EFFECT OF ACCELERATED PORTLAND CEMENT-CHITOSAN ON DENTINOGENIC/OSTEOGENIC DIFFERENTIATION OF STEM CELL FROM HUMAN EXFOLIATED DECIDUOUS TEETH

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

APC-CHT	Accelerated Portland cement-chitosan
ALP	Alkaline phosphatase
a-MEM	Alpha-Minimum Essential Medium
APS	Ammonium Persulphate
ANOVA	Analysis of Variance
BO	Bismuth oxide
BSA	Bovine serum albumin
$CaCl2.H_2O$	Calcium chloride dehydrate
cm	Centimeter
CHT	Chitosan
DFSC	Dental follicule stem cells
DISC	Dental implant stem cells
DPSC	Dental pulp stem cells
DMP-1	Dentin matrix acid phosphoprotein-1
DSPP	Dentin sialophosphoprotein
DF	Dilution factor
DMSO	Dimethyl sulphoxide
ECL	Enhance chemiluminescence (ECL) plus western blotting detection reagents
FBS	Fetal bovine serum
GFSC	Gingival fibroblastic stem cells
GI	Glass ionomer

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HRP	Horse Radish Peroxide
MEPE	Matrix extracellular phosphoglycoprotein
MSC	Mesenchymal stem cells
µg	microgram
µl	microliter
µm	micrometer
mg	milligram
ml	millilitre
mM	milliMolar
MTA	Mineral trioxide aggregate
nM	nanoMolar
nm	nanometer
OCN	Osteocalcin
OM	Osteogenic medium
OPN	Osteopontin
%	percentage
PDLSC	Periodontal ligament stem cells
PBS	Phosphate-buffered saline
PGA	Polyglycolic acid
PLA	Polylactic acid
PVDF	Polyvinylidene difluoride
PC	Portland cement

RIPA	Radio immunoprecipitation assay
RMGI	Resin modified glass ionomer
rpm	Revolutions per minutes
SRP	Scaling and root planing
SDS-PAGE	Sodium dodecyl sulphate - Polyacrylamide Gel Electrophoresis
SHED	Stem cells derived from human exfoliated deciduous teeth
SCAP	Stem cells from apical papilla
TEMED	Tetramethylethylenediamine
TBS	Tris buffer saline
T-20	Tween 20
WPC	White Portland cement
WHO	World health Organization
ZOE	Zinc oxide eugenol

KESAN PORTLAND SIMEN-KITOSAN KE ATAS PEMBEZAAN DENTINOGENIK DAN OSTEOGENIK DALAM SEL STEM DARI GIGI SUSU MANUSIA YANG TERLUPAS

ABSTRAK

Mineral trioksida agregat (MTA) adalah biomaterial endodontik yang ideal untuk masa sekarang tetapi penggunaanya dibatasi oleh kos yang mahal, masa penetapan yang lama dan kesan toksiknya kepada sel. Untuk mengatasi masalah ini, penemuan dan pengembangan biomaterial baru sangat diperlukan. Oleh sebab itu, tujuan kajian ini adalah untuk menentukan kesan Portland simen-kitosan (APC-CHT) ke atas pembezaan dentinogenik dan osteogenik dalam sel induk dari gigi gugur manusia (SHED). APC-CHT telah dihasilkan 1 minggu sebelum 14 hari rawatan ke atas SHED. Morfologi sel dikaji menggunakan mikroskop terbalik. SHED menunjukkan peralihan fenotip dari morfologi seperti fibroblast kepada morfologi spindle bermula dari hari 7 hingga hari 14. Kepekatan protein setiap kumpulan yang diperolehi oleh ujian Bradford menunjukkan peningkatan bilangan protein dari hari 3 hingga hari 14. Kepekatan protein tertinggi adalah 16.89 mg / ml, yang berada dalam kumpulan 4 (APC-2,5% CHT). Saiz protein dalam kajian ini iaitu OCN, GAPDH, ALP, DMP-1, MEPE, OPN dan DSPP dinilai menggunakan SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Keamatan jalur tertinggi diperhatikan pada hari 14 dalam kumpulan 3 (APC-1,25% CHT) dan 4 (APC-2,5% CHT). Analisis peblotan Western juga menunjukkan kemunculan jalur yang jelas pada hari ke-14 pada kumpulan 3 dan 4. Kesimpulannya, APC-CHT menunjukkan kesan yang baik terhadap pembezaan dentinogenik / osteogenik dalam SHED dan berpotensi tinggi untuk digunakan sebagai bahan alternatif dalam aplikasi pergigian.

