ISSN 2218-6263 (online)

World Journal of *Stomatology*

World J Stomatol 2019 January 15; 7(1): 1-19

Published by Baishideng Publishing Group Inc

World Journal of Stomatology

Contents

Irregular Volume 7 Number 1 January 15, 2019

REVIEW

World Journal of Stomatology
Volume 7 Number 1 January 15, 2019

Contents

NAME OF JOURNAL
World Journal of Stomatology

ISSN
ISSN 2218-6263 (online)

LAUNCH DATE
December 31, 2011

FREQUENCY
Irregular

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PUBLICATION DATE
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World J Stomatol 2019 January 15; 7(1): 1-19

DOI: 10.5321/wjs.v7.i1.1

ISSN 2218-6263 (online)

REVIEW

Dental pulp stem cells: Novel cell-based and cell-free therapy for peripheral nerve repair

Nessma Sultan, Laila E Amin, Ahmed R Zaher, Ben A Scheven, Mohammed E Grawish

ORCID number: Nessma Sultan (0000-0003-0965-9170); Laila E Amin (0000-0002-2357-0810); Ahmed R Zaher (0000-0003-2198-7839); Ben A Scheven (0000-0002-3219-3906); Mohammed E Grawish (0000-0003-4732-8022).

Author contributions: Sultan N generated the figure, table and wrote the manuscript; Grawish ME, Amin LE, Zaher AR and Scheven BA contributed to writing of the manuscript, provided intellectual contribution to the content, and made critical revisions; all authors reviewed and approved the final version of the manuscript.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Manuscript source: Invited manuscript

Received: August 21, 2018
Peer-review started: August 22,

Nessma Sultan, Laila E Amin, Ahmed R Zaher, Mohammed E Grawish, Department of Oral Biology, Faculty of Dentistry, Mansoura University, Mansoura 35516, Egypt

Ben A Scheven, School of Dentistry, Oral Biology, College of Medical and Dental Sciences, University of Birmingham, Birmingham B5 7EG, United Kingdom

Corresponding author: Mohammed E Grawish, PhD, Professor, Department of Oral Biology, Faculty of Dentistry, Mansoura University, El-Gomhoria Street, Mansoura 35516, Egypt.
grawish2005@yahoo.com
Telephone: +20-50-2200706

Abstract

The regeneration of peripheral nerves comprises complicated steps involving a set of cellular and molecular events in distal nerve stumps with axonal sprouting and remyelination. Stem cell isolation and expansion for peripheral nerve repair (PNR) can be achieved using a wide diversity of prenatal and adult tissues, such as bone marrow or brain tissues. The ability to obtain stem cells for cell-based therapy (CBT) is limited due to donor site morbidity and the invasive nature of the harvesting process. Dental pulp stem cells (DPSCs) can be relatively and simply isolated from the dental pulps of permanent teeth, extracted for surgical or orthodontic reasons. DPSCs are of neural crest origin with an outstanding ability to differentiate into multiple cell lineages. They have better potential to differentiate into neural and glial cells than other stem cell sources through the expression and secretion of certain markers and a range of neurotropic factors; thus, they should be considered a good choice for PNR using CBT. In addition, these cells have paracrine effects through the secretion of neurotrophic growth factors and extracellular vesicles, which can enhance axonal growth and remyelination by decreasing the number of dying cells and activating local inhabitant stem cell populations, thereby revitalizing dormant or blocked cells, modulating the immune system and regulating inflammatory responses. The use of DPSC-derived secretomes holds great promise for controllable and manageable therapy for peripheral nerve injury. In this review, up-to-date information about the neurotrophic and neurogenic properties of DPSCs and their secretomes is provided.

Key words: Dental pulp stem cells; Secretomes; Cell-based therapy; Cell-free therapy; Peripheral nerve injury

2018
First decision: October 14, 2018
Revised: November 15, 2018
Accepted: January 8, 2019
Article in press: January 8, 2019
Published online: January 15, 2019

Core tip: The distinct developmental pathway of dental pulp stem cells (DPSCs) from neural crest cells results in a cell type that can be participate in neural tissue regeneration. The efficacy of using DPSCs for peripheral nerve repair (PNR) is strongly influenced by boosting trophic factors that promote axonal growth and regeneration and provide direct and indirect protection against cell death. Recently, encouraging results from different studies indicate that DPSC secretomes have reparative and protective properties comparable with their cellular counterparts in PNR. The use of DPSC secretomes as a safe and possibly more valuable substitute for cell-based therapy approaches is a novel therapeutic perspective.

Citation: Sultan N, Amin LE, Zaher AR, Scheven BA, Grawish ME. Dental pulp stem cells: Novel cell-based and cell-free therapy for peripheral nerve repair. *World J Stomatol* 2019; 7(1): 1-19
URL: https://www.wjgnet.com/2218-6263/full/v7/i1/1.htm
DOI: https://dx.doi.org/10.5321/wjs.v7.i1.1

INTRODUCTION

Structurally, the nervous system comprises two main components, the peripheral nervous system (PNS) and the central nervous system (CNS). The CNS involves the spinal cord and brain and acts as the motor output and center for all sensory perception. The low regenerative capacity of the CNS makes injury to these regions permanent because the damaged neurons undergo degenerative cell death and are not substituted[1]. The PNS includes the sensory nerves, motor nerves and ganglia outside the spinal cord and brain. The peripheral nerves transfer signals throughout the body and the spinal cord. These signals are sent to the brain and provide sensory information when a reflex response is provided[2]. The proper function and maintenance of peripheral nerves are primarily controlled by cells other than neurons, specifically, Schwann cells (SCs) that surround the nerves and release important trophic factors, such as nerve growth factor (NGF) which is important during the process of nerve repair and is responsible for proliferation, growth regulation and survival of target neurons[3].

Peripheral nerve injury (PNI) may result in the loss of motor function, sensory function, or both. Such injury leads to neurapraxia, axonotmesis or neurotmesis and may occur as a result of acute compression, trauma, iatrogenic induction during surgical procedures, diabetes or other health conditions such as Guillain-Barre syndrome. Patients with PNI encounter several challenges, ranging from mild discomfort to long-term functional defect. During end-organ denervation, reinnervation can occur in two ways: through collateral branching of unbroken axons or through regeneration of the damaged axon[4]. Collateral branching occurs in cases where 20%-30% of the axon' cells within a nerve are damaged and is considered the main recovery mechanism. In injuries disturbing more than 90% of the axon' cells within a nerve, axonal regeneration is the primary method of recovery[5]. To accomplish full recovery, the axon undertakes three main processes: clearing of the distal stump or Wallerian degeneration, axonal regeneration, and end-organ reinnervation. Poor functional consequences usually experienced by patients with PNI result from the failure of any of these processes[6].

SCs play fundamental roles in the maintenance and survival of healthy axons and in axonal regeneration, and they transfer essential molecules across the axons. They produce a variety of neurotrophic factors that interact with tyrosine kinases and other receptors and modify the neuron gene expression profile to enhance regeneration[7]. Within healthy nerves, NGF has a low expression level, but it is upregulated in SCs during injury. This factor promotes proliferation and growth of certain target neurons[6]. Many neurotrophic factors have been discovered in neurons and in SCs, and they function to improve cell survival through apoptosis prevention mechanisms and enhancing regeneration processes[8]. Furthermore, studies have evaluated SCs for engraftment and myelination of injured nerves in animal models, providing a foundation for axonal regeneration and functional recovery[9]. However, SC harvesting is limited for multiple reasons; among them the need to sacrifice one or more functional nerves for SC isolation with subsequent neuroma formation, donor site morbidity and loss of sensation, thereby leading to a demand for other cell sources[10].

Adult stem cells are considered multipotent undifferentiated cells that have the ability to self-renew and differentiate into several cell lineages. These adult cells have been successfully isolated from different tissues, such as neural, bone, retina, skin and dental tissue. Adult mesenchymal stem cells (MSCs) can be isolated from a wide range of tissues, such as bone marrow (BMSCs), adipose tissue (AMSCs) and dental tissues. Postnatally, dental stem cells can be isolated from different dental tissues for example from dental pulp tissue (DPSCs), human exfoliated primary teeth (SHED)[11], the tissue of the apical papilla (SCAP)[12] and periodontal ligament surrounding the roots of teeth (PDLSC)[13]. The ability of MSCs to restore injured tissue is generally related to their chemokine surface receptors which enable them to migrate toward injured tissue under the influence of growth factors or chemokines secreted by the damaged target organ. MSCs have immune-modulatory, anti-inflammatory and multilineage differentiation potential [14,15].

MSCs and their secretomes, which include paracrine factors secreted into the extracellular matrix, have been extensively studied for their potential to promote nerve regeneration. In 2013, Teixeira *et al*[16], suggested that MSCs isolated from Wharton jelly of the umbilical cord, bone marrow or adipose tissue are capable of nerve repair due to their differentiation ability and multipotency. In addition to their differentiation potential, MSCs are capable of secreting neuroregulatory factors that promote neurogenesis and survival of glial cells and neurons. However, the transplanted cells cannot survive long; therefore, most of the attention has been focused on the bioactive molecules secreted by MSCs, including growth factors and cytokines. These factors are secreted into the extracellular matrix which is actively involved in the guidance, regulation and control of tissue homeostasis, development and regeneration[17].

DPSCs are a neural crest derived cells that possess MSC properties[18]. DPSCs are easily harvested and isolated from extracted teeth. Storage and cryopreservation of DPSCs are essential steps for banking of these cells for future application and use[19,20]. DPSCs have the ability to differentiate into multiple cell lineages with the potential to differentiate into neural cells. Surprisingly, DPSCs in an undifferentiated state can express neural markers, such as S100, β-III-tubulin and NGFR p75[21]. Under appropriate conditions, DPSCs have been successfully differentiated into SCs and acquire both neuronal morphology and function. It was found that DPSCs express characteristic SC markers, such as laminin and CD104[22]. Moreover, they promote neurite outgrowth of trigeminal neurons and rescue motor neurons in spinal cord injury models and exhibit typical SC interactions with neurons, such as neuritis myelination. Furthermore, they are able to secrete a range of neurotrophic factors including vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell line-derived neurotrophic factor (GDNF) and NGF. These properties together with their availability make DPSCs an auspicious tool for cell-based therapy (CBT) for PNI[23].

DPSC-conditioned medium (CM), *i.e*., the cell culture supernatant (Figure 1), was previously regarded as waste that contains cell debris, but now, it recognized that CM contains the regenerative ambience of secretomes. Immune-regulation, anti-fibrotic, and anti-apoptotic properties; the ability to stimulate angiogenesis and neurogenesis; and a variety of biological activities have been attributed to secretomes[24-28]. In this review, we will discuss therapeutic application of DPSCs and their secretomes for peripheral nerve repair (PNR). Where relevant, comparisons between CBT with DPSCs and cell-free therapy (CFT) using secretomes will be noted. We will briefly summarize the neurogenic and neurotrophic properties of DPSCs and their secretomes and then summarize the main differences between CBT and CFT.

MECHANISMS OF PHYSIOLOGIC PNR AND TREATMENT METHODS

The PNS has an intrinsic repair and regeneration capability; however, this ability is restricted and depends on many factors, such as the mechanism of injury, age of the patient and especially the proximity of the injury to the nerve cell body[29]. PNI accompanied by sensory disturbance or pain in the orofacial region is considered a major clinical challenge and may lead to permanent disability[30]. Distal nerve stump denervation for prolonged periods, especially in large gaps, is often accompanied by a decrease in the number of SCs; therefore, the outcomes following PNI remain poor. The cell bodies of axons after nerve transection start to swell in an attempt to address the increased metabolic demand necessary for regeneration and some nerve cells will shift to neuronal cell apoptosis. Then, SCs, which are considered the principal glial cells of the PNS, start to proliferate, convert to a phagocytic phenotype and begin to

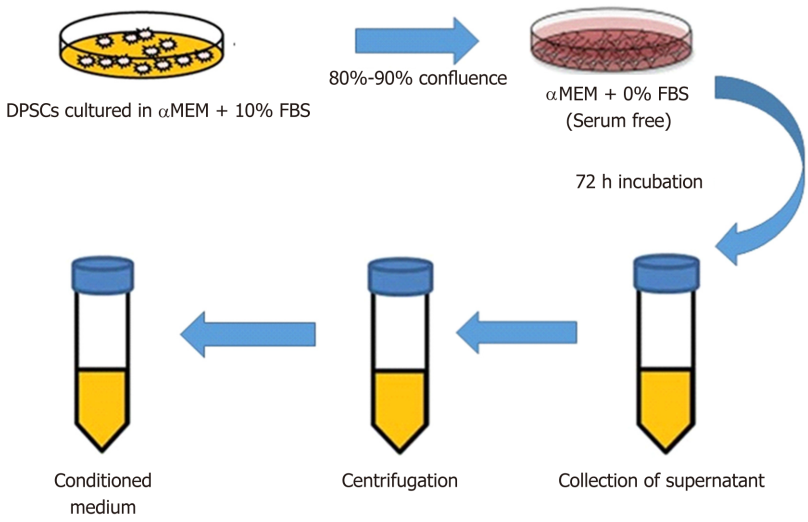

Figure 1 Schematic diagram representing the steps of obtaining cell-free conditioned medium from dental pulp stem cells. The dental pulp stem cells (DPSCs) were plated onto culture vessels and grown in culture medium containing 10% fetal bovine serum. When adherent cells reach 80%-90% confluence, the medium was discarded and the cells washed with phosphate-buffered saline. Then, the DPSCs were incubated in α MEM serum-free medium and incubated for 72 h. The supernatant representing the conditioned medium was collected and centrifuged, filtered and concentrated.

attract circulating macrophages to the site of damage. These important steps serve to remove axonal and myelin debris from the distal stump in an attempt to prepare the injury site for the regenerating axon. At the same time, SCs start to secrete growth factors, which create favorable environmental conditions for nerve regrowth toward the target tissue[31,32]. Thus, nerve regeneration is a complicated process that is regulated through the interplay of complex cell signaling processes. These signaling processes direct SC migration and axonal outgrowth to bridge the nerve gap between two ends of transected peripheral nerve stumps.

