Regenerative endodontic

A project submitted to College of Dentistry, Baghdad University, Department of Conservative Dentistry in partial fulfillment of the requirement for B.D.S. degree

By

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

يَرْفَعِ اللهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ ۚ وَاللهُ بِمَا تَعْمَلُونَ خَبِيرٌ ﴿١١﴾

صَدَقَ اللهُ الْعَظِيمُ

Supervisor Declaration

This is to certify that the organization and preparation of this project have been made by the under graduated student " **Saja Hussein Ali** " under my supervision at the College of Dentistry, University of Baghdad in a partial fulfillment of requirements of the degree of B.D.S in Preventive Dentistry.

Signature

Dr. Anas Falah Mahdee

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1 INTRODUCTION

Dental pulp is a unique soft tissue that resides in a hard tissue compartment of its own making. Its cell maintain an intimate spatial and functional relationship with surrounding tissue throughout the life. This rigid compartment of dentin –enamel in the crown and dentin- cementum in the root offers the required mechanical and microbial protection for the soft tissue of the pulp and gives the pulp its distinctive anatomical features (Goldberg and Smith 2004) . The unique nature of the dental pulp, relates most importantly to the presence of highly specialized odontoblast at its periphery and extension of cytoplasmic processes within dentinal tubules. In addition to forming dentin, odontoblast may have sensory function and they may sense and respond to microbial antigens in the same manner as immunological cells. Both of these functions indicate that odontoblast are more active in the regulation of the defensive reactions of the dentin – pulp complex (Tjäderhane, Nascimento et al. 2013) . If the rigid chamber loses its structural integrity, the pulp is at a risk of adverse stimulation from the oral cavity, Caries, tooth wear, cracks, fractures, and open restoration margins provide an open route for the oral micro flora and their toxins to access the pulp (Tjäderhane and Haapasalo 2009). Recent advances in biotechnology and translational research have made it possible to provide treatment modalities that protect the vital pulp, allow manipulation of reactionary and reparative dentinogenesis and more recently permit revascularization of an infected root canal space. The dental pulp is a complex specialized connective tissue that is enclosed in a mineralized shell and has a limited blood supply; these are only a few of

the many obstacles faced by the clinician researchers attempting to design new therapeutic strategies (Diogenes, Ruparel et al. 2016).

Regenerative dental procedures have a long history originating around 1952 , when   Dr.B.W.Hermann reported on the application of Ca(OH)2 in case report of   vital pulp amputation (Holland, Pinheiro et al. 1982). The management of immature permanent teeth with pulpal necrosis is challenging as the root canal system is often difficult to debride and the thin dentinal walls are at an increased risk of a subsequent cervical fracture.This result in a restorative problem since implant is generally contra indicated in young patients with a growing craniofacial skeleton (Hargreaves, Diogenes et al. 2013) .

This review provides an overview of regenerative endodontic and its goal, and describes possible techniques that will allow regenerative endodontic to become a reality. These potential approaches include root canal revascularization , postnatal (adult) stem cell therapy , pulp implant , scaffold implant , three dimensional cell printing , injectable scaffolds ,and gene therapy clinical use (Araújo, Silva et al. 2017) .

The purpose of this review is to evaluate the effects of regenerative endodontic treatment for necrotic immature permanent teeth and to discuss recent treatment approaches.

2 Pulp biology

2.1 Odontoblast

Odontoblasts (Od) are derived from embryonic connective ectomesenchymal cells from the cranial neural crest (Mitsiadis and Graf 2009). Signaling molecules from the primary and secondary enamel knots regulate the differentiation of the post-mitotic outer cells of the dental papilla into Odontoblasts (Thesleff, Keranen et al. 2001).

The main function of the Od is secretion of dentine matrix to form tubular, primary dentine, and both physiological secondary and reactionary tertiary dentine. These functions may be preserved throughout life. The Ods secrete extracellular type I collagen-rich matrix to form predentine, in addition to a number of non-collagenous proteins including glycoprotein, proteoglycans, and dentine phosphoproteins to biomineralise the dentine organic matrix (Tjäderhane and Haapasalo 2009).

Odontoblasts also serve inflammatory, sensory and reparative roles in response to aging and injury, including dental caries, tooth wear and operative dental intervention. The position of the Ods at the interface between the pulp and dentine with their cellular processes extending far within the dentinal tubules, and the presence of the partially impermeable barrier as a pseudo-epithelial layer, makes them the first cell line to encounter microorganisms and their products from the oral cavity (Magloire H 2010) . It has been reported that the Ods provide an innate immune barrier by expressing TLR2 and TLR4 receptors (inflammatory receptors on the cell membrane of mammalian cells activated during

contact with the bacteria) on their dentine interface. This suggests that the pro-inflammatory cytokines and innate immune responses to tooth injury could be initiated from TLR4 signaling of the Odontoblasts (Pevsner-Fischer M 2007).

2.2 Odontoblast processes

The Odontoblast cell process is a gradually narrowing cytoplasmic process that penetrates into mineralized dentin, filling the lumen of dentinal tubule. The Odontoblast process lacks cell organelles but contains secretory vacuoles, numerous longitudinally arranged actin filaments, and microtubules (Sigal, Aubin et al. 1984). One of the longest-lasting and most controversial questions in research on the dentin–pulp complex has been the extent of Odontoblast processes into dentinal tubules.

The controversies are often related to the techniques used to study the processes. Numerous studies, involving a range of methods including; scanning electron microscopy (SEM), transmission electron microscopy, confocal laser microscopy, and immunofluorescence microscopy have suggested that the Odontoblast processes are limited to the inner third of the dentinal tubules. Other studies have reported the existence of the OPs in peripheral dentine. It is an interesting area for further investigation to understand the role of these processes after tooth development such as sensing, maintaining dentine integrity, responding to different stimuli, and the consequential physiological and pathological reactions (Tjäderhane and Haapasalo 2009).

2.3 Dentinal tubules

Tubularity is a central characteristic of dentin, affecting, for example, its mechanical properties, its ability to withstand occlusal forces, and its behavior in dentin bonding. The understanding of dentin three-dimensional structure may have a significant impact on optimal cavity design and restorative procedures (Zaslansky, Zabler et al. 2010). The number of dentinal tubules in different locations in relation to the DEJ or cementum does not vary except under the cuspal area, where the number of dentinal tubules close to the DEJ is significantly higher. This may relate to the regulation of the pulp– dentin defensive systems against wear (Pashley 1996).

In addition to the main tubule, dentinal tubules have numerous branches and ramifications. The number of branches is higher in areas where the density of the main tubules is low, forming an abundant anastomosing system of canaliculi very much like osteocytes in bone (Mjör and Nordahl 1996; Lu, Xie et al. 2007).

A collar of highly mineralized dentine called peritubular dentine surrounds dentinal tubules. This type of dentine measures about 1μm in thickness, is more highly mineralized than intertubular dentine (about 40% more), with little or no collagen but rich in phosphoproteins (Gotliv and Veis 2009).

The thickness of this type of dentine varies greatly depending on the distance from the pulp, being very thin or completely absent near the pulp and with greatest thickness near the DEJ. Peritubular dentine has a porous nature, and the boundaries of dentinal tubules are fenestrated by numerous tiny pores, which, together with the branches of the dentinal tubules, facilitate the movement of fluids and other matrix components across the peritubular dentine in all directions (Mjör and Nordahl 1996) .

