

# **Comparison of the Effects of Different Pulp Capping Materials on Viability, Morphology and Ageing of Dental Pulp Cells**

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## Abstract

A significant amount of research has been directed towards the development of minimally invasive and/or regenerative therapeutic approaches to the maintenance of dental pulp vitality. Direct pulp capping is an example of such a therapeutic approach where the success of the treatment is largely affected by the biological characteristics of pulp capping agent. Tissue engineering approaches also include the use of human dental pulp cells (HDPCs) seeded on biocompatible scaffolds to regenerate lost hard, or soft dental tissues. Such processes may require a large number of cells which can only be produced by expansion of isolated HDPCs *in vitro*. Under these conditions, biological characteristics of the cells may be altered and their proliferation and differentiation capacity affected as a result of biological or cellular ageing. In this study we have investigated the effects of various pulp capping agents on HDPCs. Additionally we have looked at cell ageing and explored avenues to reduce the effects of this *in vitro*. HDPCs isolated from extracted human third molars were exposed to pulp capping agents through serial passage in culture. Dycal exhibited highly cytotoxic effects on the HDPCs, whereas, MTA facilitated the maintenance of cell viability and appeared to encourage proliferation of HDPCs. The use of propolis, a natural product of bees that has been reported to have uses in dentistry, resulted in slightly lower cell viability in comparison to the control and MTA groups. MTA and propolis appeared to enhance the osteogenic differentiation potential of HDPCs in comparison to control as indicated by the up-regulation of *BMP-2*, *ALP*, *RUNX-2* and *OCN* and the undetectable expression of *DSPP* and *DMP-1*.

We also clearly show that the biological characteristics of HDPCs (for example viability, cell proliferation and differentiation) were altered in later passages. A decrease in viability of cells treated with propolis seen in later passages, appeared to coincide

with the up-regulation of *BCN1*, a marker for for autophagy. Serially passaging of HDPCs also resulted in telomere shortening as measured by a progressive decrease of telomere restriction fragment (TRF) and this may lead to replicative senescence or cell ageing. Telomere shortening might be expected as expression of the telomerase associated gene (*TERT*) was undetectable. Contrary to expectations, propolis appeared to cause the greatest shortening of telomere length. Addition of the oligonucleotide “Telome 3”, which was designed to facilitate exogenous priming of telomeric DNA synthesis, to serially passaged HDPCs maintained high cell-viability throughout. Additionally, TRF measurements suggested that “Telome 3” may indeed aid in the maintenance of telomere length in serially passaged HDPCs. These data suggest that within the constraints of this *in vitro* model, the viability, proliferation and differentiation of HDPCs are largely affected by the cell passage number and also the type and concentration of the pulp capping agent used.

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# List of abbreviations

A (in nucleotide sequence)	Adenine
AD	Atosomal Dominant Dyskeratosis
ALP	Alkaline phosphatase
BMP	Bone Morphogenic Protein
BSP	Bone SialoProtein
cDNA	Complementary DNA
C (in nucleotide sequence)	Cytosine
Ca (OH) <sub>2</sub>	Calcium Hydroxide
CaO	Calcium Oxide
CAPE	Caffeic Acid Phenethyl Ester
Ct	Cycle threshold
DC	Dyskeratosis Congenital
DMEM	Dulbecco's Modified Eagle's Medium
D loops	DNA double loops
DMSO	Dimethylsulphoxide
DMP-1	Dentine Matrix Protein-1
DNA	Deoxyribonucleic Acid
DSPP	Dentine Sialophosphoprotein
DPP	Dentine Phosphoprotein
EDTA	Ethylene Diamine Tetra Acetic Acid
ESC	Embryonic Stem Cells
EtBr	Ethidium Bromide
FCS	Foetal Calf Serum

FGF	Fibroblast Growth Factor
FPIA	Flow Particle Image Analyzer
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
G-CSF	Granulocyte Transforming Growth Factor
GIC	Glass Ionomer Cement
G (in nucleotide sequence)	Guanine
GMTA	Grey MTA
HBXIP	Hepatitis B X interacting protein
HSCs	Hematopoietic stem cells
HDPCs	Human Dental Pulp Cells
ICAM-1	Inter-Cellular Adhesion Molecule 1
IGF-1	Insulin-like Growth Factor-1
IPA	Isopropanol
IRM	Intermediate Restorative Material
KDa	Kilo Dalton
MDS	Myelodysplastic Syndrome
mRNA	Messenger RNA
MTA	Mineral Trioxide Aggregate
MSCs	Mesenchymal Stem Cells
NCAM-1	Neural Cell Adhesion Molecule
NEC	Novel Endodontic Cement
NFkB	Nuclear Factor kappa B signaling pathway
OCT4	Octamer Motif Homeodomain Transcription factor
OCN(BGLAP)	Osteocalcin
OD	Optical Density

PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PGA	Polyglycolic Acid
PCR	Polymerase Chain Reaction
PLA	Polylactic Acid
PLGA	Polyactic-co-Glycolic Acid
PMN	Polymorphonuclear
Pot1+	Protection of Telomere Protein 1
PS	Penicillin Streptomycin
Q-PCR	Quantitative Polymerase Chain Reaction
Q RT-PCR	Quantitative Reverse Transcription PCR
ROS	Reactive Oxygen Species
RUNX-2	Runt-related transcription factor 2
rhIGF-I	Recombined Human Insulin-like Growth Factor I
SCAP	Stem Cells Apical Papilla
STRO1	Stromal Precursors 1
T (in nucleotide sequence)	Thymine
TAE	Tris Acetate EDTA
TE	Trypsin
TGF	Transforming Growth Factor
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase protein component
T loops	Telomeric loop
TRAP	Telomere Repeat Amplification Protocol
TRF	Terminal Restriction Fragment

TRF1	Telomeric Repeat Protein binding factor
tRNA	Transfer RNA
UV	Ultra Violet
VCAM-1	Vascular Cell Adhesion Molecule-1
WMTA	White MTA
ZnP	Zinc Phosphate
$\alpha$ - MEM	Alpha Modified Minimum Essential Medium

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# Chapter 1: Introduction

## 1.1 Tooth development, structure and function

Teeth constitute approximately 20% of the surface area of the mouth where they serve several functions including mastication, speech and aesthetics. The teeth consist of a hard, acellular enamel formed by epithelial cells supported by the less mineralised, vital tissue called dentine. Dentine is formed from and supported by the dental pulp, a soft, vascularised, innervated connective tissue. In mammals, teeth are attached to the bones of the jaw by tooth-supporting connective tissues consisting of the cementum, periodontal ligament and alveolar bone (Nanci, 2014). The complex process of tooth development commences with embryonic cell growth in the first brachial arch, followed by eruption into the oral cavity (Goldberg and Smith, 2004). In order for natural teeth to be functional in the oral environment the development of enamel, dentine, cementum, pulp and periodontal ligament are essential. Enamel tends to be more translucent as a result of its complex hydroxyapatite crystal prismatic structure, whereas dentine is a more porous compound comprised of hydroxylapatite crystal particles in collagen matrix (Smith, 2003). The more highly mineralized outer enamel layer protects the more porous dentine and pulp tissue but the pulp and dentine combined can, in turn, serve to protect and maintain the integrity of the whole tooth when caries or trauma intervenes (Figure 1). The many intricate biological reactions and interactions, within the pulp-dentine complex particularly, can be harnessed by the dental practitioner as they apply techniques and materials that might help to maintain the vitality and health of the dental pulp (Pashley, 1996).

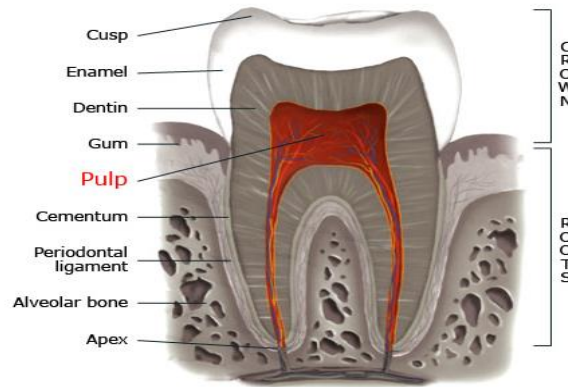


Figure 1. Tooth structure Adapted from (Marcovitch, 2005).

## 1.2 Human dental pulp (HDP)

### 1.2.1 Biology of the pulp

Dental pulp is sometimes colloquially known as the tooth nerve but the function of the pulp is wider ranging, including dentine formation and defence through the dentine-pulp complex, as well as providing sensory feedback (Goldberg and Smith, 2004). Pulp is a loose connective tissue within the central pulp chamber (Sloan and Smith, 2007). It is comprised of a mixture of mesenchymal and para-axial mesenchymal cells, as well as cells from the cranial neural crest, which are present at the site of the dental papilla and tooth bud development (Goldberg and Smith, 2004). It was assumed that the source of pulp cells listed within the crown and root of the tooth is the same but there is continuing debate. There is no positive evidence to support this assumption, in fact there is differing nerve development and vascular morphogenesis in these two sites (Takahashi, 1985). While a tooth is forming, the epithelial and dental papillae cells stimulate the mesenchymal cells to differentiate into odontoblasts and produce primary dentine for development of tooth morphology (Murray *et al.*, 2001). Pulp outer margins are populated by these columnar cells with irregular nuclei. Their long cytoplasmic

processes run through dentine in the dentinal tubules (Gronthos *et al.*, 2000). They are responsible for secretion of primary dentine during tooth development, secondary dentine after complete tooth growth and physiological secondary dentine is secreted after complete root formation. Irregular secondary dentine or tertiary osteodentine is formed in response to different stimuli (Bluteau *et al.*, 2008). The tertiary dentine acts as a barrier against irritating stimuli to defend the dentine-pulp complex (Smith, 2003). After tooth eruption the reparative dentine is formed in response to mechanical stimuli. Odontoblasts themselves are post mitotic and are not able to repair or regenerate damaged dentine. Instead new odontoblasts derived from tertiary dentine migrate to the damaged dentine surface to seal and protect the pulp against injury (Smith *et al.*, 1995). Reactionary dentine is formed in response to mild stimulus characterized by surviving post mitotic odontoblast cells by which a tertiary dentine matrix is secreted. On the other hand, the reparative dentine is formed as a response to strong stimuli, from new generation odontoblast like cells to form tertiary reparative dentine matrix (About *et al.*, 2000). Terminology and different types of dentine formation are described in Table 1.

Table 1 A list of the different types of dentine formation

<b>Terms</b>	<b>Definition</b>	<b>Types</b>
Primary dentine	Dentin that is laid down before and during tooth eruption	Mantle Circumpulpal Peritubular Intertubular
Secondary dentine	Dentine that is formed after root formation is complete. It is responsible for the reduction of the pulp chamber over time	
Tertiary dentine	Dentine that is formed in response to insults to the dentin/pulp complex. Called reparative, reactionary or irregular secondary dentine	Reparative: Regular dentine laid down by odontoblasts in response to a low grade insult. Reactionary: Irregular dentine formed by odontoblasts-like cells in response to acute injury that results in necrosis of odontoblasts

Fibroblasts, widely found in the pulp, are capable of synthesizing collagen and extracellular matrix and include cells which have the ability to differentiate. They are particularly involved in the process of wound healing in pulp tissue. These cells are established within the cell rich zone, which lies under the odontoblast layer and their prominent cytoplasmic process change just like odontoblast cells (Goldberg and Smith, 2004, Walton and Torabinejad, 1996, Cohen *et al.*, 2006). In a study by Fitzgerald *et al.* determined that in damaged tissue fibroblasts may be stimulated to differentiate into replacement odontoblast cells (Fitzgerald *et al.*, 1990). Pulp cells and fibres as well as the blood vessels and nerves which enter and leave through the apical and other foramina are embedded within gel-like ground substance. Odontoblasts are the most abundant cell phenotype in the pulp. They have a critical function of supplying nutrients during dentine formation, hydration of mature dentine via dentinal tubules and modulating host defence mechanism through the pulp-dentine complex by triggering inflammatory and immune responses (Pashley, 1996, Ricketts, 2001). A positive tissue pressure produces an ultra-filtrate of blood which leaves the pulp via the tubules in an outward flow, known as dentinal fluid. The defence reaction depends on the concentration of bacterial by-products ingressing, the permeability of dentine that change with age and the pulpal fluid pressure. The initial inflammatory response is by infiltration of inflammatory cells against the bacterial by-products that are diffusing down open tubules (Goldberg and Smith, 2004). Dentine permeability may then be moderated by deposition of apatite crystals within the dentinal tubules to form peritubular dentine. Formation of reactionary tertiary dentine and reparative tertiary dentine by odontoblast cells can further moderate dentine permeability and influence the outward fluid flow and inflammatory response (Ricketts, 2001).

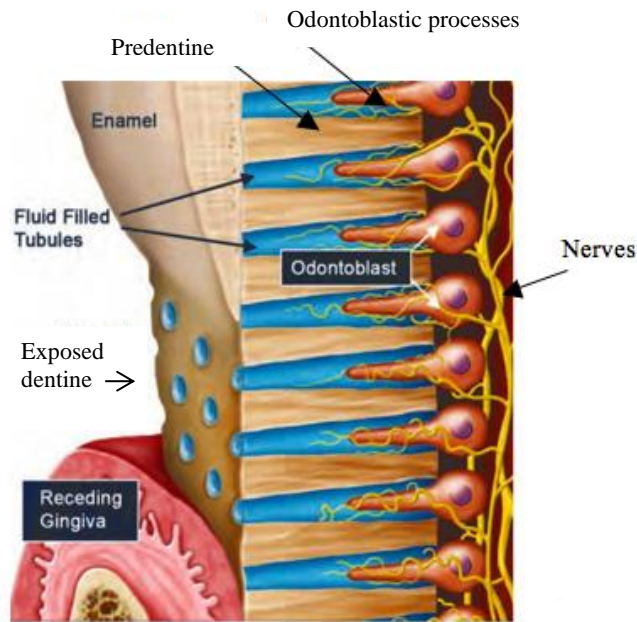


Figure 2. Pulp-dentine complex, adapted from (Strassler *et al.*, 2008).

Pulp is a highly vascular structure with vessels from the apical and sometimes other foramina dividing in the more vascularised coronal portion and forming a complex microcirculation under the odontoblast layer, to eliminate metabolic waste products and transfer oxygen and nutrients that maintain the health of the tissue (Kim, 1985). Dental pulp is innervated by myelinated sensory as well as autonomic nerve fibres. The autonomic fibres are responsible for the regulation of microcirculation and the sensory nerve fibres for determination of distinctive pains (Pashley, 1996). The types of sensory nerve fibres are classified into groups depending on their diameter and conductive velocity. They are comprised of A- $\beta$ , A- $\gamma$ , C fibres and non-myelinated sympathetic fibres. Functions of A- $\beta$  fibres are not well known but have a very low stimulation threshold and comprise 50% of the myelinated fibres in pulp (Pashley, 1996). The A- $\gamma$  fibres are myelinated and are involved in sharp and fast dentinal pain, stimulated by hydrodynamic movements in dentine. The stimulation threshold of C fibres is high, producing less localized throbbing pulpal pain. This is provoked by pulpal tissue injury. The non-myelinated fibres are post-ganglionic fibres that are stimulated by

vasoconstriction (Walton and Torabinejad, 1996, Cohen *et al.*, 2006). Usually interdental nerves are located in pulp horns (Nanci, 2007).

Dental pulp cells are a mixed population of cells that act as a mechanical support system embedded within a vascular stroma (Shi and Gronthos, 2003, Nanci, 2007). The central region of the coronal and radicular pulp contains large nerve and blood vessels. This area is lined peripherally by a specialized odontogenic area which has four layers. The innermost, the pulpal core, is comprised of many cells and an extensive vascular supply and is very similar to the cell-rich zone. Then the cell rich zone contains fibroblasts and undifferentiated mesenchymal cells. The cell free zone (zone of Weil), which is rich in both capillaries and nerve networks, is directly under the outermost odontoblastic layer which lies next to the predentine and mature dentine. Cells found in the dental pulp include fibroblasts (the principal cell), odontoblasts, defence cells like histiocytes, macrophages, granulocytes, mast cells and plasma cells, dentetic cells. The nerve plexus of Raschkow is located central to the cell-free zone (Nanci, 2007).

Besides odontoblasts, fibroblasts and undifferentiated primitive mesenchymal cells, nerve cells, dendritic cells, pericytes, endothelial cells and collagen fibrils and also defence cells like (lymphocytes, microphage), are all embedded in dental pulp (Coedeiro *et al.*, 2008). These populations of cells usually develop from the neural crest and mesoderm, and afterwards from dental follicles. Dental pulp cells were assumed to be stem cells, which have the ability to differentiate into progenitor odontoblast cells, and later on, further differentiate into odontoblast cells (Jontell *et al.*, 1987).

