PHYSICO-CHEMICAL PROPERTIES AND EFFECTS OF CHITOSAN-BASED ACCELERATED PORTLAND CEMENT ON STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

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by

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$C = 4P/\pi D^2$ **(3.1)** 91

$VHN = 2F \sin(136°/2)/d^2 = 1.854 F/d^2$ **(3.2)** 92

$D = (m_1 - m_2) / m_1 \times 100$ **(3.3)** 93

$C = Av \times 2 \times 10^4$ cell/ml **(3.4)** 98

Cell viability (%) = absorbance of samples / absorbance of control x 100 **(3.5)** 99

LIST OF ABBREVIATIONS

α-MEM	Alpha minimum essential medium
ALP	Alkaline Phosphatase
APC	Accelerated portland cement
APC-CT	Chitosan-based accelerated portland cement
APC-0.6%CT	Chitosan (0.6%)-based accelerated portland cement
APC-1.25%CT	Chitosan (1.25%)-based accelerated portland cement
APC-2.5%CT	Chitosan (2.5%)-based accelerated portland cement
ASTM	American society for testing and materials
ATR	Attenuated total reflectance
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
$CaCl_2.2H_2O$	Calcium chloride dihydrate
COL1A1	Collagen Type 1 Alpha 1
CT	Chitosan
DD	Degree of deacetylation
DEPC	Diethyl Pyrocarbonate
DMP-1	Dentin matrix protein 1
DMSO	Dimethyl sulphoxide
DPSC	Dental pulp stem cells
DPP	Dentine phosphoprotein
DSP	Dentine sialoprotein
DSPP	Dentin sialophosphoprotein
EBA	Super-ethoxy benzoic acid

EDX	Energy dispersive X-ray microanalysis
Eff	Amplification efficiency
ELISA	Enzyme-linked immunosorbent assay
EO	Enamel organ
ERRM	EndoSequence Root repair material
ESE	European society of endodontology
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscopy
FTIR	Fourier transform infra-red
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	genomic DNA
HDPCs	Human dental pulp cells
HPDLCs	Human periodontal ligament cells
HV	Vickers hardness
IEE	Inner enamel epithelium
IRM	Intermediate restorative material
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
LB	Lithium borate
Mg	Magnesium
MEPE	Matrix extracellular phosphoglycoprotein
MMPs	Matrix metalloproteinases
MSC	Mesenchymal stem cell
MTA	Mineral trioxide aggregate
MW	Molecular weight

Na_2HPO_4	Disodium phosphate
NCPs	Non-collagenous proteins
ng	Nanogram
NH_3	Ammonia
nM	Nanomolar
NTC	Non-template control
OCN	Osteocalcin
OD	Optical density
OEE	Outer enamel epithelium
OH	Hydroxide
OM	Dentinogenic/osteogenic medium
ONC	Osteonectin
OPC	Ordinary portland cement
OPG	Osteoprotegerin
OPN	Osteopontin
PBS	Phosphate buffered saline
PC	Portland cement
PDL	Periodontal ligament
PGs	Dentin proteoglycans
pH	Potential hydrogen
PI	Propidium iodide
qPCR	Quantitative polymerase chain reaction
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RMGI	Resin-modified glass ionomer

RUNX2	Runt-related transcription factor 2
R^2	Correlation coefficient
SCPP	Secretory calcium-binding phosphoprotein
SHED	Stem cells from human exfoliated deciduous teeth
SI	Stratum intermedium
SIBLINGs	Small integrin-binding ligand N-linked glycoproteins
Si	Silicon
SR	Stellate reticulum
Ti	Titanium
TGF	Transforming growth factor
UV	Ultraviolet
WMTA	White mineral trioxide aggregate
WPC	White portland cement
ZOE	Zinc oxide eugenol

PENILAIAN SIFAT FIZIKO-KIMIA DAN KESAN SIMEN ACCELERATED PORTLAND BERASASKAN KITOSAN TERHADAP SEL TUNJANG DARIPADA GIGI SUSU MANUSIA YANG TERLUPAS

ABSTRAK

Kemajuan dalam bidang endodontik seperti teknik, peralatan dan bahan telah meningkatkan penjagaan kesihatan mulut dan menjadikan rawatan pergigian lebih berkesan, serta menjimatkan kos dan masa. *Accelerated Portland* simen (APC) adalah bahan berpotensi dengan sifat kimia, fizikal dan biologi yang baik. Ia telah dikaji sebagai bahan alternatif untuk mengatasi kelemahan utama mineral trioksida agregat (MTA) dan simen portland (PC) seperti tempoh pengerasan dan kos yang tinggi. *Chitosan* (CT) juga telah digunakan dalam banyak aplikasi perubatan kerana kepelbagaian sifat biologinya. Dalam kajian ini, APC disediakan dalam kombinasi dengan CT dan dinamakan APC-CT. Kajian ini bertujuan menilai sifat kimia, fizikal dan mekanikal APC-CT dan kesannya terhadap kebioserasian, pemineralan dan potensi pembezaan dentinogenik/osteogenik sel tunjang dari gigi susu manusia yang terlupas (SHED). APC-CT disediakan dengan pelbagai kepekatan larutan CT (0.625%-, 1.25%- dan 2.5%) dan APC telah digunakan sebagai kawalan. Sifat kimia dinilai menggunakan FTIR dan FESEM/EDX selain sifat fizikal dan mekanikal seperti masa pengerasan, kekuatan mampatan, kekerasan mikro permukaan, pH dan keterlarutan. Kemudian, kesan ekstrak APC-CT terhadap kebolehhidupan, pelekatan sel dan apoptosis juga dinilai. Aktiviti pemineralan SHED dinilai oleh pewarnaan merah Alizarin dan Von Kossa. Akhirnya, pembezaan dentinogenik/osteogenik SHED dianalisis dengan menilai pengekspresan gen penanda dentinogenik/osteogenik terpilih iaitu *DSPP, MEPE, DMP-1, OPN, OCN, OPG, RANKL, RUNX2, ALP* dan *COL1A1* dengan “real-time PCR”. Hasil kajian FTIR yang disahkan menunjukkan

kehadiran hablur halus CT yang tersebar dan mengisi ruang struktur APC menghasilkan fasa yang lebih homogen. Komposisi kimia APC dan APC-CT adalah hampir sama dengan kehadiran O, C dan Si yang lebih tinggi dalam APC-CT. Julat nilai tempoh pengerasan, kekuatan mampatan, kekerasan mikro, pH dan keterlarutan adalah di antara 46.6-48.5 min, 51.3-39.1 MPa, 44.89-38.57 HV, 11.02-11.04 (24 jam) dan 3.23-2.44%. CT meningkatkan pH dan keterlarutan APC dan memanjangkan tempoh pengerasannya. Walau bagaimanapun, kekuatan mampatan berkurang dan memberi kesan minimum terhadap kekerasan mikro, Ujian kesitotoksikan menunjukkan bahawa APC-CT menyokong proliferasi dan interaksi SHED terhadap bahan tersebut; serta tidak menunjukkan kesan apoptosis. Pewarnaan merah Alizarin dan Von Kossa menunjukkan peningkatan aktiviti pemineralan SHED apabila dirawat dengan APC-CT. Pengekspresan gen penanda *DSPP, MEPE, DMP-1, OPN, OCN, OPG* dan *RANKL* meningkat dalam SHED yang dirawat APC-CT. Sementara itu, pengekspresan gen penanda *RUNX2, ALP* dan *COL1A1* berkurang. Penemuan ini menunjukkan bahawa APC-CT memperlihatkan sifat kimia, fizikal dan mekanikal yang baik. APC-CT adalah tidak toksik dan menggalakkan pembezaan dentinogenik/osteogenik dan aktiviti pemineralan; ini menunjukkan potensi aplikasi APC-CT dalam kejuruteraan tisu gigi/tulang.