The main bulk of dentine is composed of intertubular dentine, which fills the spaces between the rings of peritubular dentine. About half of its composition is organic, mainly made up of collagen fibers that run circumferentially around each tubule. Because of this organic matrix, intertubular dentine is usually preserved after pathological or laboratory decalcification (Beniash, Traub et al. 2000).

2.4   Dentinogenesis

In different mineralised tissues, biomineralisation is always a cellular responsible process in which cells direct the formation of mineralised tissue (Tjäderhane, Buzalaf et al. 2015).
 Several simultaneous events are necessary to perform biomineralization of collagenous matrix

(a) Cellular synthesis of extracellular matrix molecules, including type I collagen and non-collagenous proteins;

(b) cell-derived accumulation of mineral ions;

(c) cell-directed modification of the matrix to allow precise and controlled mineralization;

(d) Deposition of initial mineral crystals; and

(e) Regulated growth and accumulation of hydroxyapatite, perhaps accompanied by further remodeling of the matrix.

The rate of dentin formation is dependent on the age and function of the tooth and the site in which the cell is situated (Love and Jenkinson 2002).

2.5   Secondary Dentinogenesis

This is the process of dentine deposition after eruption of the tooth crown to reach its functional contact. At this time, the crown has fully formed by enamel and dentine deposition, while the root is still developing. Secondary dentine is structurally similar to primary dentine and composed of intertubular and peritubular dentine. Slight differences in the curvature and regularity of dentinal tubules has been detected (Couve 1986). The continuity in dentinal matrix secretion in predentine results in increased thickness of primary and/or physiologically deposited secondary dentine, i.e. decrease in pulp chamber volume (as show in figure 1).

This possibly promotes reduction in the cellular densities of Ods, sub Odontoblast (Sods), and pulp fibroblast. This process is radio graphically detectable especially on the floor and roof of the pulp chamber and results in limiting the space of the pulp chamber and canals in elderly patients (Slane, Helfand et al. 2002). The continuous deposition of dentine progressively decreases both the volume occupied by pulp fibroblasts and the dentine/Od interface. This age-dependent reduction in the cellular

population possibly causes a decrease in the regenerative capacity (plasticity) of the pulp (Bluteau, Luder et al. 2008).
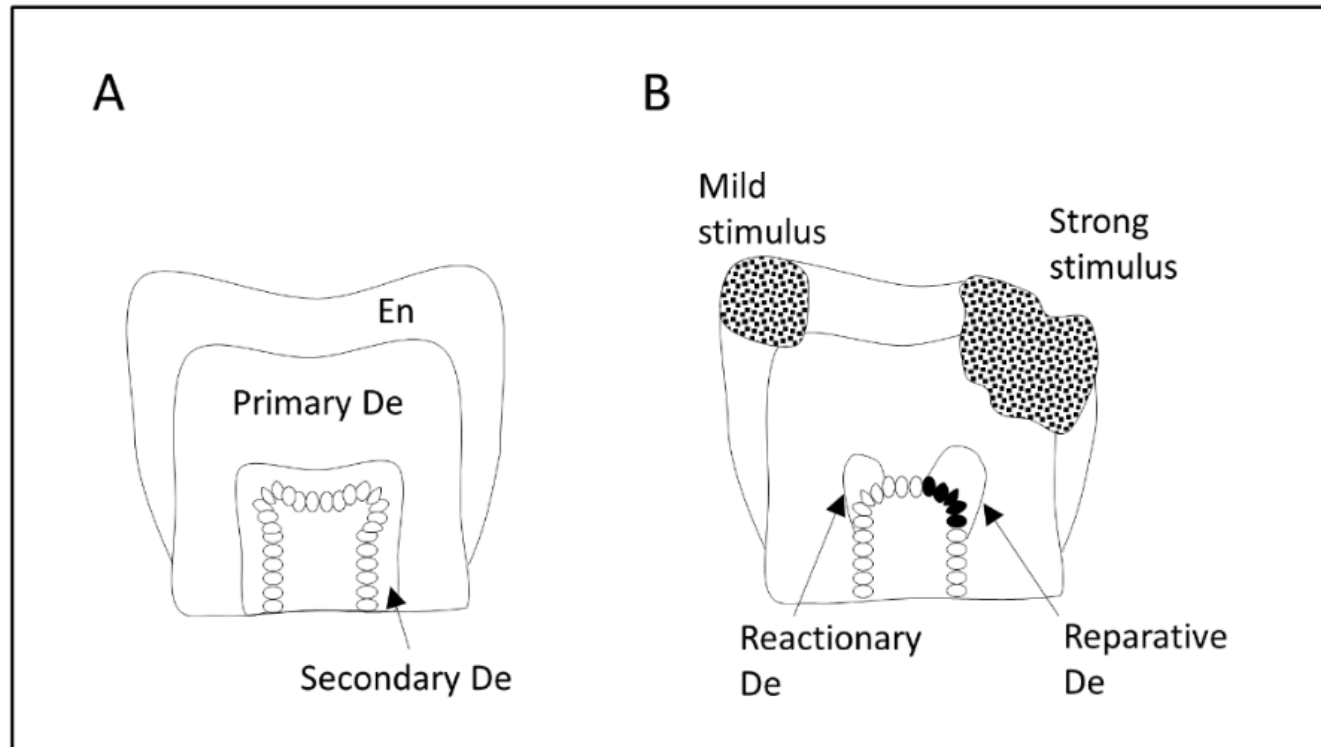
Figure 1: Schematic illustration of dentine type (Mahdee 2017)

2.6 Tertiary Dentinogenesis

Tertiary dentin forms as a response to external irritation—attrition, abrasion, erosion, trauma, caries, or cavity preparation—in order to increase the thickness of the mineralized tissue barrier between the oral microbes and the pulp tissue. It has also been called irritation dentin, irregular dentin, irregular secondary dentin, etc (Cox, White et al. 1992). Generally, two forms of tertiary dentin are recognized: reactionary dentin (produced by original primary Odontoblasts) and reparative dentin (produced by newly differentiated replacement Odontoblasts) (Mjör 1985; Yamamura 1985) (as show in figure 1). In addition, it has been

suggested that a third type of mineralization be distinguished as a merely defensive non-specific production of mineralized matrix. This would be produced by so-called fibrodentinoblasts, and the product would be called fibro- or osteodentin or pulp stone formed in regions of the pulp chamber or root canals, which may cause difficulties, or failure of root canal treatment (Magloire, Christophe Maurin et al. 2010).

The goal of the tertiary dentine deposition, in addition to increasing dentine thickness, is to decrease dentine permeability beneath an injury, and thus to isolate the irritated pulp area from further stimulation (Becerra, Ricucci et al. 2014). If a second injury occurs such as a carious lesion, the presence of reparative dentine possibly decreases the pulp's response and the defense reaction after repeated insults (Farges, Alliot-Licht et al. 2015).

2.7   Tooth innervation

  The histological examination of nerve fibers entering the tooth reveals both myelinated and unmyelinated types. The majority of these nerves are nociceptive sensory fibers, myelinated A-delta and unmyelinated C fibers. The cell bodies of these fibers are mainly located in the Gasserian (semilunar) trigeminal ganglion. The unmyelinated fibers have also shown some minor quantities of both sympathetic and parasympathetic (Abd-Elmeguid and Yu 2009). Almost all nerve fibers enter the tooth in bundles through one or more apical foramina.

These bundles pass through the radicular pulp in association with blood vessels as neuro-vascular bundles. Very few nerve fibers appear to be terminated within the root and these fibers may reach the radicular Od layer. However, the rest of the nerve fibers terminate within the tooth crown (Mahdee 2017).