Any interruption during this process might lead to down regulation of the growth factors and loss of axonal regeneration[33,34]. On the other side, the denervated target organ will undergo a fatigue of trophic factors, atrophy of muscle fibers and apoptosis of satellite cells[35]. Treatment modalities such as braces or splints, electrical stimulators, physical and occupational therapy and exercise are insufficient[36]. Surgical treatments of PNI with autologous nerve grafts is considered the gold standard; however, this treatment has the disadvantage of donor site morbidity, and available grafts may be limited in length and there is a potential for neuroma formation. Today, complete recovery is rare despite all the types of available treatment modalities, and these limitations increase the demand for alternative modalities (Figure 2) for nerve reconstruction[37-40]. Currently, meticulous microsurgical repair is the best choice, especially with the use of tensionless epineural sutures. Nevertheless, in the presence of a wide nerve gap at which end-to-end suturing is impossible, autologous nerve grafting should be considered the optimal solution[41]. Nerve injuries should be repaired as quickly as possible because any delay in the repair process may have a significant detrimental effect on sensory and motor recovery[42].

SOURCES OF STEM CELLS FOR PNR

Stem cells, undifferentiated cells that are capable of differentiating into specific and specialized cell types, can be allocated into several categories: (1) Embryonic stem cells (ESCs) obtained from the embryoblast of a blastocyst; (2) induced pluripotent stem cells (iPSCs) created directly from adult cells reprogrammed to become embryonic-like pluripotent cells; and (3) adult stem cells, including hematopoietic stem cells and MSCs. ESCs, iPSCs, MSCs and neural stem cells (NSCs) have been studied *in vitro* and *in vivo* for their ability to aid in nerve repair. ESCs promoted the repair of a 10-mm gap in rat sciatic nerve in a histological, electrophysiological and molecular study[43]. SCs-like precursors were generated from ESCs in models of PNR *in vitro*, and these cells expressed myelin protein[44]. When NSCs were seeded in chitosan, they give results comparable with autografts in repairing a 10-mm nerve

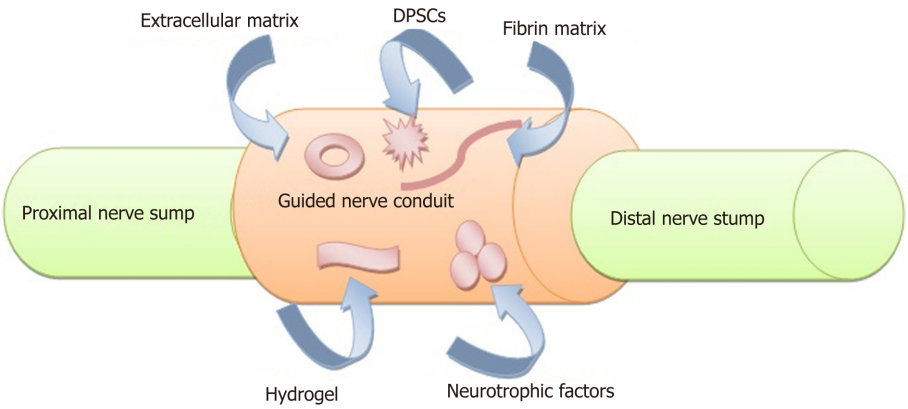

Figure 2 Schematic diagram showing a strategy to promote regeneration of bisection peripheral nerve injury using guided nerve conduit with incorporation of dental pulp stem cells and neurotrophic factors. The proximal and distal nerve stumps (light green color) are sutured into the two ends of artificial nerve conduit (peach color). The conduit mimics the structures and components of autologous nerves and bridging the nerve gap to support the growth and regeneration of neural cells. The microenvironment of the conduit should contain nutrients, cytokines and growth factors (extracellular matrix, hydrogel and neurotrophic factors) as well as cellular elements (dental pulp stem cells).

gap[45]. Additionally, iPSCs can be used to efficiently repair a 10-mm nerve gap and produce functional neural crest cells[46].

Despite the fact that ESCs have unique characteristics, such as an unlimited quantity and pluripotency, the clinical application of these cells has been restricted by safety problems, such as immunogenic reactions, ethical concerns, low efficiency, tumorigenicity and inadequate availability[47]. Compared with ESCs, the use of MSCs in regenerative medicine is accompanied by fewer ethical issues, considering the risk of teratoma formation, the sourcing of the cells and undesired cell differentiation. The ability of MSCs to differentiate into different cell lineages with specific and predetermined environmental conditions and to exhibit immunosuppressive properties has enabled their fruitful transplantation into a well-matched donor[14,15]. Allogeneic MSCs are comparable to autologous MSCs from nonhuman primates and were not rejected[48].

Recently, in CBT for PNI, emphasis has been given to creating satisfactory environmental conditions for axon regeneration. The goal is to increase SCs number and activity because they are the orchestrators of PNR and to prevent their senescence[34]. Achieving sufficient numbers of autologous SCs for culture requires healthy nerve scarification and extended periods of expansion and purification that subsequently delay the repair process. Prolonged denervation leads to loss of SC-mediated axonal support and apoptosis of the cell body in the peripheral nerve. Therefore, autologous transplantation of SCs is considered impossible[49,50]. Optimal stem cell criteria for PNR should include that cells are easily accessible, rapidly expanded *in vitro*, easily integrate into host tissue and are capable of survival *in vivo*[51].

The potential of MSCs to regenerate injured tissue is basically related to three mechanisms: "homing", which refers to the ability of stem cells to migrate to the target organ due to chemical gradients[52]; their ability to self-renew and the potential for multilineage differentiation; and a paracrine mechanism *via* secretion of a broad array of bioactive factors[53,54]. In tissue engineering and/or regenerative medicine applications, engrafted stem cells are susceptible to ischemic attack, and this may lead to limited paracrine secretion and function and poor survival of grafted cells[55]. Recent research has shown that the paracrine effects of MSC secretomes are an important factor in CBT and may have a direct or indirect influence on the surrounding microenvironments[56-58].

DENTAL PULP STEM CELLS FOR PNR AND REGENERATION

Alge *et al*[59], compared DPSCs and BMSCs and reported that DPSCs were superior in all examined properties, including proliferation, differentiation and mineralization potential. Additionally, Mead *et al*[60], confirmed that DPSCs exhibit significantly more neuroprotective and neuritogenic effects on retinal ganglion cells than BMSCs or

AMSCs; they also concluded that DPSCs secreted higher amounts of numerous growth factors, such as containing BDNF, NGF, VEGF and GDNF, which play pivotal roles in neuroprotection and neuritogenesis. They concluded that MSCs have distinctive neurotrophic gene expression profiles, but specifically, DPSCs expressed prostaglandin E2 receptor (EP2) at higher levels than both BMSCs and AMSCs. EPs have a role in the release and synthesis of neurotrophins from different cell types.

DPSCs share a common origin with peripheral nerve glial progenitor cells[61]. This feature makes these cells a very interesting choice for PNR. Nestin expression in DPSCs is well documented and suggests the ability of these cells to differentiate into neuronal lineages because they originate from neural crest cells. DPSCs express neuronal markers, such as neurofilament (NF), BIII-tubulin and glial fibrillary acidic protein (GFAP). This indicates that there is a great similarity in membrane properties between DPSCs and neuronal cells[62,63]. Moreover, when DPSCs were grown on non-adherent culture plastic, they reorganized from a uniform cell monolayer and switched to a more quiescent state distinguished by the presence of spheroid structures similar to neurospheres, which stained positive for nestin[64-67]. Therefore, DPSCs are a heterogeneous population ranging from neuroblast-like to fibroblast-like cells[68,69]. The high expression of neurotransmitter receptors and neural markers by DPSCs suggest that these cells vigorously respond to neural environmental signals, promoting re-establishment of functional nerve conductivity[70-72].

Martens *et al*[23], confirmed the potential of DPSCs to differentiate into SCs *in vitro* that efficiently myelinated dorsal root ganglion neurons, a result confirmed by an ultrastructural TEM analysis. Regarding the significant role that SCs play in axonal peripheral nerve regeneration and protection and the obstacles in their maintenance and harvesting, the use of DPSC-derived autologous SCs may be considered an important step in designing new treatments for PNI. One of the DPSC-derived neurotrophic factors is GDNF which reverses the symptoms associated with neuropathic pain and exerts a powerful analgesic effect[73]. Small fiber neuropathy was treated using a small molecule modulator of ligand-induced GFRα/RET receptor signaling through a topical application[74].

A literature search was performed in September 2018 in the PubMed database. The following keywords were used: dental pulp stem cells[Title/Abstract] AND nerve repair[Title/Abstract], and the search retrieved 6 results and searching with keywords dental pulp stem cells[Title/Abstract] AND nerve regeneration[Title/Abstract] retrieved 12 results. Exclusion of duplicate and review articles yielded 15 results[14,23,60,75-86]. Overall, 8 studies[14,75-81] were found that tested *in-vivo* and *in-vitro* application of DPSCs (Table 1), 2 studies[82,83] were conducted *in-vivo* with animal models (Table 2) and 5 studies[23,60,84-86] only performed cell culture tests (Table 3). The source of stem cells, target tissue, study model, and objective and outcomes of the retrieved studies were included and are described in the abovementioned tables.

PARACRINE EFFECT OF MESENCHYMAL STEM CELLS

The paracrine effect of MSCs is mediated through their secretomes to promote the repair processes[87,88]. Secretomes contain a broad range of bioactive soluble factors with anti-apoptotic, anti-fibrotic, angiogenetic, chemo-attractive and immunomodulation properties, and these components include free nucleic acids, soluble proteins, extracellular vesicles and lipids. Extracellular vesicles can be subdivided into microvesicles (MVs) and exosomes (Figure 3)[89].

Exosomes, MVs and apoptotic bodies are secreted vesicles that can be distinguished from each other by morphology and size. Exosome sizes range from 40-100 nm whereas MV sizes range from 100-1000 nm and apoptotic bodies are more than 1000 nm in size[90]. Exosomes are usually derived from multivesicular bodies, while MVs are formed by plasma membrane budding and anti-apoptotic bodies *via* blebbing of the plasma membrane of dying cells[91]. Exosomes and MVs usually contain proteins, lipids, mRNAs and microRNAs which are important in cell-cell communication, Exosomes exert their action by delivering their contents directly into cells without the need for specific receptor expression. Bilayer membrane encapsulation provides protected environmental conditions that allow them to travel within the body without degradation[92]. Mead *et al*[93], highlighted more than 40 surplus miRNAs in MSCs compared with fibroblast exosomes, suggesting that the combination of miRNAs may responsible for exosome mediated neuroprotection[94].

Nakano *et al*[95] designed genetically engineered MSC secretomes and reported that these cells were able to secrete hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), transforming growth factor-β (TGF-β), and VEGF, which are closely related to neurite outgrowth and neuronal survival *in vitro*. After MSCs

Table 1 Studies retrieved from PubMed database evaluating the effect of DPSCs on peripheral nerve repair or regeneration both *in-vivo* and *in-vitro*

Author (publication year)		Source of stem cells	Target tissue	Study model	Objective	Outcome
Carnevale *et al*[14], 2018	*In-vivo*	Human STRO-1$^+$ /c-Kit$^+$ /CD34$^+$DPSCs expressing P75^{NTR}, nestin and SOX-10	Sciatic nerve defect	Animal rat model	To demonstrate the ability of human STRO-1$^+$ /c-Kit$^+$ /CD34$^+$ DPSCs expressing P75^{NTR}, nestin and SOX-10 to promote axonal regeneration.	The cells promoted regeneration and functional recovery of sciatic nerve defects after injury.
	In-vitro	Human STRO-1$^+$ /c-Kit$^+$ /CD34$^+$DPSCs expressing P75^{NTR}, nestin and SOX-10	To differentiate into SC-like cells	*In-vitro* culturing of DPSCs and their differentiation to SCs	To demonstrate the ability of Human STRO-1$^+$ /c-Kit$^+$ /CD34$^+$ DPSCs expressing P75^{NTR}, nestin and SOX-10 to differentiate into SC-like cells.	Under appropriate conditions, the cells differentiated into SC-like cells
Kolar *et al*[75], 2017	*In-vivo*	Adult rat SCs; Human SCAP, DPSCs and PDLSC	10 mm nerve gap defect in a rat sciatic nerve	Sciatic nerve injury model	To demonstrate the ability of human SCAP, DPSCs and PDLSC to promote axonal regeneration using nerve guidance conduit of 14 mm length.	All the stem cell types significantly enhanced axon regeneration after two weeks. SCAP are the optimal dental stem cell type for peripheral nerve repair.
	In-vitro	CM from unstimulated or stimulated human SCAP, DPSCs and PDLSC	Differentiated human neuroblastoma SH-SY5Y cell line	*In-vitro* neurite outgrowth assay	To examine the biological activity of the conditioned medium for unstimulated and stimulated human SCAP, DPSC and PDLSC.	Quantification of the neurite outgrowth showed that unstimulated and stimulated human SCAP, DPSCs and PDLSC increased both the percentage of cells producing neurites and the total neurite outgrowth length.
Omi *et al*[76], 2017	*In-vivo*	DPSCs isolated from the incisor teeth of 6-wk-old male rats	Sciatic nerve; Sensory nerve fibers; Sural nerves	Streptozotocin-induced diabetic rats.	Investigated whether the transplantation of DPSCs ameliorated long-term diabetic polyneuropathy in streptozotocin-induced diabetic rats.	Significant reductions in the sciatic motor/sensory nerve conduction velocity, increases in the current perception threshold, and decreases in capillary density in skeletal muscles and intra-epidermal nerve fiber density. Sural nerve morphometrical analysis revealed that the transplantation of DPSCs significantly increased the myelin thickness.
	In-vitro	DPSCs isolated from the incisor teeth of 6-wk-old male rats	Dorsal root ganglion neuron were cultured for use in neurite outgrowth with DPSC-CM; Immortalized adult Fischer rat SCs were cultured with DPSC-CM	*In-vitro* neurite outgrowth assay; Cell viability assay	Evaluation of neurite outgrowth. Analysis of myelin-related protein formation in immortalized adult Fischer rat SCs.	DPSCs-CM promoted the neurite outgrowth of dorsal root ganglion neurons. Increased the viability and myelin-related protein expression of SCs.

