomy. Suggested future studies needed to incorporate the efficacy of antibodies, growth factor and nanoparticles incorporated with stem cells and GelMA within full root segment. Moreover, a greater need to establish a canine model based on the previous studies is required in order to facilitate clinical application.

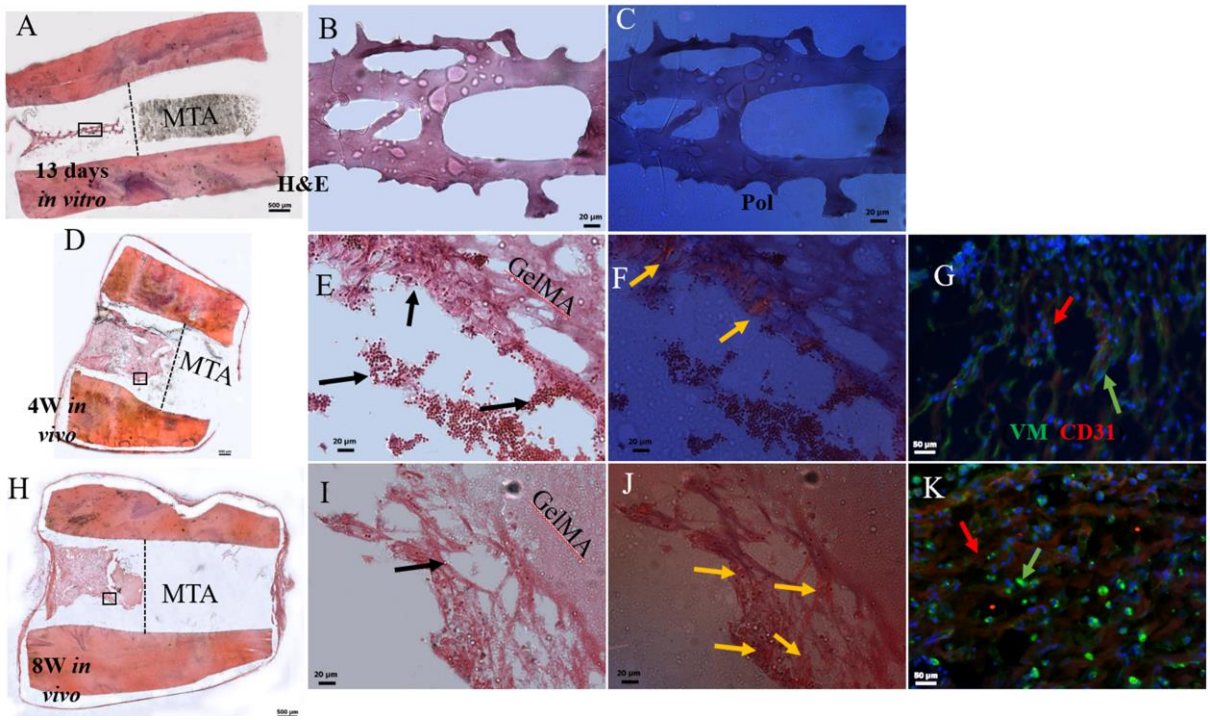
Figures



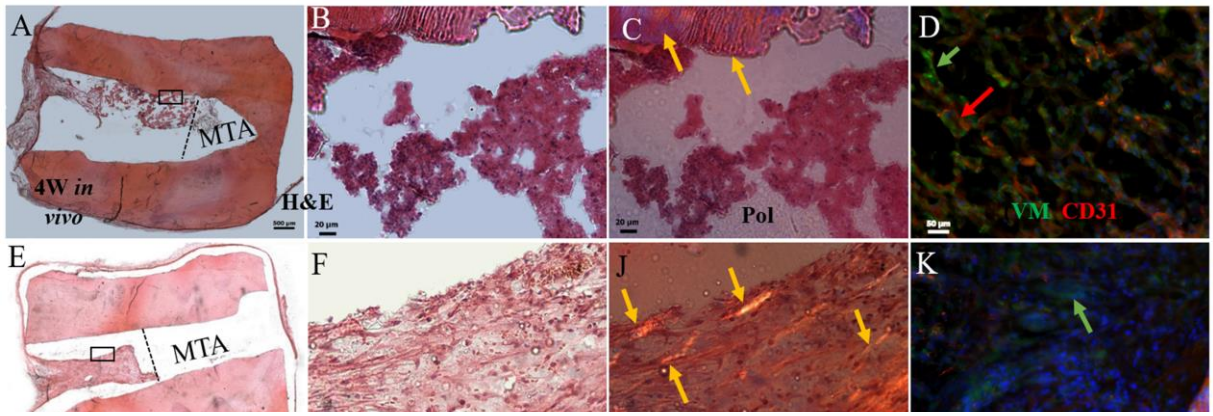
(Figure 3) Research Design: (A) in the (G1) GelMA Encapsulated Cells, both cell lines hDPSCs and HUVECs were trypsinized, then encapsulated within 5% of GelMA followed by injecting the mixture into the RS. After injecting the Acellular GelMA with RS, both groups were photocrosslinked by OmiCure Machine for (30sec.). Empty RS didn't receive any treatment in this stage. (B) all the 3 groups were placed in OM for 13 days followed by plant them subcutaneously at rat's backs for 4&8 weeks in nude rats aged between 4-6 weeks (4 RS were placed per rat). (C) represent the timeline of the study, starting with cell seeding tell removing them from OM (13 days) considered to be *in vitro* period, then 4 & 8 weeks *in vivo*.