PHYSICO-CHEMICAL PROPERTIES AND EFFECTS OF CHITOSAN-BASED ACCELERATED PORTLAND CEMENT ON STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

ABSTRACT

Advancement in the field of endodontic such as techniques, instrumentations and materials have considerably improved the oral health care and have made the dental treatment more efficient, as well as cost and time effective. Accelerated Portland cement (APC) is a potential material with favourable chemical, physical and biological properties. It was studied as an alternative material to overcome the major limitations of mineral trioxide aggregate (MTA) and portland cement (PC) such as delayed setting time and high cost of MTA. Chitosan (CT) has also been used in numerous medical applications due to its various biological properties. In this study, APC was prepared in combination with CT and designated as APC-CT. This study aimed to evaluate the chemical, physical and mechanical properties of APC-CT and to evaluate its biocompatibility, mineralization activity and dentinogenic/osteogenic differentiation potential on stem cells from human exfoliated deciduous teeth (SHED). APC-CT was prepared with various CT concentrations of 0.625%-, 1.25%- and 2.5%-CT solutions, and APC was used as control. The chemical characterizations by FTIR and FESEM/EDX were evaluated, in addition to the physical and mechanical properties such as setting time, compressive strength, surface microhardness, pH and solubility. Then, the effect of APC-CT on cell viability, attachment and apoptosis were assessed. The mineralization activity of SHED was evaluated by Alizarin Red staining and Von Kossa stain. Finally, the dentinogenic/osteogenic differentiation of SHED was analysed by evaluating the gene expression of selected dentinogenic/osteogenic markers i.e. *DSPP, MEPE, DMP-1, OPN, OCN, OPG, RANKL, RUNX2, ALP* and

COL1A1 by real-time PCR. The results confirmed the interaction of CT with APC by FTIR spectra. The surface morphology of APC-CT was characterized by the presence of CT crystallites which spread and filled the spaces in APC structure that resulted in more homogeneous phases. The chemical compositions of APC and APC-CT were almost identical with intensified O, C and Si in APC-CT. The setting time, compressive strength, microhardness, pH and solubility obtained ranged between 46.6-48.5 min, 51.3-39.1 MPa, 44.89-38.57 HV, 11.04-11.02 (24 hrs) and 3.23-2.44%, respectively. CT improved the pH and solubility of APC and extended its setting times. However, compressive strengths were reduced and minimum effect on microhardness was observed. Cytotoxicity assays demonstrated that APC-CT supported the cell proliferation and interaction of SHED to the materials; as well as no apoptotic effect was observed. Alizarin Red and Von Kossa stainings demonstrated increased mineralization activity of SHED when treated with APC-CT. The expressions of *DSPP, MEPE, DMP-1, OPN, OCN, OPG* and *RANKL* markers were up-regulated in APC-CT-treated SHED. While, the expressions of *RUNX2, ALP* and *COL1A1* markers were down-regulated. These findings demonstrate that APC-CT exhibits good chemical, physical and mechanical properties. APC-CT is non-toxic and promotes dentinogenic/osteogenic differentiation and mineralization activity; which provides potential applications of APC-CT in tooth/bone tissue engineering.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Advancement in the field of endodontic such as techniques, instrumentations and materials have considerably changed the quality of dental treatment. These advancements have improved the oral health care and have made the dental treatment more reliable, predictable, as well as more cost and time effective (Lababidi, 2013).

The progress in the field of regenerative materials has significantly highlighted on the research in tooth mineralization and biological behaviour of the dentin-pulp complex. Dentin-pulp complex has the ability to adapt with the stimuli invoking defence responses to preserve the tooth vitality. The main function of dental pulp is to secrete dentin during tooth development and maintain self-protection by reinitiating dentinogenesis when exposed to the external injuries. The concept of vital pulp therapy involves the process and procedure which aim to maintain the pulp vitality. The technique is based on biological approach where more focus on the understanding of patho-physiological processes of dentin-pulp complex. This concept is applied in the research and studies on the development of new materials which simulate the physiological factors of restored tissues (Akhlaghi and Khademi, 2015).

In dental treatment, a tissue response to injuries involves complex cellular and molecular biological process which result in tissue repair or regeneration (Lin and Rosenberg, 2011). Treatment of the endodontic diseases such as irreversible pulpitis or apical periodontitis involves wound healing by tissue repair or combination of tissue repair and regeneration (Lin and Rosenberg, 2011). In indirect pulp capping treatment,

the application of a potential material can induce a reactionary dentinogenesis by stimulating the surviving primary odontoblast and the release of growth factors from dentin matrix (Song *et al.*, 2017a; Tomson *et al.*, 2017). Whereas, in direct pulp capping, pulpotomy and apexogenesis, the application of the material can induce the dentin bridge formation by stimulating a reparative dentinogenesis through the recruitment and differentiation of progenitor/stem cells in pulp into odontoblast-like cells and the release of growth factors from the dentin matrix (Chogle *et al.*, 2012; Tomson *et al.*, 2017). Moreover, in periapical wound healing, the progenitor/stem cells are recruited and differentiated into PDL fibroblasts, cementoblast-like cells and osteoblasts to form PDL ligament, cementum and alveolar bone, respectively (Han *et al.*, 2014). A regenerative healing with some fibrosis of the periapical tissue is induced after surgical and nonsurgical endodontic treatments. In apexification, the formed calcified barrier at the blunt open apex was described as cementum-like tissue or osteodentin (Lin and Rosenberg, 2011).

The introduction of mineral trioxide aggregate (MTA) is one of great advancement in endodontic. MTA is a portland cement-based material which was introduced in an endodontic field in the year 1993. The material was used for root end filling and root perforation repair (Lee *et al.*, 1993; Torabinejad *et al.*, 1993). Several studies have demonstrated that MTA shows good physico-chemical, mechanical and biological properties (Asgary *et al.*, 2012; Kim *et al.*, 2013; Kaup *et al.*, 2015) as well as enhances the mineralization activity and dentinogenic/osteogenic cell differentiation (Wang *et al.*, 2014b; Yan *et al.*, 2014; Saberi *et al.*, 2019). In addition, its behaviour in clinical applications has been widely investigated (Parirokh *et al.*, 2018). As for the clinical and treatment purposes, MTA is very useful for restoring root perforations and

resorption (Ikhar *et al.*, 2013; Yadav *et al.*, 2013; Mente *et al.*, 2014b), apexification procedure (Damle *et al.*, 2016) and as a lining in the vital pulp therapy (Mente *et al.*, 2014a). However, MTA is not routinely used in the clinical practice due to the high cost and could be unaffordable for some patients (Foley, 2011).