Sanen *et al*[77], 2017	*In-vivo*	SCs derived from differentiated human DPSCs	15-mm rat sciatic nerve defects	Sciatic nerve injury model	Evaluated the performance of SCs derived from differentiated human DPSCs in a rat model of PNI.	Immunohistochemistry and ultrastructural analysis revealed in-growing neurites, myelinated nerve fibres and blood vessels along the construct.
	In-vitro	SCs derived from differentiated human DPSCs	Human microvascular endothelial cell line (HMEC-1)	Alamar Blue cell proliferation assay; Transwell migration assay; Tube formation assay	Investigated the neuroregenerative and the proangiogenic capacities of SCs derived from differentiated human DPSCs.	The endothelial cell line HMEC-1 had proliferated significantly more in the presence of conditioned medium from human DPSCs and differentiated human DPSCs compared with those in control medium.
Hei *et al*[78], 2016	*In-vivo*	Schwann-like cells were derived from human DPSCs; Human DPSCs	3 mm - wide crush injury was inflicted at a distance of approximately 10 mm from the mental foramen	Male Sprague-Dawley rats crush-injury site	To investigate the effect of Schwann-like cells combined with pulsed electromagnetic field on peripheral nerve regeneration.	Schwann-like cells, human DPSCs with or without pulsed electromagnetic field, and pulsed electromagnetic field only improved peripheral nerve regeneration.
	In-vitro	Schwann-like cells were derived from human DPSCs; Human DPSCs	Schwann Cells	Cell culture dishes	To demonstrate the ability of hDPSCs to differentiate into Schwann - like cells and demonstrate glial character with expression of CD104, S100, GFAP, laminin and $p75^{NTR}$.	Successful morphological differentiation of hDPSCs toward Schwann - like cells.
Yamamoto *et al*[79], 2016	*In-vivo*	Human mobilized DPSCs	5-mm gap of the left sciatic nerve	Rat sciatic nerve defect model	To investigate the effects of human mobilized DPSC transplantation on peripheral nerve regeneration using 9-mm collagen conduit.	Human mobilized DPSCs promote axon regeneration through trophic functions, acting on SCs and promote angiogenesis.
	In-vitro	CM of human mobilized DPSCs	Rat SCs (RT4-D6P2T)	Migration, proliferation, and anti-apoptotic assays	To investigate the trophic effects of mobilized human DPSCs on proliferation, migration and anti-apoptosis in SCs	The human mobilized DPSCs-CM significantly enhanced proliferation and migratory activity and decreased apoptosis of RT4-D6P2T cells.
Askari *et al*[80], 2014	*In-vivo*	Human DPSCs transfected with a tetracycline-inducible system expressing oligodendrocyte lineage transcription factor 2 gene	Sciatic nerve demyelination experiment	Mouse model of local sciatic demyelination damage by lysolecithin	To investigate if the tetracycline-regulated expression of oligodendrocyte lineage transcription factor 2 gene transfected in human DPSCs can lead to mouse sciatic nerve regeneration upon transplantation.	Human DPSCs-derived oligodendrocyte progenitor cells have relevant therapeutic potential in the animal model of sciatic nerve injury.

	In-vitro	Human DPSCs	Oligodendrocyte	*In-vitro* plasmid construct and transfection	DPSCs were transfected with oligodendrocyte transcription factor 2 which play important role in differentiation of DPSCs to oligodendrocyte progenitor cells.	Exogenous expression of the oligodendrocyte lineage transcription factor 2 gene by a tetracycline-regulated system could be used as an efficient way to induce the differentiation of DPSCs into functional oligodendrocytes.
Dai *et al*[81], 2013	*In-vivo*	SCs, AMSCs, DPSCs, and the combination of SCs with AMSCs or DPSCs	15-mm-long critical gap defect of rat sciatic nerve	Sciatic nerve injury model	To test their efficacy in repairing PNI 17-mm nerve conduit.	Co-culture of SCs with AMSCs or DPSCs in a conduit promoted peripheral nerve regeneration over a critical gap defect.
	In-vitro	SCs, AMSCs, DPSCs, and the combination of SCs with AMSCs or DPSCs	Neuronal cells	RT-PCR analysis of the coculture *in-vitro*	To verify if the combination of cells led to synergistic neurotrophic effects NGF, BDNF, and GDNF.	Results confirmed the synergistic NGF production from the co-culture of SCs and ASCs.

SCAP: Stem cells of apical papillae; DPSC: Dental pulp stem cells; PDLSC: Periodontal ligament stem cells; CM: Conditioned medium; AMSCs: Adipose mesenchymal stem cells; SCs: Stem cells; NGF: Nerve growth factor; BDNF: Brain derived neurotrophic factor; GDNF: Glial derived neurotrophic factor.

transplantation, they were able to secrete NGF, NT-3, GDNF and a high level of BDNF, leading to *in vivo* axonal growth. Comparable results were also reported by Neuhuber *et al*[96], who found that MSC secretomes could promote axonal growth and recovery of neuron function owing to the presence of neurotrophic factors[97,98].

DENTAL PULP STEM CELLS AND THEIR SECRETOMES

DPSC secretomes include immunomodulatory, anti-inflammatory, anti-apoptotic and angiogenic regulatory and neurotrophic factors (Figure 4).

Immunomodulatory and anti-inflammatory effects

The immunomodulatory function of DPSCs is mediated through T-lymphocyte function inhibition and this occurs through the action of prostaglandin E2, interleukin-6 (IL-6), TGF-β and HGF secreted from DPSCs[99,100]. Other studies have implicated HGF and TGF-β as DPSCs mediators due to their anti-proliferative effect on T cells. These results were further supported by as study showing upregulation of TGF-β and HGF transcripts during MSCs/T cell cocultures[101].

Anti-apoptotic effects

Neuronal apoptosis or programmed cell death is an important process after nerve injury. Basically, there are two apoptosis pathways, the extrinsic or death receptor pathway, which is activated by tumor necrosis factor-α (TNF-α) overexpression; and the intrinsic pathway, which occurs through mitochondrial damage. DPSCs can prevent TNF-α overexpression and maintain the level of Bcl-xl, thus blocking the extrinsic and intrinsic mechanisms and subsequently decreasing neuronal apoptosis[102].

Angiogenic regulation

Angiogenesis is regulated by both inhibitory and stimulatory molecules[103]. Several studies have shown that MSC/DPSCs are able to express angiogenic factors, such as FGF-2, VEGF, IGF-1, PDGF and TGF-β. Additionally, numerous anti-angiogenic factors have been detected in cultures of DPSCs such as plasminogen activator inhibitor-1, chemotactic protein-1 and endostatin[104-106]. Moreover, DPSCs are capable of inducing *in vitro* migration of endothelial cells and *in vivo* formation of blood vessels and exhibit a higher angiogenic capability than BMSCs/AMSCs[99]. Application of DPSC secretomes to endothelial cells enhanced tubulogenesis and cell migration, demonstrating the paracrine and pro-angiogenic effect of the cell secretomes[107]. Angiogenesis appears to be of prime importance in nerve repair

Table 2 Studies retrieved from PubMed database evaluating the effect of DPSCs on nerve repair or regeneration *in-vivo*

Author	Publication year	Source of stem cells	Target nerves	Study model	Objective	Outcome
Ullah *et al*[82], 2017	2017	Human DPSCs; Differentiated neuronal cells from DPSCs	5-mm gap sciatic nerve transection	Animal rat model	To evaluate the *in-vivo* peripheral nerve regeneration potential of human DPSCs and differentiated neuronal cells from DPSCs.	*In-vivo* transplantation of the undifferentiated hDPSCs could exhibit sufficient and excellent peripheral nerve regeneration potential.
Spyridopoulos *et al*[83], 2015	2015	DPSCs isolated from second lateral incisor pigs	Transected fifth and sixth intercostal nerves	Animal pig model	Examined the potential of DPSCs for peripheral nerve regeneration, using biodegradable collagen conduits.	The nerves where DPSCs were injected exhibited morphological and functional recovery.

DPSCs: Dental pulp stem cells; hDPSCs: Human dental pulp stem cells.

because newly formed blood vessels act as tracks/guiding paths for SCs to cross the bridge gap taking regrowing axons with them[108].

Neurotrophins and their receptors

Neurotrophins are a group of proteinaceous substances that induce the development, function and survival of neurons. Neurotrophins activate and bind to a family of receptor tyrosine kinases (TRKs). NT-3 binds to TrkC, BDNF to TrkB and NGF to TrkA. Binding of these receptors to their factors presents a survival signal to neurons. Another receptor, named $p75^{NTR}$, also binds to neurotrophins but with lower affinity[109]. $P75^{NTR}$ is an indispensable receptor that works in coordination with the TRK family, transducing the signals from NGF, BDNF and NT-3 to regulate a broad array of processes essential to maintenance and development of the nervous system[110].

DPSCs from both rats and humans release neurotrophins, including NGF, GDNF, BDNF and CNTF. Neurotrophins enhance neurite guidance, promote growth of neurons both *in vivo* and *in vitro*, stimulate rescue survival of neurons and induce neurogenesis at the site of injury. They recruit endogenous cells to differentiate into specific cell types necessary for nerve regeneration at the site of damage and stimulate the endogenous cells to secrete neurotrophic factors, promoting tissue regeneration. In animal models and in spinal cord injury, the production of neurotrophins by DPSCs has been shown to rescue motor neurons and mediate the survival of sensory and dopaminergic neurons in addition to the survival of trigeminal ganglia and sympathetic neurons[22].

NGF involved in differentiation and survival of sympathetic and sensory neurons. Its role in neural development has been comprehensively studied. Zhang *et al*[111] found that low NGF concentrations are effective in promoting stem cell proliferation. NGF was also shown to guide the migration of SCs in the PNS, and this is mediated through $p75^{NTR}$[112,113]. BDNF is also one of the neurotrophins that is intensely involved in numerous developmental events in the nervous system, including proliferation, differentiation, migration, apoptosis and survival[114]. This factor helps in neuronal survival and encourages growth and differentiation of new neurons[115]. In the developing visual cortex, exogenous use of BDNF promotes the complexity of pyramidal neurons, with an increase in dendritic length in a layer-specific manner, suggesting that BDNF modulates a specific form in dendritic growth in addition to enhancing neuronal growth[116,117].

In experimental animal models, NT-3 has been verified to promote regeneration of injured axons, enhance neurite outgrowth and improve axon function. Cells overexpressing NT-3 migrated more and displayed longer neurites *in vitro* and *in vivo* than other cells[118,119]. CNTF has powerful therapeutic effects on nerve apoptosis, neuro-inflammation and neuronal proliferation[120,121]. Additionally, GDNF potently promotes the survival of many types of neurons. The most prominent feature of GDNF is its ability to support the survival of motor and dopaminergic neurons[22].

Table 3 Studies retrieved from PubMed database evaluating the effect of DPSCs on nerve repair or regeneration *in-vitro*

Author	Publication year	Source of stem cells	Target tissues	Objective	Outcome
Geng *et al*[84], 2017	2017	Human DPSCs	Differentiation of hDPSCs.	To demonstrate the differentiating ability of resveratrol on DPSCs.	Resveratrol induced DPSCs differentiation into neuroprogenitor cells. DPSCs might be an important cell population for neurological disease treatment.
Hafner *et al*[85], 2017	2017	Human DPSCs	Spider dragline silk fibers	To evaluating adhesion and alignment of dental pulp stem cells to a spider silk substrate for tissue engineering applications.	Natural drawn spider silk acted as an effective substrate for cellular adhesion and alignment of DPSCs and could be used in neural differentiation applications.
Chang *et al*[86], 2014	2014	Human DPSCs	Medium preparation for the induction of spinal motor neuronal differentiation; Medium preparation for the induction of dopaminergic neuronal differentiation	To evaluate the efficacy of dopaminergic and motor neuronal inductive media on transdifferentiation of human DPSCs (hDPSCs) into neuron-like cells.	Human DPSCs-derived dopaminergic and spinal motor neuron cells after induction expressed a higher density of neuron cell markers than those before induction.
Mead *et al*[60], 2014	2014	Human DPSC, human BMSCs human AMSCs	Axotomised adult rat retinal ganglion cells	To evaluate the therapeutic potential for neurodegenerative conditions of retinal ganglion cells.	Human DPSCs promoted significant multi-factorial paracrine-mediated retinal ganglion cell survival and neurite outgrowth compared with Human BMSCs/Human AMSCs.
Martens *et al*[23], 2014	2014	Human DPSCs	Dorsal root ganglia	Evaluated the differentiation potential of human DPSCs toward SCs, together with their functional capacity with regard to myelination and support of neurite outgrowth.	Human DPSCs are able to undergo SCs differentiation and support neural outgrowth.

DPSCs: Dental pulp stem cells; BMSCs: Bone marrow stem cells; AMSCs: Adipose mesenchymal stem cells.

CELL-FREE THERAPY

As discussed above, neurotrophic factors released by DPSCs/MSCs may act as modulators of neural differentiation and survival[122-124]. Individual use of these trophic factors or even combinations of them appears to be unsuccessful and less efficient at enhancing regeneration than the full secretomes[95,125]. The possibility of repairing injured tissue with secretomes rather than cell therapy introduces a new era for therapeutic application of secretomes in regenerative medicine[126]. Mead *et al*[60] confirmed the role of neurotrophins in neuroprotection and neuritogenesis using the fusion protein Fc-NTFR to block neurotrophin receptor sites and examine the mechanism of DPSCs/BMSCs/AMSCs-mediated neuroprotection and neuritogenesis when cocultured with retinal ganglion cells. The DPSCs neuroprotective effect was considerably decreased after addition of Fc-NTFR, confirming the major role of neurotrophins (GDNF, BDNF, NGF, VEGF, NT-3 and PDGF) in neuroprotection and neuritogenesis.

