# THE EFFECT OF BIPHASIC CALCIUM PHOSPHATE AND SIMVASTATIN ON HUMAN DENTAL PULP CELLS IN VITRO ON DENTIN REGENERATION

**By**

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| | |
|---|---|
| ADM | Adrenomedullin |
| ASTM | Archimedes standard test method |
| α-TCP | Alpha-tricalcium phosphate |
| ATP | Adenosine triphosphate |
| ALP | Alkaline phosphatase |
| Anti-Anti | Antibiotic-antimycotic |
| A260/A280 | Ratio of 260 absorbance over 280 absorbance |
| BCP | Biphasic calcium phosphate |
| BMD | Bone mineral density |
| BMP-2 | Bone morphogenic protein-2 |
| bp | Base pair |
| BSP | Bone sialoprotein |
| β-TCP | beta-tricalcium phosphate |
| Ca/P | Calcium/Phosphate ratio |
| Cbf-α1 | Core-binding factor subunit alpha-1 |
| CDA | Calcium deficient apatite |
| CD | Cluster of Differentiation |
| cDNA | Complementary deoxyribonucleic acid |
| COL1A1 | Collagen type I alpha 1 |
| DEPC | Diethyl pyrocarbonate |
| DF | Dental follicle |
| DFPCs | Dental follicle precursor cells |
| DGP | Dentin glycoprotein |
| DME | Dentin matrix extract |
| DMP-1 | Dentin matrix protein-1 |

| | |
|---|---|
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxy nucleoside triphosphate |
| DPGs | Dentin proteoglycans |
| DPP | Dentin phosphoprotein |
| DPSCs | Dental pulp stem cells |
| DSP | Dentin sialoprotein |
| DSPP | Dentin sialophosphoprotein |
| DTA | Differential thermal analysis |
| EGF | Epidermal growth factor |
| ELISA | Enzyme-linked immunosorbent assay |
| FBS | Fetal bovine serum |
| FESEM | Field emission scanning electron microscope |
| FGF | Fibroblast growth factor |
| FGF-2 | Fibroblast growth factor-2 |
| FTIR | Fourier transform infra-red spectroscopy |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| Gdf11 | Growth differentiation factor-11 |
| gDNA | Genomic deoxyribonucleic acid |
| HA | Hydroxyapatite |
| HA/β-TCP | Hydroxyapatite/Biphasic calcium phosphate ratio |
| HDPCs | Human dental pulp cells |
| Hhs | Hedgehog proteins |
| HMG-CoA | 3-hydroxy-3-methylglutaryl coenzyme A |
| hTERT | Human telomerase transcriptase |
| ICDD | International centre for diffraction data |
| IEE | Inner enamel epithelium |

| | |
|---|---|
| Ig | Immunoglobulin |
| IGF-I | Insulin-like growth factor-I |
| kV | Kilovolt |
| LDL | Low density lipoproteins |
| M | Molarity |
| mA | Milliampere |
| μCT | Micro-computed Tomography scans |
| MEM | Minimum essential medium |
| MEPE | Matrix extracellular phosphoglycoprotein |
| MPa | Megapascal |
| m.w. | Molecular weight |
| MMPs | Matrix metalloproteinases |
| mRNA | Messenger ribonucleic acid |
| MSCs | Mesenchymal stem cells |
| MTA | Mineral trioxide aggregate |
| MTT | 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide |
| NCPs | Non-collagenous proteins |
| ng | Nanogram |
| nm | Nanometer |
| nmol | Nanomlar |
| OCN | Osteocalcin |
| O.D | Optical density |
| Oct-4 | Octamer-binding transcription factor-4 |
| OEE | Outer enamel epithelium |
| ONC | Osteonectin |
| OPG | Osteoprotegerin |
| OPN | Osteopontin |

| | |
|---|---|
| PBS | Phosphate-buffered saline |
| PDF | Powder diffraction file |
| PDGF | Platelet-derived growth factor |
| PDL | Periodontal ligament |
| PDLs | Population doublings |
| PDLSCs | Periodontal ligament stem cells |
| PE | Polyethylene |
| PGA | Polyglycolic acid |
| pg | Picogram |
| pH | Potential of hydrogen |
| PLA | Polylactic acid |
| PLG | Polylactide-co-glycolide |
| PLGA | Polylactic-co-glycolic acid |
| PlGF | Placenta-derived growth factor |
| *p*NPP | *p*-nitrophenylphosphate |
| *p*NP | *p*-nitrophenol |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| RANKL | Receptor activator of nuclear factor kappa-B ligand |
| rpm | Round per minute |
| RT-PCR | Reverse transcription polymerase chain reaction |
| RUNX-2 | Runt-related transcription factor-2 |
| SCAPs | Stem cells of apical papilla |
| SCPP | Secretory calcium-binding phosphoprotein |
| SGF/IGF-II | Skeletal growth factor/insulin-like growth factor-II |
| SHED | Stem cells from human exfoliated deciduous teeth |
| SIBLING | Small integrin-binding ligand, N-linked glycoprotein |

| | |
|---|---|
| SLRPs | Small leucine-rich proteoglycans |
| SLS | Selective laser sintering |
| SPARC | Secreted Protein Acidic and Rich in Cysteine |
| SRμCT | Synchrotron X-ray micro Computer Tomography |
| STRO-1 | Stromal cell surface marker-1 |
| SSEA | Stage-specific embryonic antigens |
| TGA | Thermogravimetry analysis |
| TGF-β | Transforming growth factor-beta |
| TGF- β1 | Transforming growth factor-beta1 |
| TNF | Tumor necrotic factor |
| TRA | Tumor recognition antigens |
| UV | Ultraviolet |
| VEGF | Vascular endothelial growth factor |
| WHO | World Health Organization |
| Wnts | Wingless- and int-related proteins |
| wt | Weight |
| XRD | X-ray diffraction |
| XRF | X-ray fluorescence |
| $2\theta$ | 2-Theta |