Therefore, current research and studies are looking for the lower cost of endodontic material which presenting good properties. Portland cement (PC) is made from raw materials which are low cost and widespread-available around the world such as limestone, clay minerals, sand, iron minerals and gypsum (Fernández-Carrasco *et al.*, 2012), which make the PC very cost-effective and affordable cement. PC is the main ingredient of MTA, both materials have a similar composition, except the presence of bismuth oxide in MTA. PC has similar physico-chemical and mechanical properties to MTA and it has been suggested as an available substitute to MTA due to its low cost and economically affordable (Islam *et al.*, 2006; Khan *et al.*, 2016).

The similarity in chemical composition and physical properties between white MTA (WMTA) and white PC (WPC) attracted the interest in evaluating the WPC as a clinical alternative to WMTA (Islam *et al.*, 2006; Khan *et al.*, 2016). The experimental studies, animal models and reported cases revealed that the favourable biological profile and good sealing ability of WPC are similar to that of WMTA (Al-Hezaimi *et al.*, 2011; Shahi *et al.*, 2011; Bidar *et al.*, 2014; Yildirim *et al.*, 2016a). In addition, the literature provided insight on the similarity between WPC and MTA in term of antibacterial activities (Tanomaru *et al.*, 2014) and amount of arsenic released from both materials (Duarte *et al.*, 2005). Furthermore, WPC demonstrated similar results

to MTA in preventing microleakage and it was successfully used in the repair of perforations and as retro filling material (Shahi *et al.*, 2011; Borges *et al.*, 2014a).

However, PC also has similar disadvantage like MTA, which is long setting time that results in the initial looseness of the mixture and makes the handling rather difficult (Torkittikul and Chaipanich, 2012). Thus, various additives were investigated as setting time accelerators to PC (Bost *et al.*, 2016) including calcium chloride ($CaCl_2$).

$CaCl_2$ significantly decreases the setting time of WPC (Torkittikul and Chaipanich, 2012) in addition to preserving and strengthening its favourable biological properties (Ong *et al.*, 2012). Accelerated portland cement (APC), where the PC was added with a portion of $CaCl_2$, had showed better sealing ability and increased the release of calcium ions while maintaining high pH (Bortoluzzi *et al.*, 2006a; Bortoluzzi *et al.*, 2006b). Furthermore, APC is non-toxic and may have potential to promote bone healing (Abdullah *et al.*, 2002; Hoshyari *et al.*, 2016).

The chemical composition of WPC from different countries of origin has been examined such as Egypt, Malaysia (Ahmed *et al.*, 2016), Korea (Hwang *et al.*, 2011), Thailand (Torkittikul and Chaipanich, 2012) and United Kingdom (Camilleri *et al.*, 2012). The Malaysian accelerated WPC has been reported by Ong *et al.* (2012) to exhibit a favourable cell viability comparable to that of accelerated MTA. Thus, it may be considered that the Malaysian accelerated WPC has the potential to be used as an alternative to the MTA since it will be more cost effective and affordable for dental applications.

In order to improve the properties of APC as an endodontic material and to overcome the undesirable characteristics, a modification to its chemical composition was made in this study by incorporating chitosan (CT).

CT is a natural biopolymer originated from chitin. It has been used in numerous medical applications due to its favourable properties such as biodegradable, biocompatible, non-toxic and possesses antimicrobial activity (Bano *et al.*, 2017). In addition, studies have demonstrated that CT improved the mechanical properties of cements and promoted osteogenesis (Aryaei *et al.*, 2015; Tao *et al.*, 2020). This material is also easy to manipulate, available and has natural cationic property which makes it suitable to be used in hydrogels (Ahmadi *et al.*, 2015). CT-based materials have been given great importance in the field of material development, they have outstanding characteristics such as biocompatibility, dentinogenic/osteogenic potential and formability into various structures (Hu *et al.*, 2020; Islam *et al.*, 2020). Hence, adding CT into APC may improve its properties by enhancing the biocompatibility, cell differentiation potential and mineralization activity while maintaining the physical and mechanical behaviours which would expand its possible applications. Therefore, a new bioactive material synthesized from combination of APC and CT was developed in the present study.

Biological testing of the newly synthesized endodontic material by using stem cells from dental pulp is a necessary first step prior to the introduction of such material for examination *in vivo*. Stem cells from human exfoliated deciduous teeth (SHED) are unique unspecialized cells originated from dental pulp tissue and have the capacity of extensive proliferation and multipotential differentiation. For dentin and bone tissue

regeneration, SHED were shown to differentiate into odontoblast and osteoblast cells and had the ability to induce dentin and bone formation *in vivo* (Miura *et al.*, 2003; Yamaza *et al.*, 2010). Thus, SHED were selected in this study to assess the cytotoxic and differentiation potential of the newly synthesized material.

1.2 Problem statement

MTA could be the closest to the ideal reparative material due to its excellent properties, and it is considered a gold-standard material for a variety of clinical applications. Despite its desirable properties over the conventional restorative materials, MTA is very expensive which restrict its availability and distribution among the dental practitioners. In addition, MTA has slow setting time which makes the handling rather difficult and necessitates a multiple-visit before completing the treatment (Foley, 2011; Tanalp *et al.*, 2012; Kang *et al.*, 2015; Mostafa and Moussa, 2018). These aforementioned drawbacks limit the use of MTA in its full potential. Thus, it will be of value to synthesize an alternative material with a shorter setting time and lower cost than MTA.

Interestingly, a number of studies have investigated the potential of APC for dental applications as a low cost and affordable material with shorter setting time (Bortoluzzi *et al.*, 2009; Ong *et al.*, 2012; Ahmed *et al.*, 2016). Studies also reported the ability of CT to improve the mechanical properties of cements and to promote the osteogenesis *in vivo* (Rakkiettiwong *et al.*, 2011; Aryaei *et al.*, 2015). However, the development and evaluation of a material consists of APC and CT for endodontic applications have not yet been explored.

1.3 Justification of the study

Various materials have been used for endodontic therapies such as calcium hydroxide ($Ca(OH)_2$) and MTA. However, $Ca(OH)_2$ exhibits some limitations such as poor quality of the formed dentinal bridge and lack of hermitic seal (Cox *et al.*, 1996). As for the MTA, the drawbacks are mainly due to the high cost, tooth discoloration, prolonged setting time and poor handling of the material (Parirokh and Torabinejad, 2010a; Tanalp *et al.*, 2012). Therefore, the endodontic therapies such as repair of perforations and resorption defects, vital pulp therapy and apexification might be considered controversial by the clinicians as that the usage of this material requires special training and the high cost restricts its routinely clinical use (Tanalp *et al.*, 2012). In addition, the prolonged setting time makes the handling rather difficult and increases the possibility of washout (Choi *et al.*, 2013) as well as the tooth colour change by MTA limits its application in aesthetic zone such as anterior teeth (Belobrov and Parashos, 2011).