EFFECT OF ACCELERATED PORTLAND CEMENT CHITOSAN ON DENTINOGENIC/OSTEOGENIC DIFFERENTIATION OF STEM CELL FROM HUMAN EXFOLIATED DECIDUOUS TEETH

ABSTRACT

Mineral trioxide aggregate (MTA) is a recent gold standard endodontic biomaterial but its application is limited by expensive cost, long setting time and can cause cell toxicity. To overcome this problem, the discovery and development of new potential biomaterial is crucially required. Hence, in this study we determined the effects of

accelerated Portland cement-chitosan (APC-CHT) on the dentinogenic and osteogenic differentiation in stem cells from human exfoliated deciduous teeth (SHED). The APC-CHT was prepared 1 week prior to the treatment on SHED for 14 days. The cell

morphology was accessed using an inverted microscope. SHED demonstrated phenotype transition from fibroblast-like to extended-spindle morphologies starting from Day 7 until Day 14. The highest protein concentration obtained through Bradford assay was 16.89 mg/ml which is in group 4 (APC-2.5 % CHT). The sizes of proteins

in this study *i.e*. OCN, GAPDH, ALP, DMP-1, MEPE, OPN and DSPP were evaluated using SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The highest band intensity was observed in Day 14 in group 3 (APC-1.25 % CHT) and 4 (APC-2.5 %

CHT). Western blot analysis also demonstrated the appearance of apparent bands in Day 14 in both group 3 and 4. In conclusion, APC-CHT exhibited favourable effects on dentinogenic/osteogenic differentiation in SHED and has the potential to be used as an alternative material in dental applications.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Dental diseases affect many people throughout their lives by causing pain, discomfort, disfigurement and even death. The Global Burden of Disease Study 2017 estimates that oral diseases affect approximately 3.5 billion people worldwide, with the most common condition being permanent teeth caries followed by periodontal diseases, oral cancers and dental trauma (WHO, 2020). The exposed teeth due to the diseases will allow pathogenic bacteria to enter the pulp chamber or root canals and cause severe endodontic infection. Several dental therapies, including dental implant and prosthesis are available but they fail to restore natural dentition even though they own excellent esthetic function. Hence, endodontic therapy is the treatment of choice since the goal of the therapy is to preserve the natural tooth as a functional unit within a functioning dentition (Yeng et al., 2007). The main challenge of endodontic therapy is that the existing endodontic biomaterials are limited by several drawbacks. Biomaterial such as calcium hydroxide has been widely used in dental therapy however, it was limited by poor seal and has no inherent adhesive qualities (Hilton, 2009). Mineral trioxide aggregate (MTA) is considered as an alternative to calcium hydroxide and recognized as a gold standard of dental biomaterial in endodontic therapy including root-end filling, pulp capping and pulpotomy for primary tooth. The material is a favourable substitute due to its anti-microbial properties, great biocompatibility, its regenerative effect for periodontal tissue and its ability to guide hard tissue formation (Naik et al., 2014). However, the presence of bismuth oxide (BO) in MTA can lead to cell toxicity

and damage. The long setting time of MTA also contributes to inconvenience in clinical use (Tsai et al., 2018).

Therefore, these drawbacks make MTA not meet the clinical requirements; thus it is a priority to explore or develop new endodontic materials. Portland cement (PC), which is compositionally similar to MTA has been widely explored in several studies due to its lower costs and shorter setting time (Naiana et al., 2011). Regardless of the economic benefits, PC alone is not suitable for clinical applications due to a lack of radiopacity (Rahimi et al., 2019a). However, the lack of radiopacity of the PC makes it advantageous to reduce the toxicity effects of the cements (Coutinho-Filho et al., 2008).

In the present study, a new biomaterial consists of three main components, white Portland cement (WPC), calcium chloride dehydrate ($CaCl2.H_2O$) and chitosan (CHT) was synthesized and called accelerated Portland cement with chitosan (APC-CHT). $CaCl2.H_2O$ is a common accelerator used to shorten the setting time of PC (Oey et al., 2015) and the addition of CHT may enhance the functionality of the cement (Ustinova and Nikiforova, 2016). Chitosan is an applicable additive due to its remarkable properties such as great biocompatibility and biodegradability, excellent antibacterial property and able to promote cell attachment, proliferation and differentiation (Croisier and Jérôme, 2013). By integrating APC with chitosan, it results in combination of the non-toxic properties of the cement and antibacterial properties of the polymer. Thus, APC-CHT can be a promising substitute to MTA and other current dental materials. In this study, the synthesized material was investigated for its ability to induce the dentinogenic/osteogenic differentiation of SHED to form dentin-forming cells and bone-forming cells for the development of tooth structure.

1.2 Problem statement

Current good material like MTA is widely used in dental clinical setting, however, the functions are limited by causing some toxicity and requiring a longer period of setting time (Tsai et al., 2018). Due to some other limitations of common dental biomaterials, it leads to the emergence of new biomaterial design to restore defective tissue and, at the same time to guide the formation of new tissues during the therapy. Thus, the development of biomaterials that are non-toxic, possess antibacterial properties, promote the regeneration of new healthy tissues, enhance dental cell activity, low-cost, can be used under different climatic conditions, storage-tolerate and allow easy handling is indispensable (Deb and Chana, 2015). Therefore, the purpose of the study is to investigate the effects of newly synthesized material (APC-CHT) on dentinogenic and osteogenic differentiation ability of SHED.