**KESAN KALSIUM FOSFAT DWIFASA DAN SIMVASTATIN TERHADAP SEL PULPA GIGI MANUSIA DALAM VITRO SEMASA REGENERASI DENTIN**

## ABSTRAK

Kajian ini adalah untuk menghasilkan perancah kalsium fosfat dwifasa (BCP) dengan kombinasi simvastatin pada kepekatan optimum untuk mendorong pembezaan sel-sel pulpa gigi manusia dan regenerasi tisu dentin. BCP pada nisbah 20/80 hidrosiapatit (HA) dan β-trikalsium fosfat (β-TCP) dengan liang mikro < 10 μm, liang makro 300 μm, dan keliangan 65% telah berjaya disintesiskan secara pemendapan basah dangan menggunakan mikrosfera polietilena sebagai pembentuk-liang. BCP sampel telah dicirikan menggunakan pembelauan sinar-x (XRD), spektroskopi jelmaan Fourier infra-merah (FTIR), pendarfluor sinar-x (XRF), mikroskop imbasan elektron (FESEM), pengecutan linear, dan kaedah Archimedes. Sampel BCP telah digabungkan dengan empat kepekatan simvastatin (2.0, 1.5, 1.0 dan 0.5 μM) untuk penilaian kebolehhidupan sel dengan menggunakan cerakin MTT (3-(4,5-dimetil-thiazonil)-2,5-diphenil-tetrazolium bromida). Aktiviti fosfatase alkali telah diuji dengan menggunakan cerakin fosfatase alkali. Kumpulan yang menunjukkan kebolehhidupan sel terbaik dan aktiviti fosfatase alkali telah dipilih. Kumpulan tersebut diuji bagi potensi pembezaan odontogenik jenis kolagen I alfa 1 (COL1A1), sialoprotein tulang (BSP), matriks dentin protein-1 (DMP-1), sialofosfoprotein dentin (DSPP), dan faktor-2 transkripsi Runt berkaitan (RUNX-2) analisis gen ekspresi menggunakan reaksi rantai transkripsi-polimerase berbalik (RT-PCR) dan pengesanan pemineralan matriks luar sel dengan menggunakan penstainan alizarin Merah S.

Hasil kajian menunjukkan bahawa kumpulan kombinasi BCP + 1.5 μM, BCP + 1.0 μM, dan BCP + 0.5 μM mempunyai min indeks kebolehhidupan sel tertinggi, kecuali BCP+ 2.0 μM adalah kesitotoksikan. Bagi aktiviti fosfatase alkali, kombinasi BCP + 1.5 μM, BCP + 1.0 μM, dan BCP + 0.5 μM menunjukkan min indeks aktiviti fosfatase yang tinggi dengan BCP + 1.5 μM adalah tertinggi. Bagi potensi pembezaan odontogenik, gabungan BCP + 1.5 μM menunjukkan regulasi-menaik gen COL1A1, DMP-1, BSP, DSPP dan regulasi-menurun gen RUNX-2. Bagi pemineralan matriks sel luar, gabungan BCP + 1.5 μM menunjukkan aktiviti tertinggi untuk mengaruh pemendapan mineral. Sebagai kesimpulan, gabungan BCP dan 1.5 μM simvastatin adalah induksi terpilih untuk pembezaan sel pulpa gigi manusia terhadap regenerasi tisu dentin.

**THE EFFECT OF BIPHASIC CALCIUM PHOSPHATE AND SIMVASTATIN ON HUMAN DENTAL PULP CELLS IN VITRO ON DENTIN REGENERATION**

## ABSTRACT

This study was conducted to prepare an appropriate biphasic calcium phosphate (BCP) scaffold in combination with optimal concentration of simvastatin to induce human dental pulp cells differentiation and dentin tissue regeneration. BCP scaffold of 20/80 hydroxyapatite (HA) to β-tricalcium phosphate (β-TCP) ratio with micropores < 10 μm, macropores of 300 μm, and porosity of 65 % was successfully synthesized using wet precipitation method and polyethylene microspheres as pore-creating agents. The scaffold was characterized using x-ray diffraction (XRD), fourier transform infra-red spectroscopy (FTIR), x-ray fluorescence (XRF), field emission scanning electron microscope (FESEM), linear shrinkage, and total porosity measurements. BCP sample extract was prepared and combined with four different concentrations of simvastatin (2.0, 1.5, 1.0 and 0.5 μM) for the assessment of cell viability using MTT (3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide) assay. The alkaline phosphatase activity was assessed using alkaline phosphatase assay. The groups which showed the best cell viability and alkaline phosphatase activity were selected. They were assessed for both odontogenic differentiation potential of collagen type I alpha 1 (COL1A1), bone sialoprotein (BSP), dentin matrix protein-1 (DMP-1), dentin sialophosphoprotein (DSPP), and Runt-related transcription factor-2 (RUNX-2) genes expression analysis using reverse transcription-polymerase chain reaction (RT-PCR) and extracellular matrix mineralization detection using Alizarin Red S staining. The results showed that combination groups of BCP +1.5 μM, BCP + 1.0 μM, and BCP + 0.5 μM had higher

mean cell viability index, except BCP + 2.0 μM that showed cytotoxicity. For alkaline phosphatase activity, the combination groups of BCP + 1.5 μM, BCP +1.0 μM, and BCP + 0.5 μM showed higher mean alkaline phosphatase activity index with BCP + 1.5 μM the highest. For the odontogenic differentiation potential, BCP + 1.5 μM combination group showed up-regulation of COL1A1, DMP-1, BSP, and DSPP genes and down-regulation of RUNX-2 gene. For the extracellular matrix mineralization, BCP + 1.5 μM combination group showed the highest ability to induce the mineral deposition. In conclusion, the combination of BCP and 1.5 μM simvastatin achieved together a preferable induction of human dental pulp cells differentiation toward dentin tissue regeneration.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background of the Study

The tooth pulp is a unique tissue surrounded by a protective layer of dentin. The dentin and the pulp are related to each other embryologically, histologically, and functionally and are referred together as the pulp-dentin complex. The main functions of the pulp include formation of dentin and provide it with oxygen, nutrition, and innervations (Mauth *et al.*, 2007). Pulp tissue is vulnerable to infection by external stimuli that can result in irreversible pulpal damage leading to loss of vitality and function in mature and immature tooth. Pulpless tooth becomes brittle and susceptible to cracks and fracture, and has no sensation to irritations making caries progression unnoticed by patients. In addition, the loss of pulp vitality is associated with arrested development of the root in immature tooth that will impact the long-term prognosis for tooth retention (Witherspoon, 2008).