A recent new knowledge about the cellular and molecular basis of the inflammatory and repair processes of the pulp (Sangwan *et al.*, 2013; Goldberg *et al.*, 2015; Paula *et al.*, 2020), and the advent of modern pharmacologic and bioengineering strategies such as drug delivery systems, have created many avenues for development of improved and predictable treatment methods for endodontic treatment. To the best of our knowledge, this is the first research that has been performed to synthesize and evaluate a material comprises of APC and CT for the endodontic application.

Since APC can solve one of the main drawbacks of PC which is long setting time by adding $CaCl_2$ (Torkittikul and Chaipanich, 2012), in addition to its cost-effective,

biocompatibility (Hoshyari *et al.*, 2016) and favourable physico-chemical and biological properties (Abdullah *et al.*, 2002; Ong *et al.*, 2012; Torkittikul and Chaipanich, 2012), it seems that APC can be used as a substitute to PC and MTA in dental applications. CT is biocompatible, biodegradable, polycationic, promotes tissues regeneration (Sultankulov *et al.*, 2019) and has potent antimicrobial activity against various microorganisms (DaSilva *et al.*, 2013) and anti-inflammatory efficacy (Fasolino *et al.*, 2019).

In light of the above-mentioned properties, a new bioactive material comprises of biocompatible APC together with the component of CT was synthesized in this study and evaluated as an endodontic material hypothesizing that the synthesized material will exhibit good physico-chemical properties and be able to regenerate the dentin and bone through its dentinogenic/osteogenic differentiation potential. The results of this study will provide insights into the use of chitosan-based accelerated portland cement (APC-CT) as an effective, low-cost and affordable endodontic material.

1.4 Research questions

1. What are the chemical properties of APC-CT material?
2. What are the physical and mechanical properties of APC-CT material?
3. Is APC-CT material biocompatible to SHED?
4. Does APC-CT material promote mineralization activity in SHED?
5. Does APC-CT material promote dentinogenic/osteogenic genes expression in SHED?

1.5 Research hypotheses

1. APC-CT material exhibits acceptable chemical properties.
2. APC-CT material exhibits acceptable physical and mechanical properties.
3. APC-CT material exhibits no cytotoxic effect on SHED.
4. APC-CT material promotes mineralization activity in SHED.
5. APC-CT material promotes dentinogenic/osteogenic genes expression in SHED.

1.6 Objectives

1.6.1 General objective

The general aim was to study the physico-chemical and mechanical properties of a chitosan-based accelerated portland cement (APC-CT) material and to evaluate its biocompatibility and dentinogenic/osteogenic differentiation potential on stem cells from human exfoliated deciduous teeth (SHED).

1.6.2 Specific objectives

1. To characterize the synthesized APC-CT material by FTIR, FESEM and EDX.
2. To evaluate the physical and mechanical properties of APC-CT material such as setting time, compressive strength, surface microhardness, pH and solubility.
3. To evaluate the biocompatibility of APC-CT material on SHED (cell viability, attachment and apoptosis).
4. To assess the effect of APC-CT material on mineralization activity of SHED.
5. To investigate the effect of APC-CT material on dentinogenic/osteogenic potential in SHED.

CHAPTER 2

LITERATURE REVIEW

2.1 An overview of tooth development

The same genetic and molecular mechanisms, which take place during tooth development, also occur during the reparative processes after carious or traumatic injuries (Mitsiadis and Rahiotis, 2004). Therefore, it is important to know the biological interactions during the dental development in order to have better understanding on the regenerative potential of dentin-pulp complex.

Embryologically, tooth development is formed by a series of interactions between the oral epithelium and the neural crest ectomesenchyme (Murphy *et al.*, 2019). Tooth formation is initiated by signals provided by the oral epithelium followed by the proliferation and projection of the epithelial cell into the underlying neural crest ectomesenchyme to form the dental lamina. The aggregation of these cells is known as a tooth bud (tooth germ), which gets pronounced to the cap shape and then to bell shape forming the enamel organ (EO). The EO is made up of the outer enamel epithelium (OEE), inner enamel epithelium (IEE), stellate reticulum (SR) and stratum intermedium (SI). The IEE cells differentiate into the pre-ameloblasts cells that become ameloblasts and lay down the future enamel (Chiba *et al.*, 2019).

The condensing mass of ectomesenchyme beneath this cap is called dental papilla (DP), which was differentiated by the influence of pre-ameloblasts into two types of cells; the odontoblasts which form the outer cell layer to secret the dentin and “non-neural crest” derived cells which form the central zone of cells to produce the primordium of the pulp (Linde and Goldberg, 1993; Chai *et al.*, 2000) (Figure 2.1).