### **1.2.2 Pulp disease**

Dental pulp connective tissue is mechanically protected from different stimuli by enamel, dentine and cementum and retains its health and vitality if the protective barrier

is intact (Bergenholtz, 1990). As mentioned, defence mechanisms involve inflammatory response, blocking the dentinal tubules and formation of secondary and tertiary dentine, as well as involvement of sensory fibres which give feedback on various stimuli. Despite this protection the pulp may be injured, diseased or die (Trowbridge, 1981). Pulp irritation could be due to microorganisms or viruses, mechanical, chemical and thermal insults. Microbial irritants include bacterial species such as *Streptococcus mutans* and lactobacilli which produce toxins and penetrate into the pulp through dentinal tubules. The pulp under those tubules is then infiltrated locally and primarily by chronic inflammatory cells like macrophages, polymorphonuclear (PMN) plasma cells and lymphocytes and may form an area of necrosis which bacteria may colonize. Cytokines are secreted as part of an immune system response to stimuli and microorganisms (Coil *et al.*, 2004). In the pulp not only odontoblast cells but also fibroblast cells have the potential to secrete the cytokines to initiate collagen formation and pulp repair (Barkhordar *et al.*, 2002, Veerayutthwilai *et al.*, 2007).

Pulp may be inflamed chronically or undergo necrosis eventually, depending on the virulence of bacteria, their ability to stimulate release of inflammatory fluids with increase in intrapulpal pressure, the host defence, amount of circulation and lymph node drainage (Tziafas, 2003). Dentine thickness will also influence the outcome (Dummer *et al.*, 1980).

Carious dentine is visualized in four zones, the outer destruction zone, a zone of infected tubules, a zone of demineralization and lastly the zone of sclerosis (Trowbridge, 1981). The initial pulp reaction to caries is observed in the odontoblast layer beneath the lesion, where changes are seen in the morphology of the odontoblast cell as a response to inflammation (Brannstrom and Lind, 1965). Other early changes include deposition and fibroblast proliferation of blood vessels (Reeves and Stanley, 1966, Pashley, 1996).

Besides the bacteria normally associated with caries, other gram-negative bacteria and anaerobic microorganisms may also play a part in pulp pathosis (Smith, 2002, Lopez-Marcos, 2004). Dentine pulp reaction in caries depends on many factors, the concentration of bacterial by-products, sugar consumption and acid concentration. Permeability properties of dentine regulate the rate of diffusion of irritants that initiate pulpal inflammation. Dentine changes with age; newly erupted teeth being more permeable and less mineralized, permitting rapid diffusion of acid. The dentinal tubules contain fluid, and more is present within the injured tooth in comparison to the intact tooth demonstrating active fluid shift between the matrix and tubules (Smith, 2003). The permeability of dentine is lowest at the dentinoenamel junction with 15,000 dentinal tubules per mm<sup>2</sup> and highest at the pulp, 65,000 tubules per mm<sup>2</sup>, because both density and diameter of dentinal tubules increases. Dentine permeability is reduced during cavity preparation due to accumulation of plasma protein within the dentinal tubules (Pashley, 1996). The dentinal tubules beneath the carious lesion are occluded by mineral deposit, appetite, whitlockite crystals and smear layer (Pashley, 1996, Ricketts, 2001). Intratubular dentine formation within the tubules, tertiary dentine formation by odontoblasts within the pulp chamber and irregular secondary dentine formation may eventually occlude the tubules and reduce the dentinal permeability, preventing the ingress of irritating substances (Reeves and Stanley, 1966, Pashley, 1996, Cooper *et al.*, 2010).

Pulp cannot eliminate the damaging bacteria but defence mechanisms including the immune response may diminish and reduces ability of the bacteria to cause destruction (Bergenholtz, 1990). Kakehashi, *et al.* established the significance of bacteria in pulp pathosis. They compared two groups, conventional and germ free rats whose pulps were exposed to the oral environment. Within eight days, the pulp of the conventional group



was necrosed with abscesses and granulomas present in contrast to the germ-free rats where there was no sign of pulp necrosis. After 14 days, the formation of dentine bridges was seen this was fully completed in 28 days (Kakehashi *et al.*, 1965). Caries, tooth cracks and fractures generate a passageway for bacteria to enter the tooth and cause inflammation (Brannstrom, 1984).

For the clinician, signs and symptoms are a valuable tool in diagnosis but there is a debate about how to classify pulp disease as the relation between the histopathological findings and pulp health and disease vary (Kansu *et al.*, 2009). It has been suggested that the clinical signs and symptoms are not obviously related to pulp disease (Dummer *et al.*, 1980). Clinical signs do occur with progressive pulp pathosis but accurate diagnosis can only be made from histological findings. A possible classification combines histopathological and clinical aspects (Levin *et al.*, 2009). Pulp pathology can be manifest as one of the following clinical conditions; reversible, irreversible pulpitis, hyperplastic pulpitis, pulp necrosis as well as atrophic pulp calcification, internal dentine resorption and fibrous degeneration (Lopez-Marcos, 2004). Reversible pulpitis is characterised by mild symptoms that subside when the stimulus is removed and if the source of irritation is removed the pulp will be able to retain its vitality. Reversible pulpitis may occur due to several factors including caries, trauma, dental erosion and attrition. In irreversible pulpitis the pulp is severely inflamed and could not retain its vitality even if the irritation was eliminated (Levin *et al.*, 2009). Irreversible pulpitis is categorised into symptomatic and asymptomatic, the symptomatic form occurs when the inflammatory oedema causes increase of the intra-pulpal pressure, producing sharp and spontaneous pain. The pain is the response of the A-delta and C fibres to inflammatory secretion. If the oedema is resolved or removed through dentinal tubules then the asymptomatic irreversible pulpitis occurs which is ultimately the initial stage of pulpal

necrosis. The radiographic changes include periodontal ligament widening followed by pulp necrosis producing an apical radiolucency, as inflammatory changes affect bone density as pulp cells and vasculature are destroyed (Dummer *et al.*, 1980, Lopez-Marcos, 2004, Abbott and Yu, 2007). Pulp necrosis could result from any source of pulp insult and symptoms vary, including discoloration of teeth and pain during occlusion. The necrosed pulp will not respond to most pulp tests (Ngassapa, 1996).

### **1.3 Restorative procedures and dental pulp response to the treatment**

One aim of restorative procedures is to preserve pulp vitality, retaining tooth function. Retaining pulp vitality is beneficial as the pulp supplies nutrition to dentine during and after complete tooth formation (Modena *et al.*, 2009). A fully vital pulp can function to resist infection but this ability naturally decreases with age, reducing pulp defence (Takahashi, 1990). It has been suggested that age might influence treatment choice as regeneration of pulp will be affected by reduction of cellularity and vascular supply (Murray *et al.*, 2002, Murray and Garcia-Godoy, 2007). Age affects pulp cellularity and influences the proliferation and differentiation ability of the odontoblast cells (Murray *et al.*, 2000).

As a result of restorative procedures irregular secondary dentine and tertiary dentine is laid down. If the injury is severe and causes death of odontoblast cells odontoblast-like cells produce specific tertiary reparative dentine beneath the site of injury in order to protect pulp tissue (Cox and Bergenholtz, 1986b, Mjor *et al.*, 1991). Here the reparative dentine has irregular morphology, with cellular inclusions and an atubular nature. Permeability is reduced and diffusion of irritant agents from the tubules is prohibited (Tziafas, 2003). However, in mild injury the odontoblasts survive and are stimulated to synthesize tertiary dentine which resembles primary dentine matrix and has a tubular

pattern (Tziafas, 2003). As a response to cavity preparation tertiary, reactionary dentine forms at the end of dentinal tubules to protect the pulp by reducing the ingress of noxious stimuli (Reeves and Stanley, 1966, Pashley, 1996).

The mechanical friction produced by cavity preparation is another concern in restorative dentistry. During enamel and dentine removal, heat may be generated through inadequate cooling of burs, excess pressure, the use of blunt burs or air spray desiccation which may result in odontoblast and pulp damage (Smith, 2002, Smith, 2003).

Despite the cellular changes produced iatrogenically, bacteria remain as the key factor as bacterial penetration is essential for pulp injury, even in the absence of caries (About *et al.*, 2001). In a study comparing dentinal permeability in the carious tooth with an experimentally produced cavity, it was found that the experimental cavity had no sign of dentine permeability in presence of bacteria in contrast to the carious lesions (Bergenholtz *et al.*, 1982). Dentine permeability is enhanced in the carious lesion, with rapid progression. However, to protect the pulp from caries and bacterial microleakage, restorative materials become important. They add a therapeutic dimension to retaining pulp vitality (Pashley, 1996). The clinician ideally would wish to protect the pulp in carious teeth by either arresting the process or eliminating the adverse effects of the lesion. In reality, this is achieved by careful removal of some carious dentine and the application of a suitable restorative material.

As mentioned in Section 1.2.2, a previous study by Dummer *et al.* has shown that it is difficult to accurately diagnose the pulp's status from clinical signs and symptoms (Dummer *et al.*, 1980). Currently the choice of treatment depends on the operator's assessment using best knowledge of the likely prognosis and severity of the case

(Walton and Torabinejad, 1996, Carrotte, 2004, Cohen *et al.*, 2006). The treatment of diseased pulp can be broadly classified in to root canal therapy and vital pulp therapy.

### **1.3.1 Root canal therapy**

Endodontic or root canal therapy is often the treatment of choice in permanent teeth with irreversibly inflamed or non-vital pulps. It is also used to treat other pulpal pathologies occurring as a result of internal root resorption, trauma and periodontal disease (Carrotte, 2004). The infected pulp will have decreased healing ability and a further development of the infection could produce a periapical abscess (Murray *et al.*, 2002). Treatment consists of several stages, removing infected and/or necrosed pulp and obturating the canal with compatible materials in order to retain function (Carrotte, 2004). The access preparation is the most important phase in order to achieve straight line access to the canals, preserving tooth structure and removing the pulp chamber roof. The cleaning, or debridement phase, eliminates the pulp debris and shapes the canal to provide a continuous conical shape from apical to coronal (Schilder, 1974). Canal irrigation is necessary to dissolve vital and necrotic tissue, lubricate the instruments used and disinfect the canal system. Sodium hypochlorite is the preferred solution (Zehnder, 2006). The last part of root canal treatment is the obturation phase, a three-dimensional filling to provide an impermeable fluid tight seal within the entire root canal system, to prevent oral and apical microleakage (Walton and Torabinejad, 1996).

### **1.3.2 Vital pulp therapy techniques**

#### **1.3.2.1 Indirect pulp capping**

Alternative approaches to root canal treatment aim to maintain all or part of the pulp. Indirect pulp capping retains pulp vitality by removal of carious dentine with preservation of a thin layer over the pulp, followed by the application of a compatible,

therapeutic restorative material with the intention of promoting tertiary, reactionary dentine deposition. It is the treatment of choice for permanent teeth with carious dentine and reversible pulpitis (Ranly and Garcia-Godoy, 2000). In a clinical study by Stark *et al.* it was determined that the preservation of pulp vitality depends on the inflammatory condition of the pulp (Stark *et al.*, 1976). If a pulp is inflamed without bacterial infection it has a better chance of survival than an infected pulp so keeping a dentine layer over the pulp, even if this is quite thin, is preferred to avoid bacterial infection (Massler and Pawlak, 1977). The carious process can be arrested by remineralisation and encouraging formation of sclerotic dentine (Farhad and Mohammadi, 2005). According to a study by Ricketts, minimal removal of carious dentine, known as stepwise excavation, is valuable in cases where no signs or symptoms of pulp inflammation are present. In primary teeth, because the amount of remaining dentine is less, indirect pulp capping is not commonly practiced (Ricketts, 2001).

The difficulty with indirect pulp capping is that pulp may be exposed inadvertently during the procedure, especially where the amount of carious dentine removal is extensive. Indeed, pulp exposure may occur in up to 75% of cases (Ranly and Garcia-Godoy, 2000).

### **1.3.2.2 Direct pulp capping**

When pulp is exposed, there is a tendency to assume that root canal treatment is indicated but pulp tissue may still have the ability to heal or regenerate after exposure, even if not treated immediately, through formation of a dentine bridge at the site of exposure (Cox and Bergenholtz, 1986, Stockton, 1999, Schuurs *et al.*, 2000). The success rate of direct pulp capping, where a material is placed directly over the exposure, has been thought to depend on the age of the patient, whether the apex is open or closed,

existing signs or symptoms of pulp inflammation, size of the exposure, response of pulp to stimuli and minimal pulp haemorrhage (Murray *et al.*, 2002). There has been debate regarding the relationship between the age and success rate and age should not be a reason to contraindicate pulp capping (Hiltn *et al.*, 2013). However, Horsted *et al.* determined that in the long term observation of pulp capping procedures, the success rate was improved in molar teeth and young patients (Horsted *et al.*, 1985). They did not observe any significant variation between carious and non-carious exposures or size of the exposure in retaining the tooth and pulp vitality. In contrast, other studies suggested that there is no relation between age and success rate of direct pulp capping (Haskell *et al.*, 1978, Baume and Holz, 1981). It is however, recognised that Horsted *et al.* did not set out to investigate age group but to compare mechanical exposure and carious pulp response to pulp capping (Horsted *et al.*, 1985). Others have pointed out that the young pulp comprises a larger number of pulp cells, especially odontoblasts, and greater vascularity which may influence its capacity for defence and regeneration (Lim and Ong, 1989).

Ricketts, suggested that for direct pulp capping absence of swelling and recurrent pain, a clinically pink pulp, normal vitality test, absence of periapical pathology and tenderness to percussion, are important factors (Ricketts, 2001). It has been assumed that only pinpoint exposures could be treated by pulp capping but the size of exposure may have no effect on the success of clinical outcome (Fuks *et al.*, 1987). However, Stockton has reported that the size of the exposure is critical and should be minimal with no symptoms from the pulp, which demonstrates some of the the uncertainty in this area (Stockton, 1999).

Establishing the success rate of a direct pulp capping from the literature is difficult as many studies fail to identify whether exposures are due to trauma or caries. The success

rate of direct pulp capping with mechanical exposure was 92.2% in comparison to carious exposure with 33.3%, therefore suggesting that pulp capping is the treatment of choice in cases of mechanical exposure, while root canal therapy would be the treatment choice in the carious lesions (Al-Hiyasat *et al.*, 2006).

A study on young permanent molar teeth by Mejare and Cvek suggested that 1–3 mm of pulp tissue should be removed to gain direct contact between pulp and pulp capping material and remove superficial inflamed pulp, a technique known as partial pulpotomy (Mejare and Cvek, 1993). Pashley, suggested that where the remaining dentine thickness is less than 1 mm pulp capping may be less successful, which tends to support partial pulpotomy, as the thin layer of dentine may be permeable and allow infection of the superficial pulp layer (Pashley, 1996).

It is essential then, in summary, to emphasize that although the size of traumatic exposure is not that significant the size of carious exposures should be minor, even after further exposure at the operation site. It is established that larger carious exposures will predispose pulp to bacterial contamination and necrosis and will result in a poor prognosis. A successful pulp capping procedure should retain vital pulp and dentine bridge formation within 75-90 days (Ricketts, 2001).

In the study by Coll, it was reported that in carious primary teeth with vital pulp exposure the removal of caries and a coronal portion of pulp is the best approach (Coll, 2008). According to Dean the success rate in 6-12 months follow up was 92% with placement of 35% formocresol that fixes the pulp and removes the bacteria (Dean *et al.*, 2002). Where ferric sulphate was used in pulp capping, as a clotting agent, the radicular pulp was protected from further damage and the success rate in 24 months of follow up was 85% but these materials have a cytotoxic effect on the pulp tissue and are not

widely utilized (Huth *et al.*, 2005). Debate continues and Milnes, assuming a lack of carcinogenicity and a safe dosage, suggests that the use of formocresol in pulpotomy is possible (Milnes, 2008). The mean dosage of formocresol in a cotton pellet with a 1:5 dilution would be 0.0026 mg with limited pulpal contact (Kahl *et al.*, 2008). Therefore if the correct amount was used carefully it was considered doubtful that carcinogenicity, cytotoxicity, immunogenicity would be a concern (Milnes, 2008). The other material of choice for indirect pulp capping could be antibacterial Ca(OH)<sub>2</sub> to protect the pulp and promote dentine formation. Warfvinge *et al.* compared the antibacterial properties and dentine bridge formation of two types of calcium hydroxide materials and found that calcium hydroxide paste in saline had better physical properties than the hard setting calcium hydroxide cement, known as Life (Warfvinge *et al.*, 1987). Previously, Stanley and Pameijer preferred improved calcium hydroxide known as Prisma VLC Dycal which had better physical properties (Stanley and Pameijer, 1985).

Direct pulp capping may be chosen where there is limited ability to treat the tooth endodontically (Hilton, 2009), perhaps due to patient management issues although Langeland noted that pulp capping could be a temporary treatment of choice in contrast to more definitive root canal treatment (Langeland, 1959). The success rate of endodontics is up to 90% and direct pulp capping is between 80% to 90% so a clinician may feel that pulp capping is an appropriate choice in some situations (Haskell *et al.*, 1978, Swartz *et al.*, 1983). Some authors have argued that the dentine becomes more brittle after root canal therapy and is less resistant to functional loading, predisposing the tooth to fracture which decreases the success rate of endodontic therapy (Sedgley and Messer, 1992). At the same time, better knowledge and material developments have improved the success rate of pulp capping (Baume and Holz, 1981). The proper choice of clinical case is the key to successful treatment. Failure of pulp capping is seen with



pulp inflammation and necrosis, as a result of improper sealing of the cavity and bacterial microleakage, with possible internal resorption and root destruction (Baume and Holz, 1981). This may explain why authors vary on their opinion as to whether exposure size is important or not, as the outcome may in fact be related primarily to seal against bacterial ingress. Dentine bridge formation is important but in some cases necrosis may occur after it has formed (Schuurs *et al.*, 2000).