2.8 Pulp Vascularization and Its Regulation by the Microenvironment

The main cell-forming unit of the vascular system is the endothelial cell, which forms the internal lining of blood vessels. These cells are derived from mesoderm stem cells which give rise to haemangioblast precursor cells which in turn give rise to the hematopoietic stem cells and angioblasts: the progenitors of endothelial cells (Asahara, Murohara et al. 1997). Thus, blood cells and endothelial cells share a common origin and remain tightly linked to each other during adult life (as show in figure 2). Endothelial cells connect to each other and organize into hollow and interconnecting structures forming the blood vessels of the tooth by a process called vasculogenesis(as show in figure 2) (Fong, Rossant et al. 1995).
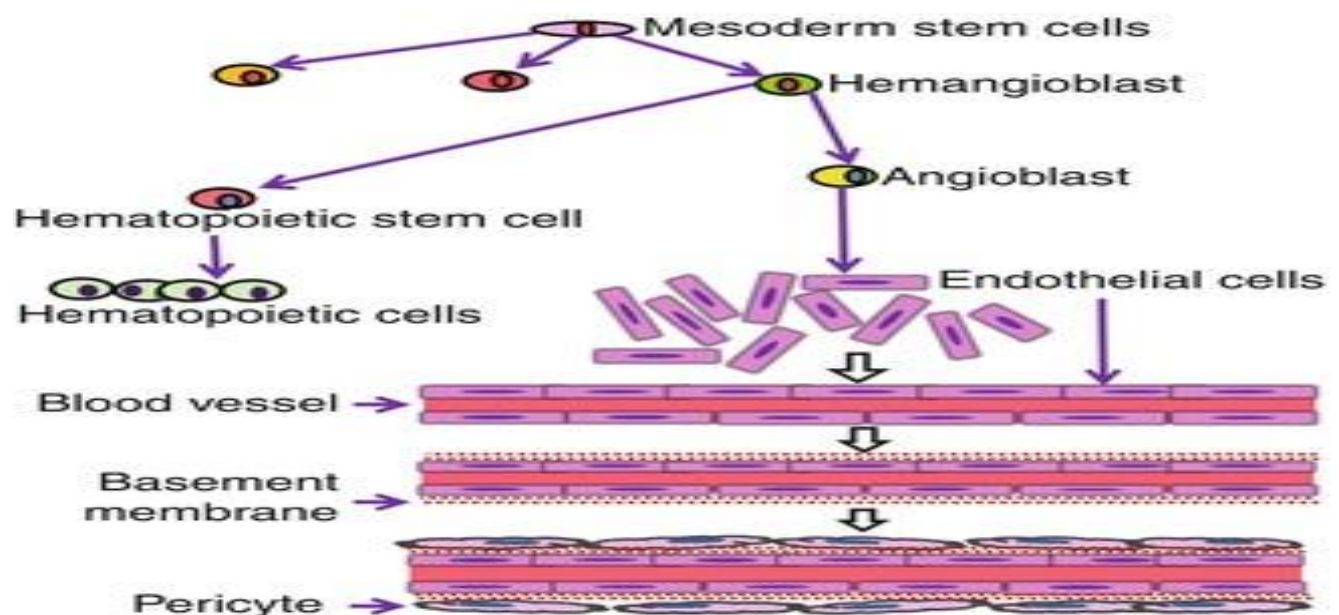
Figure .2 Origin of endothelial cells and vasculogenesis (Goldberg 2014).

At later developmental stages and in the mature pulp, endothelial cells form blood vessels by another process called angiogenesis . This process implies the formation of new blood vessels by "sprouting" from pre-existing blood vessels. It is a critical part of the wound healing process in all tissues, and the local pulpal angiogenesis is a prerequisite for successful repair in the tooth (Leung, Cachianes et al. 1989).

 It is also a key process in tissue engineering procedures. This process is regulated by many inductive and inhibitory signals (Carmeliet and Jain 2011; Bronckaers, Hilkens et al. 2013) (as show in figure 3).

 Among all the pro-angiogenic factors, VEGF is considered the most essential for the differentiation of the vascular system.

 After the initial vasodilatation of acute inflammatory reaction, which increases blood supply to the pulp, an adaptive formation of capillaries by neoangiogenesis is initiated as a reaction to hypoxia in ischemic tissues during the regeneration process (Carmeliet 2001).
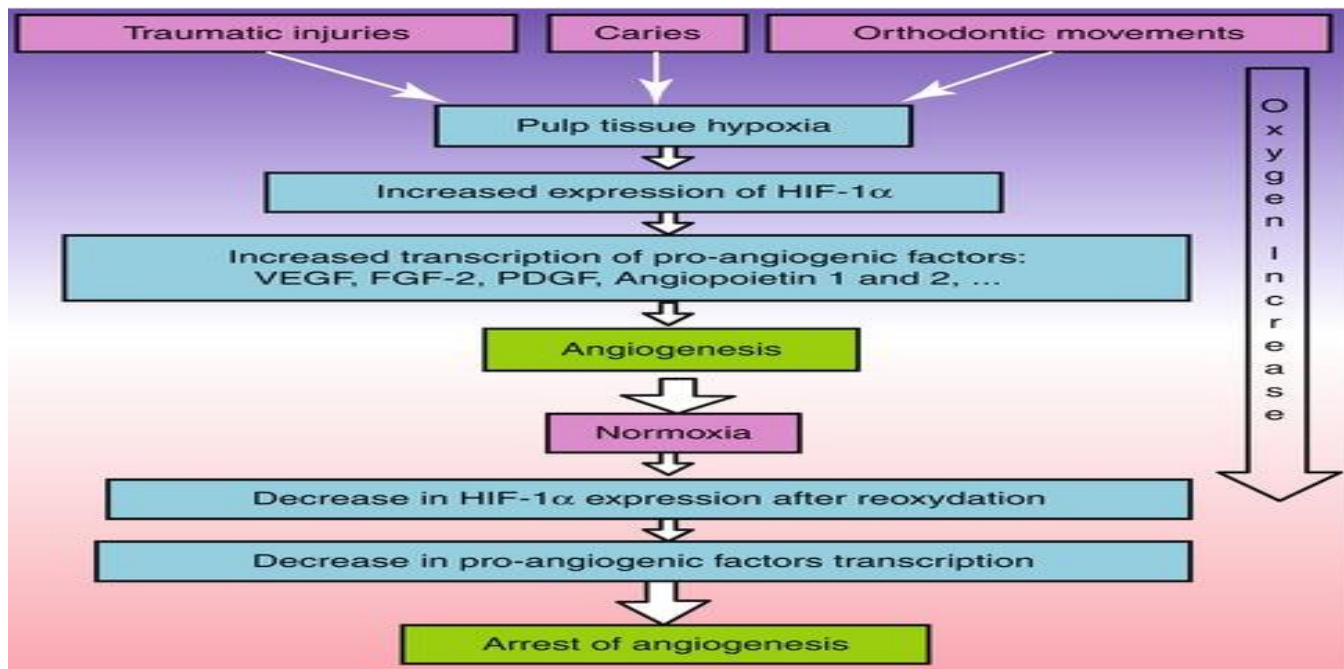
Figure 3. Pulp hypoxia induces neo-angiogenesis (Goldberg 2014). Maintenance of oxygen homeostasis is essential to all living tissues. It is regulated through the activity of hypoxia-inducible transcription factor-1 (Hif-1) (Jiang, Rue et al. 1996). Hif-1α plays a key role in angiogenesis by activating the transcription of genes encoding pro-angiogenic growth factors including VEGF, FGF-2, angiopoietin 1 and 2 (Ang1, Ang2), placental growth factor (PGF), PDGF and angiogenic receptors (Jiang, Rue et al. 1996; Aranha, Zhang et al. 2010) .