Vital pulp therapy is performed to preserve pulp vitality, functions, and ultimately, its position in the arch. It includes two well-known procedures which are pulp capping and pulpotomy. They are indicated for teeth that have a pulp exposure after trauma or caries in permanent immature or mature teeth. These procedures offer a more conservative approach and good alternatives to root canal therapy (Dean *et al.*, 2010). Appropriate pulp capping material should be used for vital pulp therapy to promote a protective hard tissue formation over the pulp exposure site. For many years, calcium hydroxide has been considered as the gold standard material for pulp therapy; however, many researchers have shown that it is not the ideal material for pulp therapy

as it has many disadvantages (Dammaschke *et al.*, 2010; Dean *et al.*, 2010; Kiba *et al.*, 2010; Willershausen *et al.*, 2011; Sangwan *et al.*, 2013).

Another pulp capping material recently developed is mineral trioxide aggregate (MTA) that has drawn much interest due to its beneficial effects. MTA has demonstrated significantly thicker, greater frequency of dentin bridge formation, and less porous dentin compared with calcium hydroxide. Researches have shown that there was no layer of necrosis and there was less pulp inflammation when MTA was used for pulp capping. However, many studies have demonstrated many drawbacks making its application limited (Bogen *et al.*, 2008; Boutsioukis *et al.*, 2008; Ling *et al.*, 2008; Mooney and North, 2008; Parirokh and Torabinejad, 2010).

To date, no synthetic materials can replace the functions and physiological normalcy of healthy tissue or can present the chemical, biological, physical characteristics and behaviors similar to the natural dental parts (Rosa *et al.*, 2012). Vital pulp therapy can preserve pulp vitality and function but the lost tooth structure replaced by artificial materials does not strengthen the tooth. Regeneration of lost dental tissues can reverse the deteriorated tooth and reduce the risk of its fracture (Huang, 2011).

In the early 1990s, the concept of “tissue engineering” was introduced by Langer and Vacanti to describe the technique for biological tissue regeneration. Tissue engineering can be defined as a multi-disciplinary science that brings together biology, engineering and clinical sciences with developing new functional healthy tissues and organs based on fundamental principles that involve the identification of appropriate cells, the development of conducive scaffolds and an understanding of the morphogenic signals required to induce cells to regenerate the tissues that were lost (Nor, 2006).

For dentin tissue regeneration, dental pulp tissue contains a unique type of cells known as dental pulp stem cells (DPSCs) that are unspecialized cells and have the ability of self renewal and multilineage differentiation potential in response to the appropriate signals (Egusa *et al.*, 2012). These signals can be either protein or gene therapy. In protein therapy, bone morphogenic protein-2 (BMP-2) is considered as the most important growth factor that has the ability to induce the differentiation of DPSCs into odontoblasts. However, the delivery of a single bolus of costly growth factor that has short half-life is not efficient to provide the required level of proteins to target cells to form hard tissue (Edwards and Mason, 2006b). Gene therapy is an alternative approach to overcome the limitations of protein therapy that involves delivering of a specific growth factor gene to the target cells that replicated together with the host genes, and provide prolonged expression of the desired protein. The main challenge for gene therapy is that it does not provide a safe long-term treatment as it leads to significant pulp necrosis in some conditions (Fischer *et al.*, 2011).

Simvastatin that is widely used as a cholesterol lowering drug specifically inhibits the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme in mevalonate pathway and induces many growth factors. Besides the induction of the BMP-2 production, it induces transforming growth factor-beta1 (TGF-β1), vascular endothelial growth factor (VEGF), alkaline phosphatase enzyme, collagene-1, bone sialoprotein, osteocalcin, osteopontin and other factors expression in osteoblasts (Chen *et al.,* 2010; Nyan *et al.,* 2010; Zhou *et al.,* 2010; Zhang *et al.,* 2011; Park, 2012; Tian *et al.,* 2013) and odontoblasts (Lee *et al.,* 2012; Karanxha *et al.,* 2013; Varalakshmi *et al.,* 2013). The distinct advantages of simvastatin for local stimulation of hard tissue formation are its efficient delivery to target cells, eliciting no antibody responses in

addition to its low manufacturing cost compared to other growth factors such as BMPs (Nyan *et al.*, 2009).

Scaffolds play a critical role in tissue engineering as temporary frameworks used to provide a three-dimensional microenvironment where cells can proliferate, differentiate and generate the desired tissue. They transport the nutrients, oxygen and waste products and serve as a carrier for morphogens and delivery vehicle to the target cells (Zhang *et al.*, 2013). Amongst many types of materials that have been used for the fabrication of scaffolds, BCP of intimate mixture of more stable phase HA and more soluble phase β-TCP is the most appropriate material for hard tissue regeneration as it resembles the inorganic part of the bone and dentin. BCP achieves balance and gradual dissolution to release both calcium and phosphate ions in the cells microenvironment. Porous BCP scaffolds are considered as a conductive and inductive material for hard tissue regeneration (Kress *et al.*, 2012).

## 1.2 Problem Statement

Vital pulp therapy can preserve pulp vitality and function but without dentin regeneration. Dentin regeneration is considered as a difficult task. In spite of the availability of accessible source of stem cells in the pulp, these cells have the ability of osteogenic, adipogenic, neurogenic, chondrogenic, fibrogenic, myogenic in addition to dentinogenic differentiation potential. The ability to stimulate the dental pulp stem cells to differentiate into odontoblasts is critical in dentin regeneration. The dental pulp stem cells differentiation to odontoblast is mainly determined by the components of local microenvironment including the signals and the scaffold. Signaling molecules that includes both protein and gene therapies have many drawbacks that can greatly affect the efficiency of DPSCs differentiation into odontoblasts and subsequently

dentin formation. Simvastatin is a low-priced pharmacological compound that can up-regulate more than one growth factor to stimulate cells differentiation. The successful use of simvastatin to promote tissue regeneration depends on its local concentration.

The property of the scaffold's chemical composition and structure is one of the major factors contributing to DPSCs morphology, proliferation, differentiation, and dentin tissue formation. In order to apply appropriate BCP for the induction of DPSCs differentiation to odontoblast, it is crucial to control the BCP chemical composition with specific HA/β-TCP ratios. Furthermore, the scaffolds' structure with controlled porosity and pore size need to be designed and optimized to ensure its functionality. Currently, several synthetic routes have been utilized for the preparation of BCP powders and for the fabrication of porous structure scaffolds. The construction of the accurate scaffold with desired characteristics is intricate task and extremely challenging.