Success may also hinge on good pulp capping technique, isolation with rubber dam to exclude moisture and bacterial contamination and use of antibacterial detergents, for example Tubulicid, which removes the smear layer blocking the dentinal tubules and entrapping bacteria. Both of these measures provide an isolated area which will reduce microleakage by enhancing the adaptation of restorative materials to cavity walls (Brannstrom, 1984). After complete isolation of the teeth, removal of caries continues with minimal exposure of the pulp. The exposed pulp may be irrigated with saline or 5.25% sodium hypochlorite solution to remove debris or remaining contamination (Stanley, 1989, Matsuo *et al.*, 1996). Controlling the bleeding is important in direct pulp capping and increases the amount of dentine bridge formation (Masterton, 1966, Schroder, 1973). Direct pulp capping ensures contact between the exposure region of the pulp and the selected material, for example, calcium hydroxide, excluding bacterial growth after drying and disinfecting (Schroder, 1985, Schuurs *et al.*, 2000). The treated tooth should be follow up clinically and radiographically for 4-5 years, 1 month after the treatment, followed by 3 months, 1 year and yearly after that (Stockton, 1999).

#### **1.4 Direct pulp capping materials**

The materials used to cap dental pulp are critical and include calcium hydroxide, resin modified glass ionomer following etch technique as well as adhesive resin based

composite and mineral trioxide aggregate (MTA) (Foreman and Barnes, 1990, Cox *et al.*, 1998).

During pulp exposure and initiation of pulp inflammation primary odontoblast cells are damaged. For formation of reparative dentine differentiated stem cells are required. Transforming growth factors (TGF) can induce differentiation of stem cells into odontoblast like cells (Kim *et al.*, 2012). Some pulp capping materials such as MTA and Ca(OH)<sub>2</sub> induce growth factor release from dentine matrix to produce reparative dentine formation (Ghoddusi *et al.*, 2014).

The acid etch technique uses phosphoric acid of pH 3.5. If 1.5 mm thickness of dentine remains the pulp is not directly injured but the microleakage through the dentine could irritate the pulp (Stanley, 1993). The technique can be used directly on the pulp with 2% sodium chloride before application of 35% phosphoric acid, followed either with composite resin or Ca(OH)<sub>2</sub> (Pameijer and Stanley, 1998). However, a lower concentration of 10% phosphoric acid caused no pulp irritation whereas 35% phosphoric acid could be destructive and irritant to the pulp (Pameijer and Stanley, 1998, Schuurs *et al.*, 2000). Several studies have examined the cytotoxic effects of endodontic materials on pulp (Dahl, 2005, Torabinejad and Walton, 2009). However, it is known that certain pulp capping materials may facilitate the formation of dentine and preservation of the vitality of the pulp by allowing proliferation of pulp stem cells and differentiation (Min *et al.*, 2009). The available pulp capping materials are now discussed.

#### **1.4.1 Calcium hydroxide**

Calcium hydroxide has been recommended as the best material for human pulp capping (Accorinte *et al.*, 2005). It was originally established in dentistry by Herman in

Germany (1920) due to its biocompatibility and therapeutic ability. It is a white powder with no scent and is usually mixed with water to give a paste (Demarco *et al.*, 2001, Farhad and Mohammadi, 2005). The formula is  $\text{Ca(OH)}_2$  and the separation of the  $\text{Ca}^{2+}$  and  $\text{OH}^-$  will result in a bactericidal effect (Fava and Saunders, 1999). It has been recommended in direct and indirect pulp capping, treatment of apexogenesis, apexification, root resorption, root fracture, intracanal dressing and tooth re-implantation and is radiopaque (Fava and Saunders, 1999, Farhad and Mohammadi, 2005). *In-vitro* studies have determined a direct relation between the vehicle, ionic separation and antibacterial activity (Estrela and Pesce, 1997). Three types of vehicles are common, aqueous, viscous and oily, depending on the clinical application. The aqueous vehicle is a water soluble substance that includes water, saline, ringer's solution and dental anaesthetic, with or without adrenaline. Water has been used as the vehicle for direct pulp capping in humans (Horsted *et al.*, 1985). Viscous vehicles include glycerine, polyethyleneglycol and propyleneglycol, and such materials are water soluble and facilitate gradual, uniform ionic separation which occurs over a longer period than in aqueous product. The other vehicles include non-water soluble substances such as olive and silicone oil, camphor and metacresyl acetate. The latter vehicle has the lowest solubility and diffusion within the tissue is rarely indicated, and only used in clinical cases where low ionic dissociation is required (Fava and Saunders, 1999). The  $\text{Ca(OH)}_2$  particle size and shape have been shown to affect penetration of microorganisms into dentinal tubules in a study using a flow particle image analyser (FPIA) (Komabayashi *et al.*, 2009).

Different forms of calcium hydroxide, powder, paste or cement, may have differing ion release and antibacterial properties (Fisher and Shortall, 1984). Calcium hydroxide cement might be preferred to pure  $\text{Ca(OH)}_2$  because of its better sealing ability and

bactericidal activity but pure  $\text{Ca(OH)}_2$  has the ability to release double the amount of calcium compared to cement, which promotes stimulation of dentine bridge formation (Shubich *et al.*, 1978). The  $\text{Ca(OH)}_2$  paste, in the absence of bacterial leakage, has a better probability of pulp tissue healing than the cement (Shubich *et al.*, 1978). Comparing the hard setting  $\text{Ca(OH)}_2$  Dycal to paste type, Dycal promoted the formation of a hard tissue barrier directly on the pulp capping material away from the necrotic zone in contrast to the paste type where the tissue formed the barrier away from the capping material (Tronstad, 1974). This was confirmed by Mjore *et al.* who showed that  $\text{Ca(OH)}_2$  cement had the ability to initiate dentine bridge formation with characteristic odontoblast like cells. The matrix formation was initiated directly on the exposed area during the 14 days of observation (Mjor *et al.*, 1991). A study comparing the effectiveness of composite resin to calcium hydroxide has suggested that although the resin has a better sealing ability than calcium hydroxide they have similar results with respect to pulp inflammation, regardless of bacterial environment (Murray *et al.*, 2002). Although  $\text{Ca(OH)}_2$  has been the material of choice for decades, some limitations are recognized. Hard setting  $\text{Ca(OH)}_2$ , Dycal, dissolves within 1-2 years and is degraded by acid etching (Schuurs *et al.*, 2000). The dentine bridge formation by  $\text{Ca(OH)}_2$  was incomplete and contained tunnels. The pulp inflammation was due to the ingress of bacteria through the incomplete bridge formed (Parolia *et al.*, 2010). Tunnels in the dentine bridge can result in 50% of pulp tissue becoming necrotic because of bacterial microleakage (Cox *et al.*, 1985).  $\text{Ca(OH)}_2$  does not have the ability to adhere to dentine, therefore there have been improvements, for example, Prisma VLC Dycal is composed of urethane dimethacrylate with initiator and accelerator. It is able to bind to dentine and is more resistant to acid etching (Farhad and Mohammadi, 2005). The chemically cured  $\text{Ca(OH)}_2$  cements cannot withstand the force under amalgam condensation and also

inhibit the polymerization of bonding resin under composite restorations (Olmez *et al.*, 1998, Schuurs *et al.*, 2000).

#### **1.4.1.1 Early studies on calcium hydroxide**

In direct pulp capping,  $\text{Ca(OH)}_2$ , with the high pH, can induce formation of dentine bridges (Lim and Kirk, 1987). A dentine bridge is a calcific hard tissue barrier laid down by the pulp, sometimes under the influence of calcium hydroxide (Pisanti and Sciaky, 1964, Attalla and Noujaim, 1969). It must be recognised that dentine bridges may develop in germ free animals with no covering of exposed pulps, as seen in the classic study by (Takehashi *et al.*, 1965). It will reduce inflammation and prevent macrophage function during remineralisation (Segura *et al.*, 1997). The amount of hydroxyl ion penetration into the dentinal tubules depends on permeability, diameter, length of dentinal tubules as well as the particle size and charge. The other factor that prevents the hydroxyl ion diffusion into dentine is the buffering capacity of the dentine and dentine thickness is also an important factor (Pashley, 1996). In a 16 day experiment of teeth filled with calcium and pulp chambers covered with saline it was determined that the dentine is able to buffer the hydroxyl ions and penetration of hydroxyl ions is very slow (Wang and Hume, 1988). Hydroxyl ion separation is responsible for the change in pH and therapeutic properties and selecting an appropriate vehicle is important to permit an environment suitable for the fibroblast and odontoblast cells to initiate the hard tissue barrier formation and promote healing of the pulp (Stamos *et al.*, 1985, Zmener *et al.*, 2007). The effect of  $\text{Ca(OH)}_2$  on microorganisms was established by Bystrom *et al.* who showed that several intracanal microorganisms could not survive the alkaline environment (Bystrom *et al.*, 1985). The toxicity is due to hydroxyl ions which damage the bacterial cytoplasmic membrane by lipid peroxidation and protein denaturation (Siqueira and Lopes, 1999). The other antimicrobial effect is

the damage to the bacterial DNA by free radicals. The hydroxyl ions of  $\text{Ca(OH)}_2$  react with the bacterial DNA and provoke splitting of the DNA strand, consequently resulting in arrest of DNA replication with gene loss (Farhad and Mohammadi, 2005).

#### **1.4.1.2 Effect of calcium hydroxide on hard tissue bridge formation**

The toxicity and irritation which results from the use of  $\text{Ca(OH)}_2$  promotes pulp healing via necrosis and three zones can be identified under the  $\text{Ca(OH)}_2$  layer (Schuurs *et al.*, 2000). Primarily, a superficial zone is initiated due to pressure, secondly an intermediate zone as a result of oedema and thirdly the coagulation zone with necrosis generated by partial neutralization of the hydroxyl ions by the pulp tissue and plasma proteins (Schroder, 1985). This was shown earlier by Zander and Glass who saw a layer of reparative dentine with odontoblast cells near the necrotic zone within four weeks (Zander and Glass, 1949, Bergenholtz, 2005). Deposition of minerals was seen in the coagulation area, where odontoblast like cells differentiated to produce a tubular like dentine, identified as a mantle dentine, which is irregular predentine formed by the elongated odontoblast like cells (Tziafas, 2004, Modena *et al.*, 2009). Similar layers are seen in pulpotomy treatments and include a mummified superficial layer, an intermediate layer which is initiated by the neutralization of hydroxyl ions and an apical layer comprised of inflammatory cells and macrophages which remove the necrosed tissue (Pitt Ford and Roberts, 1991).

After pulp exposure, inflammatory cells migrate and proliferation of the mesenchymal or progenitor cells produces fibroblast cells (Schroder, 1978). Organization of fibroblasts and endothelial pulp cells near to the necrotic zone was observed. The fibroblast cells synthesise a calcified matrix and induction of fibronectin reparative molecules promotes odontoblast like cells to differentiate and commence reparative

dentine formation (Fernandes *et al.*, 2008). Collagen was observed in four days within the firm necrotic zone and after seven days, initiation of cellular activity was seen in rat pulp as a radiopaque area (Harrop and Mackay, 1968, Schuurs *et al.*, 2000). After one month of observation, there was an irregular hard-tissue barrier initiated within the external surface. After three months, this barrier had a superficial layer of irregular tissue and a layer of dentine like tissue close to the pulp (Schroder and Granath, 1972).

The hard tissue barrier has been shown to have tunnels containing blood vessels that maintain the calcium ion supply (Goldberg *et al.*, 1984, Pereira *et al.*, 2000). There are also cells between the necrotic and calcified zone. The calcific hard tissue barrier protects the pulp but also stimulates the odontoblast cells to differentiate (Seltzer and Bender, 1958, Stanley and Pameijer, 1997). Sometimes the calcific barrier is seen after inflammation and necrosis of the pulp and would undermine the contention that hard tissue formation is a sign of pulp healing. Obtaining an intact cavity seal is important in order to protect the pulp from bacterial penetration. However, in up to 50% of cases the calcific barrier was incompletely formed meaning pulp inflammation can arise as a result of bacterial penetration (Holland *et al.*, 1979). Inflammation, due to microleakage, inadequate sealing or early mechanical exposure cannot always be prevented by dentine bridge formation, which is less efficient than intact dentine (Cox *et al.*, 1985).

Given that calcium hydroxide has the ability to induce a hard tissue barrier through irritation there have been many debates regarding the source of the calcium ions incorporated within tissues (Cvek *et al.*, 1987, Lim and Kirk, 1987). An autoradiographic animal study showed that the calcium responsible for hard tissue barrier formation was released from the pulp tissue and not from the calcium hydroxide material (Sciaky and Pisanti, 1960). In contrast, Holland *et al.* showed that in pulpotomized dog teeth, using three different pulp capping materials, the hard tissue

barrier formation was as a result of calcium release from calcium hydroxide material which was detectable in the necrotic zone (Holland *et al.*, 1982). Holland *et al.* determined that the amount of calcium released is important for dentine bridge formation and gained a 20% increase in calcific tissue formation by increasing the catalyst to paste ratio (Holland *et al.*, 1978).

#### **1.4.2 Mineral trioxide aggregate**

Mineral trioxide aggregate (MTA) has been reported as a reliable, effective pulp capping material and has been increasingly popular since around 1990 (Paranjape *et al.*, 2010). It is a type of Portland cement with bioactive properties, comprised of a fine particle, hydrophilic powder mixed with sterile water, which sets in the presence of moisture (Torabinejad *et al.*, 1995, Camilleri, 2008). It produces a permeable, solid colloidal gel with a pH of 10-12.5 (Watts *et al.*, 2007). MTA contains calcium hydroxide and calcium silicate hydrate, which gives high alkalinity (Lee *et al.*, 2004, Camilleri, 2007, Camilleri, 2008). It also contains bismuth dioxide in 4:1 ratio and exhibits radiopacity (Torabinejad *et al.*, 1995, Camilleri *et al.*, 2005). MTA is composed of 50-70% of calcium oxide and 15-25% silicone oxide (Torabinejad *et al.*, 1995). It also contains a minor portion of silicone dioxide, magnesium oxide, potassium sulphate and sodium sulphate (Dammaschke *et al.*, 2005). Compared to original Portland cement, MTA has small particle size, is less toxic and has a longer setting time allowing time for preparation, making it more applicable in clinical treatments (Islam *et al.*, 2006). The hydration reaction between tricalcium silicate and dicalcium silicate is essential for the improvement of material strength in the setting process (Dammaschke *et al.*, 2005). The compressive strength of MTA increases in moisture up to 21 days (Hachmeister *et al.*, 2002).



#### 1.4.2.1 Research studies on different types of MTA

There are two types of MTA, the first commercially accessible MTA was grey in colour, called grey MTA (GMTA) but because of tooth discoloration and aesthetic consideration white MTA (WMTA) was developed (Kratchman, 2004). Commercial MTA was introduced as ProRoot-MTA by Dentsply. The main differences between the grey and white MTA is that the grey contains tetracalcium aluminoferrite which is an iron based material causing tooth discoloration in contrast to white MTA (Moghaddame-Jafari *et al.*, 2005). Basically GMTA is comprised of dicalcium and tricalcium silicate and bismuth oxide, while WMTA consist of tricalcium silicate and bismuth oxide (Parirokh and Torabinejad, 2010). The concentration of aluminium, magnesium and iron is lower in WMTA than GMTA and lower magnesium may produce the lighter colour. WMTA has a smaller crystal size as shown by qualitative x-ray analysis (Song *et al.*, 2006, Asgary *et al.*, 2009). WMTA has higher solubility and pH after mixing than GMTA but the solubility becomes constant and pH reduces as time passes in GMTA (Fridland and Rosado, 2005). The clinical outcome of pulp capping was evaluated in permanent carious teeth, comparing non setting calcium hydroxide to GMTA in partial pulpotomy, and determining promising outcomes for GMTA, though further research was encouraged (Qudeimat *et al.*, 2007).

There have been different hypotheses regarding the antibacterial and antifungal activity between GMTA and WMTA. Studies suggested that both materials possess antibacterial and antifungal activity due to their pH level rising to 12.5 within three hours (Al-Hezaimi *et al.*, 2005, Tanomaru-Filho *et al.*, 2007), although an *in vitro* study reported that GMTA has no antibacterial activity against *E. faecalis* (Carvalho *et al.*, 2007). A study by Eskandarizadeh *et al.* established the antibacterial activity of MTA relating to its high pH level and preferred GMTA in comparison to calcium hydroxide in pulp

capping of 42 healthy dogs (Eskandarizadeh *et al.*, 2006). The previous study was supported by Asgary *et al.* using WMTA in dogs and evaluating the result by scanning electron microscopy (Asgary *et al.*, 2006).

