EVALUATION OF GYPSUM-CHITOSAN AS A BIOMATERIAL IN DENTAL PULP PROTECTION

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EVALUATION OF GYPSUM-CHITOSAN AS A BIOMATERIAL IN DENTAL PULP PROTECTION

by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

ALP	Alkaline phosphatase
BHI	Brain Heart Infusion
BMP-2	Bone morphogenetic protein 2
$Ca(OH)_2$	Calcium hydroxide
$CaSO_4$	Calcium sulfate
CHT	Chitosan
CO_2	Carbon dioxide
CPC	Calcium phosphate cement
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DPSCs	Dental pulp stem cells
DTSCs	Deciduous teeth stem cells
FBS	Fetal bovine serum
Gyp	Gypsum
GIC	Glass ionomer cement
ISO	International Standard Organization
MEM	Minimum Essential Medium
MPa	Mega Pascal
MSCs	Mesenchymal stem cells
MTA	Mineral trioxide aggregate
ml	millilitre
µg	microgram

µl	microliter
NaOH	Sodium hydroxide
OD	Optical density
PBS	Phosphate buffered saline
SEM	Scanning Electron Microscopy
SHED	Stem cells from human exfoliated deciduous teeth
TBS	Tris buffer saline
TGF-β	Transforming growth factor beta
UV	Ultra violet
VPT	Vital pulp therapy
–NH3+	Ammonia group
α-MEM	Alpha modified Eagle's medium
α	Alpha
Gyp-CHT	Gypsum-chitosan biomaterial
Gyp-CHT 0%	Gypsum-chitosan with 0% CHT solution
Gyp-CHT 1%	Gypsum-chitosan with 1% CHT solution
Gyp-CHT 2.5%	Gypsum-chitosan with 2.5% CHT solution
Gyp-CHT 5%	Gypsum-chitosan with 5% CHT solution
Gyp-CHT 10%	Gypsum-chitosan with 10% CHT solution
Gyp-CHT-BMP-2	Gypsum-chitosan-bone morphogenetic protein-2 biomaterial

Gyp-CHT 0%-BMP2	Gypsum-chitosan-bone morphogenetic protein-2 with 0% CHT solution
Gyp-CHT 1%-BMP2	Gypsum-chitosan-bone morphogenetic protein-2 with 1% CHT solution
Gyp-CHT 2.5%-BMP2	Gypsum-chitosan-bone morphogenetic protein-2 with 2.5% CHT solution
Gyp-CHT 5%-BMP2	Gypsum-chitosan-bone morphogenetic protein-2 with 5% CHT solution
Gyp-CHT 10%-BMP2	Gypsum-chitosan-bone morphogenetic protein-2 with 10% CHT solution

PENILAIAN TERHADAP GIPSUM-KITOSAN SEBAGAI BIO-BAHAN BAGI PERLINDUNGAN PULPA GIGI

ABSTRAK

Dalam bidang pergigian, pemeliharaan tisu pulpa vital menggunakan terapi pulpa merupakan sebuah pendekatan klinikal yang mencabar. Dalam prosedur pelapikan pulpa langsung, kawasan yang terdedah ditampal dengan bahan pergigian untuk mengekalkan vitaliti pulpa dan mendorong pembentukan dentin reparatif. Beberapa bahan seperti kalsium hidroksida dan mineral trioksida agregat telah digunakan dan dikaji ke atas pelapikan pulpa gigi dengan beberapa limitasi. Oleh itu, kitosan (CHT) dan kalsium sulfat (gypsum asli – Gyp) digabungkan bersama protin morfogenetik tulang 2 (BMP-2) telah dipilih bagi kajian ini untuk mengeksploitasi kelebihan bahan tersebut dan menyediakan biobahan dengan ciri-ciri biologi dan mekanikal yang tinggi. Gyp bersifat bioserasi, mouldable dan digunakan sebagai pembawa antibiotik. CHT juga bersifat bioserasi, mempunyai aktiviti antibakteria, dan merangsang pertumbuhan tisu. BMP-2 pula mendorong pembezaan sel pulpa ke dalam odontoblas dan membantu pembentukan dentin reparatif. Tujuan kajian ini adalah untuk menyediakan biobahan eksperimental berasaskan gipsum dan menguji kesan biobahan dengan beberapa nilai kepekatan CHT (10%, 5%, 2.5%, 1%, and 0% larutan kitosan) terhadap tetapan masa (min), pH, kekuatan mampatan (MPa), keterlarutan (%), aktiviti antibakteria terhadap bakteria kariogenik dan aktiviti proliferasi SHED. Di samping itu, BMP-2 telah digabungkan dengan Gyp-CHT dan kesannya telah dinilai melalui aktiviti fosfatase beralkali (ALP) SHED. Pelekatan dan proliferasi SHED ke atas biobahan eksperimental juga telah diperhati menggunakan mikroskop imbasan electron (SEM). Pelapik pergigian komersial Dycal dan Fuji IX telah disediakan mengikut arahan pengeluar dan beberapa sifat