 Interestingly, the fact that Hif expression increases in dental pulp stem cells implies that these cells may be activated under hypoxia not only to regenerate dentin but also to regenerate the pulp vasculature (Linden, Irwin et al. 2009).

3 Dental trauma and inflammation

3.1 The pulpal environment and injury responses

The nature of the bacterial challenge will vary depending on disease progression and the extent of existing tooth tissue loss. At earlier stages, relatively small bacterial products may begin to diffuse within the tubules to the pulp, but with increasing disease progression, permeability of the tissues will increase and allow intact bacteria to migrate and colonize the deeper areas of the dentin and pulp (Demarco, Conde et al. 2011). Proteomic analysis of dentin already indicates the presence of up to nearly 300 distinct proteins, and many of these display bioactive properties capable of signaling a multitude of cellular events in both tissue- resident cells and those recruited to the pulp as a part of the immune defense and wound healing processes (Park ES Res. 2009).

 Some signaling pathways are common to a number of cell types and their processes, which lead to more unpredictable effects of the combined challenges from bacterial and dentin matrix exposure. For example, p38

mitogen-activated protein kinase (MAPK) signaling has been implicated in the control of Odontoblast secretory activity during tertiary dentinogenesis (Simon S 2013).

Tertiary dentinogenesis represents a repair response of the dentin-pulp, ultimately aimed at tissue regeneration if conditions are permissive. This repair process may be further sub classified into reactionary and reparative dentinogenesis depending on whether the formative cells are surviving post mitotic primary Odontoblasts or a new generation of Odontoblasts-like cells arising from differentiation of stem/progenitor cells due to local death of the primary Odontoblasts (Veerayutthwilai O 2007) (as show in figure 1).

During reparative dentinogenesis, involvement of pulp-derived mesenchymal stem cells (MSCs) may influence defense events through their immunomodulatory properties .Thus, it is important that inflammation and repair/regeneration are considered as overlapping and interrelated processes (Leprince JG 2012) (as show in figure 4).
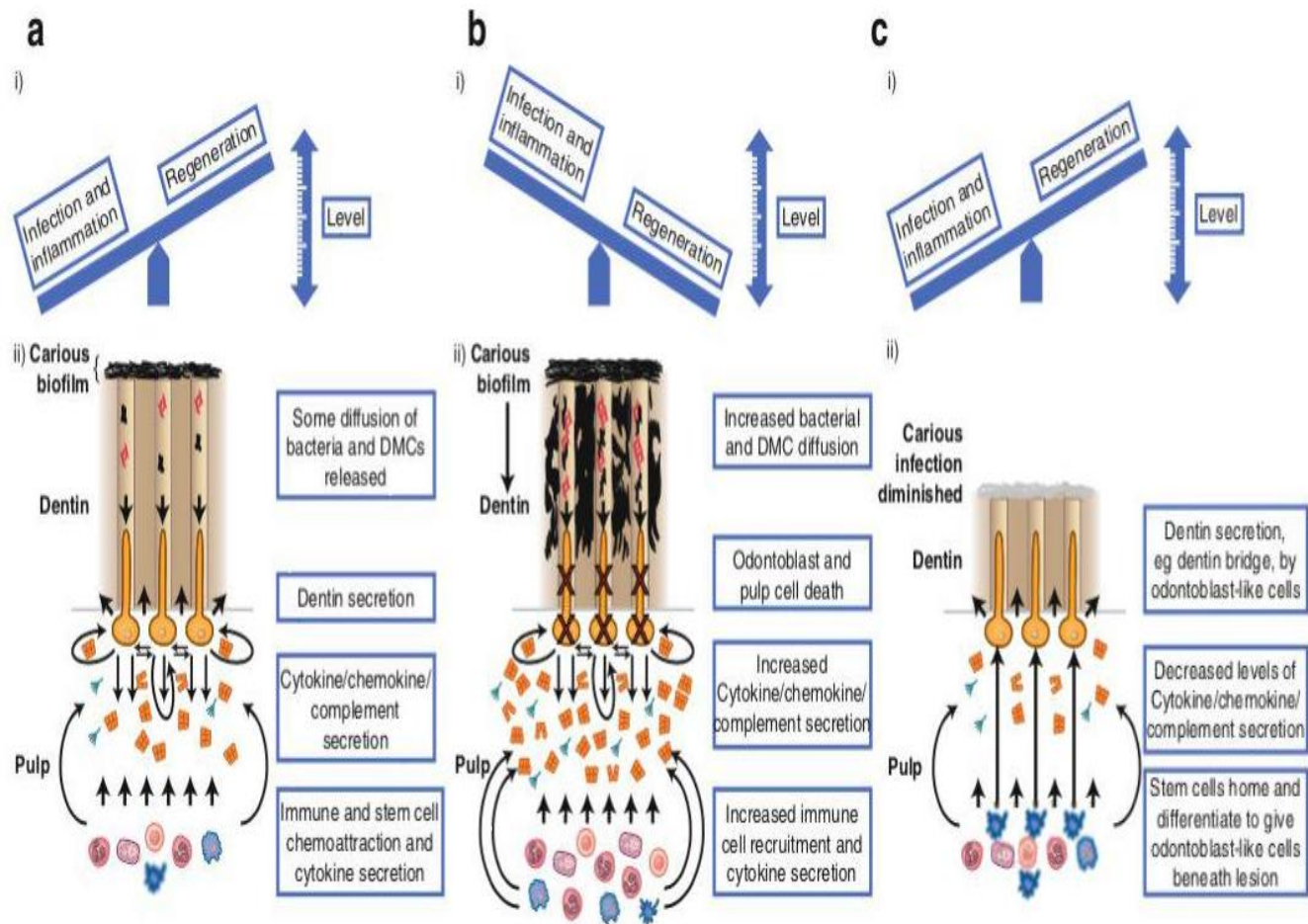
a

i)

Infection and inflammation — Regeneration

Level

ii) Carious biofilm

Dentin

Pulp

Some diffusion of bacteria and DMCs released

Dentin secretion

Cytokine/chemokine/ complement secretion

Immune and stem cell chemoattraction and cytokine secretion

b

i)

Infection and inflammation — Regeneration

Level

ii) Carious biofilm

Dentin

Pulp

Increased bacterial and DMC diffusion

Odontoblast and pulp cell death

Increased Cytokine/chemokine/ complement secretion

Increased immune cell recruitment and cytokine secretion

c

i)

Infection and inflammation — Regeneration

Level

ii)

Carious infection diminished

Dentin

Pulp

Dentin secretion, eg dentin bridge, by odontoblast-like cells

Decreased levels of Cytokine/chemokine/ complement secretion

Stem cells home and differentiate to give odontoblast-like cells beneath lesion

Figure 4. Inflammatory process of the pulp (Goldberg 2014) .

   a) Early stage of carious disease with minimal hard tissue involvement.

   b) Chronic and later stages of carious disease with increasing hard tissue involvement.

   c) Resolution of infection and modulation of inflammation.

3.2 Innate and Adaptive immune responses

Both innate and adaptive immune responses encompass a complex range of cellular and molecular events. While the earlier responses to bacteria and other injurious challenges generally reflect innate immunity, the transition to adaptive immunity is a gradual one as infections become

chronic and will vary in the same way as disease progression in each individual patient varies (Mejàre IA 2012).