The effect of WMTA on cell proliferation, viability and regeneration has been determined *in vitro* in mouse odontoblast cells and undifferentiated pulp cells exposed to WMTA. Flow cytometry analysis determined that within 24 hours there was no sign of apoptosis, instead the proliferation rate of both odontoblast and pulp cells increased. There was an enhanced DNA synthesis which could promote dentine-pulp regeneration *in vitro* (Moghaddame-Jafari *et al.*, 2005). This study was further confirmed by another *in vitro* study in rat pulp tissue using WMTA compared to Ca(OH)<sub>2</sub> as a control group. They also observed, using histochemical analysis, that WMTA was capable of initiating odontoblast like differentiation in pulp capping procedures and also tertiary dentine formation with minimum apoptosis (Masuda-Murakami *et al.*, 2009). In a study investigating tertiary reparative dentine formation in rat, the application of MTA on exposed pulp in molar teeth led to dentine bridge formation after seven days (Kuratate *et al.*, 2008). The proliferation of progenitor cells and their differentiation into odontoblast like cells was evaluated underneath MTA material. A study comparing bridge formation and pulp inflammation in human teeth capped with Ca(OH)<sub>2</sub> and WMTA, showed no obvious variation between the two materials (Iwamoto *et al.*, 2006). This is in contrast to Sawicki *et al.* who determined that pulp capping of human teeth with WMTA in comparison to Ca(OH)<sub>2</sub> provided dentine bridge formation with less pulpal inflammation in premolar and molar teeth (Sawicki *et al.*, 2008).

An *in vivo* study on HDPCs confirmed the increased proliferation with MTA within 14 days in human dental pulp compared to Ca(OH)<sub>2</sub>. It was suggested that the proliferation is due to regular release of calcium ions which initiate the mineralization (Schmittgen *et*

*al.*, 2000). The toxicity of GMTA was measured by cell viability assay in human periodontal ligament and compared with other materials. After 24 hours setting time the GMTA in high concentration presented with the lowest toxicity (Keiser *et al.*, 2000). An *in vitro* study on HDPCs cultured with MTA for up to seven days indicated that the material enhanced HDPC proliferation, differentiation capacity to odontoblast like cells and hard bridge formation. The findings of this study pointed toward possible positive effects of MTA on the repair process of injured human dental pulp (Paranjape *et al.*, 2010). All of these effects contribute to the biocompatibility potential of MTA in pulp capping procedures (Faraco Junior and Holland, 2004). The advantages of MTA were also supported by Aeinehchi *et al.* comparing GMTA with Ca(OH)<sub>2</sub> in pulp capping of permanent molar teeth. Histological assessment estimated that GMTA gave a thicker dentine bridge and less inflammation within six months of observation (Aeinehchi *et al.*, 2003).

A study has shown similar healing efficiency in healthy pulp tissue treated with GMTA and WMTA following mechanical pulp exposure and both are superior to traditional Ca(OH)<sub>2</sub> (Roberts *et al.*, 2008). Parirokh *et al.* analysed the dentine calcific bridge formation in mechanically pulp exposure and following treatment with WMTA and GMTA in dogs' teeth. After two weeks of observation, inflammation was absent with no significant difference between the WMTA and GMTA (Parirokh *et al.*, 2005). In an *ex vivo* immunohistochemical study of immature permanent human teeth treated with GMTA it was demonstrated that GMTA was capable of remineralising pulp tissue after a day, in contrast to Ca(OH)<sub>2</sub> which took 28 days to initiate remineralisation (Ford *et al.*, 1996). Response of canine pulp tissue to pulp capping material, where pulp was mechanically exposed and treated with MTA and Ca(OH)<sub>2</sub>, showed that after four months of observation, no sign of inflammation was present and a dentine bridge was

formed, compared to Dycal where inflammation occurred and formation of a calcific bridge was noticed in only a few samples (Zhu and Xia, 2003). The early review of MTA activity and function compared to  $\text{Ca(OH)}_2$  was established by Ford *et al.* in monkey teeth (Ford *et al.*, 1996). They determined the superiority of MTA compared to  $\text{Ca(OH)}_2$  in stimulating dentine bridge formation and inducing less pulpal inflammation (Faraco and Holland, 2001). Thus several studies on human teeth agree that the calcific dentine bridge formation is more rapid and results in thicker dentine with MTA in comparison to  $\text{Ca(OH)}_2$  (Aeinehchi *et al.*, 2003, Min *et al.*, 2008, Moore, 2009).

Primarily MTA has been used in endodontics because of its potential to seal natural, pathological and iatrogenic communications between the pulp space and periradicular tissues. It seals the communication pathway between the teeth and the oral cavity (Torabinejad and Chivian, 1999, Mah *et al.*, 2003, Shahi *et al.*, 2009). MTA has proven to be the most biocompatible pulp capping material in *in-vivo* and in an *ex-vivo* study in mouse fibroblast cells comparing Portland cement to MTA (Saidon *et al.*, 2003). In the study by Min *et al.* in human cultured pulp cells, they determined that Portland cement was biocompatible and not cytotoxic (Min *et al.*, 2009). MTA uses a 3:1 powder to liquid ratio and the setting time can be from five minutes to four hours; temporary placement of a damp cotton pellet on the material directly has been suggested until a follow-up appointment can be arranged (Torabinejad *et al.*, 1993, Roberts *et al.*, 2008). Increased solubility longer term has been raised as an issue in clinical applications (Fridland and Rosado, 2005).

Comparing MTA strength after 24 hours to amalgam, intermediate restorative material (IRM) and super-EBA shows reduced compressive strength but no other crucial variation after three weeks (Torabinejad and Chivian, 1999). Sealing capacity and biocompatibility is better than amalgam and equal or superior to IRM in *in vivo* and *in*

*vitro* experiments, although an *in vitro* study has shown that MTA has less retention than glass ionomer (GIC) and zinc phosphate ( $ZnPO_4$ ) and is not suitable for luting (Nakata *et al.*, 1998, Vargas *et al.*, 2004). A thicker layer of MTA increases resistance to displacement (Kogan *et al.*, 2006).

Mineral trioxide aggregate can be used for pulp capping procedures, repair of perforation and furcation, apexification and root end filling and closure (Lee *et al.*, 1993, Ferris and Baumgartner, 2004, Yildirim *et al.*, 2005, Felipe *et al.*, 2006, Nair *et al.*, 2008, Sarris *et al.*, 2008, Saunders, 2008). Histological evaluation of dentine bridge formation, using SEM, was performed on dogs' teeth after pulp capping. MTA was compared to  $Ca(OH)_2$  and acid etch dentine bonding treatment of exposed pulps, evaluated for 50 and 150 days. The inflammation of the pulp was not significantly different between the MTA and untreated control group. However, histological evaluation determined that MTA protects the pulp by complete dentine bridge formation in comparison to  $Ca(OH)_2$  and acid etch dentine bonding (Dominguez *et al.*, 2003). MTA has a faster rate of pulp tissue healing than calcium hydroxide, although these two materials show similar effects with regard to the processes of dentine bridge formation including induction of reparative dentinogenesis and stimulation of odontoblast differentiation (Moore, 2009). After 60 days of evaluation, it was demonstrated that both MTA and calcium hydroxide showed similar results with respect to success of pulp capping. Another study suggested that the CaO mesoporous-silica had a greater potential for mineralization of the pulp and viability of mesenchymal cells compared to Calcium hydroxide (Kuo *et al.*, 2009).

The effect of MTA on cells has been variously reported. Bismuth oxide release and interaction with calcium hydroxide precipitation may inhibit cell proliferation in culture (Camilleri *et al.*, 2004). However in contrast, an *in vitro* pulp capping study with MTA

material, determined that MTA stimulates proliferation (Moghaddame-Jafari *et al.*, 2005). Antibacterial activity is limited to various facultative bacteria and not anaerobic bacteria and compared to calcium hydroxide is very low (Torabinejad *et al.*, 1995). A lower concentration seems to favour antibacterial activity (Al-Hezaimi *et al.*, 2006).

An *in-vitro* study with MTA on mouse odontoblasts and undifferentiated pulp cells has shown that the MTA has the ability to initiate slight proliferation of odontoblast cells which relates to the ability of the MTA to initiate dentine bridge formation (Moghaddame-Jafari *et al.*, 2005). It may be concluded that MTA has the ability to regenerate dental pulp like tissues *in vitro*. Although the effectiveness of MTA as a pulp capping material in permanent teeth has been reviewed in several clinical studies and case reports (Aeinehchi *et al.*, 2003, Witherspoon *et al.*, 2006) further investigations to confirm that MTA could replace the Ca(OH)<sub>2</sub> are required. Few studies have evaluated the histological responses to MTA pulp capping material in human teeth (Minamikawa *et al.*, 2009, Parolia *et al.*, 2010). The development of hydroxyapatite by MTA in exposed pulps confirms the biocompatibility of this material, but further clinical investigation should be encouraged (Moretti *et al.*, 2008).

### **1.4.3 Propolis**

Propolis has been called 'bee glue' and is named from the Greek word pro-, meaning defence and polis, meaning city (Ghisalberti, 1979, Burdock, 1998b). It is a product of bees and is a natural, resinous, sticky gum made as bees harvest from different plants with addition of salivary enzymes and metabolic products (Marcucci *et al.*, 2001, Sonmez *et al.*, 2005). It has a variety of colours, from yellow, green, reddish to dark brown and a distinctive aroma (Ramos and Miranda, 2007). Consistency varies according to the source and temperature, soft at 25°C and liquid at 60-70°C.

Attempts have been made to separate propolis as “balsam propolis” and ethanol, water, 95 % ethyl ether, chloroform and methanol have all been tried as solvents (Ghisalberti, 1979, Marcucci, 1995). It was suggested that the composition of blossom exudates and propolis is alike (Bankova *et al.*, 1995). Propolis contains vitamins and some minerals, for example iron, calcium, aluminium and copper (Deblock-Bostyn, 1982). That in beehives is composed of 50% balsam resin (flavonoid and phenolic acid), 10% essential and odour oils, 30% wax , 5% pollen, 5% other materials (Monti *et al.*, 1983, Burdock, 1998). Up to 200 chemical compounds are found in propolis from different provinces with esters, fatty and aromatic acids, carbohydrates, aliphatic acids, aldehydes, amino acids, terphenoids, steroids and others, flavonoids and phenolic acid being the important constituents (Bankova *et al.*, 2000, Havsteen, 2002, Rushdi *et al.*, 2014). Antibacterial activity may originate from phenolic compounds, fatty acid esters and cinnamic acids (Xu *et al.*, 2009). The composition may or may not vary between different geographical areas (Johnson *et al.*, 1994, Marcucci, 1995a, Nieva Moreno *et al.*, 2005, Silici and Kutluca, 2005). Propolis is classified into Brazilian and European types, with p-coumaric acid by-products and flavonoids respectively, the latter is also found in Chinese propolis (Fujimoto *et al.*, 2001, Bankova, 2005). Other valuable components of propolis are histamine and serotonin that can defend against hypersensitivity reactions (Almas *et al.*, 2001).

#### **1.4.3.1 Biological characteristics of propolis**

The antibacterial activity of propolis in the oral cavity has been investigated *in vivo* and *in vitro* against 75 bacterial strains (Meresta and Meresta, 1985). Almost all the bacteria were shown to be sensitive to propolis, especially *Staphylococci* and *Streptococcus mutans* plus gram positive rods and cocci, *Escherichia coli* and human tubercle bacillus (Grange and Davey, 1990). This was confirmed by the *in vitro* clinical evaluation of

antibacterial activity of propolis (Steinberg *et al.*, 1986). This activity is due to flavonoids, phenolic, aromatic esters and acid present in resin, caffeic and ferulic acids (Meresta and Meresta, 1985, Marcucci, 1995). Anti-inflammatory flavonoids prevent the synthesis of prostaglandin and sterilize the tissue (Kujumgiev *et al.*, 1999). They stimulate and support the immune system by initiating phagocyte activity and they also reduce discharge of free oxygen radicals (Burdock, 1998a, Al-Shaher *et al.*, 2004b).

CAPE (Caffeic Acid Phenethyl Ester) is also an active component which has various biological actions, antibacterial, anticarcinogenic, anti-inflammatory (Banskota *et al.*, 2001). Antiviral and fungal action plus anti-oxidant, regenerative and anaesthetic effects have also been identified (Ghisalberti, 1979). CAPE has been used as a pulp capping material, due to anti-inflammatory and anticarcinogenic effects (Grunberger *et al.*, 1988). There has been debate about the effect of CAPE on dental pulp cells and repair after pulp capping. A study by Sabir *et al.*, determined that propolis helps regenerate and reduce human pulp inflammation (Sabir *et al.*, 2005) in contrast to another study which suggested that more than 2 mg of propolis is toxic for fibroblast cells (Al-Shaher *et al.*, 2004). A study by Djurica *et al.*, evaluated the effect of CAPE from Germany in dental pulp of pigs and compared it to Ca(OH)<sub>2</sub> to assess pulp healing and bridge formation. After 4-14 days of evaluation they determined that inflammation was present but the control group that was capped with Ca(OH)<sub>2</sub> had formed a dentine bridge with complete healing of the pulp. CAPE samples showed no sign of dentine pulp repair and instead of calcific bridge formation scar fibrous tissue was formed (Djurica *et al.*, 2008).

The cytotoxic effect of propolis was evaluated *in vitro* on different cell lines and the cytostatic effect of propolis on human cancer cells (Hladon *et al.*, 1980, Grunberger *et al.*, 1988).



Propolis has been in use for many years in supplements and natural remedies and in dentistry, both endodontically and periodontally, promoting bone regeneration (Kosenko and Kosovich, 1990, Ferreira *et al.*, 2007). Dental pulp rejuvenation was observed and histological evidence verifies that hydro-alcoholic solution propolis stimulates epithelial repair after tooth extraction. A study of six different propolis samples from different geographic areas looked into their cytotoxicity on human gingival fibroblast cells and their antibacterial effect on oral pathogens (Scheller *et al.*, 1978, Magro Filho and de Carvalho, 1990). The propolis was selected from Germany, USA, Turkey and Australia. All reduced periodontal microorganisms at low concentration. However propolis from USA and Australia was shown to retain the best antibacterial activity. Propolis from all the sources was cytotoxic to gingival fibroblast cells and now it is recognized that a lower concentration is preferable for antibacterial activity (Sonmez *et al.*, 2005). This has also been observed with the antifungal property of Brazilian flavonoid against the *Candida albican* species (Ota *et al.*, 2001, Sawaya *et al.*, 2002). Propolis was evaluated as a direct pulp capping agent by examining the morphologic, radiographic and clinical status of direct and indirect pulp capping using an alcoholic solution of propolis, which was compared with zinc oxide in 150 teeth. Morphological evidence determined that the indirect pulp capping process was usually followed by pulpitis after secondary dentine formation in comparison to direct pulp capping where reparative dentine had been formed, followed by remineralisation and no sign of pulp necrosis (Ionita *et al.*, 1990). This conclusion was supported by a recent study of direct pulp capping in rats, comparing propolis-derived flavonoids with non-flavonoid extract, using zinc oxide eugenol as a control group. They concluded that the propolis-derived flavonoid could delay pulp inflammation and promote dentine bridge formation by stimulating the reparative dentine. However, treatment with the non-

flavonoid resulted in pulp inflammation within 4 weeks and there was no sign of dentine bridge formation (Sabir *et al.*, 2005).

The effect of propolis on human fibroblast cells collected from periodontal ligaments and pulp revealed that using 0-20 mg/ml of flavonoid propolis compared to Ca(OH)<sub>2</sub>, allowed cells to retain 75% viability, showing less toxicity to propolis in comparison to Ca(OH)<sub>2</sub> after 20 hours. Propolis was thus suggested as an inter canal treatment (Al-Shaher *et al.*, 2004). The histological response of healthy human pulp after pulp capping was evaluated by Parolia *et al.* using 36 healthy human premolars with three different pulp capping materials, over 15 and 45 days. The first group was treated with 70% ethyl alcohol propolis from USA, the second group was ProRoot MTA and third group was Dycal treated. There was no significant difference between the three pulp capping materials used, although more pulp inflammation occurred with Dycal and more dentine bridge formation was seen in MTA and propolis treated groups (Parolia *et al.*, 2010).

Propolis has been shown to exhibit antiproliferative, antioxidant, anti-inflammatory, antibiotic activities. It was also found to cause chromatin condensation and caspase activation. Antitumor mechanisms of propolis have been shown to be through the induction of apoptosis and inhibiting of telomerase expression. In the study by Gunduz *et al.* they demonstrated that propolis decreased the number of malignant cells significantly compared to the propolis-free cell cultures (Gunduz *et al.*, 2005).

A study of dentine regeneration in guinea pigs documented stimulation of dental pulp stem cells by propolis. This provides evidence that this material has advantages over calcium hydroxide as a capping agent in vital pulp therapy. In addition to producing no pulpal inflammation, infection or necrosis this material induces the production of high quality tubular dentine (Ahangari *et al.*, 2012).