yang dipilih telah dinilai untuk tujuan perbandingan. Keputusan tetapan masa untuk biobahan eksperimen adalah di antara 4.1-6.6 min di mana ianya boleh diterima untuk aplikasi klinikal. Nilai pH selepas 24 jam adalah di antara 5.7-6.4 merupakan nilai yang sesuai bagi aplikasi pelapikan pulpa. Kekuatan mampatan meningkat antara 2.63-5.83 MPa apabila kepekatan CHT dalam biobahan semakin meningkat. Kadar keterlarutan semakin menurun dalam biobahan yang mempunyai CHT berbanding tanpa CHT kecuali untuk biobahan yang mempunyai Gyp-CHT 10% yang menunjukkan keterlarutan yang tertinggi. Biobahan eksperimental menunjukkan aktiviti antibakteria yang tinggi terhadap *S. mutans* dan *S. sobrinus*, dan aktiviti antibakteria ini lebih jelas dengan kepekatan CHT yang lebih tinggi dan ianya setanding dengan bahan-bahan pelapik yang lain. Biobahan tersebut menunjukkan keserasian sel yang baik terhadap SHED dalam ujian MTS langsung dan tidak langsung (ujian elusi). Biobahan tersebut juga cenderung untuk meningkatkan pembebasan ALP diluar sel. Selain itu, biobahan eksperimental tersebut telah meningkatkan aktiviti ALP SHED di mana aktiviti ALP lebih tinggi oleh biobahan yang digabungkan bersama BMP-2. Pemerhatian menggunakan SEM menunjukkan SHED tersebut sihat, melekat dengan baik serta telah merebak di permukaan sel melalui aktiviti proliferasi. Hasil kajian *in-vitro* ini menunjukkan bahawa sifat-sifat fizikal dan mekanikal biobahan gipsum berasaskan kitosan boleh diterima sebagai biobahan dalam pelapikan pulpa. Aktiviti ALP SHED yang mana merupakan petunjuk pemineralan adalah jauh lebih tinggi dalam biobahan Gyp-CHT-BMP-2 berbanding Dycal. Sifat antibakteria biobahan ini juga didapati setanding dengan bahan komersial. Justeru itu, hablur gipsum yang mengandungi matriks organik kitosan dan BMP-2 mempunyai potensi untuk diaplikasikan dalam terapi pulpa vital.

EVALUATION OF GYPSUM-CHITOSAN AS A BIOMATERIAL IN DENTAL PULP PROTECTION

ABSTRACT

In dentistry, preservation of vital pulp tissue through pulp therapy is a challenging clinical approach. In the procedure of direct pulp capping, the exposure site is sealed by dental material to maintain the vitality of the pulp and induce reparative dentin formation. Several materials such as calcium hydroxide and mineral trioxide aggregate have been used and investigated for dental pulp capping with limitations. Thus, chitosan (CHT) and calcium sulfate (pure gypsum - Gyp) incorporated with bone morphogenetic protein 2 (BMP-2) were chosen in this study to exploit their advantages and develop a biomaterial with high biological and mechanical characteristics. Gyp is highly biocompatible, moldable and used as carrier of antibiotic. CHT is biocompatible, has antibacterial activity and enhances tissue growth. BMP-2 induces differentiation of pulp cells into odontoblasts and reparative dentin formation. The aims of this study were to prepare an experimental gypsum-based biomaterial and to study the effects of the biomaterial with several concentrations of CHT (10%, 5%, 2.5%, 1%, and 0% chitosan solution) on setting time (min), pH value, compressive strength (MPa), solubility (%), antibacterial activity against cariogenic bacteria, and the proliferative activity of SHED. In addition, BMP-2 was incorporated with Gyp-CHT and its effect was evaluated on alkaline phosphatase activity (ALP) of SHED. The adhesion and proliferation of SHED on the experimental biomaterial was also observed by scanning electron microscope (SEM). Commercial dental liner Dycal and Fuji IX were prepared according to manufacturer's instructions and selective properties were evaluated for

comparison purpose. For the experimental biomaterials the results of setting time were ranged between 4.1 and 6.6 min which is considered acceptable for clinical application. The pH values after 24 hours were ranged 5.7 and 6.4 which is suitable for application as pulp capping. The compressive strength increased with higher CHT concentration in the biomaterial which ranged between 2.63 and 5.83 MPa. The solubility rate decreased with CHT incorporation compared to that without CHT except for Gyp-CHT 10% which showed the highest solubility. The experimental biomaterial showed potent antibacterial activity against *S. mutans and S. sobrinus*, which was more evident with greater CHT concentration and comparable with other lining materials. The biomaterial showed good cell compatibility to SHED in both direct and indirect MTS tests. The biomaterial tended to increase the release of ALP outside the cells. The experimental biomaterial induced the ALP activity of SHED with higher activity in biomaterial incorporated with BMP-2. SEM observations showed that the seeded SHED were apparently healthy, well adhered and were spread on the surface through proliferative activity. The *in vitro* results of this study suggest that the physical and mechanical properties of gypsum based chitosan biomaterials were acceptable in relation of pulp capping biomaterials. The ALP activity of SHED which is an indicator of mineralization was significantly higher in Gyp-CHT-BMP-2 biomaterials compared to Dycal. Antibacterial properties of experimental biomaterials were found comparable with the commercial materials. Thus, the crystalline gypsum impregnated with CHT and BMP-2 organic matrices may have the potential for application in vital pulp therapy.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Vital pulp therapy (VPT) is the treatment initiated to preserve and maintain the healthy state of the pulp tissue that has been compromised by trauma, restorative procedures, or caries. Its objective is to retain the tooth as a functional unit through the stimulation of the reparative dentin formation. This is particularly important in the incomplete development of the apical root of the young adult tooth. The focus is directed to preserve the involved pulp of the permanent tooth, based on the premise the tissue of the pulp has an innate capacity to repair when microbial contamination is absent (Ingle *et al.*, 2008).

Historically, the pulp capping procedures were first performed in 1756, when a small gold piece was packed over the vital pulp to promote its healing by Phillip Pfaff (Qureshi *et al.*, 2014).

The highly vascular tissue of the pulp has a unique distinction to be encased within the rigid pulp chamber consisting of dentin, enamel, and cementum. This rigid chamber provides mechanical support as well as protection from the microorganisms of the oral cavity (Figure 1.1). Pulp tissue functions include dentinogenesis, nutrition, proprioreceptor cognizance, and immune cell defense (Leeson *et al.*, 1988; Stockton, 1999; Yu and Abbott, 2007).