CBT *VS* CELL-FREE THERAPY

The main challenge with CBT is how to maintain cell viability and function after *in vivo* implantation because *in vivo* conditions are very different from well-controlled *in*

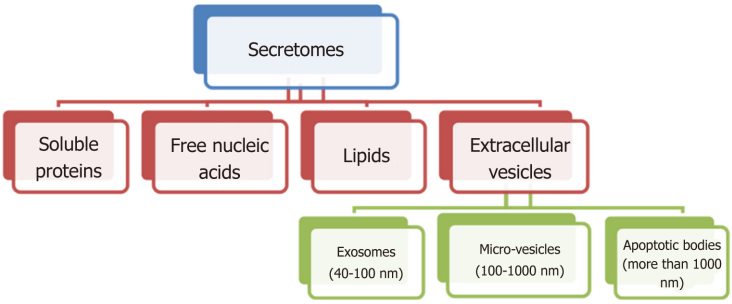

Figure 3 Organization chart representing the different components of secretomes from the mesenchymal stem cells. Exosomes, micro-vesicles and apoptotic bodies were classified according to the size and morphology. Exosomes (40-100 nm) usually derived from multi-vesicular bodies, microvesicles (100-1000 nm) by plasma membrane budding and anti-apoptotic bodies (more than 1000 nm) *via* blebbing of plasma membrane of dying cells.

vitro cultivation conditions. Factors such as tissue collagen density, blood supply and scar formation potential greatly affects the fate of transplanted cells[127]. For instance, the spreading of fibroblasts initially increases with the increase in collagen density, but beyond a specific limit, the relationship is reversed. At a high collagen density, the attempt of a cell to spread maximally is restricted by the availability of collagen binding sites and consequently, cells exert a maximal force to tightly bind with the few available sites[128]. Moreover, the molecular environment in injured tissue enhances apoptotic cell death; therefore, massive death of the transplanted cells ocurres. This is due to the elevated level of oxidative stress, mediated by reactive oxygen species, in the injured tissue, which triggers cell apoptosis[129].

Bork *et al*[130] reported that DNA methylation and epigenetic changes occurred in replicative senescence upon long term cultivation. Lack of differentiation potential, cell enlargement and eventual growth arrest occurs in replicative senescence. A similar methylation pattern was observed in MSCs from older donors. These findings support evidence showing that aging and replicative senescence represent a developmental program and are not only caused by accumulation of cellular and molecular defects, and thus, long-term culture and aging might be regulated by similar mechanisms. At the same time, the long-term cultivation process leads to stress-induced senescence changes due to high oxygen content and the artificial *in vitro* environment. This brings about a decrease in the self-renewal potential of cells, and when such cells are implanted in the host, poor growth, cell survival and paracrine effect outcomes result. Therefore, there are still many hurdles before stem CBT can be adopted for PNI and it becomes decisive to determine a strategy to overcome difficulties and the problems related to cell transplantation[131].

In the field of regenerative medicine, the use of CFT has been widely studied. The use of the secretomes overcomes a number of safety concerns that include tumorigenicity, emboli formation and immune compatibility. Secretomes can be stored for a long period without application of a potentially toxic cryopreservative and without loss of product potency[132,133]. An important feature of exosomes, which are part of the secretome, is that they are encapsulated, providing protection to their contents against *in vivo* degradation, thus possibly avoiding obstacles associated with soluble small molecules, such as transcription factors, cytokines, growth factors and RNAs, that are quickly degraded[134]. Moreover, exosomes can act as liposomes and can pass through the blood-brain barrier, making them potentially suitable for treatment of neurological disorders[135].

To sum up, secretomes contain soluble growth factors and cytokines related to protection, repair, regeneration, immunomodulation, cell proliferation, cell communication and other important functions[136]. Use of DPSCs/MSCs secretomes has several advantages, and we can avoid concerns relating to cell transplantation. Recently, a comparative study demonstrated that treatment with secretomes induced a long-lasting effect with a disease-modifying profile similar to that shown by stem cells. Wakayama *et al*[137] illustrated the effect of DPSCs and their secretomes on acute lung injury; they examined the persistence and localization of DPSCs transplanted to an injured lung. It was found that the survival of DPSCs was less than 1% one week after transplantation and the therapeutic benefit of DPSCs and DPSCs secretomes was similar two weeks after transplantation. Therefore, the researchers concluded that the therapeutic effect of DPSCs was likely mediated *via* paracrine signaling that remains for an extended period of time even after cell disappearance. This study opens new possibilities for treatment using a cell-free approach that is able to retain the benefits of cell therapy without the inherent difficulties of CBT[138].

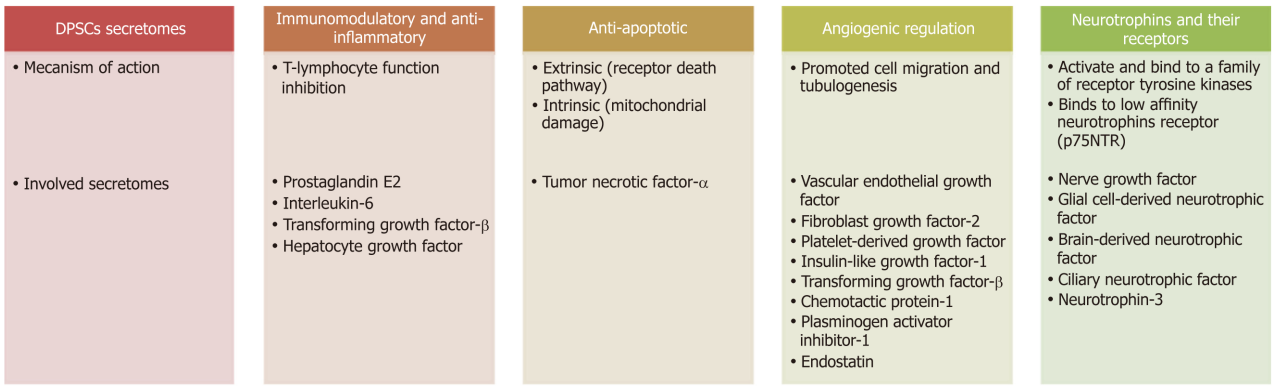

Figure 4 Horizontal bullet list for the dental pulp stem cells' secretome, their mechanism of action and the involved factors in the immunomodulatory, anti-inflammatory, anti-apoptotic angiogenic regulatory and neurotrophic function.

CELL-FREE THERAPY CHALLENGES

It is essential to consider the possible adverse effects of the therapeutic potential of exosomes against their future application. It has been stated that miRNAs that are carried by exosomes might induce cancer or tumor formation[139]. In addition, exosomes may be associated with a number of neurologic diseases related to old age, such as Parkinson's and Alzheimer's diseases[140]. Moreover, the ideal timeframe for injection/addition of exosomes to exploit their benefits and whether a single application dose is sufficient or if daily, weekly or monthly doses are necessary are still unknown. Additionally, different epitopes are expressed on the surface of exosomes released from the same cells, indicating the presence of exosome subtypes, which merits further research[141]. Further in-depth research is guaranteed to lead to an improved understanding of exosomes and their interference with unknown secreted factors.

CONCLUSION

DPSCs secretomes are a promising strategy for CBT and CFT. They can be easily isolated, purified and stored, thus avoiding complications associated with cell therapy, such as unwanted proliferation/differentiation and development of ectopic tissue. To appraise the role of the surrounding microenvironment in the biological response of DPSCs, preconditioning may be helpful to obtain tailor-made secretomes. Preconditioning achieved by subjecting cells to hypoxia, drug treatment, and specific growth factor/cytokines may help in obtaining an optimal secretome profile. The ideal timeframe for injection/addition of the secretomes to exploit their benefits is still unknown, as well as whether a single application dose is sufficient or if daily, weekly or monthly doses are necessary. Additionally, different surface epitopes are expressed on exosomes from the same cells, indicating the presence of exosome subtypes, which merits further research.

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P- Reviewer: Fatkhudinov T, Hassan AI, Pixley JS
S- Editor: Cui LJ **L- Editor:** A **E- Editor:** Bian YN

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ISSN 2218-6263 (online)

World Journal of *Stomatology*

World J Stomatol 2019 February 20; 7(2): 20-27

Published by Baishideng Publishing Group Inc

World Journal of Stomatology

Contents

Irregular Volume 7 Number 2 February 20, 2019

MINIREVIEWS

World Journal of Stomatology
Volume 7 Number 2 February 20, 2019

Contents

NAME OF JOURNAL
World Journal of Stomatology

ISSN
ISSN 2218-6263 (online)

LAUNCH DATE
December 31, 2011

FREQUENCY
Irregular

EDITORS-IN-CHIEF
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February 20, 2019

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World Journal of Stomatology

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World J Stomatol 2019 February 20; 7(2): 20-27

DOI: 10.5321/wjs.v7.i2.20

ISSN 2218-6263 (online)

MINIREVIEWS

Molar incisor hypomineralization and pre-eruptive intracoronal lesions in dentistry-diagnosis and treatment planning

Uri Zilberman, Jomanna Hassan, Shirley Leiboviz-Haviv

ORCID number: Uri Zilberman (0000-0002-8343-8686); Jomana Hassan (0000-0003-3185-7544); Shirley Leiboviz-Haviv (0000-0002-3590-8612).

Author contributions: All authors contributed to the manuscript.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported.

Manuscript source: Invited manuscript

Received: December 11, 2018
Peer-review started: December 12, 2018
First decision: January 4, 2019
Revised: January 14, 2019
Accepted: January 28, 2019
Article in press: January 28, 2019
Published online: February 20, 2019

Uri Zilberman, Jomanna Hassan, Shirley Leiboviz-Haviv, Pediatric Dental Clinic, Barzilai Medical University Center, Ashkelon 7830604, Israel

Corresponding author: Uri Zilberman, DMD, PhD, Senior Lecturer, Pediatric Dental Clinic, Barzilai Medical University Center, 2nd Hahistadrut Street, Ashkelon 7830604, Israel.
ori@barzi.health.gov.il
Telephone: +972-8-6745854

Abstract

The aim of this study is to report the diagnostic features, prevalence, mineral content, clinical significance and treatment options of molar incisor hypomineralization (MIH) and pre-eruptive intracoronal lesions (PEIR), in order to minimize miss-treatment of primary and permanent teeth in young children. MIH was defined as the occurrence of hypomineralization of one up to four permanent first molars from a systemic origin and frequently associated with affected incisors. PEIR are lesions that are located in the occlusal portion of the crown of unerupted permanent or primary teeth. The prevalence of MIH was reported between 2.5%-40% in the permanent first molars and 0%-21.8% in primary second molars. PEIR was observed in 2%-8% of children, mainly in mandibular second premolars and second and third permanent molars. A number of possible causes for MIH were mentioned, including environmental changes, diet and genetics in prenatal and postnatal periods, but all are questionable. In PEIR, the resorption of the intracoronal dentine begins only after crown development is complete and is caused by giant cells resembling osteoclast observed histologically on the dentine surface close to the pulp. The mineral content in MIH is reduced in comparison to normal enamel and dependent on the severity of the lesion. In PEIR the resorbed surface of enamel showed less mineral content. The hypomineralized enamel in MIH is not suitable for restorations with amalgam or composite materials, and the best material should be based on remineralization material like glass-ionomers. Similar, the resorbed dentin surface in PEIR should be covered by the biocompatible and re-mineralizing glass-ionomer cement.

Key words: Molar incisor hypomineralization; Pre-eruptive intracoronal lesions; Glass-ionomer cements; Enamel; Dentin

Core tip: Molar incisor hypomineralization and pre-eruptive intracoronal lesions are an

increasing concern in pediatric dentistry. Since both conditions are developmental and not caused by carious attack, correct diagnosis of the conditions is significant in order to successfully treat the teeth. Early diagnosis can improve the survival rate of these teeth, because breakdown of the enamel can occur in both lesions and subsequent carious attack may lead to pulpal involvement and extraction of teeth at a very young age. The optional restorative material should be based on the re-mineralizing properties of glass-ionomer cements.

Citation: Zilberman U, Hassan J, Leiboviz-Haviv S. Molar incisor hypomineralization and pre-eruptive intracoronal lesions in dentistry-diagnosis and treatment planning. *World J Stomatol* 2019; 7(2): 20-27
URL: https://www.wjgnet.com/2218-6263/full/v7/i2/20.htm
DOI: https://dx.doi.org/10.5321/wjs.v7.i2.20

INTRODUCTION

Molar incisor hypomineralization (MIH) was defined as the occurrence of a hypomineralization of one up to four permanent first molars from a systemic origin and frequently associated with affected incisors[1].

The cause of MIH in permanent molars or primary molars (HSPM) is a disturbance during the initial calcification and/or maturation of enamel of the affected teeth[1,2]. The term hypomineralized second primary molars (HSPM)[3-6] is currently used to describe the condition previously known as deciduous molar hypomineralisation (DMH), although the condition can be observed also in primary first molars or canines. The characteristic features of hypomineralized permanent or primary molars are: (1) Opaque stains that vary between white, yellow and brown; (2) post-eruptive enamel breakdown, (3) atypical restorations and/or extensive caries with opacities at the margins; and (4) early extracted permanent molars. Variety can be noted not only in severity between patients but also varying degrees within the mouth of a single patient[6]. The affected permanent molars are very sensitive and difficult to treat due to some degree of inflammation in the pulp[2]. A wide variation in the reported global prevalence of MIH 2.9%-44% and HSPM 0-21.8% was published[7]. In a recent published study of 1001 Egyptian children aged 8-12 years old the prevalence of MIH was very low, 2.3%[8], and the most prevalent clinical defect was demarcated opacity. In a nearby country, Israel, the prevalence of MIH was 17.1% in the Jewish population and 17.95% in the Arab population (2671 Jewish children and 2844 Arab children were examined). The most important finding was that the percentage of MIH was higher in the younger age group (6-10 year) in comparison with the older age group (11-16 year)[9], implicating that more children with MIH will be treated by dentists in the future. A number of possible causes for MIH were mentioned, including environmental changes, diet and genetics in prenatal and postnatal periods[10-14]. The most significant etiological finding was that the *ENAM* rs3796704 marker may be associated with MIH[15]. Mineral density of MIH molars showed on average about 19% reduction compared to sound enamel, and mineral content was in average only 58% vol% mineral[16,17]. In contrast protein content was 15-21-folds higher in brown enamel and 8-folds in yellow and chalky enamel compared to normal enamel[18].