#### 1.4.4 Other pulp capping materials

In an *in vitro* study, researchers looked at the regeneration of dentine pulp like tissue in cleaned root canal spaces and suggested that stem cells from the apical papilla (SCAP) had the ability to induce odontoblast cells and regenerate dentine like tissue *in-vitro* (Huang *et al.*, 2010).

In a study using rat pulp cells Emdogain was compared with Vitapex, containing calcium hydroxide, as a pulp capping agent. The results suggested that Emdogain has an effect on reparative dentine formation by inducing bone morphogenetic protein expression (Kaida *et al.*, 2008).

Direct pulp capping in animal studies determined that the development of hard tissue, such as dentine structure, may be related to initiation of biologically active and morphogenetic growth substitutes, which are extracellular matrix molecules. The potentiality of pulp capping materials for induction of dentinogenesis was evaluated on two month old rats where the mechanically exposed pulp of a molar tooth was treated with sterile 4% methylcellulose gel, including either 400ng of human insulin-like growth factor I (rhIGF-I) or saline. After 28 days it was suggested that the rhIGF-1 was capable of inducing dentinogenesis and formation of reparative dentine (Lovschall *et al.*, 2001). Stimulation and initiation of autogenous osteogenic proteins or bone morphogenic proteins (BMP) 2,4 and 7 induces calcific bridge formation and dentinogenesis in human teeth in 28 days, suggesting that BMP may be used to regenerate hard tissue in carious teeth (Nakashima, 1994). The same result was confirmed by using sialoprotein, an osteogenic protein that is bioactive, added to mechanically exposed pulps of rats. After 30 days of evaluation, homogenous formation of dentine like structure was observed near to the pulp chamber, confirming the

biocompatibility of the material (Decup *et al.*, 2000). Enamel matrix proteins caused formation of a tubular reparative dentine in pig's pulp after four weeks of evaluation (Nakamura *et al.*, 2001). Other studies have suggested that all bioactive derivatives are capable of producing dentine bridge formation and mineralization of pulp (Goldberg *et al.*, 2003).

Recent studies on MTA established a novel Endodontic cement (NEC) containing different calcium compounds, developed by Asgary *et al.* The clinical use of this material is similar to MTA and able to produce hydroxylapatite. It has adequate physical properties and sets within one hour in an aqueous environment with good sealing ability and bactericidal properties (Asgary *et al.*, 2008). The results of an *in vivo* study on dogs showed that MTA and NEC showed the same favourable results as other pulp capping materials and were preferred over calcium hydroxide. NEC, with phosphorous as a major component, has a surface composition similar to surrounding dentine (Asgary *et al.*, 2008). A cell culture study on L929 (mouse fibroblast cells) suggested that both MTA and NEC demonstrated no cytotoxic effect on cells indicating that NEC could be an alternative material to MTA according to its physical properties (Ghoddusi *et al.*, 2008). According to the results of an *in vivo* study on premolar teeth treated with NEC it was suggested that NEC, induces formation of a thicker dentinal bridge, less inflammation and presence of odontoblast cells in comparison to MTA. NEC could be considered as an alternative pulp capping material to MTA but further clinical validation is required (Zarrabi *et al.*, 2009).

Finally, there are different conclusions on the effect of EMD (Enamel matrix derivative) on pulp regeneration (Ghoddusi *et al.*, 2014). Comparing EMD to Ca(OH)<sub>2</sub>, EMD was associated with greater reparative dentine formation whilst other workers found the

opposite, less inflammation and more dentine formation in  $\text{Ca(OH)}_2$  treated teeth (Nakamura *et al.*, 2002, Kiatwateeratana *et al.*, 2009).

## **1.5 New approaches to endodontic treatment-tissue engineering**

Reconstructive surgery is a commonly prescribed treatment modality for individuals who suffered tissue loss as a result of traumatic injuries, genetic abnormalities, neoplasia or ageing. Such surgical procedures are highly costly and may have serious complications. Tissue or organ malfunction may result in the need for a replacement therapy. However, where a suitable donor organ or tissue is not available there is the need for an alternative regenerative procedure (Daar and Greenwood, 2007, Mason *et al.*, 2008). There has been great progress in science in the development of biocompatible materials that may be used to replace the natural human tissue (Wang *et al.*, 2013). More recently there has been much scientific research in the application of tissue engineering, in particular via the application of embryonic stem cells, both *in vivo* and *in vitro*, for regenerative medicine (Kaigler and Mooney, 2001). Regenerative medicine is a fundamental development in tissue engineering which has made a major contribution to our understanding of the use of biocompatible functional tissues to restore damaged organs and tissues. One of the aims of tissue engineering is to develop alternative strategies for tissue replacement using stem cells *in vitro*, combined with appropriate factors to stimulate the regeneration of specific tissue types *in vivo* (Baum and Mooney, 2000, Nakashima and Akamine, 2005). Tissue engineering has in the past been defined in various ways. One of earliest definitions described tissue engineering as “an interdisciplinary field provided to regenerate tissue resembling the natural human tissues in order to restore the injured organs” (Langer, 2000). This has subsequently been refined over the years with the advent of new developments in science and

technology associated with regenerative medicine (Rahaman and Mao, 2005). Tissue engineering can be considered as either restoring or replacing damaged tissue.

Current practice in endodontic therapy largely fails to take into consideration the important role of pulp tissue in retaining the vitality of the tooth and usually does not attempt the full restoration of tooth function. Although most materials used for replacing the pulp are considered to be biocompatible it has been shown that many endodontic sealers and filling materials may result in tooth discoloration (van der Burgt and Plasschaert, 1985, Van der Burgt *et al.*, 1986). Others study has suggested that removal of pulp tissue from the tooth will decrease the survival rate of tooth and increase the probability of tooth fracture (Doyon *et al.*, 2005). There have been various developments in regenerative endodontic therapy, including the use of dental pulp stem cells for pulp regeneration (Kabir *et al.*, 2014). Initial results suggest that this can be successful in pulp tissue regeneration and may have the potential in increasing the retention of teeth for a longer period of time (Iohara *et al.*, 2004). A study by Nakashima and Akamine, investigated alternative, biocompatible materials e.g. growing pulp cells on different types of scaffolds to repair the damaged tissue, replacing and resembling the original tissue have shown some promise (Nakashima and Akamine, 2005). Suitable pulp replacement tissues can be generated by seeding pluripotent stem cells onto porous scaffold materials (Haug, 2009). Additionally, it has been suggested that the use of these tissues can be improved by stimulating the cells to differentiate into specific tissue types (Young *et al.*, 2002, Cordeiro *et al.*, 2008). For tissue engineering to be successful three main requirements need to be met: firstly appropriate pluripotent stem cells must be obtained and combined with suitable morphogenic proteins or signals for differentiation. Secondly an appropriate biological scaffold needs to be selected and finally a set of culture conditions must be identified to facilitate the

regeneration of the required tissue characteristics (Nakashima and Reddi, 2003). Research into the use of different biocompatible scaffolds has progressed significantly. Many suitable materials have been identified that fulfil the desired characteristics, e.g. nontoxic, porous and biodegradable and that also allow the supply of nutrients and oxygen to the cells for supporting cell viability and tissue development. Four main types of scaffolds have been identified: biological, polymeric and glass or ceramic materials (Vats *et al.*, 2003). The accepted biomaterials should be biocompatible and several types of material have been used successfully. Scaffolds have many different structures and types, the types are synthetic and natural polymers and the structure can be: solid, permeable or even injectable gels. A common type of natural polymer used in cartilage and bone engineering is lyophilized collagen in association with freeze-dried bone and glycosaminoglycan (Sharma and Elisseeff, 2004). Cells grown on these scaffolds are often supplemented with desirable growth factors by means of utilizing sheet based lyophilized tissue. Such, osteo-inductive scaffolds have been used to re-establish damaged tissues within the oral cavity renovating the periodontal ligaments, cementum and alveolar bone (Kaigler and Mooney, 2001). Types of biocompatible glass ceramics or pure ceramics can also be used as scaffolds by bonding to the living tissue; however lack of porosity and long term degradation has limited their use (Malhotra *et al.*, 2009). Hydroxyapatite, calcium phosphate or bone augmentation scaffolds establish bone formation by being major constituents of bone that bind BMPs within the extracellular matrix (Jadlowiec *et al.*, 2003). Various types of polymer that enhance cell adhesion and *in vivo* metabolism have also been used as synthetic scaffold materials in tissue engineering. These include, polylactic acid (PLA), polyglycolic acid (PGA), and copolymers, polylactic-co-glycolic acid (PLGA); synthetic hydrogels include polyethylene glycol (PEG) based polymers (Lee and Mooney, 2001). Additionally,

such polymers modified with cell surface adhesion peptides such as arginine; glycine and aspartic acid have also been used. These scaffolds are usually biodegradable and their breakdown products are removed easily from the body, however, inflammation is a major side effect of the use of these polymers (Lee and Mooney, 2001, Burdick and Anseth, 2002, Almany and Seliktar, 2005). Synthetic polymer scaffolds provide attachment for bone tissue and can initiate and stimulate bone growth to develop prosthetic implants *in vivo* and have been used successfully to repair fractured mandibles *in situ* (Kaigler and Mooney, 2001). *In vivo* study of the morphogenic characteristics of human deciduous pulp cells, the cells grown in tooth slice biodegradable scaffolds and then transplanted into immunodeficient mice, has clearly shown that the new tissue generated resembled dental pulp and contained cells that were differentiated into odontoblast cells (Cordeiro *et al.*, 2008). This study was further corroborated by *in vivo* studies using rabbit dental pulp stem cells grown in poly (lactic-co-glycolic acid) scaffolds for 12 days. This study showed that these cells were capable of differentiating into a dentine like structure, which suggested that these cells maybe suitable for regenerating dentine pulp complex (El-Backly *et al.*, 2008). Developments in regenerative endodontic technology have resulted in different approaches to generating dental pulp tissues. Postnatal stem cells from different tissues like bone, skin, umbilical cord and buccal mucosa have been used (Kindler, 2005). Injecting postnatal stem cells using a hydrogel scaffold into root canals with open apices could be a new approach of pulp regeneration but the possibility of pulp regeneration is low. Although reliable, easy, fast delivery, least painful and easy cell harvest are benefits of injecting postnatal stem cells, their disadvantages including migration of cells into different locations and short lifespan of cell overcome their benefits (Murray *et al.*, 2007). The other approaches have included pulp and scaffold implantation. All of these techniques



require a reliable procedure, large amount of cells and a nontoxic scaffold. Standardization of the techniques requires additional *in vivo* and *vitro* experimental work (Fukuda *et al.*, 2006).

*In vivo* work using rat dental pulp to evaluate dentine bridge formation used gelatine hydrogel incorporated with various amounts of FGF-2 on collagen sponge. They demonstrated that only the moderate and high dose release of FGF-2 formed dentine like bridges on the surface of induced dental pulp. The dentine bridge formation was confirmed with up regulation of *DMP-1* gene expression (Ishimatsu *et al.*, 2009).

An *in vivo* stem cell transplant study in pulpectomized dog teeth demonstrated that transplantation of stem cells in combination with granulocyte transforming growth factor (G-CSF) generated larger amounts of regenerative dentine-pulp complex (Reed and Patarca, 2006).

Although all these recent approaches have provided vast knowledge into dental and medical treatments, it has been shown that using stem cells from individuals of different ages can limit the potential of the stem cells. This is because age will limit the proliferation of stem cells due to their short telomere length, resulting from telomere attrition during DNA replication (Blasco, 2007). To overcome the problems associated with cell ageing, much research into the molecular biology of telomeres and their associated enzymes in the cells used for tissue engineering has been carried out. Much of the data published has provided the potential to establish approaches to generating pluripotent stem cells able to regenerate functional tissue resembling the original (Reed and Patarca, 2006).

## **1.6 Stem cells**

Stem cells are from all the living tissues in our body and their unique ability is to, continuously self-renew, proliferate and differentiate from daughter cells into specialized cell or tissues (Rao, 2004). Stem cells could be categorized into totipotent (e.g. spores and zygotes, which are capable to differentiate into complete organism), pluripotent (e.g. embryonic stem cells, which can develop different varieties of tissues of the three primary germ layers) and multipotent (e.g. adult mesenchymal stem cells, that develop into specialized cell with specific functions) (Robey and Bianco, 2006). Numerous types of stem cells have been identified within the body and they are usually inactive, but they can be activated to repair, replace and regenerate tissues and cells that have been damaged or lost during disease and injury and many of these stem cells have been used for regeneration of dental tissues (Fortier, 2005, Casagrande *et al.*, 2006). Two main characteristics of stem cells are that they are unspecialized cells capable of self-renewal for prolonged periods of time throughout cell division and able to differentiate within certain conditions into specific tissues and cell types for regeneration and retain some undifferentiated cells. The ideal source of stem cells for therapy is the cells harvested from the donors cells (Badorff and Dimmeler, 2006, Cho and Clarke, 2008, Schwob *et al.*, 2008).

### **1.6.1 Embryonic stem cells**

Embryonic stem cells (ESCs) are derived from the inner cell mass of embryo blastocysts embryos. They are pluripotent undifferentiated cells and capable of undefined proliferation and multi-lineage differentiation into all tissue types. ESCs usually have longer telomeres and increased proliferation potential as result of the presence of telomerase activity and are normally viable for a longer time period

(Gronthos *et al.*, 2000). The other beneficial property of ESCs compared to adult stem cells is their plasticity which enables these cells to differentiate into different tissues. This allows ESCs to be valuable in advanced therapies (Gardner, 2002, Martin-Rendon and Watt, 2003).

Human ESCs were first isolated and cultured by Enders (1954) by growing polio virus in human embryonic kidney cells (Murray *et al.*, 2007). Where these cells are supplemented with appropriate growth factors *in vitro* they are able to differentiate into different tissues or can be transplanted into any damaged tissues where they may differentiate into similar tissue (Thomson *et al.*, 1998). Human ESCs can retain their differentiating pluripotent capability during each round of cell division, which will enhance their potential to undergo several cell passages while retaining their viability. The main property of ESCs is that they are derived from embryonic cells that have not differentiated into specialized cells (Thomson *et al.*, 1998). Telomere length has direct impact on ESCs proliferative potential. This was confirmed by study on transduced progenitor cells with telomerase reverse transcriptase (*TERT*) to maintain high telomerase activity without affecting differentiation potential, which therefore makes them useful for cell therapy and tissue engineering (Forsyth and McWhir, 2008). ESCs retain their self-renewal property during each round of cell division, however, influencing the proliferation capacity and pluripotency of ESCs in culture may result in genetically variant cell lines. Studies of specific protein markers on ESC such as alkaline phosphatase (ALP) and octamer binding transcription factor (OCT4) suggested that expression of these ESCs specific markers change as they progress toward differentiation (Rajasekhar and Vemuri, 2005). There are a number of limitations to the use of ESCs including; ethical considerations, unpredictability especially with ESCs grown in mouse, the need of long-term use of drugs to prevent rejection reactions,

potential tumourgenesis from rapidly growing ESCs and limited genetic stability of ESCs (Guenin, 2004). Thus, investigations of less risky options such as the use of adult stem cells have been exhaustive (Forsyth and McWhir, 2008).

### **1.6.2 Adult stem cells**

The first adult stem cell therapy was initiated in a bone marrow transplant in 1979 (Kenny and Hitzig, 1979). Adult stem cells are also referred to as postnatal stem cells and these stem cells can be found in adults, children and infants. They differentiate into cells resembling the characteristics of the tissue in which they reside and provide to maintain growth (Gronthos *et al.*, 2002). Mesenchymal progenitor cells may have a potential role in dentine/pulp regeneration by following essential factors. The essential condition not only includes proper cell isolation and characterisation but also evaluating differentiation signalling pathways. Following these criteria may enable the regeneration of pulp *in vivo* (Sloan and Smith, 2007). Adult multipotent stem cells demonstrate plasticity and therefore may initiate the formation of multiple types of tissue *in vitro* by applying appropriate cell culture conditions (morphogenic, angiogenic, osteogenic) (Barry, 2003, Camargo *et al.*, 2004, Wagers and Weissman, 2004). HDPCs are not only responsible for dentine regeneration and repair but also differentiating into adipocytes and neural like cells (Gronthos *et al.*, 2002). Adult stem cells have been identified in many different tissues in the body including umbilical cord and blood, bone marrow, peripheral blood, body fats, skeletal tissue, skin, liver, pulp tissue and periodontal ligaments (Gimble and Guilak, 2003, Bernardo *et al.*, 2007). The most valuable adult stem cells used in medical therapy are neurogenic, adipogenic, chondrogenic, and osteogenic (Safford *et al.*, 2002). The ability of adult dental pulp stem cell to regenerate the pulp has been confirmed in many studies by retaining their

multipotent activity and differentiating into odontoblast like cells for dentinogenesis and to regenerate dental pulp after exposure (Trope, 2008, Huang *et al.*, 2010).