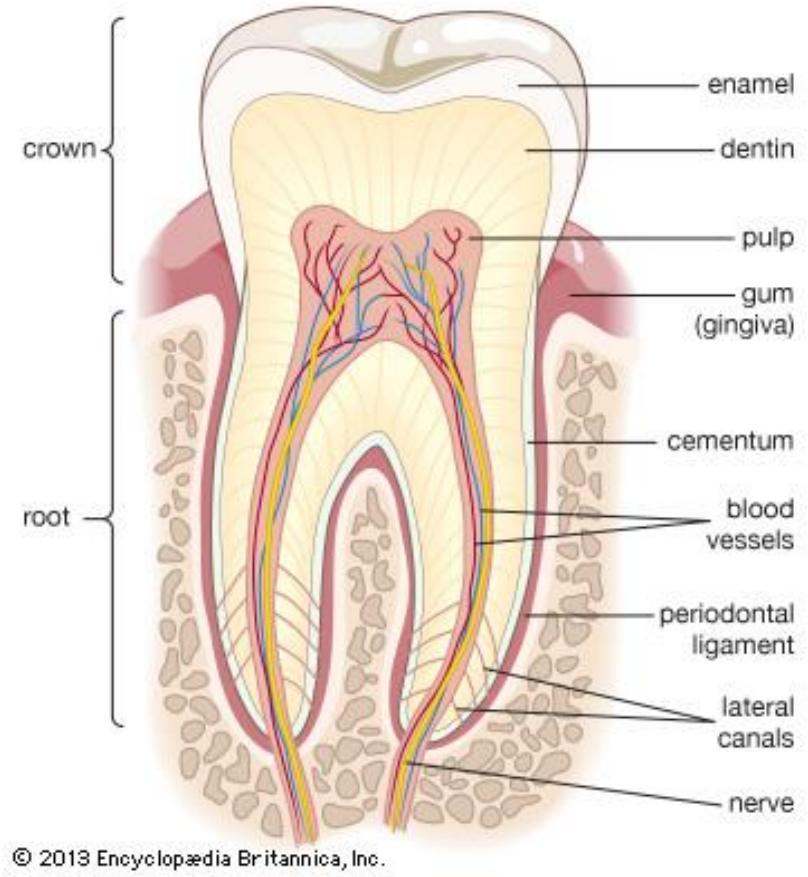

Figure 1.1 The structure of the tooth. Dental pulp tissues are imbedded in rigid chamber of hard enamel, dentin, and cementum. Blood vessels and nerves are protected against external microbes and injuries. (http://global.britannica.com/science/tooth-anatomy)

During the life of the tooth, the healthy pulp produces reparative, peritubular and secondary dentin in response to the different pathological and biological stimuli. Thus, for the tooth's long-term survival, the preservation of exposed pulp vitality is desirable (Shetty *et al.*, 2006).

When the tooth hard casing is compromised and the pulp is exposed to the microbial ingression, inflammatory changes can lead to the necrosis of the pulp and more pathologic changes, as infection and further consequences (Bjørndal and Darvann, 1998; Brännström and Lind, 1965). The purpose is to delay the aggressive therapies which could lower the prognosis of long-term function and retention of the tooth. Compromised tooth structure that have been treated endodontically and restored with post and core are susceptible to failure and fracture more owing to loss of protective mechanisms.

Endodontic treatment of teeth with caries progression or trauma involves the removal of the irreversible affected pulp tissue, and filling the root canals with a hermetic sealing material, which then followed by either, post endodontic filling or prosthetic approach (crown application) (Dostálová and Michaela, 2010).

However, in terms of survival rate, the prognosis of endodontically treated teeth is not good as the prognosis of the vital teeth, particularly in molars (hazard ratio, 7:1) (Caplan *et al.*, 2005). This could be due to the loss of proprioceptive function (Randow and Glantz, 1986), tooth sensitivity, and damping property (Ou *et al.*, 2009), which can be provided by the defensive mechanism of the vital pulp against the harmful stimuli.

VPT of the permanent teeth includes pulp capping, partial pulpotomy and full coronal pulpotomy. These therapies are employed for carious, traumatic and mechanical pulp exposure (Ward, 2002). The pulp capping refers to either indirect or direct pulp capping. In indirect pulp capping the carious dentin is adjacent to the pulp of a symptom-free, vital tooth (Camp and Fuks, 2002). While, in direct pulp capping, a vital pulp tissue is with carious or mechanical exposure due to dental procedure or trauma, and the pulp is asymptomatic and vital (Farooq *et al.*, 2000).

In direct pulp capping the exposed vital pulp is treated by the sealing of the pulpal wound with a dental material which is placed directly on the exposure site to induce reparative dentin formation and maintain the vitality of the pulp (Glickman *et al.*, 2003). With a good rule of thumb, the diameter of the exposure site is limited to less than 1.5 mm (Cohenca *et al.*, 2013) (Figure 1.2).

Two pulp exposures are considered for direct pulp capping: mechanical exposure of the pulp and exposure caused by caries. In both cases, the health of the pulp should be normal or present of reversible pulpitis. The difference in these two types of exposure is that in the mechanical exposure, the condition of the pulp is likely to be reversible pulpitis. Whereas in deep carious lesion, the condition is likely to be severely inflamed (Fouad *et al.*, 2008).

There is a perception of that unfavorable prognosis and aggressive treatment should be considered such as pulpotomy or pulpectomy to the pulp exposure in a carious field (Langeland, 1981; Tronstad and Mjör, 1972). These strategies are based on the protocols of the traditional treatment and the material that is not providing a suitable environment for the reparative bridge formation and pulpal repair.