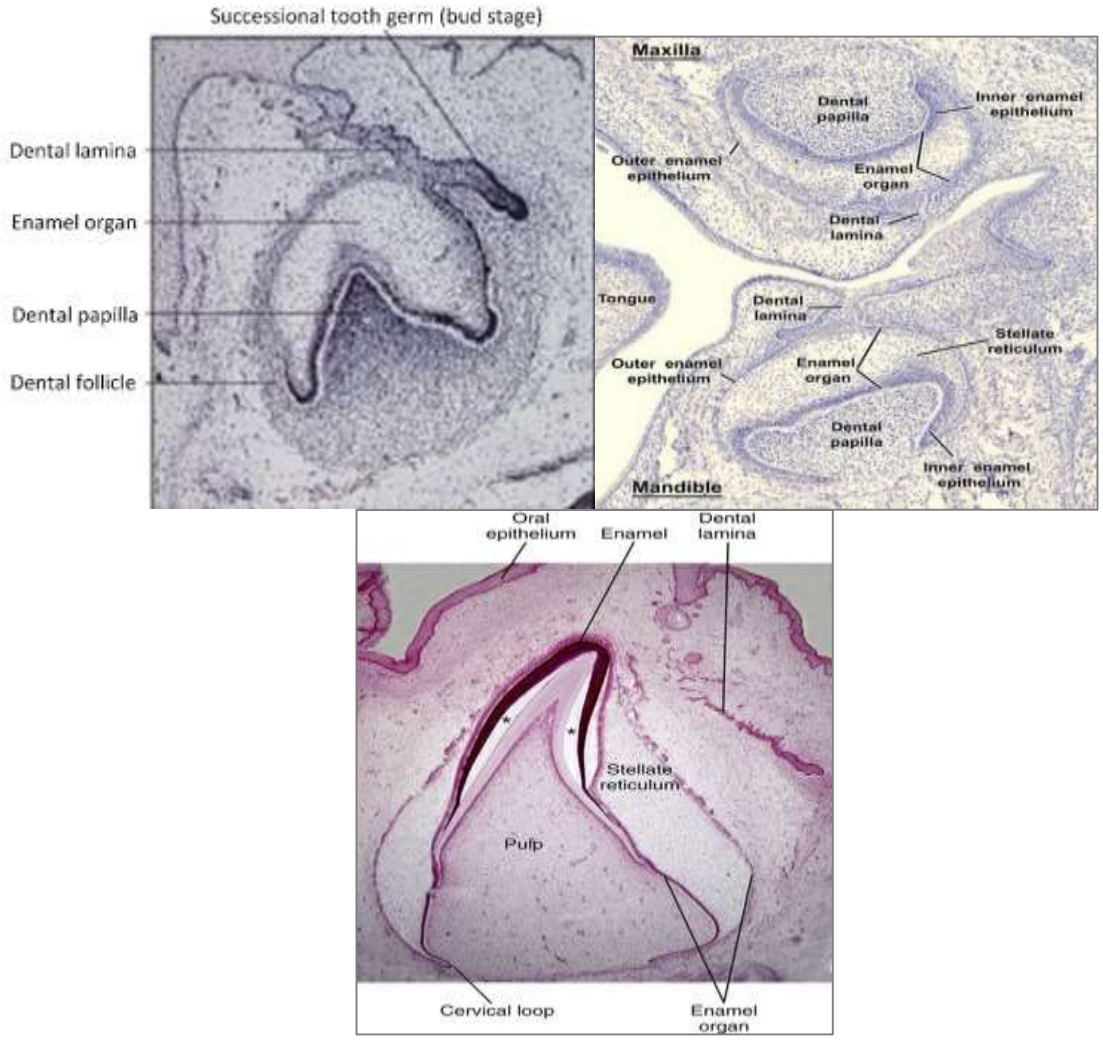

Figure 2.1: Modified images of tooth bud from Ten Cate's Oral Histology (Nanci, 2017). The tooth bud consists of enamel organ, dental papilla and dental follicle. Four cell types are yielded from the differentiation of the enamel organ: OEE, SR, SI and IEE. The OEE and IEE oppose each other forming the cervical loop and grow apically to form the epithelial root sheath of Hertwig's.

The remaining mesenchymal tissue surrounds the EO to form the dental follicle. The cervical loop is formed apically at the region where the OEE opposes the IEE and gives rise to two cell layers known as epithelial root sheath of Hertwig's, which starts the formation of the root and determines its shape. Then, a differentiation of the mesenchyme cells of the dental papilla into odontoblasts occurs to produce the root dentin and also a differentiation of the mesenchyme cells of the dental follicle into cementoblasts, fibroblast and osteoblast occurs to form cementum, periodontal ligaments and alveolar bone, respectively (Ohshima, 2008) (Figure 2.2). The membrane which separates the EO and the dental papilla becomes the site for the future dentino-enamel junction (DEJ) (Figure 2.3).

A number of signalling factors were involved in the process of embryonic tooth development such as initiation, proliferation, cytodifferentiation, distribution and morphogenesis of the cells. These factors consist of transforming growth factor (TGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) (Puthiyaveetil *et al.*, 2016). BMP family belongs to the TGFβ superfamily. BMP is the key factor in the differentiation of odontoblast and ameloblast as it is involved in communication and signalling between the epithelium and mesenchyme (Liu *et al.*, 2016).

2.2 Pulp-dentin complex

The dental pulp has a similar embryonic origin of dentin; both stay in close relationship for the whole life cycle maintaining the integrity of tooth function and shape. This dynamic relationship is considered to be "pulp-dentin complex" (Mauth *et al.*, 2007).

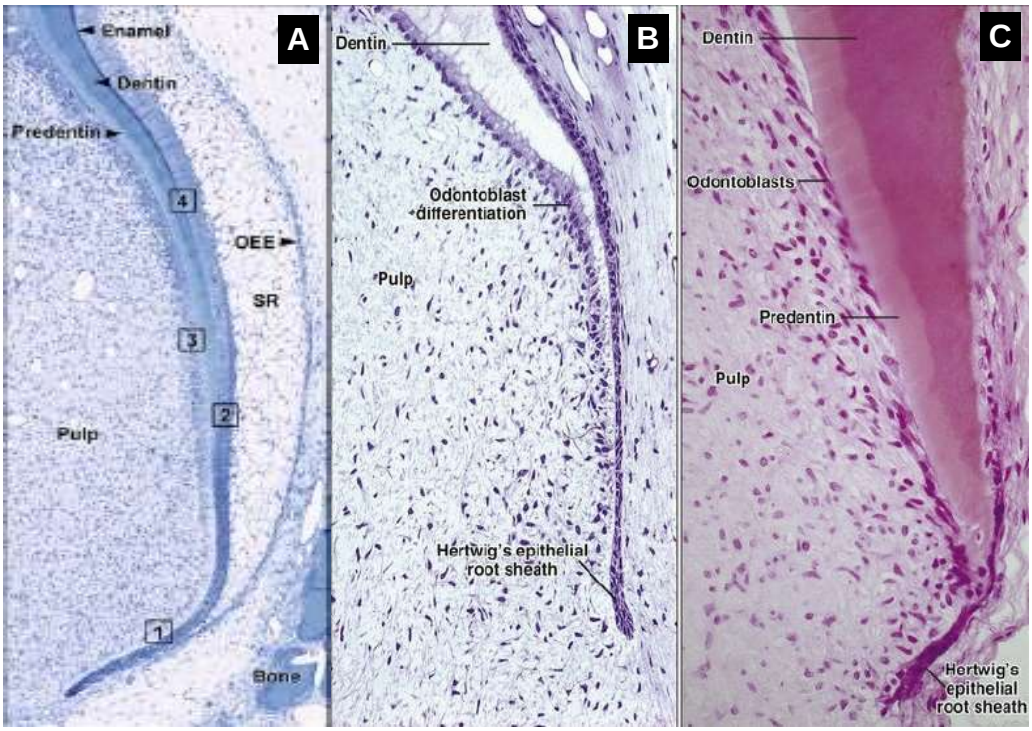

Figure 2.2: Modified images of tooth crown and root formation from Ten Cate's Oral Histology (Nanci, 2017). (A) Crown formation: At 1 Acellular zone separates the epithelium from the dental papilla. At 2 Elongation of cells at the inner enamel epithelium and elimination of the acellular zone due to the differentiation of the odontoblasts. At 3 Odontoblast movement toward the pulp leaving behind the produced dentin. At 4 Movement of ameloblasts outward leaving behind the produced enamel. (B, C) Root formation: Root formation occurs as a result of extension of the IEE and OEE in the cervical loop forming epithelial root sheath of Hertwig's, which induce the odontoblast differentiation from radicular pulp to form the root dentin.