The drawback of using the adult stem cells in tissue engineering is that the cells have already differentiated prior to their application. Another issue is the age of the patient that the cells were harvested from. For example, for an elderly patient, the stem cells isolated have gone through many cell divisions and the telomere length is usually shortened, which may result in ageing of the cells, in comparison to the cells harvested from younger patients. Additionally, there are also concerns related to viability of cells harvested from younger patients (Ho *et al.*, 2005, Mimeault *et al.*, 2007). Adult stem cells are present in a variety of tissues in the human body. The most studied adult stem cell population is the mesenchymal stem cells (MSCs). MSCs possess multi-lineage differentiation potential and produce a variety of cytokines, chemokines, growth factors that are involved in the regeneration of damaged tissues (Pittenger *et al.*, 1999). MSCs can be readily isolated from several tissues like bone marrow, adipose tissue, umbilical cord tissue and from post-natal dental pulp tissues. Multipotent mesenchymal progenitor cells known as dental pulp stromal/stem cells, have high proliferative potential for self-renewal (Suchanek *et al.*, 2009, Yan *et al.*, 2010). Looking further into the future and the clinical application of MSCs there are points to be considered prior to *ex vivo* expansion and differentiation which is essential. First, the exact mechanism through which differentiation is induced and the impact this has on cellular biochemistry and signalling needs to be delineated. Second, the way in which differentiation is assessed needs to be addressed. Care should be taken when comparing results regarding multipotentiality of MSCs as there are several factors known to increase or decrease the tendency for differentiation toward a particular lineage including, serial passage, donor age, cell isolation methodology and cell culture methodology (Thomas *et al.*, 2004,

Tunaitis *et al.*, 2010, Cheng *et al.*, 2011, Laschober *et al.*, 2011, Vater *et al.*, 2011). Cell surface antigen profile of dental pulp stem cells differs compared to other MSCs (Huang *et al.*, 2009).

## **1.7 DNA (Deoxyribonucleic acid)**

DNA is the hereditary material in in most organisms including humans. DNA is a long polymer molecule made from repeating units called nucleotides that contains coded instruction for the cells (Watson and Crick, 1953). The backbone of the DNA strand is made from the sugar carbohydrate 2'-deoxyribose and is joined together by phosphate groups that form phosphodiester bonds between the third and fifth carbon atoms of adjacent sugar rings. In the DNA double helix the direction of the nucleotides in one strand is opposite to the direction in the other strand which is referred to as an antiparallel arrangement. Asymmetric ends of DNA strands are 5'-ends (five prime) with a terminal phosphate and 3'-ends (three prime) with terminal hydroxyl groups (Lin and Yan, 2005). The DNA double helix is stabilized by hydrogen bonds between the bases attached to the two strands. The four bases are adenine (abbreviated A), cytosine (C) guanine (G) and thymine (T). They are attached to the sugar/phosphate to form the complete nucleotide. The nucleotide atoms form a three-dimensional structure connecting the nucleotides together via chemical bonds to form the DNA chains (Cerni, 2000).

Genetic information in the form of DNA is unique to an individual, except in indistinguishable twins. The main function of the DNA molecule is storage of information (Hewakapuge *et al.*, 2008). Our DNA is inherited from parents, and we resemble our parents simply because our bodies were formed using DNA we inherited

from them (Watson and Maaloe, 1953). We may resemble our parents, but we are never exactly like them (Cerni, 2000). This is because each child gets only some of the DNA each parent carries. About half our DNA is inherited from our mother, and half from our father (Watson and Crick, 1953). The DNA inheritance is basically random, and each child gets a different subset of the parents' DNA. Thus, siblings may have the same parents, but they usually do not have exactly the same DNA except for identical twins (Oh *et al.*, 2004).

### **1.7.1 Gene expression**

Gene expression is the process by which the nucleotide sequence (genetic code) of a gene is used to direct protein synthesis and produce the structure of the cells. Each time a cell divides, two cells result which, although they contain the same genome in full, can differ in which genes are turned on and making proteins. The gene is a functional segment in DNA of living organisms, the entire complement of which is called the genome (Baird *et al.*, 2006). The genome is composed of coding and non-coding sequences. The coding sequence determines the structure of proteins the latter regulate including regions involved in the expression of the genes. A structural gene involves a number of different components (exons and introns). Exons code for amino acids and collectively determine the amino acid sequence of the protein product. It is these portions of the gene that are represented in final mature mRNA molecule. The introns are portions of the gene that do not code for amino acids, and are removed (spliced) from the mRNA molecule before translation (Stryer, 1995). Upstream of the coding region of a gene there is usually also a promoter which controls the level of gene expression via transcription of the genes. Enhancers are also regulatory elements of DNA that may bind certain proteins to intensify the rate of transcription. The process of gene expression involves two main stages: transcription and translation. DNA

transcription is the process of transcribing DNA into mRNA by RNA polymerase and occurs in the nucleus in eukaryotes. There are several types of RNA including mRNA, tRNA and rRNA. The mRNA is transcribed from the DNA and is exported to the cytoplasm where it is translated into a protein by a ribosome. Ribosomes are also made of RNA (rRNA) and protein. The tRNA is used to transfer a specific amino acid to the growing polypeptide at the ribosomal active site. During the post-transcription process the pre-mRNA will be modified into mature mRNA that contains spliced exon and intron which is nucleic acid sequence encoding the protein. The tRNA and rRNA do not code for proteins, they are transcribed but not translated into protein (Cerni, 2000). In the translation process the mature mRNA is used as template to assemble a series of amino acids to produce a polypeptide with a specific amino acid sequence. Translation process occurs in the cytoplasm when the coding and non-coding sequence is transcribed from DNA into mRNA and this mRNA will result in protein synthesis. Then the introns, that are found in the eukaryotic DNA region of genes, are translated into protein by the process of intron splicing (Ausubel *et al.*, 2002, Lin and Yan, 2005). A gene that is turned on in one cell may make a product that leaves the cell and diffuses through daughter cells, turning on genes only above a certain threshold level. These cells are thus induced into a new fate, and may even generate other morphogens that signal back to the original cell. Over longer distances morphogens may use the active process of signal transduction. In parallel with this process of building structure, the gene cascade turns on genes that make structural proteins that give each cell the physical and biological properties it requires (Davidson and Levin, 2005).

Dental pulp possesses a natural tissue repair potential, which leads to the formation of reparative dentine. During reparative dentinogenesis, the original odontoblasts at the exposure site are destroyed and replaced by newly differentiated odontoblast-like cells



(Thesleff and Hurmerinta, 1981, Tziafas, 1994). Reparative dentinogenesis is often initiated by the formation of atubular fibrodentine matrix. However the molecular signalling involved in cell differentiation during reparative dentinogenesis has still not been fully characterized. During tooth development, odontoblast differentiation is controlled by specific basement membrane-mediated epithelial-mesenchymal interactions (Okiji and Yoshida, 2009). Characterization of the signalling processes responsible for the induction of odontoblast differentiation is fundamental to the understanding of both physiological dentinogenesis as well as pulp tissue repair. The key roles played by growth factors in physiological odontoblast differentiation, and the recapitulation of such events during repair (Goldberg and Smith, 2004). During reparative dentinogenesis, bone sialoprotein (BSP) and osteopontin (OPN) have been detected at the exposure site, while odontoblast-like cells were shown to express dentine sialoprotein. BSP and OPN are non-collagenous proteins that may control the mineralization process (Okiji and Yoshida, 2009). The co-localization of fibronectin and OPN at the pulp exposure site suggests their role in the migration of progenitors and their differentiation into odontoblast-like cells during reparative dentinogenesis. It is likely that the superficial layer and the fibrodentine of the newly formed dentin bridge matrix have osteogenic characteristics (Teti *et al.*, 2013). The numerous growth factors normally expressed during primary odontogenesis are transforming growth factor beta (TGF-beta) direct signalling cytodifferentiation, including several members of the bone morphogenetic protein family (e.g. BMP-2, BMP-7), and insulin-like growth factor-1 (IGF-1) appear to play a key part in the induction of odontoblast-like cell differentiation from progenitor pulpal cells (Hwang *et al.*, 2008). Dentin Matrix Protein 1 (DMP1), Dentine phosphoprotein (DPP) and Dentine Sialoprotein (DSP), as well as type I collagen are noncollagenous proteins demonstrating different phenotypes of odontoblast

mineralized matrix markers (Edwards and Mason, 2006, Teti *et al.*, 2013). DSP and DPP are unique phenotypic markers of dentine and secretory odontoblast cells. They are encoded by a single gene known as dentine sialophospho protein (*DSPP*). The expression of these genes is associated with dentinogenesis and occurs after a collagenous predentine matrix is formed. *DSPP* transcription is controlled by a series of growth factors and transcriptional factors as well as local factors. Some factors up-regulate *DSPP* expression whereas others down-regulate its transcription (Vishwakarma *et al.*, 2014). DSP and DPP may function as intracellular transducers. DPP is able to induce tooth- and bone-related gene expressions via signalling pathways while DSP binds to cell membrane proteins, resulting in activation of intracellular protein kinases. This may confirm their role in cell proliferation and differentiation besides biomineralization capability (Butler *et al.*, 1997, Goldberg and Smith, 2004b). The differentiation and mineralization of osteoblasts and odontoblasts involves an initial period of extracellular matrix proliferation and biosynthesis that is followed by cell differentiation. In the early stages of this process, the matrix matures, and specific proteins associated with the pulp cell phenotype like alkaline phosphatase (ALP) can be detected (Goldberg and Smith, 2004).

However, dental pulp MSCs display a greater propensity for osteogenic differentiation as evidenced by the level of expression of osteogenic/odontogenic markers (ALP, runt related transcription factor 2 (*RUNX-2*), osteopontin and osteocalcin) (Patel *et al.*, 2009, Karaoz *et al.*, 2010). TGF- $\beta$ 1 is the predominant isoform found in bone and responsible for matrix turnover (Centrella *et al.*, 1991) and whilst capable of stimulating *RUNX-2* expression, it is actually inhibitory to osteogenesis in the later stages (Fromiguet *et al.*, 1997). Conflicting data are available regarding the capacity for BMPs to induce osteogenic differentiation. Firstly, the effects of BMPs on MSCs have been shown to be

species specific (Osyczka *et al.*, 2004). Secondly, osteogenic differentiation in human cells by BMP-2, 4 and 7 has been shown to be both upregulated and downregulated (Cheng *et al.*, 2003, Diefenderfer *et al.*, 2003). Different isoforms have been shown to be key in the initiation of osteogenesis through up-regulation of two of the earliest markers of differentiation, the transcription factors RUNX-2 and osterix (Bennett *et al.*, 2005). RUNX-2 is a key mediator in the early phase of osteogenic differentiation. Being a transcription factor, the role of RUNX-2 is to bind to the promoter sequences of its target genes, initiating their transcription and subsequent translation to protein. *RUNX-2* has many known target genes including bone sialoprotein (*BSP*), osteocalcin (*OCN*), osteopontin (*OPN*), osteoprotegrin and type I collagen  $\alpha 1$  (Otto *et al.*, 2003, Cohen, 2009). Type 1 collagen is the major organic component of bone matrix and therefore its production is another key marker in early osteogenic differentiation. Alkaline phosphatase (ALP) is another key marker, which is expressed at a basal level by immature MSCs, gradually increasing through the first phase of differentiation, peaking, and then decreasing in expression through the second phase of differentiation (Lian and Stein, 1995, Hoemann *et al.*, 2009). *BSP*, *OCN* and *OPN* are all commonly assessed late markers of osteogenic differentiation, demonstrating maximal expression in the second phase (Oldberg *et al.*, 1986, Oldberg *et al.*, 1988, Baht *et al.*, 2008, Ogata, 2008).

Current studies are focusing on the derivation and phenotypes of the cells involved in nonspecific reparative odontogenesis and the molecular mechanisms that regulate their cytodifferentiation. Such approaches will ultimately lead to regenerative therapy and tissue engineering of the dentine-pulp complex (Haung, 2009).

## 1.7.2 DNA replication

DNA replication is the process whereby double stranded DNA is copied to produce a second identical DNA double helix. It is essential prior cell division in order to pass a full complementary DNA to the daughter cells. DNA replication is a semi-conservative process such that the finished products are two double DNA strands, each with one original and one new strand. It is an intricate process requiring an orchestrated action of several proteins. Proteins involved in replication are clustered together and anchored in the cell and the DNA helix is fed through them (Granger *et al.*, 2002, Pratt and Cornely, 2013). The process starts with unwinding and separation of the double stranded DNA into two single strands by the enzyme helicase. The point where the DNA is separated to form a single strand and a new DNA will be synthesised is called the replication fork. Single strand binding proteins (SSBs) bind loosely to the single strand and maintain the separation preventing re-annealing of DNA. The separated single strands act as a template for the production of two new complementary strands by the DNA polymerase enzyme III. However, in order for polymerase enzyme to work, RNA polymerase called primase, first creates a short complementary RNA segment, that is called a primer by copying a short segment of the DNA single strand. This is essential in the replication process as the DNA polymerase enzyme can only extend a nucleotide chain but not start one *de novo* whereas RNA polymerase can. Two DNA polymerase enzymes, working in opposite directions, are required to extend the RNA primer and synthesise nucleic acid in a 5' to 3' direction. One polymerase can remain on its DNA template and copy the DNA in one continuous strand in 5' to 3' direction (so called, leading strand), however, the other polymerase can only copy a short segment of DNA before it runs into the primer of the previously sequenced fragment in which case it repeatedly releases the DNA strand and slide further upstream to begin extension from another

RNA primer. This process leads to synthesis of the strand in short pieces (Okazaki fragments) which is known as the lagging strand (Saldanha *et al.*, 2003, Pratt and Cornely, 2013).

The lagging-strand of DNA undergoes further processing to create continuous double stranded DNA. RNA primers are degraded from Okazaki fragments by RNase H enzyme. The sequence gaps created by this process are then filled in by DNA polymerase I which extends the 3' end of the neighbouring Okazaki fragment. Finally, DNA replication process is completed when the ligase enzyme joins the Okazaki fragments together into one continuous strand (Ausubel *et al.*, 2002, Lin and Yan, 2005, Pratt and Cornely, 2013).

The ends of linear chromosomes pose a unique problem during DNA replication called “end replication problem”. Since DNA polymerase can only elongate from free 3' hydroxyl group, the replication machinery builds the lagging strand by back a stitching mechanism. RNA primers provide 3' hydroxyl groups at regular intervals along the lagging strand template, whereas the leading strand elongates continuously all the way to the end of the DNA template as show in Figure 3. However, the lagging strand is incomplete at the farthest end of the chromosome, due to lack of 3'hydroxyl group to primer DNA synthesis (Stryer, 1995). Because of a lack of ability to replicate, the end of the chromosome would progressively shorten during each replication cycle, resulting in cellular senescence. Additionally, after a certain number of cell divisions, and when telomeres reach a critically short length, cell division and growth is arrested and the cells eventually enter senescence (Granger *et al.*, 2002). Telomere shortening is believed to be a principle mechanism in cell ageing and a major contributor to cell senescence (Shawi and Autexier, 2008). In some cell types e.g. stem cells, cancer cells,

an enzyme known as telomerase can suppress this telomere shortening by causing elongation of the telomere 3' ends (Alberts *et al.*, 2013).

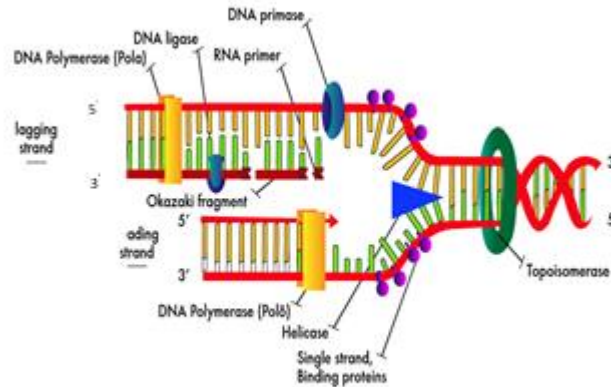


Figure 3 DNA replication diagram adapted from (Alberts *et al.*, 2013).

## 1.8 Ageing

As individuals get older, their tissues and cells accumulate signs of ageing. The mechanisms associated with the ageing process in humans and other animals are complicated and involve many biological changes that occur with time. Ageing influences the physiological and molecular responses to deterioration, or “wear and tear” of body organs and their functions. During the process of ageing, molecular changes in the genetics e.g. telomere length, and/or gene expression may occur. However, recent research has determined that some of these changes can be overcome by stem cell engineering (Nakagawa *et al.*, 2004, Ho *et al.*, 2005). Studies have also confirmed that shortening of telomere length has an effect on proliferation capacity of hematopoietic stem cells leading to replicative senescence (Allsopp and Weissman, 2002).

The potential deterioration of regenerative capacity of haematopoietic stem cells (HSCs) with age has been studied in mice where it was concluded that age adversely affected the regenerative capacity and function of HSCs. This study also showed that HSCs were

not protected from aging and exhibited several manifestations of aging including; loss of epigenetic regulation at the chromatin level and increased tendency for neoplastic change (Chambers *et al.*, 2007).