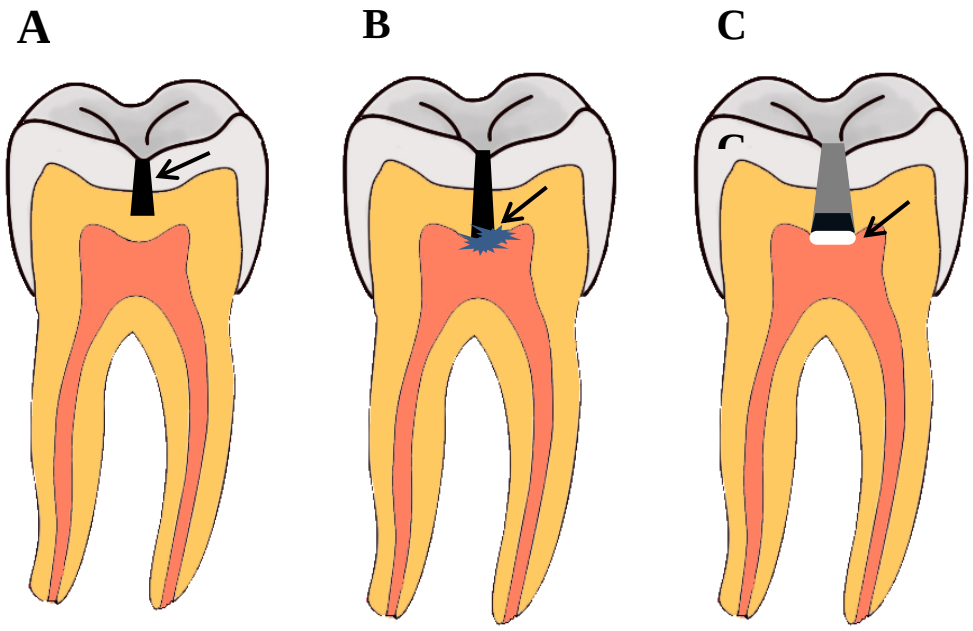

Figure 1.2 Application of pulp capping material over exposed pulp in order to maintain the vitality and function of the pulp. (arrow in figure **A**) initiation and extension of the caries. (arrow in figure **B**) exposure of the dental pulp. (arrow in figure **C**) application of pulp capping material over the exposed pulp tissue, then dental cement and final restoration were applied over the pulp capping material. (Adapted and modified figures: https://commons.wikimedia.org/wiki/File:Dentistry_logo.svg)

Many histologic studies demonstrated that the vital pulp with carious exposure is not always infected completely, depending on the severity and duration of the carious lesion (Langeland, 1987; Mitchell and Tarplee, 1960; Seltzer *et al.*, 1963). Occasionally, the inflammation remains adjacent to the carious lesion and does not spread to the whole of the radicular and coronal pulp (Langeland, 1987; Trowbridge, 2002), thus it is possible to conserve the remaining healthy pulp when the infected tissue is removed.

A systematic review recently evaluated the current evidence state of radiographic and clinical success for the clinical treatments range of the VPT in cariously exposed pulps of vital permanent teeth using $Ca(OH)_2$ or MTA. The review reported that the success rate of direct pulp capping is 87.5% in > 6 months to one year; 95.4% in > one to 2 years; 87.7% > 2 to 3 years; and 72.9% in > 3 years. The review conclusion stated that the carious exposed pulp in the vital permanent teeth can be successfully treated with VPT (Aguilar and Linsuwanont, 2011).

Also, other clinical evaluations of pulp capping to carious pulp exposure using $Ca(OH)_2$, MTA and tricalcium silicate has demonstrated success rates between 72.9% to 98% (Aguilar and Linsuwanont, 2011; Bogen *et al.*, 2008; Koubi *et al.*, 2009; Mente *et al.*, 2010).

The favorable outcomes of the direct pulp capping vary depending on the materials and techniques. For decades the researchers have strived to produce and identify a pulp capping material that would ideally exhibit the stimulation characteristics of the reparative dentin formation, maintain the vitality of the pulp, adhesion to dentin, adhesion to restorative material, resist force during the placement of the restoration, resist the force under restoration during the restoration lifetime,

maintain bacterial seal, radiopaque, sterile, and bactericidal or bacteriostatic (Cohen and Combe, 1994). Various materials have been used and investigated for dental pulp capping includes:

Calcium hydroxide

Calcium hydroxide ($Ca(OH)_2$) has been considered the "gold standard" for several decades for direct pulp capping after it was introduced in 1921 by Hermann. $Ca(OH)_2$ has excellent antibacterial activity and induces the mineralization of the reparative dentin. The drawbacks of $Ca(OH)_2$ include poor adhesion, high solubility in oral fluids, existence of tunnels in the reparative dentin, subjected to dissolution over time and degradation after acid etching (Cox *et al.*, 1995; Cox and Suzuki, 1994; Schröder, 1985).

Bonding agent

4-META-MMA-TBB resin has been investigated for pulp capping, it showed superior adhesion to the hard tissues and provide an effective seal against microleakage. However it showed poor outcome due to absence of calcific bridge formation, high cytotoxic effect, and formation of dilatation and congestion of blood vessels and also chronic inflammatory pulp response (Miyakoshi *et al.*, 1993).

Calcium Phosphate

Calcium phosphate has superior compressive strength, biocompatibility and it is transformed into hydroxyapatite over time, and thus it was suggested as an alternative material for pulp capping. Tetracalcium phosphate was demonstrated to induce the formation of dental bridge without superficial necrosis and pulp inflammation in contrast to $Ca(OH)_2$, however clinical trials are required to evaluate this material (Yoshimine and Maeda, 1995).