Pre-eruptive intracoronal lesions (PEIR) are lesions that are located in the occlusal portion of the crown of unerupted permanent or primary teeth[19-21]. The lesions may be detected incidentally on radiographs before their eruption into the oral cavity. PEIR manifest on roentgenographs as radiolucent lesions, which resemble caries. The lesions are located just beneath the dentine-enamel junction of unerupted teeth and may affect all the crown dentine[22]. It has been suggested that most of the cases previously defined as hidden or occult caries were in fact due to PEIR, but observed only after eruption of the involved tooth, and breakdown of the occlusal enamel[23]. The published prevalence of PEIR is 1.63%-8% by subject and 0.32%-2% by tooth[24-26], but two studies from Turkey reported a very high prevalence, 15.1% and 27.3%[27,28]. The highest prevalence was found in mandibular first premolars, followed by second or third molars, with no significant gender differences. The resorption of the intracoronal dentine begins only after crown development is complete[29] and results from resorption of dentin by osteoclast like giant cells observed histologically on the dentine on the pulpal surface[30] that may originate from undifferentiated cells of the developing dental follicle[31]. No systemic factors are currently known to predispose to

PEIR, but in a recent publication it was correlated with delayed dental development of at least six months[32]. The aim of the study was to examine the mineral content of teeth affected by MIH and PEIR and to describe treatment options.

MIH

One normally exfoliated first primary molar affected by hypomineralization (FPMH) and one permanent molar extracted due to MH were analyzed (Figures 1 and 2).

The primary molar showed two distinct areas of hypomineralization on the buccal surface: a yellow-brownish area (Figure 1, area 2) and an area with breakdown of enamel (Figure 1 area 3). The permanent molar showed breakdown of enamel on two-thirds of the crown height. The enamel close to the CEJ was the control area since MIH and HSPM affects only two-thirds of crown height. We used an energy-dispersive X-ray spectroscopy (EDAX EDS System) SEM (FEI Quanta 200 microscope operating at 25 keV) to compare the ion content of the surface enamel at the discolored area and the border of enamel breakdown lesion to normal enamel in the first primary molar and the border of the hypomineralized enamel breakdown in the first permanent molar to normal enamel at the cervical region. The results are summarized in Tables 1 and 2.

In the first primary molar the reduction of phosphate and calcium was related to the degree of hypomineralization- at the brown discolored area the calcium was reduced by almost 50% and at the border of breakdown enamel the reduction was by 70%. The phosphate was minimally reduced at the discolored area but significantly (by 65%) reduced at the border of breakdown of enamel. The carbon and oxygen content were increased. In the first permanent molar the reduction of calcium was by 35% and of phosphate by 60%. The oxygen and carbon content was increased by more than 300%. PEIR: The mineral content of an archeological specimen diagnosed with PEIR was analyzed[33]. The internal surface of the enamel of second permanent molar affected by PEIR was compared to intact enamel surface of first permanent molar. The calcium content was reduced by 10% but the phosphate was resorbed by 90%.

CONCLUSION

MIH is quite a new developmental entity that affects enamel formation in primary and permanent dentition. Its prevalence is low in the Africa countries and very high in the north European, Australia and South America countries. Recent publications tried to show that MIH can be found among past populations, as early as 12th century[34-37] . The main problem in the report is that the discoloration of the teeth attributed to hypomineralisation (Figures 1-3 in Garot *et al*[34] 2017, Figures 1 and 2 in Ogden *et al*[36] 2008, Figure 2B in Curzon *et al*[35] 2015) showed similar affected areas on both sides of the jaw and with similar hypoplastic areas. As Dr Manton, one of the authors of Garot *et al*[34] manuscript, described the clinical condition of MIH in a webinar, there is a great variability of severity of hypomineralisation in the same mouth between teeth developing during the same period. So, the similarity of discoloration and hypoplasia in teeth from the same jaw cannot be attributed to MIH. Pitted enamel (Figure 1 in Ogden *et al*[36] 2008, and in Kuhnisch *et al*[37] 2016) cannot be attributed to MIH, but to hypoplastic enamel. A well-known phenomenon is that taphonomic stains (Figure 1A and B in Kuhnisch *et al*[37] 2016, Figure 1B in Curzon *et al*[35] 2015) in archeological material may have a similar appearance to MIH as described by Garot *et al*[38]. The PEIR lesion can be easily diagnosed in archeological material in individuals with a caries free dentition and one tooth with an extensive resorption of the dentine with or without enamel breakdown[33].

The clinical significance of MIH can be attributed to the high prevalence of the lesion in some Western countries and the early age of the patients. The permanent teeth affected by MIH are very sensitive and hard to anesthetize due to some degree of chronic pulpitis, since the dentinal tubuli are very wide and the inflammatory factors can reach the pulp very easy. The borders of MIH lesions, even if they appear clinically fully mineralized, showed some degree of hypomineralization[39]. Amalgam restorations require sound enamel on the borders in order to prevent ditching and discoloration[40]. Hypomineralized enamel is not suitable for composite restorations since the bonding strength is reduced[41,42]. In our clinic glass-ionomer restorative materials are used in cases of MIH, HSPM or HFPM with very high success rate (Figures 3-5).

The PEIR lesions have to be treated early after eruption of the tooth or even before tooth eruption if the lesion is very large. Since the borders of the lesion are hypomineralized due to the resorption process Glass ionomer cements should be used for final restoration and remineralization of enamel and dentine[43](Figure 6).

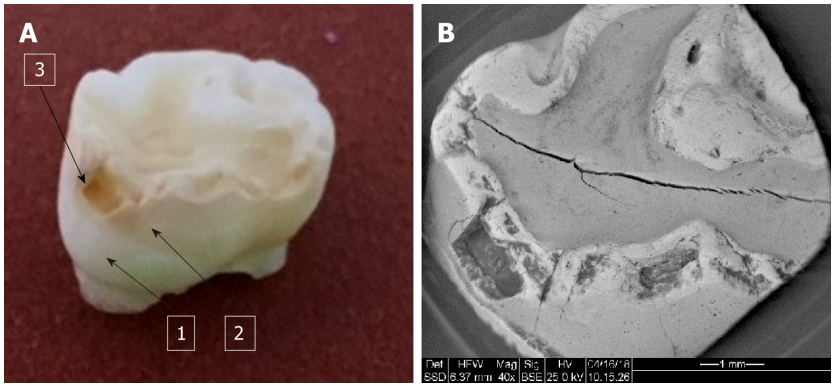

Figure 1 Clinical and SEM pictures of hypomineralized first primary molar. A: Clinical picture of hypomineralized first primary molar. 1: Normal enamel; 2: Brown discolored enamel; 3: Breakdown of enamel; B: SEM picture of hypomineralized first primary molar.

Recent meta-analysis showed that ART single surface restorations using high-viscosity glass-ionomer cements like EQUIA Forte presented high survival percentages in both primary and permanent posterior teeth[44]. More efforts should be made in order to describe the clinical features of MIH and PEIR to GPs in order to minimize the atypical restorations with amalgam or composite and to minimize the need of extractions of permanent molars in young children.

Table 1 Ion content in % of the enamel of first primary molar at different areas

	Normal enamel	Demarcated opacities	Breakdown of enamel
Carbon	17.61%	23.30%	50.79%
Oxygen	22.76%	35.24%	31.21%
Phosphate	14.74%	13.33%	5.36%
Calcium	44.87%	26.46%	12.64%

Table 2 Ion content in % of normal and hypomineralized enamel of first permanent molar

	Normal enamel	Breakdown of enamel
Carbon	3.5%	12.75%
Oxygen	11.5%	41.74%
Phosphate	36.5%	14.74%
Calcium	46.3%	30.99%

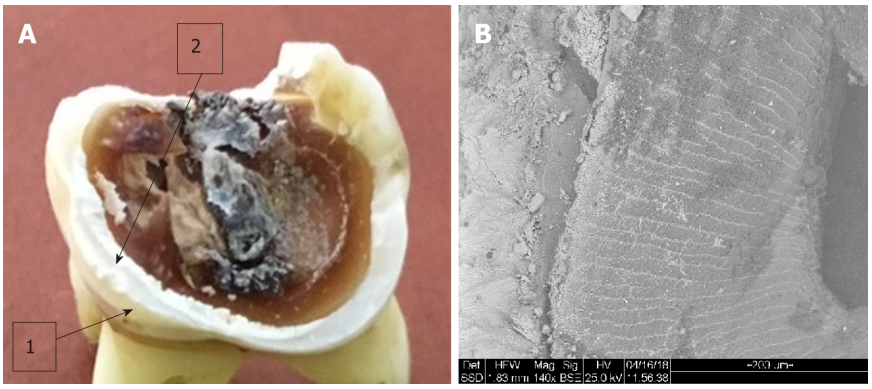

Figure 2 Clinical image of the first permanent molar affected by MH and the SEM image of the analyzed enamel. A: Clinical picture. 1: Normal enamel; 2: Hypomineralized enamel border; B: SEM picture.

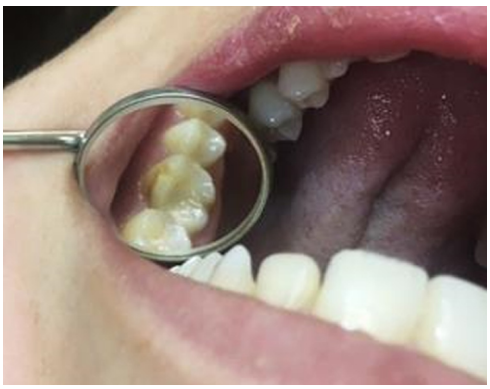

Figure 3 Restoration of disto-buccal cusp of upper permanent M1 affected by MIH with Glass-ionomer cement (EQUIA forte by GC Japan).

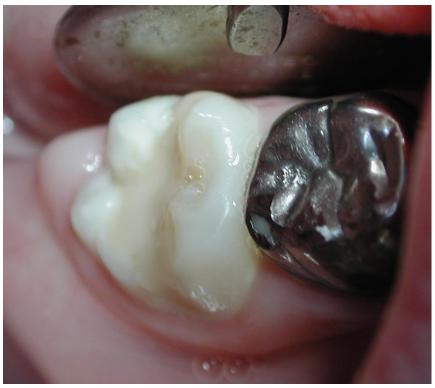

Figure 4 Bucco-occlusal restoration of permanent lower first molar affected by MIH with Glass-ionomer cement (EQUIA forte by GC Japan).

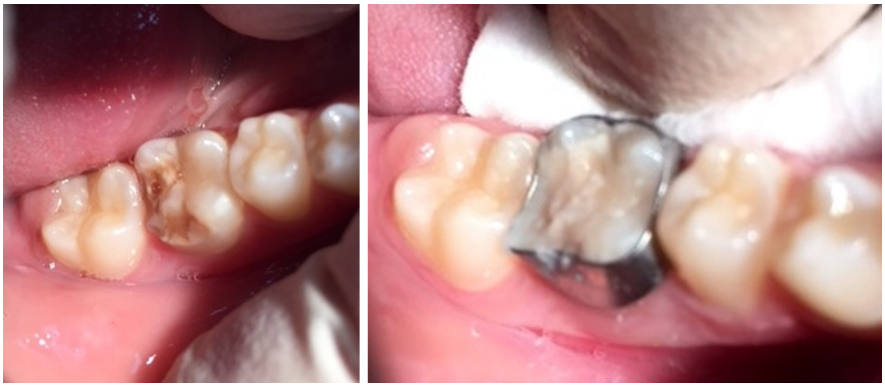

Figure 5 Breakdown of enamel of a first permanent molar due to MIH. An orthodontic ring was cemented with Fuji 1 (Glass-ionomer cement) and the lesion filled with Glass-ionomer cement (EQUIA forte by GC Japan).

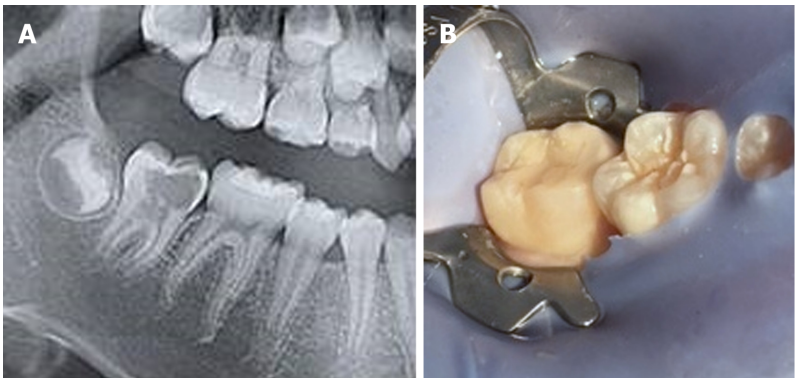

Figure 6 Pre-eruptive intracoronal lesions of unerrupted second permanent molar (A) and restoration of the lesion with Glass-ionomer cement (EQUIA forte by GC Japan). A: SEM picture; B: Clinical picture.

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World J Stomatol 2019 December 18; 7(3): 28-38

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World Journal of Stomatology

Contents

Irregular Volume 7 Number 3 December 18, 2019

CASE REPORT

World Journal of Stomatology
Volume 7 Number 3 December 18, 2019

Contents

NAME OF JOURNAL
World Journal of Stomatology

ISSN
ISSN 2218-6263 (online)

LAUNCH DATE
December 31, 2011

FREQUENCY
Irregular

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World J Stomatol 2019 December 18; 7(3): 28-38

DOI: 10.5321/wjs.v7.i3.28

ISSN 2218-6263 (online)

CASE REPORT

Autologous platelet-rich-fibrin-induced revascularization sequelae: Two case reports

Ahmed M Eltawila, Rania El Backly

ORCID number: Ahmed M Eltawila (0000-0003-3070-3746); Rania El Backly (0000-0003-3147-0032).

Author contributions: Eltawila AM performed the clinical procedures, analyzed data and wrote the manuscript; El Backly R designed the study, supervised the clinical procedures, interpreted data and revised the manuscript; all authors issued final approval for the version to be submitted.

Informed consent statement: Informed written consent was obtained from patients before clinical procedure, and for publication of this report and any accompanying images.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

CARE Checklist (2016) statement: The authors have read and prepared the manuscript according to the CARE Checklist (2016). The list is attached.