1.3 Rationale of the study

Portland cement is a biocompatible material for dental application and the cement is safer, faster setting time and more economic substitute for MTA while chitosan is a natural polymer that own outstanding antibacterial property, biodegradable and considered as non-toxic material. Thus, CHT was incorporated into Portland cement to synthesize non-toxic biomaterial with the presence of bacteriostatic effects. The APC-CHT was developed and tested in the study to investigate the effect of the material on the differentiation of SHED into odontoblasts and osteoblasts for regeneration of tooth structure. The APC-CHT may be considered as a new competent biomaterial due to its biocompatibility, biodegradability and antibacterial properties. Therefore, the aim of the study is to determine the effects of APC-CHT seeded with SHED and maintained in osteogenic media.

1.4 Objectives

1.4.1 General objective

To study the effect of APC-CHT on dentinogenic/ osteogenic differentiation on SHED

1.4.2 Specific objectives

i. To determine the expression of dentinogenic protein (DMP-1, DSPP and MEPE) by Western blot
ii. To determine the expression osteogenic protein (ALP, OCN and OPN) by Western blot

1.5 Hypothesis

APC-CHT promotes the dentinogenic/osteogenic differentiation of treated-SHED.

CHAPTER 2

LITERATURE REVIEW

2.1 Dental diseases

Tooth is the vital organ in human body where any defect has an effect on the quality of human life. Regardless of their importance, people are unconscious of the existence of hidden dangers that can be caused by dental defects. The lack of awareness among the public people can be seen through the high cases of dental caries and periodontal diseases. It is estimated that 3.5 billion people have been affected not only by caries and gum problems but also by oral cancers, oral HIV manifestations, dental trauma, cleft lip and palate and noma (Dye, 2017; WHO, 2020).

Oral health is one of the major concerns by the government because it has a devastating health burden in many countries due to the fact that dental disease not only causes pain throughout human life but can also lead to disfigurement and death (Kaye, 2007). Most dental pain occurs as a result of caries and once caries reaches dentine structure, the pain may occur in the presence of thermal stimulation or sweet-sour of dietary sources (Holt et al., 2001). Pain may also occur when dentine is exposed to trauma, erosion or abrasion. In addition, dental decay can provide pathogenic bacteria access into the root canal system and lead to endodontic infections including apical periodontitis (Persoon and Özok, 2017).

Periodontitis can cause loss of tooth which is a common infirmity in the global population. The tooth loss often results in hard tissue damage of the jaw, creating an enormous clinical challenge for dental practitioners to solve the problems (Jimi et al., 2012). The most worrisome part is that a research study reported that 9 out of 10 Malaysian adults suffer from gum disease (Abdul Aziz, 2014). Besides, as stated

by Ibrahim *et al*. (2020), all 46 chronic kidney disease (CKD) patients in Northeast Peninsular Malaysia tertiary hospital suffer periodontal problems (Ibrahim et al., 2020). These revealed that dental problems occur in both healthy and immunocompromised individuals, leading to an extra burden to be resolved.

2.2 Treatments for dental diseases

In oral disease, there are variety of treatments depending on the stage of the disease, ranging from non-surgical therapies that control bacterial growth to surgery for repairing the damaged tissues (Armitage and Robertson, 2009). In general, dental surgery are divided into three main components which are endodontic, prosthodontics and orthodontic. Endodontic is a type of surgery that deals with the root of tooth, prosthodontics is a surgery that involve dental prosthetics and orthodontic is a surgical treatment that involves of placing of devices on tooth structure to treat abnormalities (Thiti, 2017). In the surgery aspects, dental prosthesis and implant remain as conventional therapies (Garrett et al., 2004).

Non-surgical therapy such as scaling and root planing (SRP) is known to be the gold standard for the initial treatment of inflammatory periodontal disease (Myneni, 2020). Non-surgical periodontal therapy involves patient managing oral home care on a daily basis for success in maintaining dental health yet the therapy tends to fail because it relates to patient compliance (Aimetti, 2014). A previous study mentioned that a high percentage of non-compliance problems contribute to the burden of managing dental hygiene (Lin et al., 2008). The therapy is intended to control microbial infection by removing bacterial biofilm, calculus and toxins from periodontally involved root surfaces. Even so, the treatment is limited by several stumbling blocks such as long-term maintainability of deep periodontal pockets and

the risk of disease recurrence. For tooth caries, a dental filling is used but the restoration often fails over time, mainly due to recurrent caries. The failure in restoration of tooth defects is a worrisome situation because of increasing cases of dental caries from year to year (Çolak et al., 2013). Thus, there is an immense need to develop a new generation of smart biomaterials to reduce and eliminate caries and other dental problems.