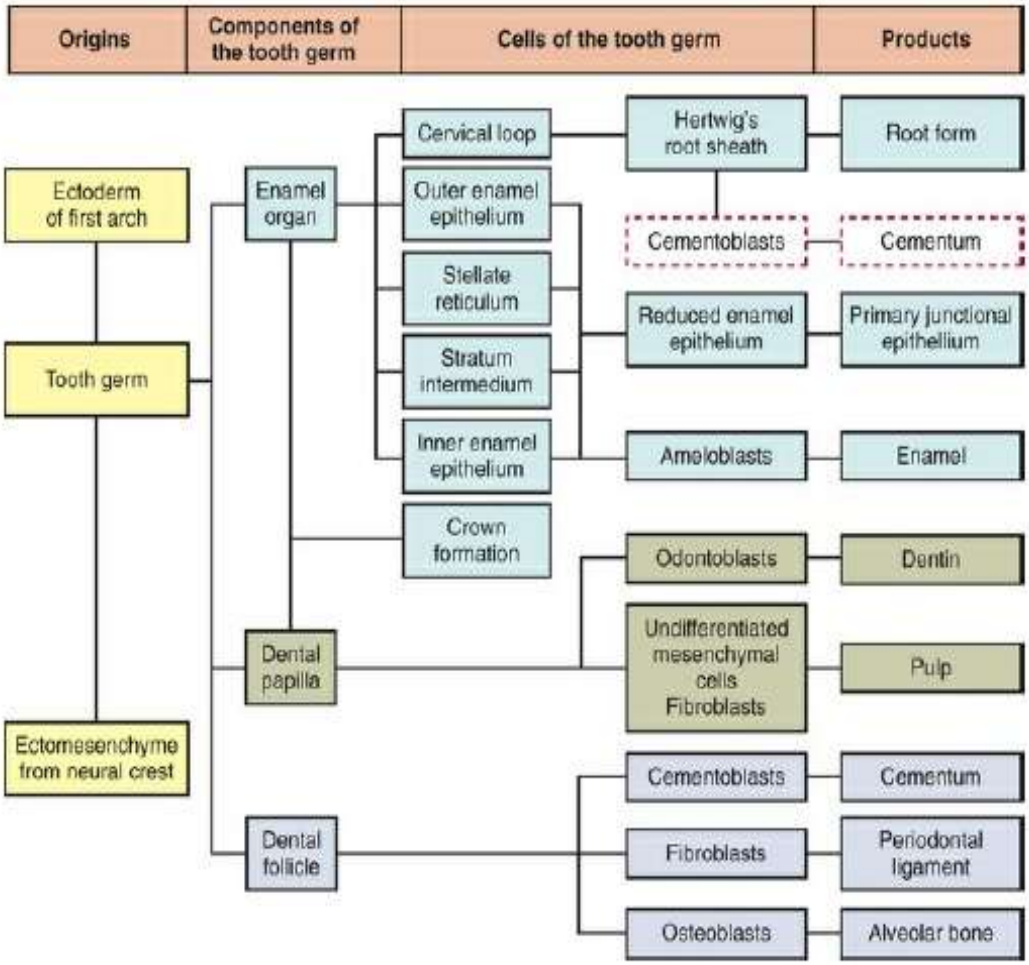

Figure 2.3: A summary of human tooth formation showing tooth germ derivatives and their secretory products (Adapted from Nanci (2017)).

2.3 Dental pulp

Dental pulp is the central portion of the tooth comprising of loose specialized connective tissue and encased by dentin. The pulp has ground substance, nerves, blood and lymph vessels and a number of cell types mainly fibroblasts (which form the fibers in the pulp) and others such as odontoblasts, blood cells, undifferentiated mesenchymal cells, schwann cells, endothelial cells, and inflammatory and immune reactions cells (Sharma *et al.*, 2010; Nanci, 2017).

The pulp consists of four different parts: (1) external layer which contains the odontoblast cells, (2) cell-free zone, a rich part of extracellular matrix and containing the nerves fibers terminal, fibroblasts cytoplasmic processes and capillary plexus, (3) cell-rich zone, made up of undifferentiated stem/progenitor cells and (4) inner layer which contains the collagen fibers, nervous and vascular plexus (D'Aquino *et al.*, 2008; Nanci, 2017) (Figure 2.4). The pulp is connected with the surrounding tissues via the apical foramen at the root apex. The foramen acts as a pathway for the blood vessels and lymph drainages supply into the pulp and vice versa (Nanci, 2017). The main function of the dental pulp is to form the dentin and maintain its vitality. The pulp supplies the dentin with the oxygen and nutrition and contains responsive sensory nervous system which detects unhealthy stimuli that is inflicted by microbial invasion, chemical irritation and mechanical trauma (Huang, 2009).

2.3.1 Odontoblasts

Odontoblasts are highly specialized and differentiated cells of about 50 μm in length. These cells originate from the neural crest and secrete the predentin and future dentin when mature (Nanci, 2017).

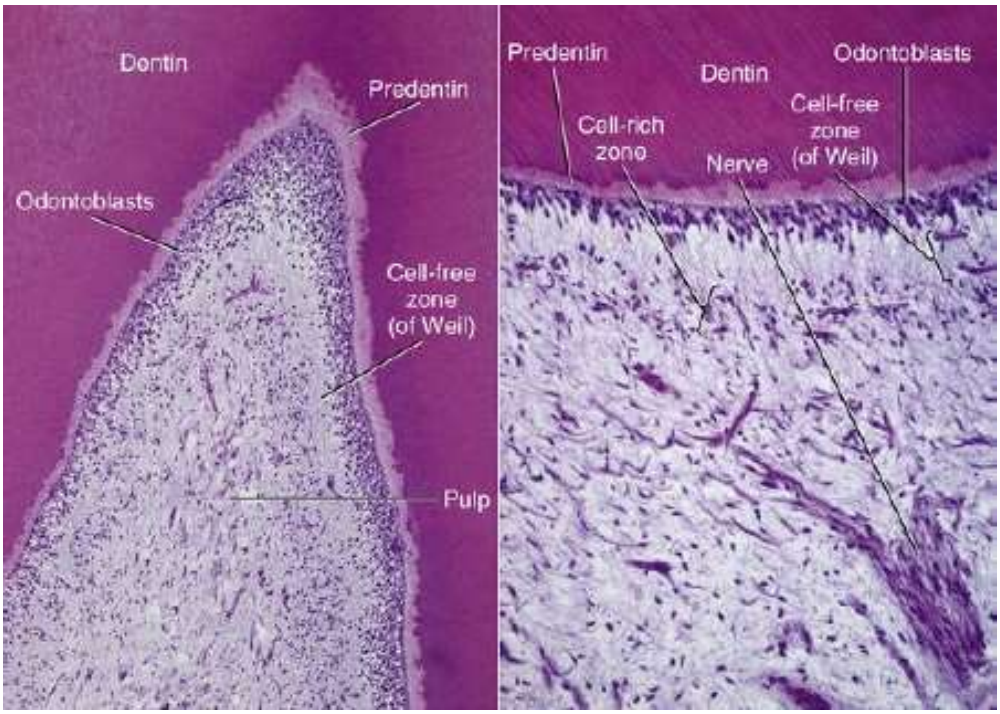

Figure 2.4: Modified photomicrograph of the pulp-dentin complex from Ten Cate's Oral Histology (Nanci, 2017). The image shows the odontoblast layer, cell-free zone, cell-rich zone and the inner layer containing the nerves.