Ahmed M Eltawila, Department of Materials Science, Institute of Graduate Studies and Research, Alexandria University, Alexandria 21526, Egypt

Ahmed M Eltawila, Rania El Backly, Tissue Engineering Laboratories, Faculty of Dentistry, Alexandria University, Alexandria 21411, Egypt

Rania El Backly, Endodontics Division, Conservative Dentistry Department, Faculty of Dentistry, Alexandria University, Alexandria 21411, Egypt

Corresponding author: Rania El Backly, BDS, MSc, PhD, Lecturer, Endodontics Division, Conservative Dentistry Department, Faculty of Dentistry, Alexandria University, Champollion Street, Alexandria 21411, Egypt. ranianoaman@gmail.com
Telephone: +2-3-4868066
Fax: +2-3-4868066

Abstract

BACKGROUND

A key requirement for biomimetic regeneration of tissues is a 3D scaffold. The gold standard scaffold for revascularization is the blood clot, however, an adequate blood clot cannot always be achieved in narrow canals or mature roots. Hereby, we document the effects of platelet-rich fibrin (PRF) for the regenerative endodontic treatment (RET) of two immature permanent teeth with necrotic pulps for up to 48 mo.

CASES SUMMARY

The first patient was a 22-year-old female with history of trauma in tooth #9 with a sinus tract and a large periapical lesion. The second was a 9-year-old male presenting with a badly decayed tooth #14. Both cases were treated with RET and PRF prepared from the patients' blood. PRF and its extract were used as a scaffold for RET. Patients were followed-up to 9 and 48 mo (4 years), respectively. Both patients, were asymptomatic after treatment. At the 9-mo-follow-up of case #1, there was radiographic evidence of periapical bone healing, however, the root apex was still open. In case #2, the roots exhibited apical closure and normal periapical bone architecture at 12-mo follow-up, while no root lengthening was observed. After 48 mo, case #2 showed extensive intracanal calcification in all root canals that complicated conventional root canal treatment.

CONCLUSION

RET with PRF and its extract could be used in revascularization of immature permanent teeth. However, proper case selection to comply with long-term follow-up is necessary and adverse events such as calcification and canal

Manuscript source: Unsolicited manuscript

Received: June 28, 2019
Peer-review started: June 29, 2019
First decision: August 2, 2019
Revised: October 2, 2019
Accepted: November 26, 2019
Article in press: November 26, 2019
Published online: December 18, 2019

P-Reviewer: Huang AHC, Paredes-Vieyra JP, Teragawa H
S-Editor: Ma YJ
L-Editor: A
E-Editor: Liu JH

obliteration should be planned for.

Key words: Revascularization; Platelet-rich fibrin; Dental pulp; Regenerative endodontics; Tooth; Case report

Core tip: This report investigated the effects of platelet-rich fibrin (PRF) for the regenerative endodontic treatment (RET). The first patient had a history of trauma in tooth #9 with a sinus tract and a large periapical lesion. The second patient presented with a badly decayed tooth #14. Both cases were treated with platelet-rich fibrin (PRF) as scaffold for RET. Patients were followed up to 9 and 48 mo, respectively. There was radiographic evidence of periapical bone healing in both cases. The second patient showed root maturation after 12 mo and extensive intracanal calcification at 48 mo.

Citation: Eltawila AM, El Backly R. Autologous platelet-rich-fibrin-induced revascularization sequelae: Two case reports. *World J Stomatol* 2019; 7(3): 28-38
URL: https://www.wjgnet.com/2218-6263/full/v7/i3/28.htm
DOI: https://dx.doi.org/10.5321/wjs.v7.i3.28

INTRODUCTION

Regenerative endodontic treatments (RETs) require a 3D biological scaffold to promote tissue regeneration[1,2]. The most common scaffold is a blood clot, created from induced periapical bleeding through the root apical foramen[3]. Periapical bleeding delivers mesenchymal stem cells (MSCs)[4] and the blood clot forms a 3-D network for endodontic tissue and root generation. However, an adequate blood clot scaffold cannot always be achieved from periapical bleeding[3]. Most practitioners believe that RETs can be more beneficial than apexification for the treatment of immature teeth with necrotic pulps[5], though they want to know how to make RETs more successful[5], by identifying a more reliable scaffold, such as platelet-rich fibrin (PRF).

PRF is a second-generation platelet-rich concentrate that allows a sustained release in a slowly degrading fibrin matrix for multiple growth factors, such as; platelet derived growth factor; transforming growth factor-β; and vascular endothelial growth factor[6], and numerous cytokines[7]. Platelet-rich concentrates are a naturally occurring cocktail of growth factors and cytokines organized in a 3D framework[7]. These bioactive factors are involved in all phases of wound healing and subsequently tissue regeneration. These properties, in addition to the ease of preparation and manipulation, make PRF an excellent candidate for RETs[8]. Additionally, the fluid obtained when centrifuging PRF (PRF-extract) can further stimulate endogenous regeneration by having synergistic action with mineral trioxide aggregate as it has been shown to enhance odontoblastic differentiation of human dental pulp stem cells[6]. Herein, we describe the successful use of PRF and its extract in conjunction with induction of bleeding, or as an alternative scaffold, for the regenerative endodontic management of two challenging cases.

CASE PRESENTATION

Preparation protocol of PRF and PRF-extract

PRF and its extract were prepared from venous blood collected from the patients' arms immediately prior to centrifugation and use[9], these steps are illustrated in (Figure 1). All procedures were performed per the institutional ethical guidelines instated by the IRB of the Faculty of Dentistry, Alexandria University and following informed patient and parent/guardian consent. 5-10 cc of the patient's venous blood was collected from their median cubital vein in a non-coated glass tube. The collected blood was immediately centrifuged at 3000 RPM for 10 min. The PRF clot was compressed against a metallic mesh to collect the fluidic extract. The PRF preparation was done chair-side and immediately before use (Figure 2A). The red corpuscle layer was gently wiped off the surface of the PRF clot set aside. At the time of insertion,

care was taken to pack the PRF clot with the platelet-plug facing the apical tissues.

Case 1

A 22-year-old female patient was referred to the conservative dentistry department clinics at the faculty of dentistry, Alexandria University complaining of a discolored upper anterior tooth. Upon examination, tooth #9 was found to be discolored with a sinus tract opening on the labial surface related to the same tooth when traced with Gutta Percha points. The patient reported a history of trauma when she was 8-years old. The periapical and panoramic radiographs revealed a large periapical lesion related to the teeth #9, #10 and tooth #11. Tooth #9 had an immature root. The tooth was tender to percussion. The cold pulp test was negative for #9 and #10. Tooth #9 responded negatively to hot and tooth #10 showed mild response when compared to the contralateral tooth. Periodontal examination revealed normal probing depths.

Case 2

A 9-year-old systemically healthy male was referred from the orthodontics department for RET of an immature permanent maxillary first molar. The patient had been complaining of toothache, then the tooth became asymptomatic except the night before treatment. Upon clinical examination the tooth appeared badly decayed, tender to percussion, and negative to both cold and hot tests, however, there was normal periodontal probing depth. Digital periapical radiographs were obtained using Sidexis software system (Sirona Dental Gmbh), and revealed a radiolucent periapical lesion in relation to the palatal root of tooth #14 which appeared to be immature while both buccal roots showed wide apices (Figure 3).

FINAL DIAGNOSIS

Case 1

A diagnosis of the immature tooth #9 was concluded as necrotic pulp and chronic periapical abscess. The patient had an irrelevant medical history and a regenerative approach was decided after appropriate consent was obtained.

Case 2

A diagnosis of necrotic pulp with symptomatic apical periodontitis was made in relation to tooth #14. Since the tooth appeared to have at least one immature root, a decision was made to treat the tooth using RETs. The risks and benefits of the procedure were explained to the parent and informed written consent was obtained.

TREATMENT PROCEDURES

Case 1

After local anesthesia and rubber dam isolation of tooth #9, the pulp chamber was accessed. Pus was drained from the canal and flushed with copious saline irrigation. Working length was determined using digital x-rays and the canal was minimally instrumented. Copious irrigation using 20 mL of 1.5% sodium hypochlorite for 5 min was done. Finally, the canal was dried with sterile paper points followed by filling the root canal with Calcium Hydroxide paste (Ultra-Cal XS, Ultradent, United States).

Three weeks later, the tooth was asymptomatic and the labial sinus tract opening had healed. The tooth was anesthetized with 3% Mepivicaine without vasoconstrictor (Alexandria Co. for Pharmaceuticals, Egypt), the canal was found to be dry and then it was copiously irrigated with 1.5% Sodium Hypochlorite followed by 10 mL of sterile saline. After drying the canal with paper points, the dentin was conditioned with 17% EDTA (Glyde) for one minute then it was rinsed with saline irrigation. The canal was dried and intra-canal bleeding was provoked using a K-file #15 extending 2 mm beyond the working length. Meanwhile, PRF was prepared as described before (Figure 2A). PRF-extract was injected inside the canal followed by packing of the PRF clot just apical to the cementoenamel junction (Figure 2B-D). A coronal barrier with MTA was made (MM-MTA™, MicroMega, France) followed by glass ionomer and composite restoration.

Case 2

The tooth was anesthetized with Mepecaine-L (2% mepivacaine HCl, levonordefrin 1:20000, Alexandria Co. For Pharmaceuticals, Egypt). Caries was removed, the pulp chamber was accessed and the tooth was isolated with rubber dam. Four root canals were identified; two mesiobuccal, distobuccal and palatal canals. Canals were

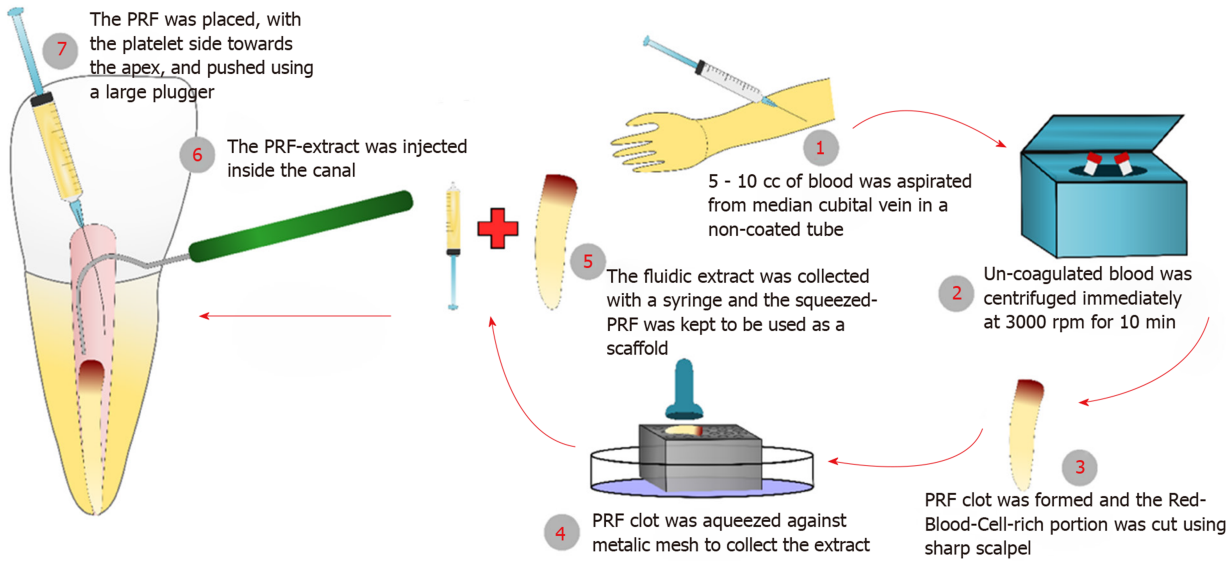

Figure 1 The preparation steps of platelet-rich fibrin and platelet-rich fibrin-extract, and how they were used for the regenerative endodontic treatments.
PRF: Platelet-rich fibrin.

irrigated with 2.5% sodium hypochlorite (NaClO) (Household Cleaning Products Company of Egypt, Cairo, Egypt) and normal saline. The working length was estimated based on the digital periapical x-rays and the canals gently instrumented with #15 K-files, then filled with Calcium hydroxide paste (Ultracal XS, Ultradent, United States), and the tooth was temporary filled (Ora-fil G, Prevest Denpro, India).

After one week, the patient had slight pain on biting and was recalled. The temporary filling was removed after the application of rubber dam. The tooth was etched (Dentsply), bonded (Excite DSC, Ivoclar-Vivadent, Liechtestein) and the missing tooth walls were restored with composite resin (Te econom, Ivoclar-Vivadent, Liechtestein). The canal orifices were enlarged using sizes 3 and 4 Gates Glidden drills and copiously irrigated with 2.5% NaClO, dried and filled with calcium hydroxide. The cavity was filled with a temporary restorative material and Cone Beam Computed Tomography (CBCT) was ordered (Figure 3E-H). After two days, the patient was recalled. The distal and palatal canals were minimally instrumented up to #15 and #20 hand K-files based on the lengths measured on CBCT. The mesial canals were instrumented up to size #30. Then, canals were irrigated, filled with calcium hydroxide paste and the access cavity was sealed with temporary filling.

After 4 wk, the patient was recalled and the tooth was asymptomatic. It was anesthetized with 3% Mepivacaine without vasoconstrictor (Mepivacaine HCl 3%, Alexandria Co. for Pharmaceuticals, Egypt), isolated with rubber dam and the temporary filling was removed. Canals were irrigated with 2.5% NaClO; 10 mL/canal with a final application of 17% EDTA (Glyde, Dentsply-Maillefer, Switzerland) for one minute then dried with sterile paper points. Bleeding was initiated by passing a pre-curved #25 K-file into the periapical tissues to create an injury milieu. Bleeding appeared minimal from the mesial canals. In parallel, PRF was prepared and used as described above. PRF-extract only was injected inside the mesial canals. A coronal barrier of white mineral trioxide aggregate (MTA) (Pro-root, Dentsply Tulsa, OK, United States) was placed. After two weeks, setting of MTA was checked and the tooth finally restored with composite resin.