Maintaining the self-renewal ability of HSC using polycomb complex protein (Bmi-1) that is encoded by Bmi-1 gene was evaluated by several studies (Park *et al.*, 2004, Chambers and Goodell, 2007). HSCs generate different cell types and are essential for durability of multicellular organisms; consequently senescence of these cells must be inhibited. *Bmi-1* maintains the stem cell pool by preventing premature senescence of HSCs, either through senescence gene repression or possibly through stimulation of telomerase associated genes to prevent telomere shortening (Park *et al.*, 2004, Chambers and Goodell, 2007).

The ability to repair and maintain injured tissues and organs is hampered by ageing and as a consequence can result in senescence, cancer or being more prone to infection due to deterioration of the immune system (Janzen *et al.*, 2006). Cell senescence is a consequence of cellular and biological ageing that also limits the capacity of cells to divide both *in vivo* and *in vitro* (Hayflick, 1974, Hayflick, 1994). The processes leading to cellular senescence as a result of ageing is complex and involves changes in gene expression and structural changes to DNA. Such changes can result from alteration of chromatin structure associated with the DNA repair and replication (Finkel and Holbrook, 2000). Throughout each cell division the shortening of telomeres and incomplete replication of the end of chromosome is apparent that may generate cell cycle arrest and alter the proliferation rate of cells (Hausmann and Mauck, 2008). Throughout life, human cells go through many rounds of cell division which requires replication of the chromosomes by DNA synthesis. During the normal process of DNA replication the linear ends of chromosomes become shorter resulting in loss of telomere

sequences. Shortening of the telomere can be indicative of cell ageing and may eventually lead to loss of function (Cawthon, 2002). A Study by Granger *et al.* demonstrated that the division of cells *in vivo* from older patients occurs slower compared to the cells from younger patients and they show a shorter telomere length (Granger *et al.*, 2002). The study of fibroblast cells *in vitro* confirmed that the age influences telomere length. Population doubling of three different age groups was estimated: embryonic tissues underwent 60-80 population doubling compared to adult cells 20-40 and elderly 10-20 it was demonstrated that proliferation rate declines and telomere length shortens during cell division with age (Granger *et al.*, 2002). This study was further corroborated by an *in vivo* study indicated that although a few cell types such as skin fibroblasts retain the ability to proliferate for longer and enter senescence later, shortening of telomeres still occurs at later passages (Gupta *et al.*, 2007).

The regenerative capacity of cells is estimated by their ability to repair and also the capacity of stem cells to restore damaged tissue by age. Therefore the age of stem cells may be critical for regeneration and improvement of damaged tissues (Ho and Punzel, 2003).

The morphology of dental pulp also changes with age resulting in narrowing of the pulp chamber with a concomitant decrease in vascularity and cellularity, and may influence the diagnosis and treatment planning for the tooth (Conboy and Rando, 2005). Age affects the dental pulp tissues, including cells, and also the dentine surrounding the pulp. This can increase the disease incidence of pulpitis, pulp calcification, dental caries and dentine sensitivity. A study comparing proliferation and differentiation rate in rat dental pulp cells has indicated age must be taken into account when using cell replacement strategies to treat pulp disease. Results confirmed that cell proliferation potential was increased in dental pulp cells extracted from young rats in comparison to cells obtained



from adult pulp (Ma *et al.*, 2009). It was suggested that the success rate of pulp capping treatment in younger human patients is higher as opposed to older patients as a result of increased vascularity and cellularity, and also a greater capacity for repair of pulp tissue in youngsters (Glass and Zander, 1949).

The results determined that the prolonged passage of pulp cells is considered a major factor in reducing the capability of pulp cells to differentiate *in vitro*. It was suggested that proliferative capacity of primary HDPCs in culture is as far as passage nine, which may cause replicative senescence (Lee *et al.*, 2006). Study on propagating HDPCs from passage three and four on 3D calcium phosphate granule scaffolds for tissue engineering, suggested that proliferation of HDPCs decreased after 14 days in culture (Nam *et al.*, 2011). Study on cell sorted human periodontal ligaments cells from three different donors, demonstrated that these cells can expand *in vitro* as far as five passages without affecting cell proliferation, osteogenic differentiation capacity and self-renewal ability (Yan *et al.*, 2014).

The importance of prolonged cultivation of HDPCs was further evaluated by Mokry *et al.* who determined that HDPCs undergo progressive telomere shortening over time *in vitro*. According to their results, they have suggested that prolonged serial passage of adult stem cells should be reduced at minimum to avoid telomere shortening and also replicative age of stem cells prior therapeutic applications (Mokry *et al.*, 2010). Although it may not be possible to turn back the clock, or reverse the ageing process, it may be possible to treat ageing associated disorders using tissue engineering approaches. One such approach may involve the repair of shortened telomeres or to identify cells with longer telomeres that may be recruited for tissue replacement therapies. It has been suggested that telomere length can be used to determine age in humans (Hausmann and Mauck, 2008). In a study to determine whether telomere length could

be used in forensic science to estimate age it was concluded that telomere shortening does not depend solely on ageing as other environmental factors can influence telomere shortening (Karlsson *et al.*, 2008). In contrast, a study to determine telomere length in DNA isolated from human dental pulp of Japanese people aged 16-70 years, has indicated that telomere length could be a useful guide to estimating age (Takasaki *et al.*, 2003). It has also been suggested that people with shorter telomeres may be more susceptible to cardiac disease (Brouillette *et al.*, 2007).

### **1.8.1 Chromosome end structures (Telomeres)**

In the 1930s Hermann Muller and Barbara McClintock described the structures of telomeres in eukaryotic cells. Their work laid the foundations for our understanding of the role of telomeres in ageing and the development of cancer (Lin and Yan, 2005). Understanding how telomere length is altered during DNA replication and the importance of telomere maintenance is fundamental in investigations relating to the use of human cells in tissue engineering. Telomeres are specialized structures located at the ends of linear chromosomes: they are DNA-protein complexes that consist of non-coding repeat DNA sequences (Jiang *et al.*, 2007). The telomeres act as caps on the ends of eukaryotic chromosomes and help to preserve the integrity of the genome by preventing a range of potential recombination events which can result from the interaction of chromosome ends. DNA binding proteins assist the telomere to fulfil this essential function. In order to retain function and preserve chromosome stability the telomere must maintain a minimum length (Blackburn, 2001, Westin *et al.*, 2007). Human telomeres consist of double stranded tandem repeat, of the sequence (TAGGG)<sub>n</sub> ending in a 3' single strand extension of the same repeat sequence (Meyne *et al.*, 1989). The single strand extension can form loops by invading local complementary areas of nucleotide sequence. At the 5' end of telomeric repeat the G nucleotide is confined by

T/A nucleotide on each side and forms loops (Saldanha *et al.*, 2003). The DNA binding proteins TRF1 and TRF2 play an important role in stabilising the telomere loop structure, thereby preserving the telomeres from attrition and fusion. The 3' single strand extension combines with TRF2 to form a D-loop by invasion of 3' single strand into an adjacent telomere double strand. This results in the formation of a larger double stranded T-loop (de Lange, 2004). TRF1 is involved in telomere elongation, however, it also acts in conjunction with TRF2 to stabilize the telomere loop structure. Consequently, they both play a role in protecting the chromosome during cell division (Griffith *et al.*, 1999, Hanaoka *et al.*, 2005, Raynaud *et al.*, 2008). Maintaining an adequate telomere length will be essential in preventing senescence of cells resulting from excessive rounds of replication (Cosme-Blanco *et al.*, 2007). In mammalian somatic cells during the replication of DNA the telomeres become shorter with each cell division. This is a consequence of eukaryotic chromosomes being linear and that DNA synthesis can only occur in the 5' to 3' direction. This results in one strand of DNA being synthesized in a discontinuous manner involving the synthesis of discrete fragments, known as Okazaki fragments. As DNA synthesis requires a nucleotide primer for initiation there is no mechanism for priming the 3' end of a DNA strand. As telomeres do not replicate fully due to this replication problem chromosomes shorten with every round of cell division. In human fibroblast and leukocyte cells telomeres shorten with each cell division by, on average, 50-100 base pairs and this can result in cellular senescence after 50-70 cell divisions (Allsopp *et al.*, 1992, Chan and Blackburn, 2004). Cellular senescence is thought to result from telomeres reaching a critical minimum length. This "telomere length hypothesis" has been proposed as a "mitotic" clock for cellular aging (Harley *et al.*, 1990, Zou *et al.*, 2004). After reaching this critical point cells undergo a crisis leading to senescence which may prevent the cell

from undergoing any more cell division (Wright and Shay, 1992). Shortening of the chromosomes does not result in the loss of genetic information as the telomeres are non-coding. However, on reaching a critical point genetic information can be lost as the telomere repeat sequences are lost (Callicott and Womack, 2006). Being able to measure telomere length, or shortening, is important and a number of methodologies have been used. Terminal restriction fragment length analysis (TRF) has been used to determine that the rate of telomere length shortening varies between different genders and ages (Slagboom *et al.*, 1994, Jemielity *et al.*, 2007). Age related changes in structure and function of adrenal cortex is related to progressive telomere shortening during cell division that might affect the proliferation *in vivo* in old age, correlating the replicative capacity and telomere length to donor's age (Yang *et al.*, 2001). In cell culture, telomeres become very short during the senescence stage however it has been shown that some of these effects can be ameliorated by activating the tumour suppressor P<sub>53</sub> or retinoblastoma proteins prior to crisis stage (Shay and Wright, 2001).

Experiments on ants to determine the correlation between the length of telomere and gender have suggested that telomere length is shorter in male ants with a shorter lifespan in contrast to females that showed longer telomeres and lived for longer. It was concluded that the maintenance of telomere length could be due to activation of a telomerase enzyme which could act to maintain telomere length (Jemielity *et al.*, 2007). Oestrogens in females are factors that can affect the telomere length by acting upon the catalytic component of the telomerase enzyme *TERT*, therefore it was suggested that because there are higher oestrogen levels in females they sustain a longer telomere length during lifespan compared to male (Hewakapuge *et al.*, 2008). Telomere length has been correlated to the age in human somatic cells using Q-PCR. The result determined as association between age and changes of telomere length, however, due to

the presence of large inter-individual variations in telomere lengths Q-PCR technique cannot be used to predict age of an individual (Hewakapuge *et al.*, 2008).

Disease has also been associated with telomere shortening. Comparing telomere length in blood lymphocytes from patients with Alzheimer's disease in old patients, compared to controls, showed that older patients had significantly shorter telomeres than control subjects (Panossian *et al.*, 2003). However, biopsy examination of the brains from older people failed to demonstrate telomere shortening in these tissues (Allsopp *et al.*, 1995). Telomere shortening has also been investigated in ageing diseases. A study of patients with Hutchinson-Gilford progeria, compared to normal controls, showed significant telomere shortening. However, after induction of *TERT* enzyme into cells, using recombinant viral vectors, telomere shortening was arrested without affecting the cellular function (Granger *et al.*, 2002).

In an experimental study on sheep, fibroblast cells the shortening of telomere length and of telomeric 3'overhang was compared. It was suggested that after cell division both telomere and telomeric 3' overhang length shortened. However, the size of telomeric 3'overhang did not determine the rate of shortening of the telomere, consequently the considerable shortening of the length and telomeric 3' overhang were associated with cellular senescence (Rahman *et al.*, 2008).

Telomere shortening has also been shown to be affected by other factors *in vivo*, including reactive oxygen species (ROS). ROS can cause cellular damage and can contribute to ageing and cell death (McCord and Fridovich, 1969). This has been confirmed by evaluating the relationship between stress and telomere length in peripheral blood cells comparing healthy women and women under stress. The results showed that cells from women under stress contained shorter telomeres compared to

healthy women, and this was correlated with the oxidative stress resulting in the reduction of telomerase activity. ROS are capable of inducing DNA strand breaks in the telomere which can lead to cell cycle arrest (Epel *et al.*, 2004). Furthermore, a study of 58 healthy premenopausal women under stress, established that the level of ROS produced was correlated to the level of stress, and that they also demonstrated shorter telomere lengths resulting from a decrease of telomerase activity (Valdes *et al.*, 2005). A comparison of telomere length in smokers and obese women with a control group showed that smokers and obese individuals had shorter telomeres, suggesting that environmental factors are capable of influencing the process of aging and telomere length (Shawi and Autexier, 2008).

Relative telomere length measurement was introduced by using Q-PCR and a primer that eliminates formation of primer dimer (Cawthon, 2002). They measured the relative telomere length using (T/S) ratio and suggested that telomere length gets shorter *in vitro* after serial passages. This method was further followed in a study by O'Callaghan and French, in which they modified the method by adding oligomer standard for relative measurement. According to their data, they have suggested that TRF measurement tends to overestimate the telomere length, due to many variable DNA components within the TRF (O'Callaghan and Fenech, 2011).

An *in vitro* study measuring telomere length in human dental pulp stem cells by Q-PCR, suggested progressive telomere length shortening of cells, regardless of the stem cell self-renewal ability. The relative telomere length (T/S) was inversely related to the doubling time of the cells (Mokry *et al.*, 2010). Telomere length measurement in human Myelodysplastic Syndrome (MDS) by Sashida *et al.* demonstrated regular telomere shortening distribution of MDS cells in metaphase in contrast to normal individual cells,

suggesting dysregulation of telomere shortening as a mechanism in MDS cells (Sashida *et al.*, 2003).

### 1.8.2 Telomerase

Telomerase is a ribonucleoprotein enzyme initially demonstrated *in vitro*, it was shown that the telomerase enzyme in Tetrahymena was apparently capable of the synthesis of telomeric repeats without a template. However, the finding of the RNA component of telomerase enzyme led to the discovery that this could act as a template (Greider and Blackburn, 1989). *In-vivo*, germ line expression of telomerase, established in early human embryonic tissue, has been shown to cease a few weeks after birth. Whilst active in some types of adult stem cells, genes for telomerase are subject to tight regulation and are generally switched off in differentiated adult cells (Forsyth *et al.*, 2002). Evaluation of telomerase activity in germ line and somatic cells, in culture, has confirmed these findings. Telomerase activity was identified in both new-born and adult testes and ovaries for up to 16-20 weeks in culture (Wright *et al.*, 1996). Senescence of cardiac stem cells has been related to the reduction of telomerase activity observed in mice myocytes with age (Torella *et al.*, 2004). Telomerase elongates telomeres by *de novo* synthesis of telomeric DNA at the end of 3'end (Greider and Blackburn, 1989, Greider, 1996), thereby restricting the shortening of telomeres that can occur in cells not expressing telomerase (Chan and Blackburn, 2004). Telomerase is comprised of both a RNA and a protein component. The protein subunit is catalytic and acts as a reverse transcriptase (*TERT*) and a RNA subunit (*TERC*) which provides a template for telomere synthesis (Kim *et al.*, 1994, Nugent and Lundblad, 1998, Shawi and Autexier, 2008). By introducing mutations into the *TERC* component and transfecting these into *TERT* expressing cells it was shown that an alteration in newly formed telomere sequences reflected the mutation in the *TERC* gene, thus confirming

that *TERC* is solely responsible for determining telomere sequence (Cerni, 2000b). *TERC* acts by annealing to the 3' single strand extension generated through lagging strand synthesis during DNA replication. By acting as a template, it allows the 3' end to be extended by translocating along the newly synthesized 3' end, the *TERC* facilitates the extension of telomeres by the enzymatic component *TERT* (Dahse *et al.*, 1997, Chan and Blackburn, 2004).

The *TERT* and *TERC* subunits are essential for telomerase activity. Telomerase activity has been shown to be highly expressed in some cancer cells possessing characteristics of immortality. This is in contrast to normal cells which do not express *TERT* and undergo cellular senescence. Therefore to prevent the development of tumour, inhibition of telomerase activity may have potential as a therapeutic intervention that could lead to shortening of telomere length in tumour cells and thereby reducing viability leading to cell senescence (Zumstein and Lundblad, 1999). In contrast, experiments using antisense oligonucleotides to reduce expression of *TERT* and *TERC* in human colon cancer cells showed that blocking telomerase activity inhibited the cell growth and resulted in the initiation of apoptosis in cancer cells. Indeed there appeared to be no significant difference between results obtained using antisense oligonucleotides to either *TERT* or *TERC* (Fu *et al.*, 2005). The *TERT* gene has been located to chromosome 5, and has been shown to be activated in many types of cancer cells including recurrent medulloblastomas and embryonic brain tumours (Fan *et al.*, 2003). In the study by Byran *et al.* *TERT* mRNA levels were measured in 50 embryonic brain tumours. They determined that almost all of the cells were *TERT* positive in brain tumour cells in comparison to early medulloblastoma in which *TERT* was rarely detected. However, in recurrent medulloblastoma more *TERT* mRNA was detected, suggesting that the increased expression level of *TERT* mRNA and telomerase enzymatic activity may be



associated with progressive tumour development. Interestingly, a study of lengthening of telomeres in human cancerous skin fibroblast cells, in the absence of *TERC*, has suggested that there may be an alternative mechanism for maintaining telomere length that is not dependent on the telomerase RNA subunit (Bryan *et al.*, 1997).