To the best of our knowledge, there is a dearth of information on the synthesis of BCP scaffold appropriate for DPSCs differentiation for dentin tissue regeneration. In addition, there has been no study conducted to determine the optimal concentration of simvastatin to be used in combination with BCP scaffold or elucidate their effects together on the DPSCs proliferation, differentiation, and dentin tissue regeneration.

### 1.3 Justification of the Study

The preparation of an appropriate extracellular microenvironment that can direct the DPSCs to differentiate into odontoblasts rather than other types of cell line is highly essential for dentin tissue regeneration. In this study, the local fabrication of highly pure BCP scaffold with precise HA/β-TCP ratio and convenient porous structure that could meet the biological needs of DPSCs differentiation to odontoblasts by using simple, reproducible, economic, and accurate method would be of great importance in the field of biomaterial synthesis. The strategy to replace the growth factor by simvastatin and the application of optimal concentration of simvastatin in combination with appropriate BCP scaffold will achieve easy, safe, efficient, cost-effective and more valuable approach for DPSCs differentiation and dentin tissue regeneration.

## 1.4 Objectives

### 1.4.1 General Objective

The general aim of this study is to prepare an appropriate BCP scaffold and determine the optimal concentration of simvastatin that can be used in combination with BCP scaffold to induce human dental pulp cells differentiation for dentin tissue regeneration.

### 1.4.2 Specific Objectives

1. To synthesize BCP scaffold of 20/80 HA to β-TCP ratio with micropores < 10 μm, macropores of 300 μm, and porosity of 65%.
2. To characterize the synthesized BCP scaffold by XRD, FTIR, XRF, FESEM, linear shrinkage, and total porosity measurements.
3. To compare the *in vitro* cell viability of human dental pulp cells exposed to BCP scaffold extract in combination with different concentrations of simvastatin using MTT assay.
4. To compare the *in vitro* alkaline phosphatase activity of human dental pulp cells exposed to BCP scaffold extract in combination with different concentrations of simvastatin using alkaline phosphatase activity assay.
5. To detect the *in vitro* odontogenic differentiation potential of COL1A1, DMP-1, BSP, DSPP, and RUNX-2 genes of human dental pulp cells exposed to BCP scaffold extract in combination with simvastatin using RT-PCR.
6. To detect the *in vitro* extracellular matrix mineralization of human dental pulp cells exposed to BCP scaffold extract in combination with simvastatin using Alizarin Red S staining assay.

## 1.5 Research Hypothesis

There is no difference among different concentrations of simvastatin used in combination with BCP scaffold extract in the induction of human dental pulp cells differentiation for dentin tissue regeneration.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Tooth Development and Structures

Tooth development is a process characterized by a series of complex reciprocal inductive and sequential interactions between oral epithelium and the underlying neural crest ectomesenchyme (Sharma *et al.*, 2010).

Oral epithelium provides signals for initiation of the tooth development after which the epithelial cells proliferate and project into the underlying neural crest ectomesenchyme forming the dental lamina. Initially, this projection is in a bud shape, gets pronounced into a cap, then bell shape forming the enamel organ that consists of two layers: outer layer of cells called the outer enamel epithelium (OEE) and inner layer of cells called the inner enamel epithelium (IEE) that differentiates into specialized cells called ameloblasts which lay down the enamel. The ectomesenchyme beneath the enamel organ condenses to form the dental papilla whose outer most layer subjacent to the IEE differentiates into specialized cells called odontoblasts which lay down dentin and the existing dental papilla matures into dental pulp (Nanci, 2007).

The inner and outer enamel epithelium grows apically to form two cell layers called Hertwig's epithelial root sheath which starts root formation and also determines the shape of the roots. Hertwig's epithelial root sheath signals adjacent mesenchyme of the dental papilla to differentiate into odontoblasts to lay down root dentin and signals mesenchyme of the dental follicle which surrounds the developing tooth germ to differentiate into cementoblasts to form cementum, fibroblasts to form periodontal ligaments and osteoblasts to form alveolar bone (Ohshima, 2008) (Figure 2.1).

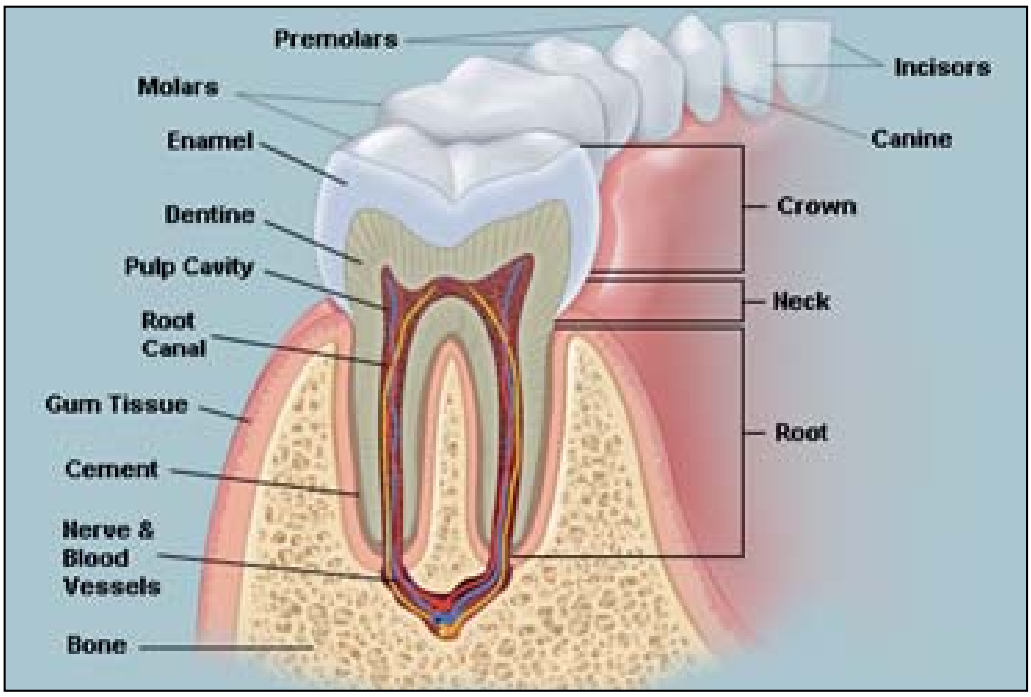


**Figure 2.1:** Tooth structure (http://www.webmd.com/oral-health/picture-of-the-teeth).

The molecular events during this developmental process such as initiation, proliferation, morphogenesis, cytodifferentiation and distribution of cells are principally controlled and mediated by the presence of growth/morphogens factors such as epidermal growth factor (EGF), transforming growth factor-beta (TGF-β) superfamily including several members of the bone morphogenetic protein family (BMP-2 to BMP-7), fibroblast growth factor (FGF), wingless- and int-related proteins (Wnts), hedgehog proteins (Hhs), and tumor necrotic factor (TNF) families (Mitsiadis and Graf, 2009).