These processes aim to both recruit circulating immunocompetent cells from the vasculature to eliminate pathogens and necrotic tissue debris and stimulate responses by resident cells in the pulp to minimize tissue damage and initiate reparative and regenerative events (Smith AJ 2012).

3.2.1 Bacterial pathogen recognition

Pattern recognition receptors (PRRs) are a group of cell membrane- and endosome-bound receptors, which can recognize ligands (pathogen associated molecular patterns or PAMPs) that are broadly shared by pathogens but which are distinct from host molecules (R 2001).

The Toll-like receptors (TLRs) are a key family of PRRs, which play a central role within the innate immune system in the recognition of their ligands or PAMPs.

TLR-1 to TLR-6 and TLR-9 expression has been detected in Odontoblasts and pulpal fibroblasts, and binding of these PRRs to their respective ligands initiates an acute inflammatory response, leading to activation of cells and release of pro inflammatory mediators (Pevsner-Fischer M 2007).

3.2.2 Early vascular response

An early feature of pulpal inflammation is changes to the vascular flow in the pulp with vasodilation and increases in blood flow. These changes are associated with increased fluid and plasma protein exudation and

recruitment of leukocytes. Fluid exudation or edema during acute inflammation classically gives rise to swelling in soft tissues, such Swelling is constrained in the pulp by the covering hard shell of mineralized dentin.

Key molecular mediators of these vascular responses may include histamine, endothelin, neuropeptides, and serotonin (Yu CY 2002). Several molecular cascade systems, based on plasma proteins, act in parallel to these cell derived mediators to further initiate and propagate the acute inflammatory response. The Complement system will likely play a role in leukocyte recruitment in the pulp as well as potentially progenitor cell recruitment for subsequent regenerative events (Chmilewsky F 2013).

3.2.3 Leukocyte recruitment

Recruitment of leukocytes to sites of inflammation is an important aspect of pathogen elimination through phagocytosis and degranulation mechanisms. Increases in vascular permeability facilitate their migration through the endothelial lining, and such extravasation is carefully regulated by the action of molecules involved in their adhesion and transmigration (Smith JG 2012). Several inflammatory mediators also show chemotactic properties including complement components C3a and C5a, the arachidonic acid metabolites, and the leukotrienes (especially leukotriene B 4 –LTB 4). Growth factors, cytokines, and chemokines also modulate chemotaxis. The chemotactic effects of some of these molecules influence migration of both pulp progenitor and inflammatory cells highlighting the interplay between inflammatory defense and regenerative events in this tissue following injury (Chmilewsky F 2013).

3.2.4 Cytokine and Chemokine Mediation of Inflammatory and Post-injury Events

PAMP recognition by TLRs on Odontoblasts and pulpal fibroblasts results in activation of the nuclear factor kappa B (NF-κB) intracellular signaling pathway, which is central to regulation of the molecular inflammatory response in many cell types (Veerayutthwilai O 2007).

A range of cytokines and chemokines are produced as a result of activation of NF-κB signaling.

These cytokines and chemokines are synthesized by a variety of immune and tissue structural cells in response to infectious and traumatic challenge and have potent cellular signaling properties. The immunomodulatory actions of these cytokines and chemokines will impact on both innate and adaptive immune processes, including extravasation, leukocyte recruitment, cell activation and differentiation, and antibody production, as well as regenerative events associated with the wound healing response (Pevsner-Fischer M 2007; Botero TM 2010).

3.2.5 Immune Cell Mediation of Innate and Adaptive Immune Responses

T- and B-lymphocytes, plasma cells, neutrophils, and macrophages are observed to infiltrate the pulp in increasing numbers as carious disease progresses. As caries extends, the immune cell infiltrate in the pulp will also increase and will change from being more focal and localized to a much more extensive presentation. Such changes reflect the transition from more acute to chronic inflammation (Hahn CL 1989; Izumi T 1995).

3.2.6 Anti-inflammatory Activities and Inflammation Resolution

In ideal circumstances, immune defense following injurious challenge to a tissue will lead to elimination of the infecting agent and ultimately provides a conducive environment within which wound healing can occur. Such circumstances are not easily achieved in the dentin-pulp with its noncompliant environment and the exposure of the tooth to the oral cavity with its complex micro flora and abundant supply of nutrient (Maderna P 2009).

Identification of several anti-inflammatory and pro-resolving mediators has started to clarify how inflammation may be suppressed after it has achieved its purpose.

Three distinct families of anti-inflammatory pro-resolving lipid mediators are now recognized: resolvins, protectins, and maresins (CN 2009).

Resolvins suppress proinflammatory mediator production and regulate neutrophil movement to sites of inflammation. Protectins can block T-cell migration and secretion of TNF-α(tumor necrosis factor alpha) and IFN-γ (interferon-gamma) and promote T-cell apoptosis as well as the chemokine receptor CCR5 on neutrophils to suppress chemokine signaling. The recently discovered maresins are produced by macrophages and inhibit proinflammatory mediator production by LTA4 hydrolase(Leukotriene A4 hydrolases) (Dalli J 2013). They represent exciting targets for the modulation of inflammatory activity, and the use of analogs may potentially provide novel therapeutic tools for clinical management of inflammation (CN. 2009).

3.2.7 Inflammation-Regeneration Cross Talk in Dentin-Pulp after Injury

Current evidence suggests that reparative and regenerative processes ensue only after significant control or resolution of infection and inflammation has occurred (G. 1981) .

The inflammation is an important prerequisite to enable repair and regeneration.

However, some cytokines, such as TNF-α, can also stimulate pro-regenerative/reparative signaling, including via p38 MAPK pathway activation, leading to Odontoblast-like differentiation of dental pulp stem cells with increased dentin phosphoprotein (DPP) and dentin sialoprotein (DSP) expression and tertiary dentinogenesis (Botero TM 2010) . Pulp-capping agents, such as calcium hydroxide and mineral trioxide aggregate (MTA), have long been used to stimulate reparative dentinogenesis following pulpal disease. Although the precise mechanisms of action of these agents remain controversial, it has been suggested that hydroxyl ion release from the material leads to high pH conditions locally in the tissue resulting in cell necrosis (H. 2002). Chemical irritation of vital pulp tissue beneath the area of necrosis was proposed to stimulate reparative processes. Other possible mechanisms of action, including the local dissolution of growth factors and cytokines from the dentin matrix (Graham L 2006), have also been proposed. It is now known that necrotic cells release low levels of proinflammatory mediators , and these may

promote regenerative/reparative events if the levels of release do not become excessive (Magalhães-Santos IF 2005).

4 Treatment

4.1 Direct pulp capping

Direct pulp capping is defined as the ''treatment of an exposed vital pulp by sealing the pulpal wound with a dental material placed directly on a mechanical or traumatic exposure to facilitate the formation of reparative dentin and maintenance of the vital pulp''(chong 2010) . The pulp wound should be cleansed of debris and the hemorrhage arrested by applying pressure using sterile paper points or cotton wool; saline and sodium hypochlorite solution can also be useful. When the wound is dry, the pulp-capping agent should be placed over the exposure, followed by a zinc oxide–eugenol or glass ionomer as a base and then a permanent restoration. Delay in placing the permanent restoration reduces the prognosis of the procedure due to the likelihood of microleakage (Barthel CR 2000).