2.3 Restorative and regenerative approach

For a long time, restorative care has been primarily involved in treating tooth defects by simply filling the hole or replacing the missing tooth structure with a material without considering any good regenerative effects (Battistella et al., 2010). Previously, the main restorative goal of dentistry was only to remove decay and recently, the primary goal of dental restoration is not just about filling the infected cavity but also to place good biomaterials that will lead to regeneration of tissues and create an environment where tissues can heal (Manivasagam et al., 2018). As to circumvent the limitations of current therapies, regenerative dentistry approach using biomaterials have been proposed as an alternative to promote dental repair. In the literature, regenerative dentistry is usually referred to as a branch of regenerative medicine that focuses on the regeneration and repair of damaged oral tissues (Tran et al., 2019). The ultimate goal of the approach is to restore defects and regenerate new healthy tissues (Mardas et al., 2014). Because of these reasons, endodontic therapy has emerged as a promising approach to treat dental disorders. Endodontic therapy involves a series of treatments, including removal of pulpal tissue, filling and shaping root canals, obturation of the root canal space and placing a permanent restoration for the teeth (Stephen, 2017). Root canal therapy including pulp extirpation, is a complex therapy

process used to treat endodontic infection but several technical complications such as root perforations and failure in identifying all pulp ramifications contribute to the treatment failure. Thus, pulp capping is often practiced as an alternative to root canal therapy since it is easier to perform and allow direct inspection of the wound area (Komabayashi and Zhu, 2010). In endodontic therapy, the crucial key to successful therapy depends largely on the endodontic biomaterials.

2.4 Biomaterial in dentistry

The ideal dental biomaterial exhibits several outstanding properties such as biocompatible, biodegradable, promote cell and tissue formation, provide sufficient structural support and allow cell proliferation and differentiation for regeneration of dental tissues including enamel, dentin, cementum, bone and other intraoral tissues (Sharma et al., 2014). Biomaterials applications in dentistry are divided into four general categories which are polymers, ceramics, metals and composites (Abdolahpour et al., 2017).

2.4.1 Polymers

Polymers contribute significantly in different aspects of dentistry (e.g. preventive, restorative, regenerative) due to their outstanding physical, mechanical and biological properties. Besides, the materials are useful for dentin regeneration or advanced drug delivery systems (Rokaya et al., 2018). Over the last decade, polymers such as alginates, polysulfides and silicone have been widely used in impression materials, the material for construction of oral structures replica (Eichmiller, 2012). Natural-based polymers (e.g. starch, chitosan, collagen) and synthetic-based polymers (e.g. polyglycolic acid (PGA), polylactic acid (PLA) are among the favourite choices as

biodegradable materials in dental application such as in implants and suture (Prakasam et al., 2017). The biodegradable polymers are also used in restoration for the local release of bone stimulating or resorptive drugs in the peri-implant region in order to achieve long-term of dental implant success (Conte et al., 2018).

2.4.2 Ceramics

Excellent esthetic properties of ceramic have led the materials to be extensively used in dental restoration mainly as dental inlays, onlays, veneers, crowns and bridges (Höland et al., 2008). Dental ceramics reveal numerous desirable material properties, including biocompatibility, chemical inertness, abrasion proof and able to reduce plaque accumulation (Sarah and Richard van, 2009). The main components of dental ceramic are Silicon-based inorganic materials (e.g. feldspar, quartz, silica). The feldspar-based and quartz-based of ceramics are mainly used in prosthetic dentistry because they can mimic the various colours and shades of natural teeth (Ho and Matinlinna, 2011). Polycrystalline ceramics, such as yttria-stabilized zirconia, are commercially available for use as all-ceramic preformed crowns in pediatric applications (Platt, 2016). Since ceramics materials are incapable of withstanding functional forces present in the oral cavity, metal ceramic approach has emerged as an alternative. The idea of metal ceramic system is to combine the noble esthetic properties of ceramics with the excellent mechanical properties of metals. Unfortunately, some metals used in the restorative materials may affect patients through several adverse effects such as allergies, gum staining and gingivitis. These drawbacks urge researchers to return to metal-free ceramic systems in restoration and regeneration of oral tissues (Shenoy and Shenoy, 2010).

2.4.3 Metals

Metallic biomaterials are well-known in hard tissue application including in field of dentistry. Metallic biomaterials exhibit unique structural functions, superior than ceramic and polymeric biomaterials and they have been employed extensively in substituting damage parts of human body (Peter et al., 2018). Among the bio-inert metallic materials, Titanium alloys, Cobalt and steel are well known in orthodontics and dental implant owing to their excellent lasting stability and reliable mechanical strength however long-term presence of the metals in the body is associated with an increased risk of development of cutaneous and systemic hypersensitivity reactions. (Prasad et al., 2017).

2.4.4 Composites

The term composites material is generally understood to mean a material consist from two or more constituent materials with different physical or chemical properties and the combination resulted in material with characteristics different from the individual components. Dental composites such as resin becomes one of several alternatives to dental amalgams due to their tooth-colored properties (Food and Drug Administration, 2017). Moreover, most of endodontic biomaterial are categorized under the composite group. The composite cement based on calcium silicate like Portland cement and mineral trioxide aggregate (MTA) are extensively used as a material in pulp capping and root canal-filling material. Both Portland cement and MTA able to result in remineralizing effects because of the presence of tricalcium and dicalcium silicate which suitable for enamel and dentin repair (Osorio and Toledano, 2016).