Amongst these growth factors, BMP family seems to be the key factor in controlling and regulating tooth development. BMP signals are involved in communication and signaling between epithelium and mesenchyme during odontoblast and ameloblast differentiation (Nanci, 2007).

## 2.2 Pulp-Dentin Complex

In the process of tooth development, pulp and dentin have the same embryonic origin and stay in a close relationship for the whole life cycle of the vital tooth maintaining the integrity of tooth shape and function. Any physiological or pathological reactions occurring at one part will affect the other. This dynamic relationship is considered to be “pulp-dentin complex” that makes it difficult to separate their functional behaviors (Mauth *et al.*, 2007).

### 2.2.1 Dental Pulp

Dental pulp is a loose specialized connective tissue that is located in the central portion of the tooth and enclosed on its outer surface by dentin. It contains a variety of cell types, fibers, ground substance, blood and lymph vessels, and nerves. The basic cells of the pulp are fibroblasts which lay down fibers of the pulp. Other cells present in the pulp are odontoblasts, undifferentiated mesenchymal cells, blood cells, endothelial cells, schwann cells and cells of inflammation and immune reactions when present (Sharma *et al.*, 2010).

The pulp is divided into four layers, from the outer to the inner part: (1) the external layer made up of odontoblast producing dentin subadjacent to the predentin (2) The second layer, called “cell free zone”, poor in cells and rich in extracellular matrix contains capillary plexus, terminal branches of nerve fibers, and cytoplasmic processes of fibroblasts (3) the third layer, called “cell rich zone” contains undifferentiated stem/progenitor cells that display plasticity and pluripotential capabilities and serves as a reservoir for the replacement of destroyed odontoblasts and fibroblasts and (4) the inner layer comprises the collagen fibers, vascular and nervous plexus (Aquino *et al.*, 2008; Tziafas, 2010).

Anatomically, the dental pulp is almost fully encapsulated by hard dentin and the only connection between the dental pulp and the surrounding tissue is through the tiny root apexes. All of the main blood vessels and lymph drainages of dental pulp pass through the tooth root apexes, which make the apex the main pathway for tooth nutrition and waste exchange (Mauth *et al.*, 2007).

The primary function of the pulp is to produce dentin by odontoblasts, and maintain the biological and physiological vitality of dentin. The highly vascularized tissue of the pulp provides oxygen and nutrition to dentin, and the abundant myelinated and unmyelinated responsive sensory nerves function as a bio-sensor to detect unhealthy stimuli caused by mechanical trauma, chemical irritation or microbial invasion, and generate unbearable pain (Huang, 2009).

Odontoblasts that are associated with the formation and maintenance of dentin are highly specialized and fully differentiated tall columnar cells of about 50 to 60 μm in length. These cells form a layer of cells lining the periphery of the pulp at the inner surface of dentin and perpendicular to the basement membrane called “pulpo-dentinal membrane”. This zone of attachment maintains the cells phenotype, prevents the entrapment of odontoblasts in the predentin matrix and ensures that the developing surface of dentin remains relatively flat and in one direction (Nanci, 2007). These cells are characterized by a unique polarized morphology with highly polarized distribution of cytoplasmic organelles in which a large nucleus is located in the basal portion of the cell, close to the pulp, while the well-defined Golgi apparatus, rough endoplastic reticulum, and several mitochondria are located in a dentinal direction from the nucleus (Tjaderhane and Haapasalo, 2009).

The odontoblast cell is divided into two parts (Figure 2.2), the cell body and the odontoblastic process. The cell body is located at the periphery of the pulp outside the predentin/dentin layer and involved in the synthesis of dentin extracellular matrix while the odontoblastic process that is a unique long unidirectional process crossing the predentin and extending inside dentin tubules up to the dentin-enamel junction is involved in the secretion of extracellular matrix molecules (MacDougall and Javed, 2010). The extracellular matrix molecules of collagen and proteoglycans are secreted in the predentin, whereas other extracellular matrix molecules that are associated with biomineralization are secreted more distally near the mineralization front, or even further within the lumen of tubules (Goldberg *et al.*, 2011).

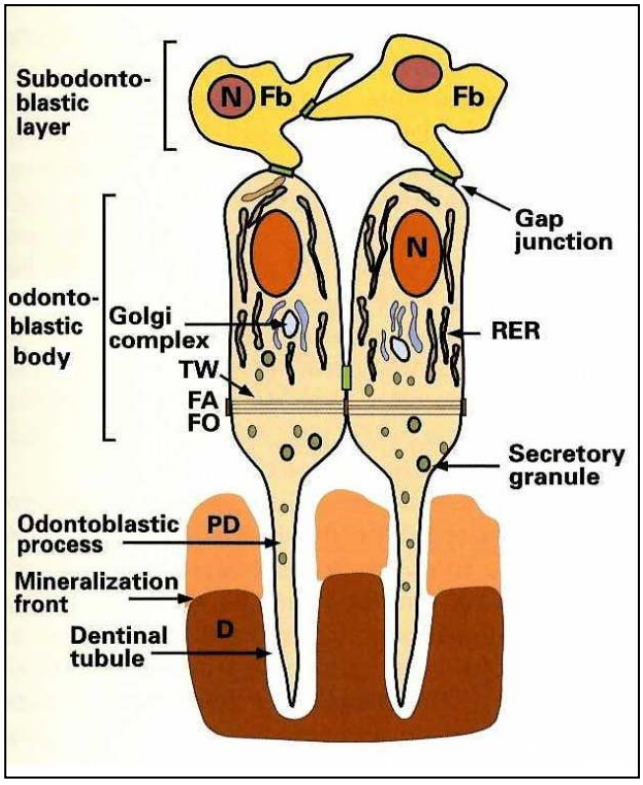


**Figure 2.2:** Mature secretory odontoblast. D = dentin; PD = predentin matrix; FO = fasciaoccludens; FA = fascia adherens; TW = terminal web; RER = rough endoplasmic reticulum; N = nucleus; Fb = fibroblast (Nanci, 2007).