Odontoblasts form a layer at the inner surface of dentin lining the periphery of the pulp called “pulpo-dentinal membrane”. Mature odontoblasts have long and polarized cell morphology consists of a large nucleus located at the basal portion toward the pulp, and also the presence of golgi apparatus, endoplasmic reticulum and numerous mitochondria. In addition, several nucleoli and dispersed chromatin are located in the nucleus (Nanci, 2017).

The odontoblast cell is characterized by two portions, the cell body and odontoblastic process. The cell body which involved in the synthesis of dentin extracellular matrix is located outside the predentin/dentin layer at the pulp periphery. Whereas, the odontoblstic process which involved in secretion of extracellular matrix molecules is located inside the dentin tubules crossing the predentin (MacDougall and Javed, 2010). (Figure 2.5). The collagen and proteoglycans molecules of the extracellular matrix are secreted in the predentin, while other biomineralization molecules of the extracellular matrix are secreted near the mineralization front (Goldberg *et al.*, 2011).

2.3.2 Odontoblast differentiation

The initiation of odontoblast differentiation takes place by the interaction of pre-odontoblast with inner dental epithelium and the effects of growth factors which present in close proximity to pre-odontoblasts in the basement membrane (Ruch *et al.*, 1995; Thesleff and Sahlberg, 1996). Growth factors are a complex of proteins in the extracellular matrix of the inner dental epithelium. These proteins have mitogenic properties and are responsible for the differentiation and polarization of the odontoblast (Kawashima and Okiji, 2016; Chang *et al.*, 2019). The growth factors in human dentin extracellular matrix are shown in Table 2.1.

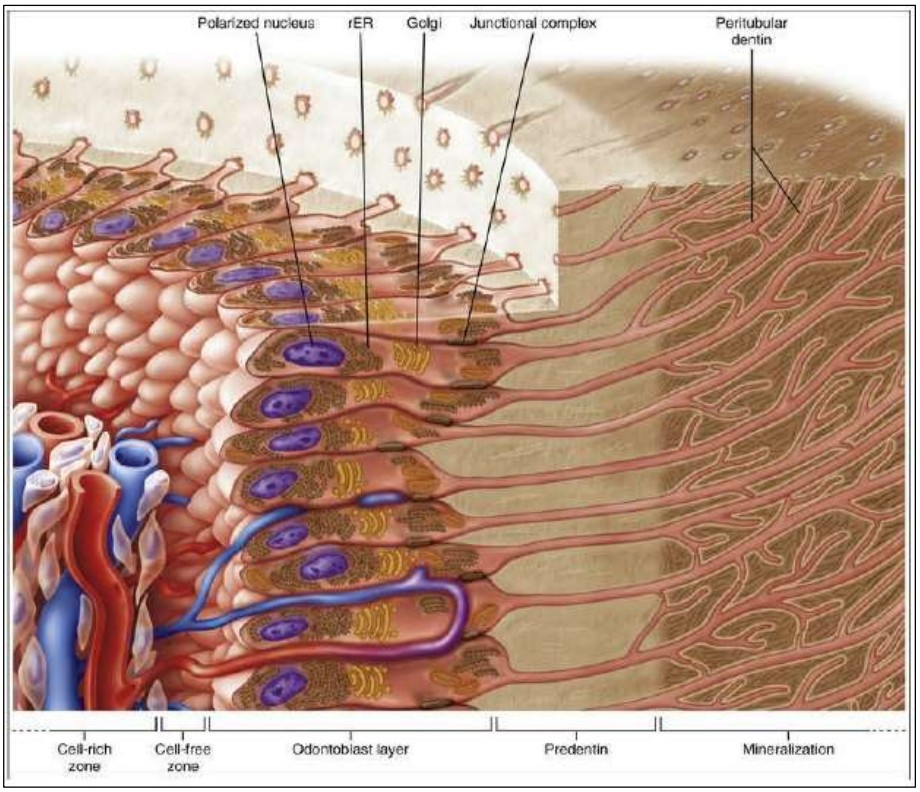

Figure 2.5: Odontoblast cells bordering the pulp (Adapted from Nanci (2017)).

The process of odontoblast differentiation occurs as; the mitotic spindles of pre-odontoblasts align in close to the basement membrane during the last round of cell division. Then, the daughter cells from the pre-odontoblasts lie in contact with the basement membrane and elongated becoming in a polarized fashion. Subsequently, a cell flattening occurs making them in parallel position to the long axis of the basement membrane. After that, the granular endoplasmic reticular system is developed, and the cells become ready to secrete the predentin and then dentin components (Ruch *et al.*, 1995; Nanci, 2017) (Figure 2.6).

The primary dental epithelium is thought to regulate the differentiation of these cells by controlling the cell cycles (Ruch *et al.*, 1995). It has been determined that pre-odontoblasts in animals must have a specific number of cell cycle to be able for differentiation (Holyfield *et al.*, 2005). Nevertheless, it is not of importance in human cells to respond to inductive signals to start differentiation (Begue-Kirn *et al.*, 1992).

2.4 Dentin

Dentin is a collagen-based mineralized connective tissue. It constitutes the main bulk of the tooth and is covered by the enamel in the crown portion and by cementum in the root portion. The dentin consists of inorganic apatite crystals embedded in the extracellular matrix (Goldberg *et al.*, 2011; Orsini *et al.*, 2012). Dentin is formed by a process called dentinogenesis which occurs in two steps: (1) the formation of collagenous network and (2) the precipitation of the inorganic mineral phase in the form of hydroxyapatite crystal (Goldberg *et al.*, 2011).

Table 2.1: Non-collagenous proteins (NCPs) in human dentin extracellular matrix. (Adapted from Orsini *et al.* (2012))

Proteoglycans (PGs)	*Small leucine-rich proteoglycan (SLRP) family* 1. Decorin 2. Biglycan 3. Fibromodulin 4. Lumican 5. Osteoadherin *Large aggregating PGs* Versican
Glycoproteins	*Vitamin K-dependent glycoproteins* Osteocalcin *Secretory calcium-binding phosphoprotein (SCPP) family* Osteonectin (SPARC) SIBLING proteins: a. Osteopontin b. Dentin matrix protein 1 c. Bone sialoprotein d. Dentin sialophosphoprotein e. Matrix extracellular phosphoglycoprotein
Serum proteins	1. Albumin 2. IgG 3. Transferin 4. Fetuin-A
Enzymes	*Metalloproteinases and cathepsins* Matrix metalloproteinases (MMPs) a. MMP-8 (collagenase-2) b. MMP-2 (gelatinase A) c. MMP-9 (gelatinase B) d. MMP-20 (enamelysine) Cysteine Cathepsins a. Cathepsin B
Growth factors	a. Insulin-like growth factor-I (IGF-I) b. Skeletal growth factor/Insulin-like growth factor II (SGF/IGF-II) c. Transforming growth factor-beta1 (TGF-β1) d. Platelet-derived growth factor (PDGF) e. Vascular endothelial growth factor (VEGF) f. Placental growth factor (PIGF) g. Fibroblast growth factor-2 (FGF-2) h. Epidermal growth factor (EGF) i. Adrenomedullin (AM)