Response of Human Dental Pulp Capped with Calcium Hydroxide Powder, Mineral Trioxide Aggregate, Biodentin and Bioactive-glass

Numerous studies have shown that Ca(OH)2 should be the material of choice among the available pulp capping materials (Iwamoto CE 2006). However, it has been reported that Ca(OH)2 does not adhere to dentin and dissolves over time, and dentin bridges adjacent to the material may contain multiple tunnel defect (Cox CF 1996). Studies have shown that mineral trioxide aggregate (MTA) may be used as an alternative to

Ca(OH)2 for treating pulp wounds (Min KS 2008) (Eskandarizadeh A 2011) . MTA stimulates formation of dentin bridges faster than calcium hydroxide , consequently leading to pulp healing, and results in high success rates in clinical procedures (Eskandarizadeh A 2011) (Zarrabi MH 2010).

In research by Iwamoto et al, show that there is no significant differences in clinical and histologic results between MTA and calcium hydroxide (Iwamoto CE 2006).

In addition, 2 randomized controlled studies have shown that MTA may result in similar clinical outcomes as calcium hydroxide after capping caries pulp exposures (Qudeimat MA 2007; Tuna D 2008) .

MTA is a bioactive, biocompatible, antibacterial material with unique stability and high sealing ability (Iwamoto CE 2006; Eskandarizadeh A 2011). However, MTA is reportedly difficult to use because of its long setting time, poor handling properties, high material costs, and the discoloration potential of dental tissue   (Parirokh M 2011). Many attempts made to improve the clinical manageability of MTA by adding a setting accelerator or a dual functional modifier (Bortoluzzi EA 2008; Villat C 2010). The addition of CaCl2 to MTA enables an increased immediate pH value and decreased setting time and improves the mechanical properties (Parirokh M 2011). To prevent discoloration, the manufacturer introduced a new MTA formula with an off-white color (Damamaschke T 2005), but white MTA has a significantly slower setting time compared with gray MTA (Islam I 2006).

Biodentine is a new calcium silicate–based restorative cement with dentin-like mechanical properties, which used as a dentin substitute on crowns and roots similar to how MTA is used. It has a positive effect on vital pulp cells and stimulates tertiary dentin formation (Zanini M 2012). In direct contact with vital pulp tissue, it also promotes formation of

reparative dentin (Tran XV 2012). Biodentine had a similar efficacy in the clinical setting and may be considered an interesting alternative to MTA in pulp-capping treatment during vital pulp therapy (Zanini M 2012) .

 Bioactive-glass (B-G) has become a valuable adjunct to promote hard-tissue healing in many clinical situations and is of particular interest for endodontic care because of its biocompatibility, regenerative and antimicrobial properties as well as chemical composition (silicium, sodium, calcium and phosphorus oxides with specific weight percentage) that closely resembles the mineral make-up of human bone and dentine (Prucher 2017).

4.2 Regenerative endodontic

The management of immature permanent teeth with pulpal necrosis is challenging as the root canal system is often difficult to debride and the thin dentinal walls are at an increased risk of a subsequent cervical fracture (M 1992) . Regenerative endodontic therapy provides an alternative treatment approach that builds on the principles of regenerative medicine and tissue engineering. The aim of the therapy is to successfully treat these challenging cases by regenerating functional pulpal tissue utilizing protocols referred to as regenerative endodontic procedures (REPs). Regenerative endodontic therapy has been defined as "biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex'' (Murray PE 2007). Regenerative endodontics comprises research in adult stem cells, growth factors, organ-tissue culture, and tissue engineering materials. Often these disciplines are combined , rather than used individually to create regenerative therapies (as show in figure 5). There are two strategies for dental pulp tissue regeneration:(a) cell-

based approaches, which involve the transplantation of cells into the root canal, and (b) cell-free approaches, which is centered around the recruitment of host cells into the pulp chamber (Goldberg 2014).
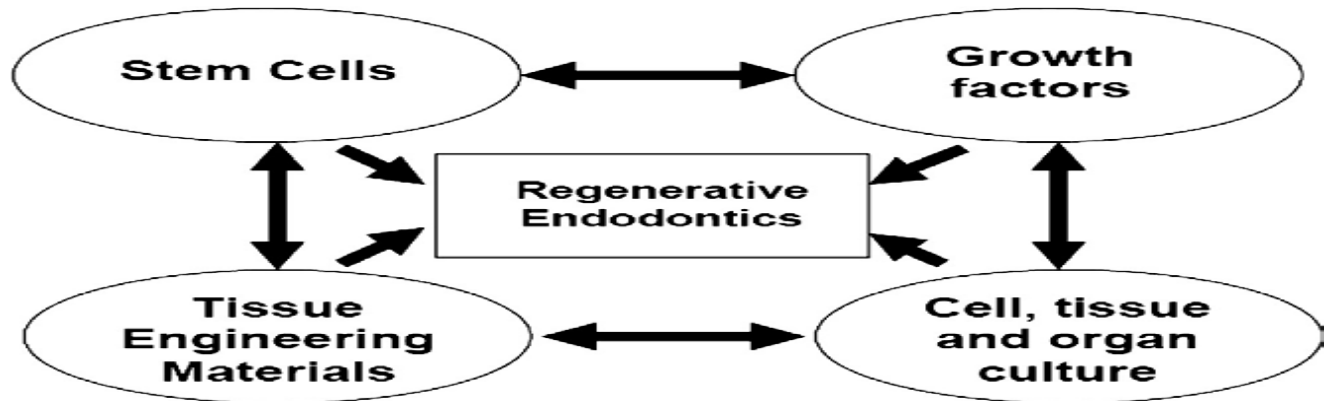

Figure 5. The major domains of research required to develop regenerative endodontic procedures (Peter E. Murray 2007).

There are three key elements for tissue engineering: Responsive cells, Scaffolds and morphogens (JE 2006).

 Responsive cells are generally stem cells. They are undifferentiated cells with varying degrees of potency and plasticity, capable of both self-renewal and multilineage differentiation (M. 2003). There are two categories of stem cells classified according to their potential of differentiation: Embryonic Stem Cells (ESC) and somatic stem cells (also called adult stem cells or mesenchymal stem cells). While ethical issues limit the use of embryonic stem cells, somatic stem cells constitute a more favorable cellular source to be used in tissue engineering (Morsczeck C 2008). Embryonic stem cells are capable of developing more than 200cell types. In contrast, an adult stem cell can divide and create another cell like itself, and also a cell more differentiated than itself, but the capacity for differentiation into other cell types is limited. This is described as being "multipotent" and is a distinguishing feature of

adult stem cells compared to the "pluripotent" or "omnipotent" properties seen in embryonic stem cells (Sedgley CM 2012).

Postnatal stem cells have been isolated from several tissues including, brain, skin, hair follicles, skeletal muscle, bone marrow and dental tissue (Volponi AA 2010). five types of dental mesenchymal stem cells were isolated an characterized: Dental pulp stem cells (DPSC) from pulp of permanent teeth; stem cells of human exfoliated teeth (SHED) and immature dental stem cells (IDPS) from primary teeth; periodontal ligament stem cells (PDLSC); stem cells from apical papilla (SCAP), and dental follicle progenitor cells (DFPC). We have shown that dental pulp stem cells are capable of differentiating in odontoblast-like cells or endothelial cells (IH 2010).

Scaffolds may serve as a 3-D framework for cells serving as an extracellular matrix for a finite period. Scaffolds provide an environment that allows both cell migration and proliferation, and may be fabricated in pre-determined shapes and composition. Natural (collagen, hyaluronic acid, chitosan and chitin) or synthetic(polylactic acid, polyglycolic acid, tricalcium phosphate, hydroxyapatite) have been largely employed for this purpose (Barnes CP 2007). Natural polymers in general provide better biocompatibility whilst synthetic polymers allow for improved control of physicochemical properties, such as degradation rate, microstructure, and mechanical strength (Yang S 2001).