2.5 Current status of endodontic biomaterials

Numerous biomaterials have been developed for endodontic application such as zinc oxide eugenol (ZOE), glass ionomer (GI), resin modified glass ionomer (RMGI), adhesive systems, calcium hydroxide, mineral trioxide aggregate (MTA) and biodentine (Moussa and Mostafa, 2018). Unfortunately, ZOE, GI, RMGI and adhesive system have been identified as cytotoxic materials towards dental pulp cells (Hilton, 2009; Murray et al., 2002). Calcium hydroxide was introduced in 1921 and has been acknowledged as a gold standard of direct pulp capping materials for several decades, which was due to its outstanding antibacterial properties. Even so, the material cannot provide a proper seal and has insufficient adhesive qualities (Hilton, 2009). Consequently, MTA is used as an alternative to the calcium hydroxide. The clinical performance of MTA is more effective compared to calcium hydroxide, where MTA has been shown to have a higher success rate in maintaining long-term tooth vitality, able to enhance better dentinogenic process and inferior in causing pulpal inflammation. However, the antibacterial properties of MTA is debatable as the material exhibit the bactericidal effect only on facultative bacteria but no effect on any of the strictly anaerobic bacteria. It shows that bacteria suppression by MTA may not be as excellent as conventional calcium hydroxide (Moussa and Mostafa, 2018). In addition to the long setting time of MTA, which takes approximately 2 hours and 45 minutes can expose the material to bacterial contamination therefore the procedure is inconvenient and impractical to both dentist and patient. Providentially, biodentine has been reported to have similar efficacy with MTA in direct capping application and it may be considered as a best substitute to MTA in human teeth (Mali and Mangala, 2020). Nevertheless, biodentine has an unfavourable radiopacity as compared to MTA

(Kaur et al., 2017) and can cause significant leakage at the dentine-material interface (Malkondu et al., 2014).

2.6 Potential biomaterial candidate: APC-CHT

Since the current dental biomaterials are incapable to fulfil the clinical requirement and possessed several limitations, it leads to the urgency for the development of new ideal biomaterial. Additionally, the increased longevity of the population has elevated the demands for improved dental biomaterial function. The creation of new material consists of both restorative and regenerative properties is crucial. In the present study, APC-CHT were synthesized using three main components which are white Portland cement (PC), chitosan (CHT) and calcium chloride dehydrate ($CaCl_2.H_2O$).

2.6.1 Portland cement (PC)

Portland cement consists of calcium phosphate, calcium and silicon oxide and it has been effectively used as an apical plug material, perforation repair, root end filling material, pulp capping and pulpotomy in several studies. In point of fact, the main composition of MTA which is a current gold standard material is 80% of PC along with the addition 20% of bismuth oxide (Naiana et al., 2011). The *in vitro* toxicity evaluation of bismuth oxide on various cell line type by Abudayyak *et al*. (2017) identified that the nanoparticles of the compund resulted in high cell apoptosis and decreased the cell viability. The compound also able to trigger the induction of oxidative damage of cells (Abudayyak et al., 2017). Due to the toxicity effects of bismuth oxide, many attempts had been initiated by many researchers to search for new material as substitute to MTA.

Moreover, Farhad Mollashahi *et al*. (2016) have conducted an *in vitro* study to determine the effect of PC on osteogenic/odontogenic differentiation of SCAP and they revealed that the cement was able to induce mineralization process and stimulated osteogenic and odontogenic differentiation (Farhad Mollashahi et al., 2016). A previous study reported that PC enriched with zirconium oxide and zinc oxide showed increase alkaline phosphatase activity and calcium ion release by DPSC which indicated the induction of osteo/odonto differentiation (Rahimi et al., 2019b). A case report on the application of white Portland cement as an apical plug in a tooth with a necrotic pulp and wide-open apex revealed positive clinical resolution thus encouraged the use of WPC for root canal treatment (De-Deus and Coutinho-Filho, 2007).

Taking consideration of similar composition of MTA and PC, both of them exhibit similar effects in dental application. These materials produce a similar result in extracellular matrix neoformation by odontoblast cell line and formation of reparative dentin. In actual fact, PC is better than MTA because of inexpensive, shorter setting time and lesser toxicity towards dental cells (Naiana et al., 2011). The time reduction in setting time of PC is due to the addition of $CaCl_2.H_2O$, the common accelerator. $CaCl_2$ provides better compressive strength when added into tricalcium silicate cement in comparison with cement composite alone (Wang et al., 2008). From the industrial point of view, the process of biomaterial manufacturing must be simple, fast and cost-effective (Gathani and Raghavendra, 2016) thus APC may satisfy the demands.