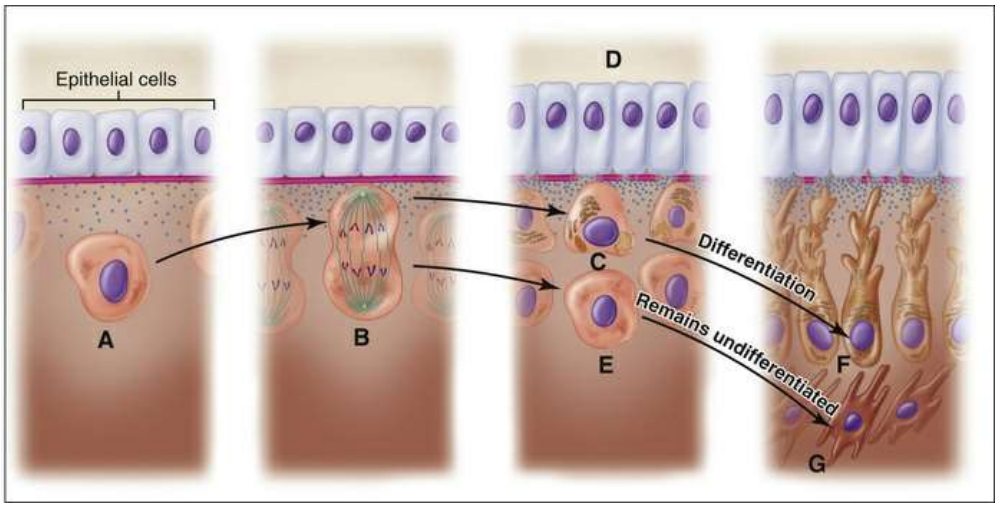

Figure 2.6: Differentiation of odontoblast. (A) Undifferentiated ectomesenchymal cell. (B) Mitotic spindle. (C, E) daughter cells. (D) epithelial cells. (F) Differentiated odontoblast. (G) Subodontoblast cells (Adapted from Nanci (2017)).

Tubular structures called dentinal tubules are crossing the dentin making it a permeable tissue, that spread from the dentino-enamel or dentino-cementum junction to the pulp. These tubules contain the odontoblast processes and dentinal fluid (Nanci, 2017).

2.5 Composition of Dentin

Seventy percent of dentin is composed of hydroxyapatite crystals, 20% of organic matrix and 10% of water (by weight) (Goldberg *et al.*, 2011). The inorganic minerals consist of inorganic phosphate and calcium which precipitate as mineral crystals to mineralize the organic matrix and form mature dentin (Luz and Mano, 2010). The organic components of dentin mainly consist of type I collagen and non-collagenous proteins (NCPs). These proteins play important roles in various structural formative, signalling and homeostatic processes (Smith *et al.*, 2012).

Collagen, a dominant fibrous protein, is found in all connective tissues including hard tissues such as dentin, cementum and bone. The odontoblast cells lay down an organic matrix of unmineralized collagen-rich layer called as predentin which mature to dentin after the deposition of minerals. The collagen of the dentin is mainly type I collagen which forms approximately of 90% of the organic material, although trace amounts of type III and type V collagen have been found. NCPs in the extracellular matrix constituting approximately 10% of matrix (Goldberg *et al.*, 2011; Orsini *et al.*, 2012).

NCPs are laid down from the distal part of odontoblast process. NCPs initiate and control the mineralization of extracellular matrix converting the predentin to dentin in "mineralization front" area during the dentinogenesis process (Butler, 1998). NCPs are

then bound to the hydroxyapatite crystals after the mineralization. NCPs in extracellular matrix of human dentin are summarized in Table 2.1. NCPs include:

2.5.1 Glycoproteins

The glycoprotein includes two groups of a small integrin-binding ligand N-linked glycoproteins (SIBLINGs) and a secretory calcium-binding phosphoprotein (SCPP) (Orsini *et al.*, 2012).

2.5.1(a) SIBLINGs family

SIBLINGs family is a multibiofunctional molecules that play important functions in regulating a promotion and inhibition of the mineralization process of dentin and bone. In mineralization process, SIBLINGs serve as nucleating factors due to their highly acidic nature through the precipitation of calcium ions and regulation of hydroxyapatite crystal formation (Toyosawa *et al.*, 2012).

All the SIBLINGs share the genomic structures which are located on the human chromosome 4q21-23 as well as share the common gene structure features (Toyosawa *et al.*, 2012). In addition, an Arg-Gly-Asp (RGD) integrin binding site which plays a role in adhesion and migration of cells is found in their protein (Suzuki *et al*., 2014). Major SIBLINGs in dentin tissue are dentine matrix protein-1 (*DMP-1*), dentine sialoprotein (*DSP*), dentine phosphoprotein (*DPP*), osteopontin (*OPN*), bone sialoprotein (*BSP*) and matrix extracellular phosphoglycoprotein (*MEPE*).

2.5.1(a)(i) Dentine matrix protein1 (*DMP-1*)

DMP-1 is considered as a specific protein for dentin in spite of its expression in bone (Huang *et al.*, 2008a). It was found in peritubular dentin, odontoblasts and around the mineralizing globules (Massa *et al.*, 2005). *DMP-1* helps in initiating the apatite nucleation and mineral deposition through nucleation of hydroxyapatite within the collagenous matrix as well as it induces odontoblast cells differentiation (Almushayt *et al.*, 2006; Narayanan *et al.*, 2006).

2.5.1(a)(ii) Dentin sialophosphoprotein (*DSPP*)

DSPP is cleaved into three proteins after secretion; dentin sialoprotein (*DSP*), dentin glycoprotein (*DGP*) and dentin phosphoprotein (*DPP*) (Fujisawa and Tamura, 2012). *DSPP* was expressed in odontoblasts and considered as a specific protein of odontoblast. *DSPP* was also expressed in bone but with lower level of approximately 1:400 as that in dentin (Qin *et al.*, 2003). *DSP* and *DPP* proteins play a role in the regulation of hydroxyapatite nucleation onto matrix collagen and hydroxyapatite crystals growth (Suzuki *et al.*, 2009) and they were identified as components of extracellular matrix of dentin (Yamakoshi *et al.*, 2003).

DPP, the most abundant NCPs in dentin extracellular matrix, is a cleavage product from C-terminal side of *DSPP* that contains extended triplet amino acid repeat sequences. It is important in biomineralization through binding to calcium and presenting it to collagen fibers to initiate and modulate the hydroxyapatite formation of dentin (Prasad *et al.*, 2010; Fujisawa and Tamura, 2012).