OUTCOME AND FOLLOW-UP

Case 1

The patient was followed-up at 2-wk, 1, 3 and 9 mo (Figures 2 and 4) and (Table 1). The patient did not return for later follow-up. The sinus tract had completely resolved at the second visit. There was mild pain which subsided after 1 mo, after that, the tooth was asymptomatic and periodontally healthy at all visits (Figure 2E-H). All pulp tests were negative for tooth #9. The periapical radiograph showed root thickening and progressive apical closure of #9. The panoramic radiograph after 9 mo (Figure 2I and J) showed the increase of bone density and diminution of the size of the lesion especially around #9 and at the peripheries of the lesion. However, the lesion had not

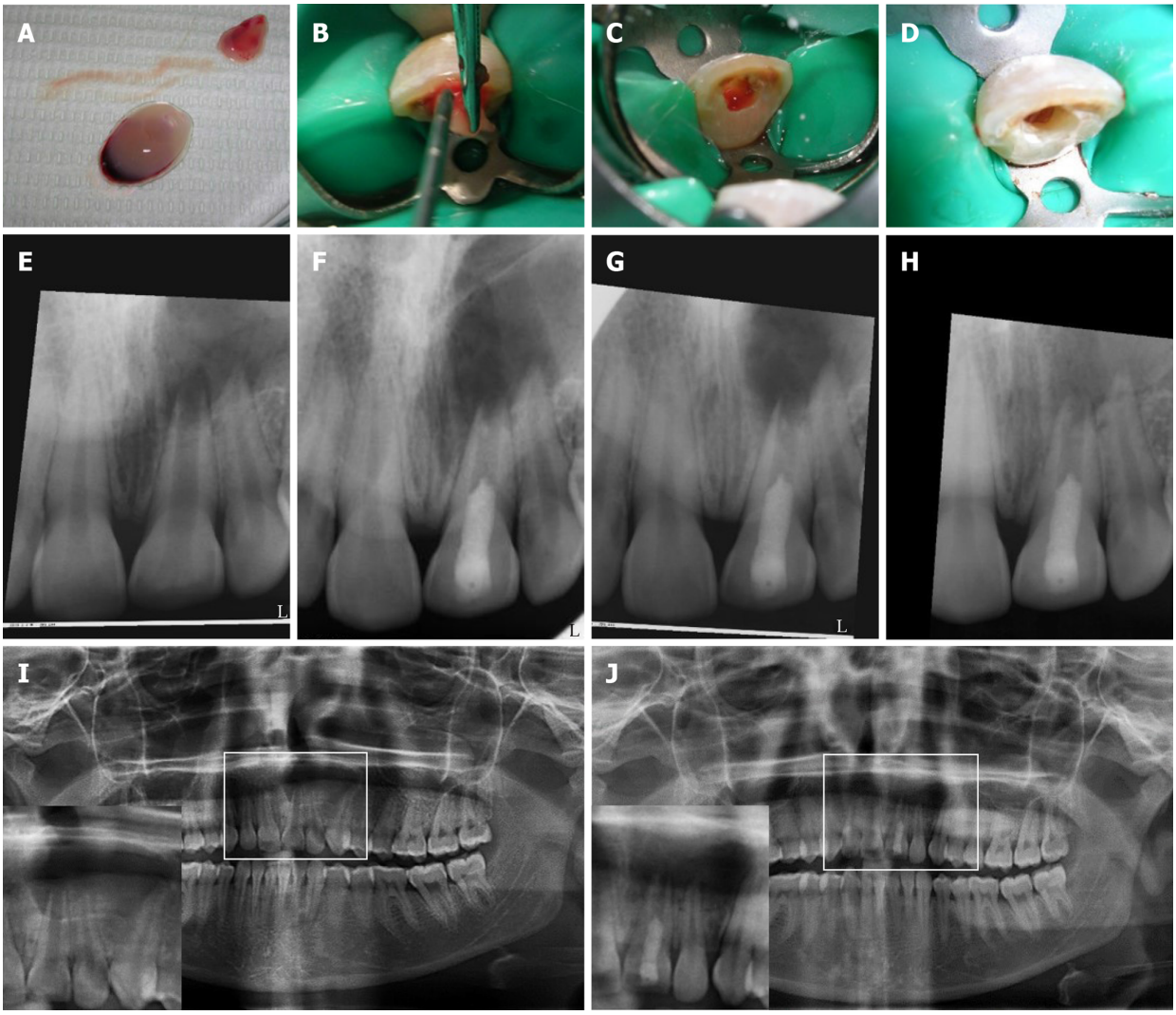

Figure 2 Revascularization procedure using platelet-rich fibrin and follow-up digital periapical radiographs and orthopantograms for case #1 (tooth #9). A-D: Prepared platelet-rich fibrin (PRF) clots in A with insert showing squeezing of clots in a metallic mesh to release supernatant (extract); B: Packing of PRF clot into the root canal with platelet-plug facing apical tissues using sterile cotton pliers and endodontic pluggers; and removal of excess extract and cleaning of pulp chamber in C and D, respectively; E, F: Periapical radiographs of the case showing in E pre-operative radiograph; F: after 1 mo; G: After 3 mo and; H: After 9 mo; I, J: Show pre- and 9 mo post-revascularization panoramic radiographs using the same device, respectively. Inserts represent magnifications of the selected regions of interest shown in I and J, respectively. Follow-up radiographs show progress in healing of the periapical lesion in terms of both size and density. The lesion originally extended from the maxillary left central incisor to the mesial root surface of the left canine. Teeth were asymptomatic. The case was unfortunately lost to follow-up after 9 mo. All digital periapical radiographs were aligned using the Turboreg plugin- image J software (http://bigwww.epfl.ch/thevenaz/turboreg/).

healed completely. The analysis of bone density of the periapical radiographs (Figure 4) revealed an overall increase in bone density except at the center of the lesion. Details are explained in the supporting information.

Case 2

The patient was followed up at 2 wk, then at 1, 3, 6, 12, and 48 mo (Table 2). The patient reported normal function and did not complain about pain at any of the recall visits. There was no pain on palpation or percussion. All pulp tests, cold and hot, were negative throughout the follow-up period. The radiographs showed increased bone density and periapical healing with palatal root maturation (Figure 3). Upon comparing the pre-operative with the CBCT obtained at 12 mo, all roots showed apical closure, narrowing of canal space and complete resolution of the periapical lesion with normal periapical bone architecture. However, no root lengthening was observed in any root. Imaging parameters and image processing details are available in the supporting information. The patient was recalled after 48 mo to initiate orthodontic treatment and the tooth was asymptomatic. However, recurrent decay was noticed at the distal surface of the tooth and it was extending underneath the composite restoration but with normal periapical tissues in the digital periapical radiograph (Figure 5A). The caries, composite restoration and the MTA plug were removed. The canals appeared clean and dry with minimal fragments of tissues

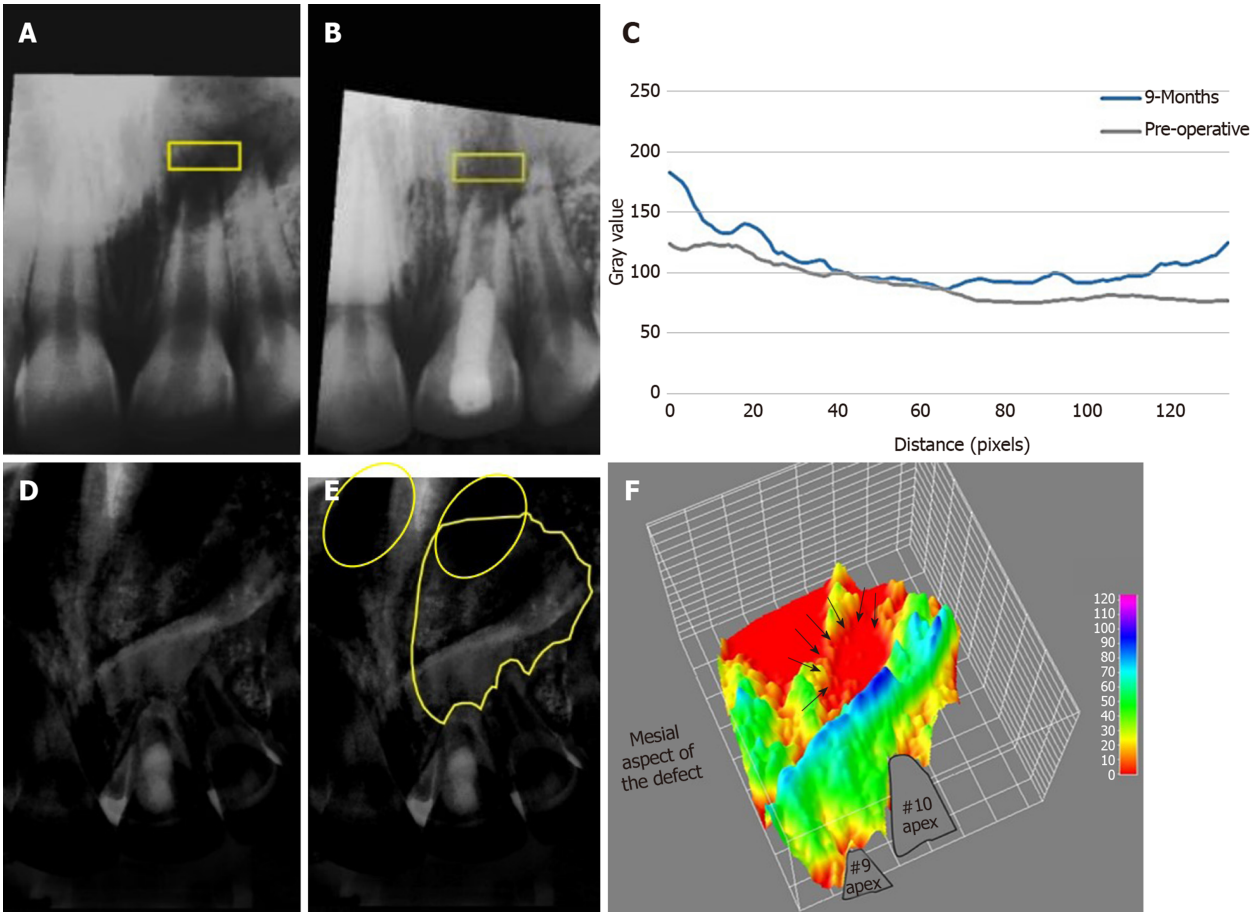

Figure 3 Showing average density changes of the periapical lesion in case#1. A, B: Average density profile measurement of periapical lesion pre-operatively and after 9 mo, respectively in the selected region of interest (ROI); C: Graph displaying the grey value changes reflecting the increase in bone density after 9 mo; D, E: Image subtraction of occlusal radiographs before and after 9 mo, where E shows the traced ROI to be measured using 3D surface plotting. Note that the left nostril is superimposed with the upper border of the defect circles; F: 3D plot showing change in bone density corresponding to ROI selected in E. Areas in red represent those with the lowest density such as the nostril area and the center of the defect (arrows). The detailed method for measurement of bone density is available in the supplementary material.

retrieved from the pulp chamber and canals (Figure 5B), but appeared with varying degrees of intracanal calcification (Figure 5C). The maximum length reached using a #10 K-file even following negotiation with ultrasonics (E-25 tip, Satelec, Acteon, France) was 16 mm for the palatal canal, 14 mm for the first mesiobuccal canal and 3 mm for the distobuccal canal while only the canal orifice was negotiated for the second mesiobuccal canal. The canals were instrumented to these lengths using manual files to remove any residual biofilm, copiously irrigated with 5.25% NaClO, dried and finally filled with gutta-percha followed by placement of glass ionomer and composite restoration (Figure 5D). The patient was recalled after 6 mo and reported the tooth as being asymptomatic with the coronal restoration intact and he was scheduled to start orthodontic treatment.

DISCUSSION

The present report is the first to document the long-term efficacy of RET for up to 48 mo. Clinical case reports documenting RET normally only extend to one year, with the longer-term outcomes being unpredictable[3], and the reliance on a small number of short-term studies, raises RET failure concerns among dentists[5]. This is also one of the rare clinical studies of using PRF and PRF-extract as scaffolds for RETs. Most RET case reports investigated blood clots[3]. There are a few unorthodox RET cases; which range from the treatment of elderly patient's teeth to the re-treatment of failed cases[2,10]. Since apical diameter and patient age appear to be two important factors for the success of REPs[11], such novel applications will undoubtedly require modifications to the originally proposed protocol in order to fully encompass the triad of tissue engineering. One interesting observation was that the RET with PRF can initiate root canal wall thickening and lengthening, which is self-limiting over the long-term as the

Table 1 The follow up outcomes of case #1

	Sinus tract	Pain on percussion	Radiographical			Pulp test	
			Dentin thick	Apical closure	Bone	Hot	Cold
Day 0	+	+	NA	NA	NA	-	-
2 wk	-	+	-	-	-	-	-
1 mo	-	-	-	-	-	-	-
3 mo	-	-	-	-	-	-	-
9 mo	-	-	+	-	+	-	-

roots did not continue growing abnormally long. This observation resolves a concern among clinicians, about the long-term problems of using RET.

The use of platelet-rich concentrates for RETs including both platelet-rich plasma (PRP) and PRF has been successful in the regenerative applications. While with PRP, there is a release of growth factors within a few hours, PRF scaffolds entrap these factors within the fibrin mesh and slowly release them with a peak at 14 d[12]. In addition, no external chemicals, *i.e*., thrombin or $CaCl_2$, are needed to prepare PRF.

Additionally, PRF can be divided to a clot and to a liquid (PRF-extract). The PRF-extract itself is a reservoir of bioactive cues that can on its own trigger the migration, proliferation, and differentiation of stem/progenitor cells[6,9,13]. Furthermore, Chai *et al*[14] recently reported that liquid PRF (prepared by centrifugation at 700 g for 3 min, the upper plasma layer is considered as liquid PRF) promotes dental pulp cellular migration, proliferation, and differentiation. The liquid PRF has also a partial immune defense against the induced inflammation.