Hydroxyapatite

Hydroxyapatite (HA) is a synthetic calcium phosphate ceramics with a stable thermo dynamical. It can be involved as scaffold for newly formed mineralized tissue. HA has neutral pH 7 with good biocompatibility, but it causes mild inflammation of the pulp with superficial necrosis (Hayashi *et al.*, 1999).

Glass Ionomer cement (GIC)/Resin Modified Glass Ionomer cement (RMGIC)

GIC showed good biocompatibility, fluoride release, bond to both dentin and enamel, and excellent seal against microleakage when used in approximate to the pulp. However it is cytotoxic when used with direct cell contact, slow setting rate, highly solubility. RMGIC showed chronic inflammation to the pulp when used as direct pulp capping material and lack of dentin bridge formation was exhibited, RMGIC is more cytotoxic than conventional GIC and contraindicated in direct pulp capping (Tarim *et al.*, 1998).

Mineral Trioxide Aggregate (MTA)

MTA was introduced early in 1900s by Torabinejad. It is radiopaque, biocompatible, and release bioactive dentin matrix proteins and has antibacterial property. In addition, MTA causes less inflammation to the pulp and more predictable formation of hard tissue barrier in comparison to $Ca(OH)_2$. However, MTA has many drawbacks including long setting time, poor handling, expensive, grey MTA caused tooth discoloration, high solubility, and two step procedure (Bogen *et al.*, 2008).

Novel Endodontic Cement

Novel Endodontic Cement (NEC) consists of calcium oxide, calcium carbonate, calcium phosphate, calcium silicate, calcium chloride and calcium sulfate. It is biocompatible and induced thicker dentinal bridge formation than MTA with less pulp inflammation (Hasan Zarrabi *et al.*, 2009).

NEC has shorter setting time, good handling characteristics compared to MTA; also it does not cause tooth discoloration. However further investigation is required to assess the inflamed pulp response to this material (Asgary *et al.*, 2008a; Asgary *et al.*, 2006; Asgary *et al.*, 2008b).

Propolis (Russian penicillin)

Propolis contains flavonoids, iron, zinc, phenolics, and other various aromatic compounds (Sabir *et al.*, 2005). Propolis showed similar dental bridge formation like MTA and superior to Dycal. Propolis is antioxidant, antibacterial, antiviral, antifungal, and anti-inflammatory. It stimulates reparative dentin formation, forms dental pulp collagen, reduces the inflammation and degeneration of the pulp. However, it showed mild to moderate inflammation after 2 - 4 weeks with formation of partial dentinal bridge (Parolia *et al.*, 2010).

Although several materials like Zinc oxide eugenol cement and Polycarboxylate cement were used in pulp treatment, they showed several drawbacks (Dummett and Kopel, 2002; McWalter *et al.*, 1976) and the evidence-based data on the above materials are very limited as well as application of those materials are very rare.

MTA and $Ca(OH)_2$ are most commonly used for pulp capping (Ulucan *et al.*, 2013) with several limitations.

For more effective biological and protecting mechanism of the dental pulp, laboratory-designed pulp lining biomaterials have been prepared by organic matrices and are being extensively investigated.

Gypsum (Gyp) is highly biocompatible, nontoxic (Mamidwar *et al.*, 2008), cheap, easily moldable, and widely used as filler and carrier of antibiotic to treat bone defects (Hesaraki *et al.*, 2009a). Chitosan (CHT) is biocompatible, nontoxic, has antibacterial activity against various microorganisms and spoilage germs. It can prevent infection, accelerate healing of wound, and enhance tissue growth (Hafdani

and Sadeghinia, 2011; Kumar, 2000). Bone morphogenetic protein 2 (BMP-2) is responsible for dentinogenesis. It induces differentiation of dental pulp stem cells into odontoblast-like cells (Lianjia *et al.*, 1993).

Thus, the impregnation of CHT and BMP-2 with the Gyp would be promising in development of gypsum-based chitosan (Gyp-CHT-BMP-2) biomaterials for dental pulp protection. The outcome of our preliminary *in vitro* study revealed evidence of high cell proliferation and high alkaline phosphatase activity of stem cells from human exfoliated deciduous teeth (SHED), which indicates the osteogenic/odontogenic differentiation of SHED and mineralization around the application site(Low *et al.*, 2015; Mahshim *et al.*, 2013). We hypothesize that the incorporation of BMP-2 will also enhance the mineralization of the dental pulp stem cells. The properties of the above composite biomaterials for dental pulp liner have not been investigated yet.

1.2 Justification of the study

Currently, the dental pulp therapy of the tooth involved by caries, trauma, or wear lesion (attrition, erosion, abrasion) is limited to the conventional restoration as root canal treatment or pulp capping. Therefore, research directed toward VPT might provide a promising alternative to the extirpation of dental pulp and preserve the function of the tooth. The success of pulp capping is based on potential cell sources and biocompatible pulp capping materials which can be employed as a carrier of signaling molecules. The pulp has stem/progenitor cells possessing the capability of differentiation into odontoblast and regeneration the degraded dentin. Thus the design of pulp capping materials should focus on the healing potential through stimulating the dentin regeneration and enhance the formation of tissue by biomolecules release.

Although, $Ca(OH)_2$ is considered the most commonly used dental material for pulp capping (Estrela *et al.*, 2001; Queiroz *et al.*, 2005), it has several drawbacks and limits the outcome of VPT as the material does not directly influence dentinogenesis.

Clinical application of MTA is restricted due its high cost (Hilton, 2009), prolonged setting time (Islam *et al.*, 2006; Torabinejad *et al.*, 1995d), poor handling properties (Johnson, 1999; Mooney and North, 2008), and high pH values (De Deus *et al*., 2005) and also has conflicting reports on its antibacterial effects (Lovato and Sedgley, 2011) .