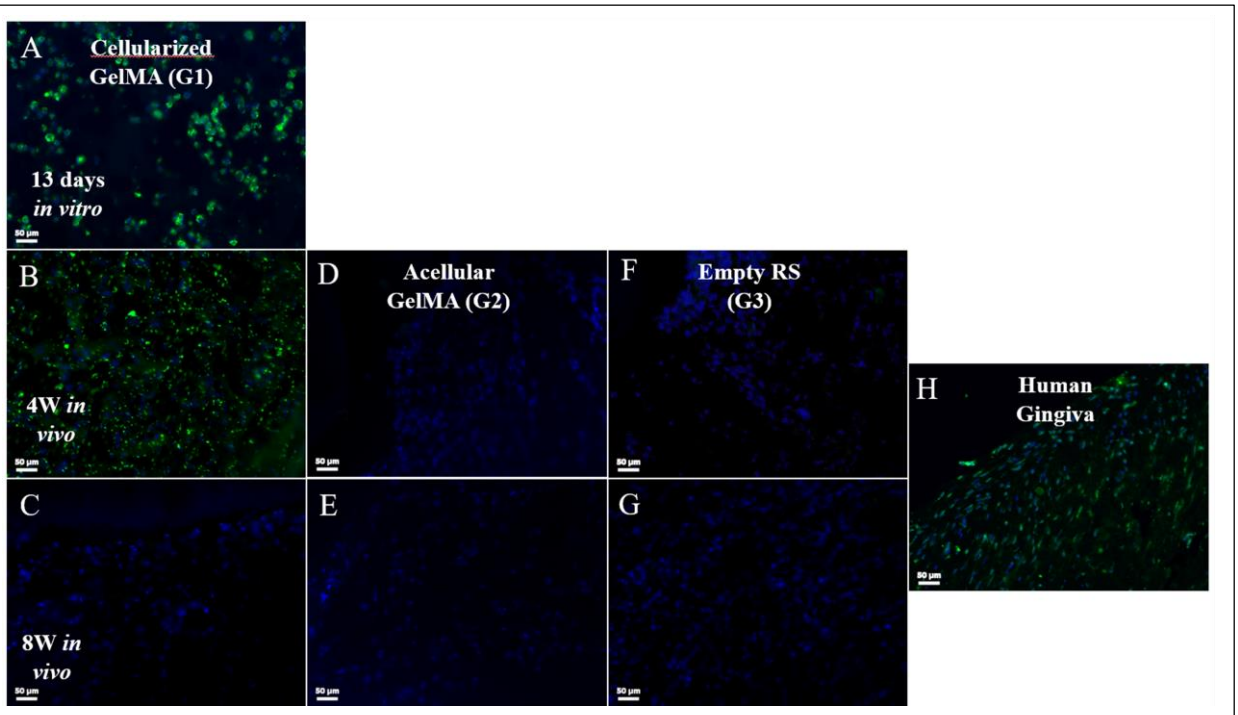
(Figure 4) GelMA Encapsulated Cells (G1) Analysis: (A) 2.5X for the entire RS of 13 days *in vitro* samples, it showed the level of the bioengineered pulp up to the MTA. Scale bar = 500 µm (B) 40X for the selected spot represent cellularity was maintained in 13 days *in vitro* within 5% of GelMA, remnant of GelMA was found in the construct as labeled by the black arrows. Scale bar = 20 µm (C) 40X (Pol) showed no ECM deposition at this stage. Scale bar = 20 µm (D) 20X of double IF CD31 in red detected the HUVECs while the Vimentin in green detected the hDPSCs. Scale bar = 50 µm. (E) 2.5X for the entire RS of 4W *in vivo*, sample were encapsulated by host cellularity. Scale bar = 500 µm (F) 40X showed odontoblast like was attached into the dentin surface (blue arrow), and neovascularization was noticed with red blood cells (green arrow). Scale bar = 20 µm. (J) (Pol) showed an obvious ECM deposition in compare to 13 days *in vitro* (yellow arrows). (H) 20x of double IF showed the cellularity been increased over the time from both cell lines. Scale bar = 50 µm (I) 2.5X for the entire RS from 8W *in vivo*, cellularity been increased in compare to 13 days *in vitro* and 4W in vivo. Scale bar = 500 µm (J) at 40X vascularization become more distinguished –Endothelia lining- in compare to 4W *in vivo*, with more red blood cells (black arrows). Scale bar = 20 µm (K) More collagen deposition was noticed in compare to 4W *in vivo* samples (yellow arrows). (D) Double IF at 20X showed both cell lines were detectable. Scale bar = 50 µm