The injectable forms of PRF may be used in inaccessible and narrow canals, which is of special importance when there is limited space for placement of a PRF clot and when induced bleeding is insufficient. In retrospective meta-analysis done by Murray[15] on a 222 immature permanent teeth, he found that PRF scaffolds showed higher success rate in terms of periapical lesion healing, root lengthening, and apical closure. While there was no difference in the thickening of dentinal walls. However, the superiority of PRF over blood clot in RETs is still controversial[16,17].

In this report, we presented two challenging regenerative endodontic cases. In case#1, the older age of the patient in addition to the presence of a large long-standing periapical lesion posed significant challenges. For case#2 presented with a permanent maxillary molar with 4 root canals and roots at different developmental stages. Hence, we used PRF and PRF-extract as a natural ameliorate for tissue regeneration. It is also noteworthy to state that induction of bleeding, although expected to be insufficient, was still done to create an injury stimulus to trigger stem/progenitor cell recruitment. The PRF clot was placed so the platelet-plug will face the apical tissues, which may have provided a higher gradient of cytokines and growth factors to enhance the healing process[18]. Moreover, antimicrobial properties of PRF have also been revealed[19]. This coupled with previously mentioned properties of PRF may have contributed to the improved healing outcome in this report.

Incomplete root maturation was observed in case#1 after 9 mo. The patient did not complain about pain, and the bone density had improved. This is analogous to two cases with periapical lesions in middle-aged patients[20,21]. The first reported by Saoud *et al*[21] involved a large periapical lesion in a 23-year-old patient. The authors found that after one year there was more trabecular bone deposition but incomplete resolution. The second case by Wang *et al*[20], demonstrated a 39-year-old patient with an extensive bilateral apical radiolucency. The radiolucency resolved 30 mo after treatment. This slow healing could be due to size of the lesion, the long-standing infection and age of the patient. RET outcomes appears to improve if the pulp necrosis occurred less than 6 mo prior to treatment[22].

The effectiveness of disinfection needed for RET is more critical than that needed in traditional endodontics, hence, in these cases, we used lower concentrations of NaClO to avoid killing the stem cells[23,24]. In addition, calcium hydroxide was selected as an intracanal medicament because it could enhance the attachment, viability and proliferation of apical papilla cells in contrast to other medicaments[24].

The use of PRF and its extract could have accelerated the healing in the first case; unfortunately the patient was lost to follow-up prohibiting long-term assessment. Age has also been shown to influence the outcome of regeneration. Dental pulp stem cells from aged individuals have shown impaired proliferation and neuronal differentiation capacities[25]. This may be one factor explaining the delayed healing

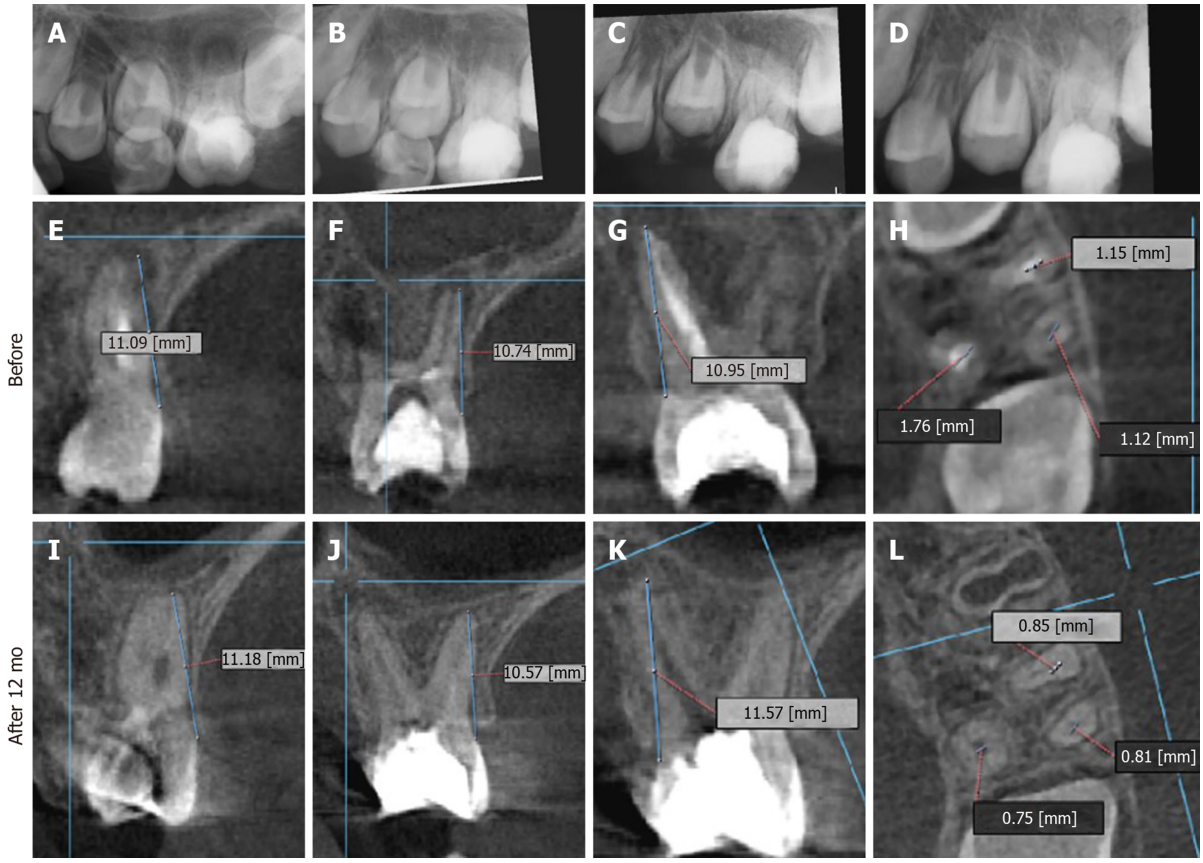

Figure 4 Twelve months periapical radiographs and cone beam computed tomography images of case #2 (tooth #14). A-D: Digital periapical radiographs showing in an immediate post-operative radiograph; B: After 3 mo; C: After 6 mo and; D: After 12 mo; E-H: Cone beam computed tomography images (CBCT) images before the revascularization procedure (but after placement of intracanal medication); I-L: Follow-up after 12 mo; E, I: Mesial root; F, J: Distal root; G, K: Palatal root; H, L: Coronal section at apical third level showing the diameters of each root canal. All roots showed apical closure with complete periapical bone healing. Again, progress of periapical healing is evident along with root thickening and closure. The tooth remained asymptomatic throughout the follow-up period. All digital periapical radiographs were aligned using the Turboreg plugin- image J software (http://bigwww.epfl.ch/thevenaz/turboreg/). Details on the CBCT parameters and method are available in the supplementary material.

with age and encourage the use of bioactive materials to enhance stem cell homing and endogenous tissue regeneration.

After 12 mo, in case#2, where PRF-extract was used solely as the bleeding was not successful in the two mesiobuccal canals, demonstrated the greater promise of the PRF approach. Although the roots did not show any root lengthening, they showed maturation and wall thickening. This is with accordance with a meta-analysis by He *et al*[26], they suggested that apical closure is not necessarily associated with root lengthening. After 4 years, the tooth was asymptomatic and radiographically successful, however, recurrent caries at the distal surface was extensive which necessitated re-intervention. This highlights the importance of proper patient selection and the importance of patients' oral hygiene for the success of the treatment. Perhaps the most interesting result is the extensive intracanal calcification noted in case #2. This may be attributed to remnants of calcium hydroxide pastes which are difficult to remove and could remain inside root canals. Song *et al*[27] found that 61.5% of cases (8 of 13 cases) with complete canal obliteration had occurred when $Ca(OH)_2$ was used as an intracanal medication, compared to (30.8%, 4 of 13) when antibiotic pastes were used.

The source of cells appears to plays a key role in root maturation and calcification, where PDL and bone marrow stem cells may contribute to extensive deposition of cementum/bone-like tissues[27] also found that cases with induced bleeding had higher frequency of calcification (69.6%, 16 of 23 cases) than those without bleeding when vital tissues were found in the canals. Documentation of long-term outcomes such as the extensive intracanal calcification seen in case#2 is crucial, particularly for long term treatment planning. RETs assisted by PRF and PRF-extract did help retain tooth#14 until orthodontic treatment commenced. Natera *et al*[28] reported a similar case that received orthodontic treatment one year after REPs in tooth #20. Although the tooth lacked further root maturation, it remained asymptomatic clinically and radiographically after 4 years and the tooth responded to orthodontic treatment as

Table 2 The follow-up outcomes of case #2

	Sinus tract	Pain on percussion	Radiographical			Pulp test	
			Dentin thick	Apical closure	Bone	Hot	Cold
Day 0	-	++	NA	NA	NA	-	-
2 wk	-	+	-	-	-	-	-
1 mo	-	-	-	-	-	-	-
3 mo	-	-	-	-	-	-	-
6 mo	-	-	+	-	+	-	-
12 mo	-	-	+	+	+	-	-
48 mo	-	-	+++	++	+	-	-

non-endodontically treated teeth.

A case report has been recently published by Chaniotis *et al*[29] who started orthodontic treatment 3 years and half after RET in tooth #8. The patient had skeletal Class II division 1, which had subjected tooth #8 to multiple traumas that lead finally to a periapical lesion and fracture. By the end of orthodontic treatment that lasted for 2 years, the periapical radiographs revealed a marked remodeling of the periapical tissues; the fractured apical third showed signs of resorption, along with the resolution of the periapical lesion. The presence of tooth #8 was essential for the success of the orthodontic treatment, however, the orthodontic forces may have disturbed the reattachment of the fractured root part. Similarly, retention of the tooth#14 in case#2 in this report was essential in spite of the need to later retreat the tooth. The decision to retreat calcified/obliterated root canals is based on multiple factors yet calcification itself may not be a failure per se[30] unless coronal seal is lost and bacterial re-infection is a risk as was the case in this report.

Both the RETs in this present report exhibited negative responses to pulp testing. However, this does not negate the possibility of re-vitalization[31]. A longer-term follow-up may be required to accurately assess the pulp re-vitalization parameters.

Some previous histological studies have shown that the use of PRP did not change the nature of the regenerated tissues, when compared to blood clots within root canals[32]. It was not possible to conduct a histologic analysis of the teeth following RET, due to the lack of patient consent to extract healthy functioning teeth.

In conclusion and within the limitations of this report, it was observed that: RET with PRF can generate the root structure of immature permanent teeth with a necrotic pulp following caries and trauma. RET with PRF can initiate root canal wall thickening and lengthening, which is self-limiting over the long-term as the roots did not continue to grow abnormally long. RET with PRF could benefit patients by saving immature teeth with a necrotic pulp from subsequent fracture and requiring extraction.

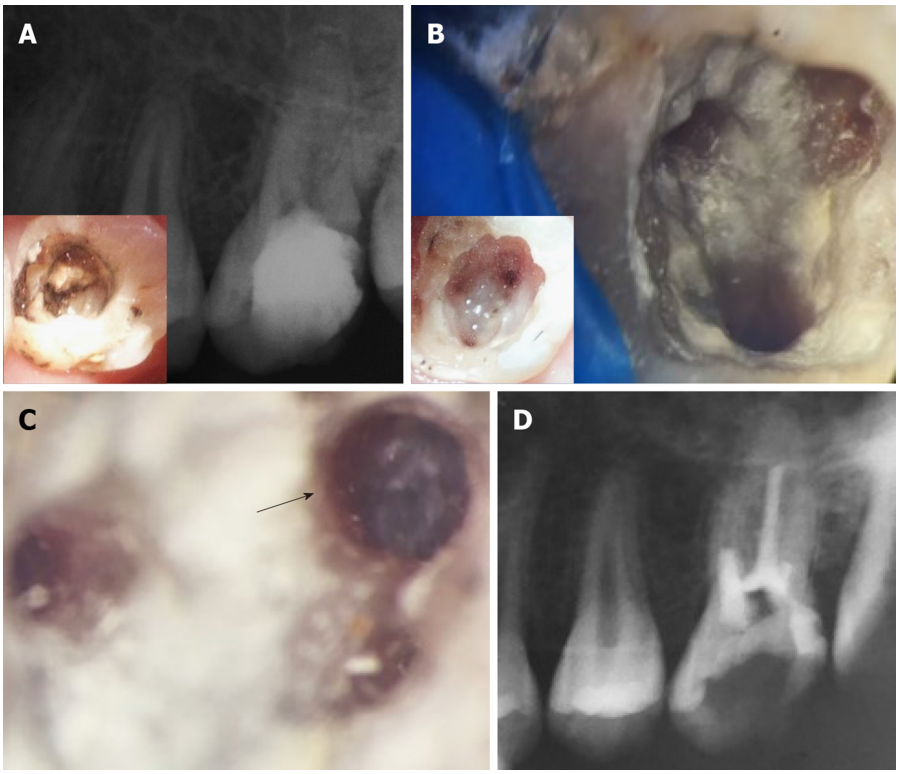

Figure 5 Four-year treatment outcome and re-treatment procedures for case #2 (tooth #14). Figure shows the condition of the tooth after 4 years. A: The 4 years periapical radiograph showing recurrent decay on the distal surface of the tooth underneath the composite restoration. Insert in A shows the extent of the decay and the pulp chamber prior to complete removal of the MTA coronal plug. Note that the periapical tissues appear healthy; B: Shows the pulp chamber after removal of residual MTA material taken using a Kaaps dental operating microscope (x 10, Original magnification). Insert in B shows location of orifices. Minimal traces of fragmented tissues were retrieved from the pulp chamber; C: Higher magnification of the first mesiobuccal canal (x 25, original magnification) shows diffuse calcification and an obliterated canal beyond this level; D: Immediate post-operative radiograph showing root canal filling with gutta percha till the level of canal obliteration for the 4 canals. The maximum length reached was 16 mm for the palatal canal, 14 mm for the first mesiobuccal canal and 3 mm for the distobuccal canal while only the canal orifice was negotiated for the second mesiobuccal canal.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Mohamed Hesham for his assistance in patient follow-up, Dr. Ayat Hamdy and Dr. Noha El Shazley for their assistance in radiographic assessment, and Dr. Semha El Naggar for assistance with clinical procedures.

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