The recent studies in dental research aim to develop a suitable biomaterial with favorable functional characteristics to exceed the drawbacks of the conventional pulp capping materials.

Materials with unique properties were chosen in this study for development of a new biomaterial to exploit their advantages and provide an ultimate biomaterial with high biological and mechanical characteristics. CHT was incorporated into calcium sulfate (pure gypsum) and combined with BMP-2. Each of the above material has its own beneficial properties particular to pulp tissue compatibility and regeneration process.

Gyp was employed as the main constituent of the biomaterial, as it is biocompatible, nontoxic, cheap, moldable, widely used as filler and calcium rich material. The material was used as a scaffold biomaterial to carry and release the other components such as antibiotic and growth factors. CHT is biocompatible, has potent antimicrobial activity, activate host defense, prevent infection, promote tissue growth, and accelerate wound healing. BMP-2 induces odontoblast-like cells differentiation from pulpal mesenchymal cells to obtain tubular dentin deposition and responsible for dentinogenesis. However, the employment of CHT and BMP2 at the pulp wound requires a carrier/scaffold material. Gyp has been successfully used in our initial studies, thus pure Gyp was used as a scaffold like biomaterial and the base component of the experimental biomaterial.

1.3 Objectives

1.3.1 General objective

To develop gypsum based chitosan experimental biomaterial and to evaluate its physical, mechanical, antibacterial properties and its effects on the growth of stem cells from human exfoliated deciduous teeth (SHED).

1.3.2 Specific objectives

1. To determine the effects of chitosan on setting time, pH, compressive strength and solubility of experimental gypsum-based chitosan biomaterial and compare with Dycal.
2. To evaluate the antibacterial properties of the experimental gypsum-based chitosan biomaterial against *Streptococcus mutans* and *Streptococcus sobrinus* and compare with Dycal and Glass ionomer cement.
3. To evaluate the effects of experimental gypsum-based chitosan on proliferative activity of SHED using direct and indirect methods and compare with Dycal (for direct method).
4. To evaluate the effects of gypsum-based chitosan with/without BMP-2 on alkaline phosphatase activity and compare with Dycal.
5. To observe the adhesion and proliferation of SHED on gypsum-based chitosan using scanning electron microscope (SEM) and compare with Dycal.

CHAPTER 2

LITERATURE REVIEW

2.1 Reparative dentinogenesis and direct pulp capping

The odontoblasts have the ability to respond to the iatrogenic injury during cavity preparation and/or pathological carious lesions progression near the pulp, and deposit the reactionary dentin by upregulating their secretory activity (Smith, 2002; Smith and Lesot, 2001). In case of odontoblasts death due to the severe intensity of the injury, the necrotic cells are replaced by a new generation of odontoblast-like cells and the secretion of a reparative dentin matrix will take place. This reparative dentin matrix will isolate the exposure site from the underlying healthy pulp (Tziafas, 1995). In this case, the exposure site is clinically treated by applying the pulp capping materials on the pulp core directly.

Many studies suggested the existence of progenitor stem cells in the pulp tissue. These cells include undifferentiated mesenchymal cells from the pulp core, fibroblasts, vascular-derived pericytes, and the sub-odontoblast cells in the layer of Höhl (Smith and Lesot, 2001). It has been shown that the pulp progenitor stem cells are multipotent.

The adult human dental pulp is shown to contain a rapidly proliferative cells subpopulation that has the ability to differentiate into neural–like cells and adipocytes (Gronthos *et al.*, 2002),and also can differentiate into odontoblast-like cells and secrete a mineralized matrix that have the molecular and mineral characteristics of dentin (About *et al.*, 2000). The above activities of progenitor stem

cells need appropriate biological environment to differentiate and the role of biomaterial application at the exposure site are issues of recent scientific interests.

2.2 Scaffold

A scaffold is a biocompatible structure to obtain a suitable environment for the tissue formation and cells growth. The scaffolds should provide cell attachment, differentiation, proliferation, migration, mechanical support and positive biological impact on the generation of extracellular matrix (Du and Moradian-Oldak, 2006; Goldberg *et al.*, 2004). In addition, scaffold must have the ability to deliver the necessary biological molecules for growth of the cells, differentiation or accelerate the tissue healing. The ideal scaffold in dental tissue engineering should be biocompatible, have suitable porosity, conductive, sterilizable, economical, and have good mechanical properties (Slaughter and Evans, 2007; Taylor, 2007; Yen and Sharpe, 2008).

Biological tissue shows amazing characteristics and has functions that cannot be easily reproduced by artificial synthesis. Learning from biological systems gives us tremendous opportunities to fabricate materials that we have not yet been able to produce.

Searching for suitable scaffold for tissue regeneration of the pulp and dentin tissue has started many years back. For dentin, pulp, or dentin-pulp regeneration, it is important for the scaffold to provide an *in vitro* environment suitable for growth, attachment, and differentiation into odontoblast cells. The ability of cell-scaffold construct to form tubular dentin like tissue is focused in *in vivo* studies when implanted in animals (Casagrande *et al.*, 2010; Sakai *et al.*, 2010).

Biphasic calcium phosphate which is a mixture of tricalcium phosphate and hydroxyapatite was widely investigated as a potential scaffold for the regeneration of the pulp and dentin tissue. When this material used with dental stem cells, the dentin-like tissues regeneration was observed(Ando *et al.*, 2009; Batouli *et al.*, 2003; Gronthos *et al.*, 2002; Sonoyama *et al.*, 2006).