2.6.2 Chitosan (CHT)

Chitosan is the second most abundant of natural polysaccharide that derived from deacytlated chitin which mainly found in the exoskeleton of crustaceans such as shrimp, crabs and lobsters (Cicciù et al., 2019). The polymer can be applied in all fields of dentistry including preventive dentistry, conservative dentistry, endodontics, surgery, periodontology, prosthodontics and orthodontics (Wieckiewicz et al., 2017).

The purpose of chitosan addition in the present study is because of their significant properties such as antibacterial activity, mucoadhesive, haemostatic, biocompatible and biodegradable (Croisier and Jérôme, 2013). The chitosan-based materials have been explored broadly in dental applications since chitosan can easily blend with other materials (Husain et al., 2017). Chitosan has been added in several material developments such as glass ionomer cement, calcium hydroxide and it resulted in enhancement of antibacterial activity of the composites (Erpaçal et al., 2019). The antibacterial activity of chitosan covers a broad spectrum of gram-negative and gram-positive bacteria as well as fungi. An *in vitro* study by Aliasghari *et al*. (2016) proved that chitosan is able to suppress the growth of cariogenic streptococci (Aliasghari et al., 2016). Moreover, another previous study suggested that the addition of chitosan to cement mortar increased the fungicidal effect because the polymer was able to suppress several types of oral fungi including *Aspergillus* spp. and *Penicillium* spp. that caused periodontitis (Ustinova and Nikiforova, 2016). Furthermore, Suzuki *et al*. (2014) reported in their study that chitosan enhanced the function of citrate solution as a root canal irrigant. Chitosan-citrate solution also exhibited antibacterial effect against *Enterococcus faecalis*, one of the agent of endodontic infection (Suzuki et al., 2014).

Several studies demonstrated the ability of chitosan to serve as a vehicle in oral drug delivery to dental structure like periodontal tissue (Pichayakorn and Boonme, 2013; Qasim et al., 2017). Chitosan is also known as a hemostatic agent, thus the polymer render a benefit in invasive dental surgery that can cause bleeding disorders (Kmiec, 2017). In addition, chitosan was able to increase the regeneration capability of the dentin pulp complex and promoted osteogenesis in guided tissue regeneration (Erpaçal et al., 2019). The previous comparative study revealed that nano hydroxyapatite-chitosan cements have better bioactivity in comparison with MTA, calcium enriched mixture and hydroxyapatite (Hosseinzade et al., 2016). For these countless reasons, chitosan can be considered as a suitable choice to be an additive in the present study.

2.7 SHED

Human teeth become a rich source of mesenchymal stem cell (MSC) populations with high potential in dental studies. Dental stem cell types consist of dental pulp stem cells (DPSC), dental follicule stem cells (DFSC), stem cells from human exfoliated teeth (SHED), gingival fibroblastic stem cells (GFSC), stem cells from apical papilla (SCAP), periodontal ligament stem cells (PDLSC) and dental implant stem cells (DISC). Each of these dental MSC is named on the basis of the origin tissue (Figure 1). Of these dental stem cells, SHED was selected for in the present study because of several important reasons. SHED was identified by Miura *et al.* (2003) as a highly proliferative and multipotent MSC that capable of differentiating into a variety of cell types including odontoblast and osteoblast (Miura et al., 2003). Numerous studies have demonstrated the ability of SHED in exhibiting dentinogenic and osteogenic properties (Brar and Toor, 2012). SHED have been shown to possess a high capacity in bone and

dentin formation when injected to immune-deficient mice (Wang et al., 2018). Miura *et al*. (2003) also demonstrated that the regenerated dentin from SHED was immune-reactive to dentin-specific antibody indicating that the cells can differentiate into odontoblasts *in vivo* (Miura et al., 2003). Since SHED are accessible with the least ethical concern, multi-differentiation potentials and high proliferation rate, it will contribute advantages in determining the effect of APC-CHT in the present study.