The physico -mechanical characteristics of scaffolds characteristics (e.g. shape and size of pores, rate of porosity, interconnectivity) are critical determinants of cell behavior and, consequently, tissue formation (Graziano A 2008).

Morphogen/growth factors   Morphogen can be used to control stem cell activity, such as by increasing the rate of proliferation, inducing differentiation of the cells into another tissue type, or stimulating stem cells to synthesize and secrete mineralized matrix. A variety of growth factors have successfully been used for dentin-pulp complex regeneration, including transforming growth factors (TGFs), bone morphogenetic proteins (BMPs), platelet-derived growth factor (PDGF) , insulin-like growth factor (IGF) (Wei G 2006). Growth factors, especially those of the transforming growth factor beta (TGFβ) family, are important in cellular signaling for Odontoblast differentiation and stimulation of dentin matrix secretion. Thus, the growth factors should be used in conjunction with postnatal stem cells to accomplish the tissue engineering replacement Of diseased tooth pulp (Arana-Chavez VE 2004).

Potential Technologies for Regenerative Endodontics

1. Root Canal Revascularization via Blood Clotting

Several case reports have documented revascularization of necrotic root canal systems by disinfection followed by establishing bleeding into the canal system via over instrumentation (Banchs F 2004). An important aspect of these cases is the use of intracanal irrigants (NaOCl and chlorhexdine) with placement of antibiotics (e.g. a mixture of ciprofloxacin, metronidazole, and minocycline paste) for several weeks. The selection of various irrigants and medicaments is worthy of additional research, because these materials may confer several important effects for regeneration in addition to their antimicrobial properties. For example, tetracycline enhances the growth of host cells on dentin, not by an antimicrobial action, but via exposure of embedded collagen fibers or growth factors (Terranova VP 1989). There are several advantages to a revascularization approach. First, this approach is technically simple and can be completed using currently available instruments and medicaments without expensive biotechnology. Second, the regeneration of tissue in root canal systems by a patient's own blood cells avoids the possibility of immune rejection and pathogen transmission from replacing the pulp with a tissue-engineered construct (Llames SG 2004).

Regenerative endodontics often involves a two- or multi-step procedure. The first appointment is centered on proper access and disinfection of the pulp space. Upon confirming the absence of clinical signs and symptoms. The second appointment focuses on removing the antimicrobial medicament, releasing growth factors from the dentin (e.g., by irrigating with ethylene diamine tetra acetic acid (EDTA)), delivering stem cells into the root canal by stimulating

bleeding. Then creating a scaffold (e.g., blood clot or platelet-rich plasma), sealing the tooth by placing a pulp space barrier (e.g., MTA or resin-modified glass-ionomer) and permanent coronal restoration to prevent bacterial reinfection. At the second appointment, the use of local anesthetic without a vasoconstrictor may better facilitate stimulation of apical bleeding (TM. 2012; A. 2013).

2. Postnatal Stem Cell Therapy

The simplest method to administer cells of appropriate regenerative potential is to inject postnatal stem cells into disinfected root canal systems after the apex is opened. Postnatal stem cells are derive from multiple tissues, including skin, buccal mucosa, fat, and bone (V. 2005). A major research obstacle is identification of a postnatal stem cell source capable of differentiating into the diverse cell population found in adult pulp (e.g., fibroblasts, endothelial cells, odontoblasts). Technical obstacles include the development of methods for harvesting and any necessary ex vivo methods required to purify and/or expand cell numbers sufficiently for regenerative endodontic applications. There are several advantages to an approach using postnatal stem cells. First, autogenous stem cells are relatively easy to harvest and to deliver by syringe, and the cells have the potential to induce new pulp regeneration. Second, this approach is already used in regenerative medical applications, including bone marrow replacement. However, there are several disadvantages to a delivery method of injecting cells. First, the cells may have low survival rates. Second, the cells might migrate to different locations within the body , possibly leading to aberrant patterns of mineralization (Brazelton TR 2005). A solution for this latter issue may be to apply the cells together with a fibrin clot or other scaffold material. This would help

to position and maintain cell localization. Therefore, the probability of producing new functioning pulp tissue by injecting only stem cells into the pulp chamber, without a scaffold or signaling molecules, may be very low. Instead, pulp regeneration must consider all three elements (cells, growth factors, and scaffold) to maximize potential for success (Nakashima M 2005)

3. Pulp Implantation

In pulp implantation, replacement pulp tissue is transplanted into cleaned and shaped root canal systems. The source of pulp tissue may be a purified pulp stem cell line that is disease or pathogen-free, or is created from cells taken from a biopsy, that has been grown in the laboratory. The cultured pulp tissue is grown in sheets in vitro on biodegradable polymer nanofibers or on sheets of extracellular matrix proteins such as collagen I or fibronectin (Venugopal J 2005). So far, growing dental pulp cells on collagens I and III has not proved to be successful, but other matrices, including vitronectin and laminin, require investigation. The advantage of having the cells aggregated together is that it localizes the postnatal stem cells in the root canal system. The disadvantage of this technique is that implantation of sheets of cells may be technically difficult. The sheets are very thin and fragile, so research is needed to develop reliable implantation techniques. The sheets of cells also lack vascularity, so they would be implanted into the apical portion of the root canal system with a requirement for coronal delivery of a scaffold capable of supporting cellular proliferation (Fukuda J 2006). Cells located more than 200 micrometer from the maximum oxygen diffusion distance from a capillary blood supply are at risk of anoxia and necrosis (Helmlinger G 1997).

4. Scaffold Implantation

To create a more practical endodontic tissue engineering therapy, pulp stem cells must be organized into a three-dimensional structure that can support cell organization and vascularization. This can be accomplished using a porous polymer scaffold seeded with pulp stem cells (M. 2005) . A scaffold should contain growth factors to aid stem cell proliferation and differentiation, leading to improved and faster tissue development (RJ 2002). The scaffold may also contain nutrients promoting cell survival and growth, and possibly antibiotics to prevent any bacterial in-growth in the canal systems. In addition, the scaffold may exert essential mechanical and biological functions needed by replacement tissue (Boccaccini AR 2005). In pulp-exposed teeth, dentin chips have been found to stimulate reparative dentin bridge formation. Dentin chips may provide a matrix for pulp stem cell attachment and be a reservoir of growth factor . The natural reparative activity of pulp stem cells in response to dentin chips provides some support for the use of scaffolds to regenerate the pulp dentin complex (Silva TA 2004).

Scaffolds must meet some specific requirements:

Biodegradability is essential, since scaffolds need to be absorbed by the surrounding tissues without the necessity of surgical removal

(Schopper C 2005). A high porosity and an adequate pore size are necessary to facilitate cell seeding and diffusion throughout the whole structure of both cells and nutrients (Sachlos E 2003). The rate at which degradation occurs has to coincide as much as possible with the rate of tissue formation.

The types of scaffold materials available are natural or synthetic, biodegradable or permanent. The synthetic materials include polylactic acid (PLA), polyglycolic acid (PGA), and polycaprolactone (PCL), which are all common polyester materials that degrade within the human body (Taylor MS 1994). These scaffolds have all been successfully used for tissue engineering applications because they are degradable fibrous structures with the capability to support the growth of various different stem cell types. The principal drawbacks are related to the difficulties of obtaining high porosity and regular pore size. This has led researchers to concentrate efforts to engineer scaffolds at the nanostructural level to modify cellular interactions with the scaffold (Tuzlakoglu K 2005).