(Figure 5) Acellular GelMA (G2) Analysis:(A) 2.5X showed acellular RS at 13 days *in vitro*, early degradation of GelMA was noticed and remnant of MTA was found in this sample. Scale bar = 500 µm (B) 40X at the selected area showed the GelMA. Scale bar = 20 µm (C) Pol didn't detect any ECM at this stage. Scale bar = 20 µm (D) 2.5X for 4W in vivo sample showed host encapsulation, more cellularity in compare to 13 days *in vitro*. Scale bar = 500 µm (E) 40X at the selected area, showed host cellularity attachment and vascularity, red blood cells were detected (black arrows) Scale bar = 20 µm. (F) Pol was able to detect some ECM from the host (yellow arrows) Scale bar = 20 µm. (G) 20X double IF showed cross reaction of the host tissues with CD31 and Vimentin Scale bar = 50 µm. (H) 2.5X showed the cellularity and host cell encapsulation for 8W *in vivo* samples. Scale bar = 500 µm. (I) 40X of the selected area showed GelMA still persisting in 8W *in vivo,* with host cellularity next to it (black arrow). Scale bar = 20 µm (J) Pol revealed more ECM deposition from the host cells in compare to 4W *in vivo* (yellow arrows). Scale bar = 20 µm. (K) Double IF (20X) showed host cross with CD31 and Vimentin similar to 4W *in vivo*. Scale bar = 50 µm



(Figure 6) Empty RS (G3) Analysis: (A) 2.5X of 4W *in vivo* showed host encapsulation and cellularity within the pulpal space. Scale bar = 500 µm (B) 40X at the selected area showed host cellularity attached to the dentin surface, and there is cellular infiltration within the pulpal space. Scale bar = 20 µm (C) Pol detected ECM from the dentin rather than the host (yellow arrow). Scale bar = 20 µm (D) Double IF at 20X showed the cross reaction of the previously antibodies with the host tissues. Scale bar = 50 µm (E) 2.5X for 8W *in vivo* samples showed better cellularity and cell encapsulation from the host. Scale bar = 500 µm (F) More distinguishable cellularity from the host in compare to 4W *in vivo* samples. Scale bar = 20 µm (J) Pol revealed more ECM deposition from the host in compare to 4W *in vivo* (yellow arrow). Scale bar = 20 µm. (K) Double IF at 20X showed the same result as 4W *in vivo* samples. Scale bar = 50 µm



(Figure 7) Rh-Mitochondria Analysis (IF): (A) rh-Mitochondria stained GelMA encapsulating hDPSCs and HUVECs at 13 days *in vitro*. Scale bar = 50 µm (B) Our implanted cells were alive on 4W *in vivo* samples within 5% of GelMA. Scale bar = 50 µm (C) revealed very week signal of rh-Mitochondria, which suggest the implanted cells were died and replaced by the host cells. Scale bar = 50 µm (D), (E) are 4-8W *in vivo* acellular GelMA, showed rh-Mitochondria was capable to differentiate between the human origin cells (hDPSCs and HUVECs) and the rat's tissues. Scale bar = 50 µm. (F), (G) are 4-8W *in vivo* empty RS has a comparable result to the acellular GelMA group, where both of them been infiltrated by the host tissues and they didn't show a (+) signals by the rh-Mitochondria. Scale bar = 50 µm. (H) Human gingiva used as a (+) for this rh-Mitochondria antibody, which obviously was capable to detect the human origin cells. Scale bar = 50 µm

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