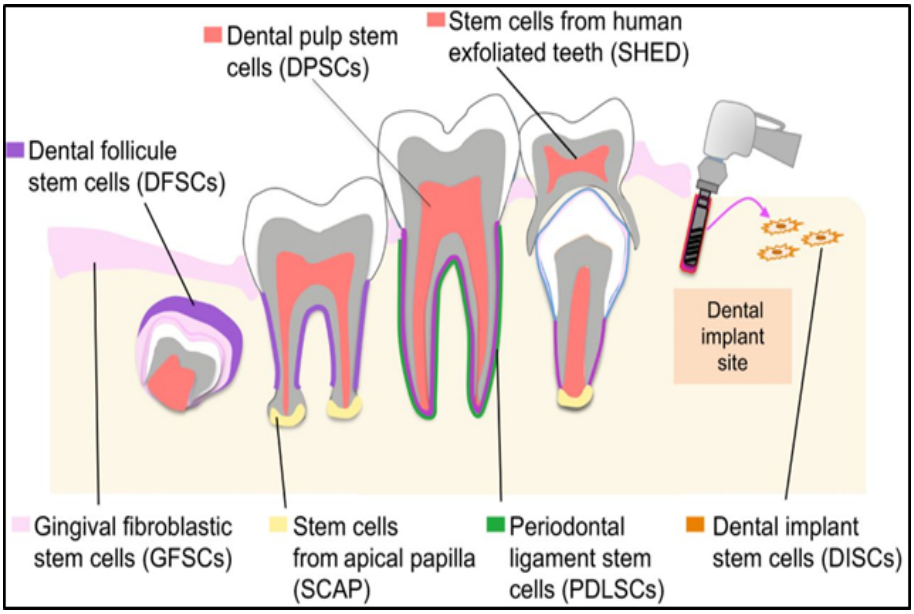

Figure 2.1 Dental stem cell. The name of dental MSC are depends on the extraction site which consist of dental pulp stem cells (DPSC), dental follicule stem cells (DFSC), stem cells from human exfoliated teeth (SHED), gingival fibroblastic stem cells (GFSC), stem cells from apical papilla (SCAP), periodontal ligament stem cells (PDLSC) and dental implant stem cells (DISC). (Adapted from Sharpe, 2016).

2.8 Protein expression analysis using Western blot

The evaluation of dentinogenic and osteogenic differentiation of SHED is commonly performed by determining the expression of certain related markers. In this study, a several common markers such as alkaline phosphatase (ALP), dentin matrix acid phosphoprotein-1 (DMP-1), dentin sialophosphoprotein (DSPP), matrix extracellular phosphoglycoprotein (MEPE), osteocalcin (OCN), and osteopontin (OPN) were used for western blot analysis. DSPP, DMP1, and MEPE are dentinogenic markers while ALP, OCN and OPN are osteogenic marker, all of which play significant roles during proliferation and differentiation of cells (Ching et al., 2016). In Western blot, the isolation and purification of cell protein is used to determine the presence of protein in cell differentiation. The greatest advantage of the technique is its sensitivity in the protein detection as little as 0.1 nanograms of protein in the sample (National Diagnostics, 2011). The greater sensitivity, the fewer antibodies are required for analysis which also provides an advantage in terms of cost (Mishra et al., 2017).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study design

This study involved material preparation, cell culture technique and detection of protein expressions by Western blot assay. All the experimental works were carried out at the Advance Molecular Biology Laboratory, Craniofacial Science Laboratory and Culture Laboratory, at the Health Campus of Universiti Sains Malaysia. Flowchart of the study is shown in Figure 3.1.