### 2.2.2 Dentin

Dentin is one of the main mineralized tissue components of the tooth that surrounds the pulp and is covered peripherally by enamel on the crown and by cementum on the root surfaces. It is a collagen-based mineralized connective tissue consisting of inorganic apatite crystals embedded in an extracellular matrix (Huq *et al.*, 2005).

Dentin formation procedure termed dentinogenesis occurs simultaneously by two processes: (a) the formation of a collagenous network followed by (b) precipitation of the inorganic mineral phase in the form of hydroxyapatite crystals in complex mechanisms that control the site and rate of apatite formation during the biomineralization process (Goldberg *et al.*, 2011).

#### 2.2.2.1 Composition of Dentin

The proportions of dentin's components are 70% mineral phase, 20% organic phase and 10% water by weight and 50% mineral phase, 30% organic phase and 20% water by volume (Mauth *et al.*, 2007). The dentin mineral phase includes inorganic calcium and phosphate that deposit as mineral crystals to mineralize the dentin organic matrix and form mature dentin (Luz and Mano, 2010). The dentin organic matrix consists of type I collagen and non-collagenous proteins. It determines dentin morphology and is believed to be the key in the formation of the mineral phase during the different stages of biomineralization process (Smith *et al.*, 2012).

Collagen is the dominant fibrous protein in all connective tissues including the hard tissues of bone, dentin, and cementum. In dentin, odontoblasts secrete a layer of unmineralized collagen-rich organic matrix termed predentin that lies between the mineralized dentin and the inner most pulpal portion. Predentin is then transformed

into dentin after mineral deposition. The dentin collagen fibrils are primarily type I collagen, with smaller amounts of type V collagen, and type III collagen. Type I collagen contributes up to 90% of the organic material (Orsini *et al.*, 2007).

Non-collagenous proteins (NCPs) are synthesized and secreted by odontoblasts that constituting approximately 10% of the dentin matrix (Butler *et al.*, 2003). They are believed to be instrumental in mineral deposition within the extracellular dentin matrix as they play fundamental roles in actively promoting, controlling, and regulating collagen fibrillogenesis, crystal growth, and mineralization within predentin to convert into dentin during dentinogenesis (Qin *et al.*, 2004). They include:

(a) Glycoproteins

Glycoproteins consist of two families which are small integrin-binding ligand, N-linked glycoprotein (SIBLING) family, and secretory calcium-binding phosphoprotein (SCPP) family (Orsini *et al.*, 2009). SIBLING family includes:

(i) Dentin matrix protein-1 (DMP-1)

DMP-1 was first isolated and characterized by George *et al.* in 1993. Originally, DMP-1 is localized in dentin along the wall of dentinal tubules and in peritubular dentin as well as in odontoblasts (Massa *et al.*, 2005; Lu *et al.*, 2007). It was proposed to be dentin specific protein, however its expression had also been observed in bone (Huang *et al.*, 2008a). It is a multifunctional protein that regulates cell attachment, induces differentiation of dental pulp stem cells into odontoblasts and plays a pivotal role in biomineralization through a co-operative interactions between DMP-1 and type I collagen that initiate apatite nucleation and mineral deposition (Narayanan *et al.*, 2003; Ye *et al.*, 2004; Almushayt *et al.*, 2005; Narayanan *et al.*, 2006).

It has been found that hypophosphatemia caused defective in the phosphorylation of DMP-1 which in turn affected their capacity to stimulate dentin development and mineralization (Boukpessi *et al.*, 2006; Chaussain-Miller *et al.*, 2007). The roles of DMP-1 in mineralization are supported by observations of delayed conversion of predentin to dentin and osteoid to bone in DMP-1- deficient mice (Peng *et al.*, 2008).

(ii) Dentin sialophosphoprotein (DSPP)

DSPP is a gene discovered by MacDougall *et al.* in 1997. It gives rise to three proteins immediately cleaved after secretion into dentin sialoprotein (DSP), dentin glycoprotein (DGP), and dentin phosphoprotein (DPP) (MacDougall *et al.*, 1997; Qin *et al.*, 2003a; Tsuchiya *et al.*, 2011). Originally, DSPP is expressed in odontoblasts and at a much lower level in bone of about 1:400 that in dentin suggesting that DSPP can be considered as a specific marker of odontoblasts (Qin *et al.*, 2002). The two proteins DSP and DPP are abundantly and independently identified as extracellular matrix components of dentin (Yamakoshi *et al.*, 2003). They play regulatory roles in the nucleation of hydroxyapatite onto matrix collagen and in the subsequent growth of the hydroxyapatite crystals (Suzuki *et al.*, 2009). DPP is the most abundant NCPs in the dentin extracellular matrix discovered by Veis and Perry in 1967 as a cleavage product from the C-terminal side of DSPP which contains unique extended triplet amino acid repeat sequences (large amounts of aspartic acid and phosphoserine), forming ordered carboxyl-phosphate interaction ridges that is essential in the biomineralization process by binding calcium and presenting it to collagen fibers initiating and modulating of dentin hydroxyapatite formation (Prasad *et al.*, 2010). DSP is the second most abundant NCPs in the dentin extracellular matrix discovered by Butler *et al.* in 1981 originating from the N-terminal part of DSPP. DSP is also involved in the initiation of

dentin mineralization (Suzuki *et al.*, 2009). DGP is the middle portion of DSPP discovered in the pig dentin extract and it has not been determined whether this molecule is present in other species and consequently if it has any role in the process of dentinogenesis (Goldberg *et al.*, 2011).

(iii) Bone sialoprotein (BSP)

BSP was isolated from bovine cortical bone and subsequently characterized by Fisher *et al*. in 1983. BSP is present in mineralized tissues including bone, dentin, mineralizing cartilage, and cementum. Its quantity in bone is similar to that in dentin. BSP acts as a nucleator of the initial apatite crystals; then, as this mineral grows on the collagen matrix, it acts as an inhibitor in directing the growth of the crystals (Zhang *et al.*, 2009a).

(iv) Osteopontin (OPN)

OPN is characterized by the presence of polyaspartic acid sequence and sites of phosphorylation that mediate hydroxyapatite binding. It has the potential to bridge between cells and hydroxyapatite. OPN is present in bone in relatively large quantities and in relatively small quantities in the matrix of reparative dentin but not in the matrix of physiological and reactionary dentin (Huang *et al.*, 2008b).