2.3 Materials used for pulp capping

2.3.1 Calcium hydroxide

Calcium hydroxide $Ca(OH)_2$ was introduced long before as a direct pulp capping agent in endodontics (Hermann, 1920). It is an odourless powder with white color and its molecular weight is 74.08 (Farhad and Mohammadi, 2005). It has low solubility in water (around 1.2g L-1 at 25 ºC) and the solubility decreases with higher temperature (Siqueira and Lopes, 1999). The dissociation coefficient of $Ca(OH)_2$ is 0.17 which controls the slow release of both ions calcium and hydroxyl (Rehman *et al*., 1996). This low solubility is useful in clinical application as the extended period is essential before it is solubilized when in direct contact with fluids of the vital tissues (Spangberg and Haapasalo, 2002).

The $Ca(OH)_2$ is insoluble in alcohol (Farhad and Mohammadi, 2005) and has a pH of approximately 12.5-12.8. Chemically, the material is classified as a strong base, and the main actions of the material are generated from the ionic dissociation of Ca^{2+} and OH^- ions and their effects on the tissues which induce the deposition of the hard tissue and antibacterial property (Siqueira and Lopes, 1999).

2.3.1.1 Antibacterial activity of $Ca(OH)_2$

The antimicrobial activity of $Ca(OH)_2$ is related to hydroxyl ions release in aqueous environment (Siqueira *et al.*, 2001). The hydroxyl ions are highly oxidant free radicals which show extreme reactivity with many biomolecules. This reactivity of hydroxyl ions are indiscriminate and high, rarely diffuses away from the generation sites (Siqueira and Lopes, 1999). The action of hydroxyl ions are responsible for bacterial cytoplasmic membrane damage, denaturation of protein and damage to the bacterial DNA (Siqueira and Lopes, 1999).

2.3.1.2 Mineralization activity of $Ca(OH)_2$

Calcium hydroxide induced a calcified barrier when used as a pulp capping agent(Eda, 1961). A necrotic superficial layer occurs to a depth of up to 2 mm due to high pH of pure $Ca(OH)_2$ (Estrela and Holland, 2009). Only a mild inflammatory response is seen beyond this layer. When the material was placed, hard tissue may be formed if the operating field is free from bacteria (Estrela *et al.*, 1995).

The most important component of $Ca(OH)_2$ is hydroxyl group, which encourages active calcification and repair as it provides an alkaline environment. The lactic acid from osteoclasts is neutralized by the alkaline pH preventing the dissolution of mineral components of the dentin, and also the alkaline phosphatases is activated in alkaline pH which play a role in the formation of hard tissue (Estrela *et al.*, 1995). However, many studies have shown less frequent, thinner bridge formation and more inflammation of the pulp that capped with $Ca(OH)_2$ when

compared with pulp capped with MTA (Abedi *et al.*, 1996; Aeinehchi *et al.*, 2003; Faraco and Holland, 2001).

2.3.1.3 Clinical applications of $Ca(OH)_2$ as pulp-capping agents for VPT

As a direct pulp capping agents, several materials and drugs have been introduced. One of the most popular and effective agent is $Ca(OH)_2$ (Farhad and Mohammadi, 2005). $Ca(OH)_2$ was reported to be used as a material for exposed pulp treatment, and its success was related to the high alkalinity of the material (Zander, 1939).

Calcium hydroxide is suitable for pulp capping due to its alkalinity, antimicrobial activity and biocompatibility. However, a good coronal seal is required because of its solubility in fluids (Mohammadi and Dummer, 2011). Also, complete calcification of the root canal tissue may occur and persistence of inflammation induction causes internal resorption eventually (Seltzer and Bender, 1975).

The studies have demonstrated the formation of dentinal bridge in about 50-87% of treated cases with $Ca(OH)_2$ (Hargreaves and Goodis, 2002). The structural examination of coronal surface of calcium hydroxide-induced bridges has shown tubular openings surrounded by bundles of collagen similar to those in predentin (Schröder and Granath, 1970).

Calcium hydroxide induces the formation of dentinal bridge and repair by causing superficial coagulation on the pulp tissue (Schröder and Granath, 1970). It maintains an alkalinity state to its immediate application site due to its high pH

which is necessary for dentin and bone formation. The region under coagulation necrosis is calcium ions saturated region and cells from underlying pulp tissue are differentiating into odontoblast-like cells and elaborating matrix (Farhad and Mohammadi, 2005). However, the extreme pH of the materials will cause harm to adjacent cells, as altered cell morphology and decreased cell viability were observed in previous research, in which materials with high pH were placed adjacent to the cells (De Deus *et al.*, 2005).

2.3.2 Mineral Trioxide Aggregate

Mineral Trioxide Aggregate (MTA) is a dental material with exciting clinical applications. In the field of dentistry, MTA is represented as one of the most versatile material in this century. It was introduced at Loma Linda University (California, USA) by Mahmoud Torabinejad (Lee *et al.*, 1993). In 1993, the first literature about the material was appeared and it has been approved by U.S. Food and Drug Administration in 1998 (Schwartz *et al.*, 1999). Many appreciable properties of MTA have been approved including its ability to stimulate the tissue regeneration, good pulp response as well as good physical properties (Rao *et al.*, 2009).

MTA consists of tricalcium silicate, tricalcium oxide, silicate oxide, tricalcium aluminate and bismuth oxide. The composition of MTA is similar to composition of Portland cement except the bismuth oxide which is included to MTA (17-18wt%) to improve the radiopacity and other properties (Torabinejad *et al.*, 1995d). Two types of MTA are available, white and grey. They differ mainly in the content of aluminum, iron and magnesium oxides, in which less quantity of these oxides are present in white MTA (Asgary *et al.*, 2005).

The compressive strength of MTA within 24 hours of mixing is about 40.0MPa and increases after 21 days to 67.3 MPa which is significantly less than the amalgam and reinforced zinc oxide cement (Super-EBA) but similar to intermediate restorative material (IRM) (Torabinejad *et al.*, 1995c). No signs of solubility have been shown to the set MTA and the solubility might increase when more water is added during mixing (Budig and Eleazer, 2008).