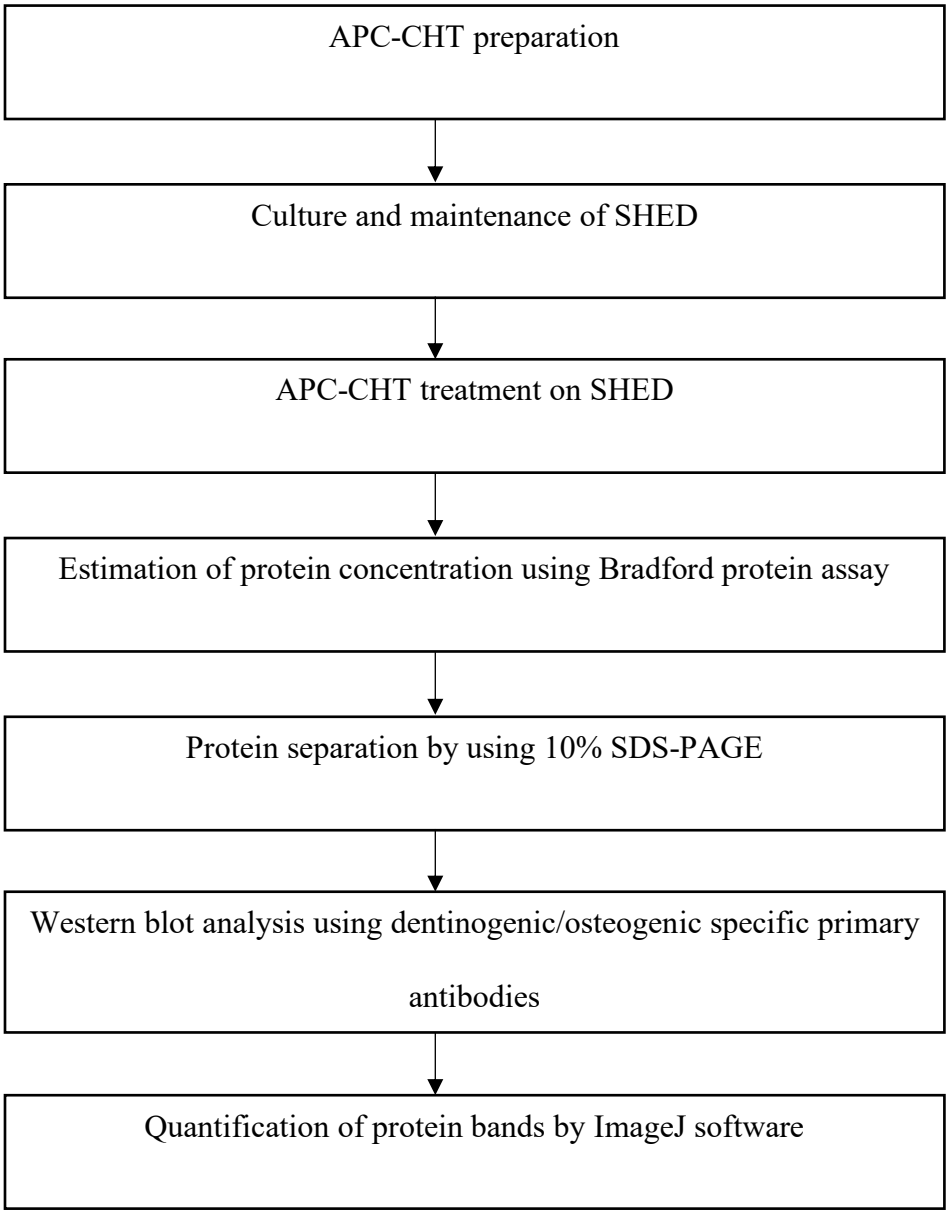

Figure 3.1 Flowchart of the study

3.2 Materials

3.2.1 Reagents and chemicals

List of reagents and chemicals used in the study are listed in Table 3.1.

Table 3.1 List of reagents and chemicals

Item	Manufacturer
0.25% Trypsin-EDTA	Gibco, USA
2-mercaptoethanol	Bio-Rad, USA
30% Acrylamide	Novus Biologicals, USA
Absolute Ethyl Alcohol (Ethanol)	HmbG, German
Acetic acid	Merck, Germany
Ammonium persulfate	Novus Biologicals, USA
Bio-SafeTM Commasie G-250	Novus Biologicals, USA
Calcium chloride dehydrate ($CaCl_2.H_2O$)	Merck, Germany
Chitosan	Sigma-Aldrich, Iceland
Clarity Western ECL Substrate	Novus Biologicals, USA
Dexamethasone	Sigma-Aldrich, USA
Dimethyl sulphoxide (DMSO)	Merck, Germany
Enhance chemiluminescence (ECL) plus western blotting detection reagents	Amersham, USA
Fetal bovine serum (FBS)	Gibco, USA
Glycine	Amresco, USA
L-ascorbic acid	Sigma-Aldrich, USA
Methanol	Thermo Fisher Scientific, USA
Penicillin-streptomycin	Gibco, USA
Phosphate-buffered saline (PBS)	Gibco, USA
Polyvinylidene difluoride (PVDF)	Merck, USA
Protease inhibitor	Nacalai Tesque, Japan
Resolving gel buffer	Bio-Rad, USA
RIPA buffer	Amresco, USA
Skimmed milk	Sunlac, Australia
Sodium dodecyl sulfate (SDS)	Amresco, USA
Stacking gel buffer -TRIS-HCl buffer	Bio-Rad, USA

TEMED	Novus Biologicals, USA
Trypan blue	Sigma-Aldrich, USA
Tween-20 (T-20)	Amresco, USA
White Portland Cement	Aalborg, Malaysia
α-Minimal Essential Medium (a-MEM)	Gibco, USA
β-glycerophosphate	Sigma-Aldrich, USA

3.2.2 Antibodies

Antibodies used in the study are listed in Table 3.2.

Table 3.2 List of antibodies

Antibody	Name	Manufacturer
Mouse monoclonal anti human primary antibody	Alkaline phosphatase (ALP)	R & D Systems, USA
	Dentin matrix protein-1 (DMP-1)	Santa Cruz Biotechnology, USA
	Dentin sialophosphoroprotein (DSPP)	Santa Cruz Biotechnology, USA
	Glyceraldeyhde 3-phosphate dehydrogenase (GAPDH)	R & D Systems, USA
	Matrix extracellular phosphoglycoprotein (MEPE)	Santa Cruz Biotechnology, USA
	Osteocalcin (OCN),	Santa Cruz Biotechnology, USA
	Osteopontin (OPN)	R & D Systems, USA
Secondary antibody	Horseradish Peroxidase (HRP)	R & D Systems, USA

3.2.3 Consumables

List of consumables used in the study are listed in Table 3.3.

Table 3.3 List of consumables

Item	Manufacturer
0.20µm sterile filter	PALL corporation, Life Sciences, USA
1.5 ml microcentrifuge tubes	Greiner bio-one, Austria
75-cm2 flasks	Biologix, China
Disposable syringe	Terumo Corporation, Japan
Falcon Tube (15ml)	Fisher Scientific, China
Falcon Tube (50ml)	Fisher Scientific, China
Micro-centrifuge tubes (1.5ml)	Labcon, USA
Micro-centrifuge tubes (2ml)	Labcon, USA
Pipette tips Eppendorf 10µl, 20µl, 200µl and 1000µl	Eppendorf AG, Germany
Sterile serological pipette 10ml	Greiner bio-one, Austria