*In vitro* studies have shown that the polyaspartate-containing OPN is an effective inhibitor of apatite formation and growth (Boskey *et al*., 2002) while highly phosphorylated OPN promotes hydroxyapatite formation concluding that OPN phosphorylation is an important factor in the regulation of OPN-induced mineralization process (Gericke *et al.*, 2005).

(v) Matrix extracellular phosphoglycoprotein (MEPE)

MEPE is highly expressed in bone and dentin matrix proteins (MacDougall *et al.*, 2002; Trueb *et al.*, 2007). It is associated with mineralization process. It has been observed that the acidic, serine and aspartate-rich MEPE is an effective mineralization inhibitor and the dephosphorylated MEPE has no effect on mineralization while the phosphorylated intact protein is an effective promoter of mineralization (Boskey *et al.*, 2010). MEPE is effective in promoting the differentiation of pulp cells into odontoblasts/osteoblast progenitors (Goldberg *et al.*, 2011).

SCPP family includes:

(i) Osteocalcin (OCN)

OCN is a vitamin K-dependent gamma-carboxylated protein. It is a small Ca-binding protein. It was demonstrated in dentin for the first time by Linde *et al.* in 1982. It is present in human odontoblasts and throughout the length of odontoblast processes, reaching the dentino–enamel junction. It has been found in smaller amounts in dentin than in bone (Papagerakis *et al.*, 2002; Onishi *et al.*, 2005). OCN binds hydroxyapatite controlling hydroxyapatite crystallization and affecting the growth or maturation of the calcium phosphate mineral phases (Goldberg *et al.*, 2003).

(ii) Osteonectin (ONC)

ONC is also referred to as SPARC: Secreted Protein Acidic and Rich in Cysteine. Its distribution is present in unmineralized predentin for intertubular and peritubular dentin. ONC plays important roles in collagen fibrillogenesis and maturation, binds to collagen, hydroxyapatite and growth factors, regulates cell proliferation, stimulates angiogenesis and matrix metalloproteinases production, and modulates cell–matrix

interactions (Martinek *et al.*, 2007; Delany and Hankenson, 2009). In developing human teeth, ONC was already expressed in the initial stages of cytodifferentiation, whereas OCN only appeared during the later stages (Orsini *et al.*, 2009).

The absence of specific molecules of DSP, DPP, DMP-1, OCN, and ONC in the pulp which have been identified in dentin can explain the lack of mineralization tissue in the pulp (Goldberg and Smith, 2004).

(b) Dentin proteoglycans (DPGs)

DPGs consist of two groups: small leucine-rich proteoglycans (SLRPs) family that includes decorin, biglycan, fibromodulin, lumican and osteoadherin, and large aggregating proteoglycans family that includes aggrecan, versican, neurocan, and brevican (Orsini *et al.*, 2009). DPGs play structural, metabolic, and functional roles in soft and calcified tissues and they seem to be key components in the mineralization process of dentin and bone (Goldberg *et al.*, 2005). DPGs that are present in predentin play important roles in matrix formation, regulate the mineralization process and prevent premature mineralization (Schaefer and Schaefer, 2010).

(c) Growth factors

Dentin contains different types of growth factors within the mineralized extracellular matrix that are available for release during demineralization caused by external injury such as caries and trauma. The diffusion of growth factors into the pulp–dentin complex stimulates reactionary dentinogenesis or reparative dentinogenesis (Smith *et al.*, 2012). These factors include TGF-β1, insulin-like growth factor-I (IGF-I), skeletal growth factor/insulin-like growth factor-II (SGF/IGF-II), platelet-derived growth factor (PDGF), VEGF, placenta-derived growth factor (PlGF), fibroblast growth

factor-2 (FGF-2), and very low concentrations of epidermal growth factor (EGF) and adrenomedullin (ADM) (Mazzoni *et al.*, 2012).

(d) Serum/plasma proteins

These proteins include albumin, transferrin, immunoglobulins (IgG, IgA and IgM) and Fetuin-A. It is thought that the close relationship between odontoblasts and the vasculature during dentinogenesis leads to the presence of these proteins within the dentin extracellular matrix. It is speculated that albumin is liberated only after demineralization of extracellular matrix while transferrin is present only during tooth development which is necessary for cell proliferation, morphogenesis, and differentiation of embryonic teeth. Then, the requirement for transferrin is lost as the teeth proceed from the early cap stage to the bell stage. Immunoglobulins are important in binding to cariogenic bacteria and facilitating their clearance by immune system cells. In addition, Fetuin-A prevents undesirable ectopic calcification without inhibiting bone or dentin mineralization (Mazzoni *et al.*, 2012; Smith *et al.*, 2012).

(e) Enzymes

Dentin demonstrates the presence of several matrix metalloproteinases (MMPs) enzymes that are immobilized within the intact dentin matrix, whereas these enzymes are released and activated when local demineralization of dentin occurs by acids released from cariogenic bacteria (Pashley *et al.*, 2004). Their release is important in activation of growth factors and other signaling molecules incorporated within the dentin. The proteins encoded by DSPP gene are processed by MMP-20 and MMP-2 to produce the functionally active peptides (Yamakoshi, 2009). In addition, a bioactive peptide which promotes differentiation of dental pulp progenitor cells is generated by MMP-2-cleavage of DMP-1 (Chaussain *et al.*, 2009). The other SIBLINGs and SLRPs

can also bind to form specific complexes for being activated (Fedarko *et al.*, 2004). IGFs and TGF-β1 are modified and activated directly by enzymatic digestion (Dallas *et al.*, 2002; Miyamoto *et al.*, 2004). Recent studies have demonstrated that MMPs accelerate wound healing following dental pulp injury by their controlled degradation of the extracellular matrix which enables immune and progenitor cells to be recruited to the site of injury (Joo and Seomun, 2008).

#### 2.2.2.2 Types of Dentin

There are three types of dentin:

(a) Primary dentin

It is the earliest and most prominent dentin formed during tooth development giving rise to the crown and root structure of the tooth (pattern of tooth organ). It lies between the enamel and the pulp. The most outer layer adjacent to the amelo-dentinal junction is known as mantle dentin. It is formed by newly differentiated odontoblasts and forms a layer approximately 150 μm wide. Mantle dentin is less mineralized as it lacks phosphoryn and has loosely packed collagen fibrils. Below mantle dentin, circumpulpal dentin lies that is a more mineralized dentin which makes up most of the dentin layer and is secreted by the odontoblasts at the rate of about 4 to 8 μm per day after the mantle dentin secretion before the root formation is completed (Nanci, 2007).