The sealing property of MTA is very superior. It has proved to be superior in bacterial leakage test by preventing the bacteria entry, and also no gaps were detected in experimental samples (Torabinejad *et al.*, 1995e).

2.3.2.1 Antimicrobial activity of MTA

Antibacterial is defined as a natural or synthetic substance that destroys bacteria or inhibits their growth (King and Brucker, 2010). No antimicrobial activity was shown against any of the anaerobes but some effects against *S.mutans*, *S.mitis, S.salivarius, S.epidermidis* and *Lactobacillus* were shown. MTA as a direct antibacterial agent may not be a beneficial in endodontic practice. But, it is proclaimed as an antibacterial agent by virtue of preventing microleakage and providing a good seal (Al-Hezaimi *et al.*, 2006; Torabinejad *et al.*, 1995a).

2.3.2.2 Mineralization Activity of MTA

MTA can induce the formation of dentin bridge (Rao *et al.*, 2009). Calcite crystals were found adjacent to the dentinal tubules opening near to MTA layer (Holland *et al.*, 1999). The tricalcium oxide in MTA was theorized to react with

tissue fluid forming calcium hydroxide, and results in formation of hard tissue in a similar manner to that of $Ca(OH)_2$, but the dentin bridge formation is faster, more complete and with good structural integrity (Faraco and Holland, 2001). It proved to be better in maintaining the integrity of the pulp and inducing the formation of reparative dentin (Dominguez *et al.*, 2003; Tziafas *et al.*, 2002), and less inflammation, necrosis and hyperemia were demonstrated. Also, more frequent odontoblastic layer formation and thicker dentinal bridge than that with $Ca(OH)_2$ (Aeinehchi *et al.*, 2003).

The main disadvantages of MTA include difficult handling characteristics, high material cost, long setting time, an absence of a known solvent for this material, the difficulty of its removal after curing and discoloration potential (Parirokh and Torabinejad, 2010).

The placement of MTA on the tissue of the pulp causes proliferation, migration, and differentiation of odontoblast-like cells and collagen matrix formation (Garber *et al.*, 2009; Tziafas *et al.*, 2002), this produced matrix is mineralized then and forming osteodentin initially followed by the formation of tertiary dentinal bridge a few months after the pulp capping.

Several clinical studies and case reports have concluded successful outcomes after using MTA as a pulp capping agent in cariously and mechanically exposed pulps (Parirokh and Torabinejad, 2010).

In a previous study, cariously exposed teeth with reversible pulpitis were capped using MTA as a pulp capping agent and the teeth were followed up at 6, 12, 18, and 24 months. In the mentioned study, 93% of the teeth showed radiographic

and clinical success after 24 months (Farsi *et al*., 2007). Another study investigated the histological finding of MTA capped human third molar or lined with $Ca(OH)_2$; dentin bridge formation was shown in all the MTA capped pulps, while, 60% of the capped pulps with $Ca(OH)_2$ revealed formation of hard tissue. Pulps capped with MTA showed significantly thicker dentinal bridge formation than the pulps capped with $Ca(OH)_2$ (Min *et al*., 2008).

2.4 Calcium sulfate

Calcium sulfate (CS) has a long history of clinical use more than most of the recent available biomaterials. The raw material of CS is abundant, inexpensive and well tolerated when used to fill bone defect. CS can be used as a vehicle to deliver growth factors, pharmacologic agents, and antibiotics. It is widely used in orthopedics and dentistry in many clinical applications includes treatment of osteomyelitis, repair of periodontal defect, an adjunct to dental implant placement, and sinus augmentation (Thomas and Puleo, 2009).

Calcium sulfate or "gypsum" ($CaSO_4$ $2H_2O$) is a mineral that consists of calcium sulfate dihydrate. The raw material must be screened for impurities, as lead, strontium, silicates, and other materials naturally occurring before using in medical application (Ricci *et al.*, 2000). When heated to 110 ºC, Gyp loses water in a calcination process, and results in formation of calcium sulfate hemihydrate, also known as Plaster of Paris.

$$(CaSO_4 .2H_2O \xrightarrow{\text{Heat}} CaSO_4.\ ½H_2O + 1½\ H_2O)$$

Calcium sulfate hemihydrate exists in two forms, α and β, which differ in surface area, crystal size, and lattice imperfections. These materials differ in physical properties although they are chemically identical. The α-hemihydrate form "dental stone" is quite hard and when compared with the β-hemihydrate form, it is relatively insoluble. The dihydrate form is produced in a mild exothermic reaction when the hemihydrate is mixed with water (Anusavice, 2003).

$$(CaSO_4 .½H_2O + 1½\ H_2O \longrightarrow CaSO_4 . 2H_2O + Heat)$$

A minimal inflammatory response has been observed in implantation of CS. The interactions between host cells and a substrate is the way to evaluate biocompatibility. The migration response of the cultured human gingival fibroblasts over the chemotactic stimulus was assessed (Payne *et al.*, 1996), the tested materials were CS, poly (lactic acid) (PLLA), and ePTFE, with polystyrene serving as a control. The migration ability of the cells was further on the CS substrate than on the other tested barriers. The examination of the cells using scanning electron microscope indicated that the morphology of the cells appeared normal on the CS substrate, whereas the cells on the polylactic acid and ePTFE showed abnormal morphology and did not appear to be migrating. This property was suggested by the authors to be important in the sites where the closure of primary wound cannot be attained.

The mechanism of CS in enhancing formation of the bone has not been elucidated completely. It is suggested that the particles of the CS bind to the adjacent bone and then resorbed providing a mechanism to guide the growth of the bone (Coetzee, 1980). During the dissolution of CS, calcium ions are released, the