5. Injectable Scaffold Delivery

In root canal systems a tissue engineered pulp is not required to provide structural support of the tooth. This will allow tissue engineered pulp tissue to be administered in a soft three-dimensional scaffold matrix, such as a polymer hydrogel. Hydrogels are injectable scaffolds can be delivered by syringe (Trojani C 2005). Hydrogels have the potential to be noninvasive and easy to deliver into root canal systems. In theory, the hydrogel may promote pulp regeneration by providing a substrate for cell proliferation and differentiation into an organized tissue structure (Alhadlaq A 2005). Past problems with hydrogels included limited control over tissue formation and

development, but advances in formulation have dramatically improved their ability to support cell survival (F. 2000; Luo Y 2004). To make hydrogels more practical, research is focusing on making them photopolymerizable to form rigid structures once they are implanted into the tissue site (Luo Y 2004).

6. Three-Dimensional Cell Printing

The three-dimensional cell printing technique can be used to precisely position cells , and this method has the potential to create tissue constructs that mimic the natural tooth pulp tissue structure (Barron JA 2005). In theory, an ink-jet-like device is used to dispense layers of cells suspended in a hydrogel to recreate the structure of the tooth pulp tissue. The ideal positioning of cells in a tissue-engineering construct would include placing odontoblastoid cells around the periphery to maintain and repair dentin, with fibroblasts in the pulp core supporting a network of vascular and nerve cells (Sanjana NE 2004).

Theoretically, the disadvantage of using the three-dimensional cell printing technique is that careful orientation of the pulp tissue construct according to its apical and coronal asymmetry would be required during placement into cleaned and shaped root canal systems (Barron JA 2004) (as show in figure 6).

7. Gene Therapy

One use of gene delivery in endodontics would be to deliver mineralizing genes into pulp tissue to promote tissue mineralization. However, a literature search indicates there has been little or no research in this field, except for the work of (RB. 2001)Rutherford. He transfected ferret pulps with cDNA-transfected mouse BMP-7 that failed to produce a reparative response, suggesting that further research is needed to optimize the potential of pulp gene therapy (RB. 2001).
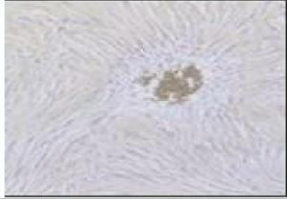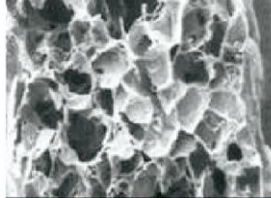
Technique	Image	Advantages	Disadvantages
Root-canal revascularization: open up tooth apex to 1 mm to allow bleeding into root canals		✓ Lowest risk of immune rejection ✓ Lowest risk of pathogen transmission	➢ Minimal case reports published to date ➢ Potential risk of necrosis if tissue becomes reinfected
Stem cell therapy: autologous or allogenic stem or cells are delivered to teeth via injectable matrix		✓ Quick, ✓ Easy delivery ✓ Least painful ✓ Cells are easy to harvest	➢ Low cell survival ➢ Cells do not produce new functioning pulp ➢ High risk of complications
Pulp implant: pulp tissue is grown in the laboratory in sheets and implanted surgically		✓ Sheets of cells are easy to grow ✓ More stable than an injection of dissociated cells	➢ Sheets lack vascularity so only small constructs are possible ➢ Must be engineered to fit root canal precisely
Scaffold implant: pulp cells are seeded onto a 3-D scaffold made of polymers and surgically implanted		✓ Structure supports cell organization ✓ Some materials may promote vascularization	➢ Low cell survival after implantation ➢ Must be engineered to fit root canal precisely
3-D cell printing: ink-jet-like device dispenses layers of cells in a hydrogel which is surgically implanted		✓ Multiple cell types can be precisely positioned	➢ Must be engineered to fit root canal precisely ➢ Early-stage research has yet to prove functional in vivo
Injectable scaffolds: polymerizable hydrogels, alone or containing cell suspension are delivered by injection		✓ Easy delivery ✓ May promote regeneration by providing substitute for extracellular matrix	➢ Limited control over tissue formation ➢ Low cell survival ➢ Early-stage research has yet to prove functional in vivo
Gene therapy: mineralizing genes are transfected into the vital pulp cells of necrotic and symptomatic teeth		✓ May avoid cleaning and shaping root canals ✓ May avoid the need to implant stem cells	➢ Most cells in a necrotic tooth are already dead ➢ Difficult to control ➢ Risk of health hazards ➢ Not approved by the FDA

Figure 6 Developmental approaches in regenerative endodontic (Peter E. Murray 2007)

What is the Biological Basis for Regenerative Endodontic Therapy?

Historically, long-term calcium hydroxide treatment was used to induce apexification of the immature tooth with pulpal necrosis before placing an obturation material such as gutta-percha in the root canal system (AL. 1966). While the success rate of calcium hydroxide apexification is reported to be as high as 95%, there are several associated problems (Kerkis I 2006). These include 1. The time required for formation of the calcified barrier (3-24 months) 2. Multiple appointments needed for reapplication of calcium hydroxide 3. The effect of long-term (several months or more) calcium hydroxide on the mechanical properties of dentin (Shabahang S 1999) .It has been proposed that exposure to calcium hydroxide denatures the carboxylate and phosphate groups in dentin (Andreasen JO 2002). Mineral trioxide aggregate (MTA), used as a root-end filling material, offers an alternative treatment for apexification. When placed adjacent to the periradicular tissues it induces the formation of cementum-like hard tissue and offers several advantages over calcium hydroxide apexification (Shabahang S 1999). These include a reduction in treatment time and fewer patient visits, which in turn facilitate the timely restoration of the tooth. Studies on MTA apexification report that the success rate of the treatment is as high as 94% (Sarris S 2008).

However, neither of the apexification treatments fosters further root development and immature teeth remain vulnerable to cervical root fractures. In contrast, regenerative endodontic therapy has the potential for increased root development, and thus, may confer a better long-term prognosis. In addition, successful regeneration of the pulp-dentin complex would likely result in vital tissue capable of mounting an immune response and signaling tissue damage by sensory neurons (Peter E. Murray 2007).

What Could Regenerative Endodontics Look Like in the Future?

The majority of human case studies have shown good clinical outcomes (absence of clinical signs and symptoms, radiographic evidence of resolution of periapical infections, continued root development and increased canal wall thickness) for immature permanent teeth with pulpal necrosis following REPs (Hargreaves KM 2013). A recent retrospective analysis of radiographic and survival outcomes of 61 immature teeth treated with either REPs or apexification found significantly greater increases in root length and thickness following REPs in comparison with either calcium hydroxide apexification or MTA apexification (Jeeruphan T 2012).

In the future, the challenge of generating tissues that mimic the original pulp and dentin-like structure might be more effectively addressed by using tissue-engineering approaches under more controlled clinical conditions (Galler KM 2011). Such approaches might rely more on therapies that utilize autologous stem cells combined with customized scaffolds and delivery of appropriate growth factors at the right time and in the right sequence. It is evident that recent advances have opened the door to exciting new opportunities for healing immature teeth with pulpal necrosis. Extension of these advances to the treatment of mature teeth with pulpal necrosis would provide significant therapeutic benefits by enabling retention of the natural dentition in a larger patient pool (Smith AJ 2001).

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