(b) Secondary dentin

Secondary dentin deposition starts immediately as the tooth becomes functional and the contacts between antagonistic cusps are established at slower rate of about 1 to 2 μm per day. At this time, the tooth is still immature with incomplete root formation, wide open apex, and relatively thin root dentin (Figure 2.3). The root of newly erupted

tooth will take up to 5 years before its growth is completed (Nanci, 2007; Cameron and Widmer, 2008).

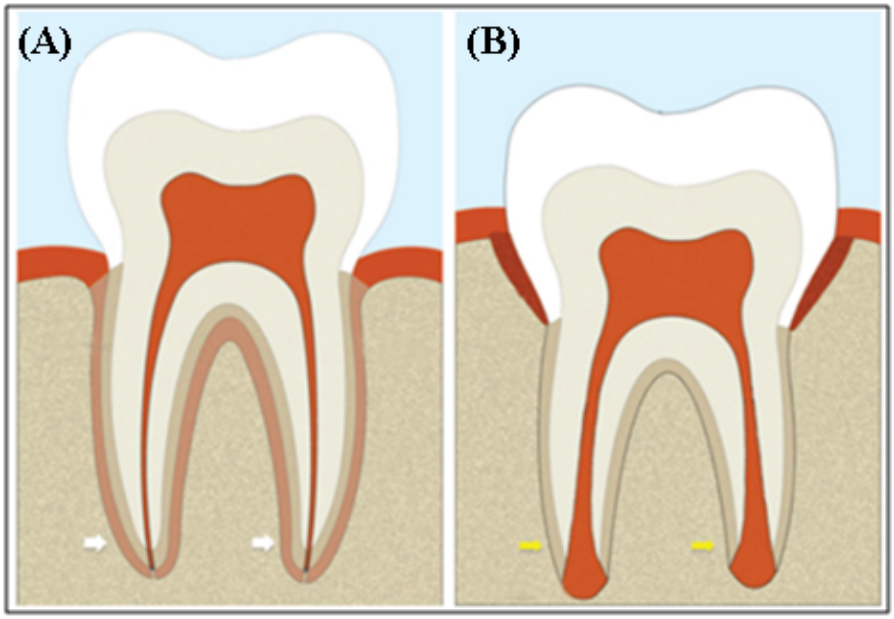


**Figure 2.3:** The structure of (A) mature and (B) immature tooth. The white arrow represents a mature closed root apex. The yellow arrow represents an immature wide root apex (Jamal *et al.*, 2011).

Secondary dentin deposition continues for the whole life of the tooth causing the decrease in the size of the pulp cavity with age as long as the pulp remains vital. It is characterized by a regular arrangement of dentinal tubules, usually in direct continuity with those of the primary dentin. Microhardness measurements indicate that secondary dentin is about 30% to 40% softer than primary dentin (Nanci, 2007; Goldberg *et al.*, 2011).

(c) Tertiary dentin

Tertiary dentin is formed as a reaction to external stimuli. It is of two types: (i) Reactionary dentin is formed from a pre-existing odontoblast when the stimuli are mild or progress slowly, such as mild caries, attrition, erosion, abrasion, superficial fracture or restorative dentistry procedure protecting the remaining odontoblasts and allowing the underlying soft pulp tissue to retain its function. (ii) Reparative dentin is formed from newly differentiated odontoblast-like cells of a pulpal progenitor cells due to the death of the original odontoblasts by the effect of the stimuli that are strong and/or rapidly progressing, such as deep dentin caries, severe attrition, erosion, abrasion, deep fracture or dental operative procedure producing excessive heat. Tertiary dentin does not have the well-organized histological structure of dentinal tubules of primary or secondary dentin (Nakashima, 2005; Nanci, 2007).

## 2.3 Vital Pulp Therapy

Dental pulp tissue is vulnerable to infection by external stimuli. When infected, it is difficult for the immune system to eradicate the infection owing to the lake of a collateral blood supply except from the root apical end. The continuous stimuli can result in irreversible pulpal damage causing necrosis of the pulpal tissues and the pulp loses its vitality and function in mature and immature teeth (Witherspoon, 2008).

The common age-group exposed to injuries is the school-aged children between 7 and 10 years old where the permanent teeth is still immature with open apical foramen (>1.1 mm). Studies have shown that 25% of school-aged children have experience some kind of dental trauma and 25% to 65% of school-aged children have untreated dental caries (Garcia-Godoy and Murray, 2012). World Health Organization (WHO) data banks demonstrate that dental caries is still prevalent in most countries worldwide

(100% incidence in some populations) (Yan *et al.*, 2011). Pulp necrosis of an immature permanent tooth, from the trauma or caries, arrests further root development and root dentin deposition leave a weak root of thin dentinal walls that is more prone to fracture with open apex making its restoration difficult. This leads to a poorer crown-to-root ratio, with possible periodontal breakdown as a result of increased mobility, and ultimately tooth loss (Neha *et al.*, 2011).

Many studies have shown that pulp vitality can be successfully protected from external stimuli and preserved its function by using vital pulp therapy (Flores *et al.*, 2007; Sawicki *et al.*, 2008; Witherspoon, 2008; Dean *et al.*, 2010; Waterhouse *et al.*, 2011). The primary goal of vital pulp therapy is to maintain the dental pulp vitality and function in mature and immature permanent teeth and to stimulate apexogenesis for complete root complex growth in immature teeth (Cameron and Widmer, 2008). Apexogenesis involves the continued normal development of the radicular root dentin and apical closure optimizing the root anatomy and strength that result in normal root thickness and length with normal apical morphology (Huang, 2008c).

### 2.3.1 Types of Vital Pulp Therapy

Clinically, there are two well-established methods for vital pulp therapy which are:

(a) Pulp capping that includes indirect and direct pulp capping. Indirect pulp capping has been defined as a procedure of coarse removal of caries that retained a small amount of carious dentin is in deep areas of cavity preparation to avoid pulp exposure. A medicament is then placed over the carious dentin to produce bacteriostatic condition, and stimulate and encourage pulp recovery. Many studies have given a low prognosis to indirect pulp therapy (Pinkham *et al.*, 2005). Direct pulp capping is a procedure in which a protective agent is placed directly on the pin point pulp exposure