The effect of telomerase activity in cancer cells with increased proliferative capacity has been evaluated in *TERC* subunit knockout mice (Artandi *et al.*, 2000). By excluding the gene that encodes the *TERC*, they determined that telomere length shortens in these mice and their mortality is increased. The rate of the tumourgenesis declined in all the mice lacking telomerase activity (with the exception of p53 genetically deficient mice) (Artandi *et al.*, 2000, Gonzalez-Suarez *et al.*, 2001). Furthermore this result was correlated with a study on immunosenescence phenotype in telomerase deficient mice. The study of knockout *TERC* deficient mice determined that old mice developed significantly shorter telomeres and a reduction in T and B lymphocytes and early death which is similar to that reported in human ageing. It was suggested that maintenance of telomere length by telomerase activity is essential for immune function and cellular immortality (Blasco, 2002). To confirm the effect of telomerase enzymatic activity on ageing, mice with increased expression of *TERT* were chosen and mated with mice that were resistant to cancer, producing mice with long telomere length and significant increase in telomerase activity which increased the lifespan (Tomas-Loba *et al.*, 2008). To investigate the efficiency of *TERT* in immortalization of HDPCs and differentiation capacity, human dental pulp cells were transfected with the human *TERT* gene. The results showed that the HDPCs were viable even after 120 passages. In comparison to odontoblasts, HDPCs have been shown to have a greater capacity for differentiation and a significantly higher level of expression of *TERT* (Kitagawa *et al.*, 2007). Although it is known that *TERT* is capable of lengthening the telomere, studies using recombinant

vectors have shown that expressing both the *TERT* and *TERC* genes simultaneously increased the telomerase activity significantly (Wang *et al.*, 2008). This is supported by a study on dyskeratosis congenita (DC) skin fibroblast cells where a combination of both *TERT* and *TERC* expression was shown to increase telomere length. This study was done on autosomal dominant dyskeratosis (AD) DC and confirmed that in AD DC cells expression of *TERC* alone did not activate the telomerase but when *TERT* gene was used telomere length extended and telomerase activity was observed. However when both genes were expressed together a significant amount of telomerase activity was expressed and the telomeres extended (Westin *et al.*, 2007). Telomerase activity and its effect on cell differentiation and tissue proliferation is related to *TERT* and *TERC* genes, and both will be essential for immortalization of cells in order to obtain the full benefits from the application of tissue engineering (Shawi and Autexier, 2008). In a study of adult myoblast cells investigating the influence of *TERT* gene on proliferation and immortalization, the TRAP assay was used to demonstrate that cells grown *in vitro*, for tissue engineering purpose, which were transfected with *TERT*, had longer telomeres, expressed telomerase and they initiated the formation of myotubes for muscle markers and additionally showed no sign of tumorigenicity *in vivo* (Di Donna *et al.*, 2003). Although telomerase activity is expressed in cancer cells, telomerase expression is not adequate to transform normal cells into tumour cells by elongating the telomeres during DNA replication (Granger *et al.*, 2002, Serakinci *et al.*, 2006).

## **1.9 Aims and objectives**

### **1.9.1 Aims**

The aim of this project was to investigate the effects of various pulp capping agents on viability, proliferation, differentiation and ageing of human dental pulp cells (HDPCs) *in vitro*. The project aims to investigate the changes at the genetic level that may be induced by such materials and thereby understand their effect on HDPCs. The translational aim of this project was to determine if pulp capping materials can promote or suppress dental pulp tissue regeneration with a view to improving their clinical applications. Furthermore, it was aimed to investigate the possibility of using HDPCs as a source for stem cells that can be used for tissue engineering based treatment modalities.

### **1.9.2 Objective**

- Specific objectives include examining the efficiency of different methods to isolate HDPCs from extracted teeth and also to investigate the most appropriate method to culture HDPCs with various pulp capping materials.
- Investigating the biological effects of commercially available and widely used pulp capping materials Dycal, MTA and a novel agent, propolis, on serially-passaged HDPCs at different time points. This will be achieved by examining the effect of pulp capping materials on the expression profile of genes controlling programmed cell death, differentiation and ageing within HDPCs at different time points and in different passages.
- As a result of data generated regarding the biological changes in HDPCs undergoing serial passages, there was a redirection of the objectives to concentrate on cellular ageing of HDPCs *in vitro*.

- The effect of pulp capping materials on telomere length shortening within serially passaged HDPCs will be investigated. The potential use of custom designed oligonucleotides to suppress telomere shortening was investigated.

# Chapter 2: Materials and Methods

## 2.1 Cell culture

### 2.1.1 General materials and culture media

Cell culture plastics were purchased from Corning (Lowell, MA). Antibiotics, 1× trypsin/ethylenediamine tetra acetic acid (EDTA) was obtained from Sigma (Poole, UK). The alpha-modified minimum essential medium without L-glutamine ( $\alpha$ -MEM), Dulbecco's phosphate buffer solution (PBS), and foetal bovine serum (FBS) were all purchased from Lonza (Switzerland).

### 2.1.2 Isolation and culture of human dental pulp cells

Dental pulp tissue was isolated from extracted healthy third molar teeth provided from the Department of Oral Surgery and One Day Unit at Leeds School of Dentistry and Dental Hospital. Teeth were obtained with patient's informed consent and with ethical approval from the Leeds (East) Research Ethics Committee (Human tissue ACT-050210/MR/34). A total of 16 teeth were collected from 10 individuals for this study, out of which 12 teeth were selected and cultured. Only eight teeth had pulp tissue of a workable size and they were successfully cultured. For this study, pulp tissue from four teeth out of eight was used and the rest was stored in tissue bank. The four teeth used were third molars (two upper and two lower). All donors were females from age group of 22-28 years. The tissue isolation process was as follows: the external tooth surfaces were cleaned and washed with sterile 1× PBS twice. The attached gingival tissues were removed from the tooth surface using a sterile scalpel. The external tooth surfaces were then thoroughly cleaned again using 1× PBS. The teeth were then cracked open using a sterile vice. The cracked tooth was placed in the tissue culture hood and was rinsed

with Hank's buffer saline solution (HBSS). Pulp tissue was harvested from the pulp chamber of the teeth and immersed in sterile Petri dishes containing 1× PBS. Pulp tissue from each tooth was split in halves. For each donor, HDPCs isolation was performed using collagenase P on one half of the pulp tissue and using the explant outgrowth method on the other half. Detailed explanation of each technique is provided in sections 2.1.2.1 and 2.1.2.2.

#### **2.1.2.1 HDPC isolation using explant out growth culture method**

Isolation methodology was adapted from previous work (Yang *et al.*, 2004). The pulp tissue was cut into small fragments (1-2 mm<sup>3</sup> sized explants) by surgical blade and cultured in T25 tissue culture flasks in basal culture media consisting of  $\alpha$ -MEM supplemented with 1% of P/S and 10 % FBS and 100 unit/ml penicillin/10 mg streptomycin in 0.9% NaCl and 5 mM L-glutamine. Tissue fragments were incubated at 37°C and 5% CO<sub>2</sub>. Culture medium was changed twice a week for one month. Cell migration from the tissue explant reaching 80-90 % confluence they were considered as passage 1 (P1).

#### **2.1.2.2 HDPCs isolation via collagenase digestion**

Isolation of individual cells from various tissues requires disruption of the extracellular matrix. Various protein-digesting enzymes and proteases are required for this process. Collagens are among the most abundant proteins in the extracellular matrix. Thus, the use of various collagenase enzymes for cell isolation has been widely used and reported (Davis, 1994).

After harvesting the pulp tissue from the pulp chamber, the pulp tissue was minced and immersed in a filter sterilized cocktail of collagenase P (Roche, USA) (3 mg/ml) and dispase (4 mg/ml) dissolved in 2.5 mg/ml of calcium chloride containing 1× PBS for 1-

2 hours at 37°C and 5% CO<sub>2</sub>. After total digestion of the tissue which was indicated by total dissolution/disappearance of the pulp tissue mince, the solution was passed through a 70 µm cell strainer (Nalgene, US) and centrifuged at 1100 × G for 5 minutes. The pellet was then re-suspended in α-MEM to obtain a cell suspension. The cells within this suspension were considered as passage 1 (P1). The cells were then cultured in basal medium in T25 flasks at 37°C and 5% CO<sub>2</sub> incubator.

### **2.1.3 Amplification of HDPCs *in vitro***

When the isolated HDPCs (P1) reached 80-90% of confluence, they were harvested with 1× trypsin/EDTA (Sigma, Gillingham, UK) and incubated for 5 minutes in a CO<sub>2</sub> incubator. Trypsin was deactivated by the addition of 10% of FBS to the detached cells. Cells were then pelleted by centrifuging for 5 minutes at 1100 × G. The cell pellet was then re-suspended in the fresh culture medium and transferred to 75 cm<sup>2</sup> tissue culture flasks at approximate density of 3500 cells/cm<sup>2</sup>. These cells were designated passage 2 (P2). HDPCs were serially passaged until passage number 12 using similar methods.

### **2.1.4 Cell freezing**

Cell freezing is a cryopreservation technique that is used to maintain stocks of primary cells and cell lines and preserve their viability and characteristics for future use. The most commonly used cryoprecipitant is dimethyl sulphoxide (DMSO) used in conjunction with FBS and/or culture medium.

At each passage, HDPCs in one flask were split into two other flasks and the remaining cells were preserved as follows; confluent cell cultures were detached as described previously and harvested by centrifuging at 1100 × G for 5 minutes. The cell pellet was re-suspended in 3 ml FBS with 10% DMSO that had been passed through the sterile filter (0.25 µm pore size). The cell suspension was stored in small aliquots in cryotubes

placed inside a cryobox which contained 300 ml ethiproponal (to allow the temperature reduce at a rate of a proximately -1°C per minute, reducing ice crystal formation within the cells) (NALGENE, USA) and were stored in a -80°C freezer for 24 hours, after which the vials were stored in liquid nitrogen over the course of the study.

### **2.1.5 Counting cells (total & viable)**

Cell suspensions were transferred to a centrifuge tube and centrifuged at  $1100 \times G$  for 5 minutes. Supernatant was carefully aspirated. The pellet was then re-suspended in 10 ml of culture medium and aliquots of 40  $\mu$ l were loaded on improved Neubauer (Assistant, Germany) 0.1 mm haemocytometer slides under light microscope (ULWC 0.30 Olympus) for cell counting. The Neubauer haemocytometer (Assistant, Germany) is a thick glass slide with a ridge in the middle, dividing the slide into two slots. Each slot is then further divided by grids into nine squares as shown in Figure 3 with a total capacity volume of 0.1  $\text{mm}^3$  each and an area of  $1\text{mm}^2$  and depth of 0.1 mm (Davis, 1994).

The total cell number per ml (X) was determined as follows; cells (Y) in all the five squares labelled 1-5 were counted (Figure 4). The total cells number per ml in the suspension can be calculated using the formula:

$$X = Y \times \frac{\text{Dilution factor}}{\text{Number of squares}} \times 10^4 \text{ cell/ml}$$



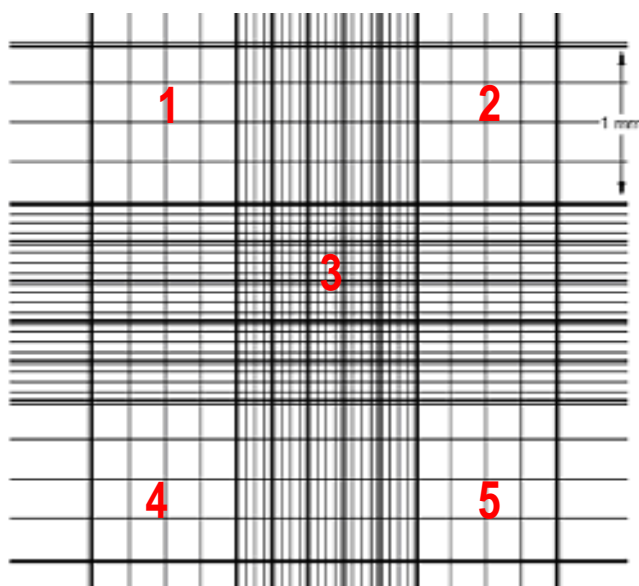


Figure 4. Improved Neubauer 1mm<sup>2</sup> haemocytometer squares for cell counting. The chamber was divided into 9 squares and each major square was divided into 16 smaller squares. Each of the 25 squares in the central big square were further divided into 16 smaller squares. Adapted from Rezapour (2008).

#### 2.1.5.1 Cell viability count estimation

Viability of the cells was determined by Trypan blue exclusion assay. Cells were re-suspended in 10 ml of the required medium. 200 µl was transferred to a tube which contained 20 µl of 0.4% Trypan blue (Sigma, Poole) and mixed thoroughly. The cells were then counted as described in 2.1.5. The blue stained cells indicated damage to cell membrane and were considered as dead cells and the cells that were not stained were viable cells. The formula for counting the viability of cells is as follow:

$$\% \text{ Viability} = \frac{\text{Total number of viable cells}}{\text{Total number of viable and non - viable cells}} \times 100$$

#### 2.1.5.2 Doubling time estimation for HDPCs

Cell number from HDPCs was determined following Materials and Methods section 2.1.3. The number of the dead/floating and attached cells within the culture medium

was counted. Doubling time was calculated using an online available doubling time calculator (<http://www.doubling-time.com/compute.php> [Accessed 20/08/2014]). The calculator utilises the initial and final cell concentration and the time lapsed to achieve the final cell concentration (Ruth, 2006).

## **2.2 Capping materials used in this study**

Pulp capping is a restorative technique to preserve pulp tissue using pulp capping materials (Qureshi *et al.*, 2014) The pulp capping materials used in this study were: Dycal (Dentsply Caulk Milford, DE, USA), Mineral Trioxide Aggregate (MTA) (Dentsply Caulk Milford, DE, USA) and propolis (Ecuadorian Rainforest LLC, USA).

### **2.2.1 Preparation of pulp capping materials**

#### **2.2.1.1 Dycal**

Dycal consists of base paste (1,3-Butylene glycol disalicylate, zinc oxide, calcium phosphate, calcium tungstate Iron and oxide pigments) and catalyst paste (calcium hydroxide N-ethyl-o/p toluene sulphonamide, zinc oxide, titanium dioxide, zinc stearate and iron oxide pigments). Dycal is available in chemically cured or light cured formulas. Dycal, chemically cured, was mixed according to the manufacturers' instructions on a sterile glass slab using a spatula. Each sample was layered on a cover slip (15mm) to obtain a disk which was the same size as the 6 well cell culture plates. A Vibrax Shaker Platform (Thermo Fisher Scientific,UK) was used to distribute the material on the disc uniformly. It was left to set for 24 hours at 37°C, 5% CO<sub>2</sub>, in a 100% humidity incubator. The material was set prior to addition in to the well cell culture plate containing cultured cells with density of ( $1 \times 10^5$  cells/well) that had been seeded overnight. Due to difficulty handling the microscope cover slip and mixing the Dycal,

the protocol was modified and Transwell<sup>®</sup> inserts (0.4 µm pore size, Greiner bio-one, UK) were used instead of the cover slip and the material was transferred into the Transwell<sup>®</sup> inserts using an MTA applicator. The Transwell<sup>®</sup> inserts containing Dycal were then placed in a 24 well cell culture plates (Greiner bio-one, UK).

#### **2.2.1.2 MTA preparation**

Mineral trioxide aggregate (MTA) is a fine powder available in single use sachets of 1 gram with provided premeasured water bottle. MTA consists of tricalcium silicate, tricalcium aluminate, tricalcium oxide, silicate oxide and bismuth oxide, similar to Portland cement except for the absence of bismuth oxide in Portland cement. Bismuth oxide is added (17-18 wt%) to improve the properties and the radiopacity.

A 2 g/ml mixture of MTA (Dentsply Caulk Milford, DE, USA) was prepared by mixing MTA powder with distilled water, the material was then placed into each Transwell<sup>®</sup> insert (0.4 µm pore size, Greiner bio-one, UK) using MTA applicator. However, due to difficulty in mixing the MTA and loading the applicator the protocol was modified. For the modified procedure, the powder was measured and mixed with the distilled water in a sterile 5-ml syringe to get a homogenous mixture. 200 µl of the material (concentration 2g/ml) was injected into the bottom of the Transwell<sup>®</sup> inserts which were then placed in a 24 well cell culture plate. The material was left to completely set for 24 hours at 37°C in 5% CO<sub>2</sub> incubator.

#### **2.2.1.3 Propolis preparation**

In general, Propolis is a resinous substance, varying in colour from yellow-brown to dark brown. It is composed of 50% resin and balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% organic debris depending on the place and time of its origin. The main chemical compounds are flavonoids, phenolic.

A 2 g/ml mixture of propolis (Ecuadorian Rainforest LLC, USA) was prepared by mixing propolis powder with 70% ethyl alcohol on a sterile glass slab with the aid of a spatula and then placing into the syringe where further mixing of the material was performed. 200 µl of the material (concentration 2 g/ml) was injected into the bottom of the 0.4 µm pore size permeable membrane Transwell® inserts (Greiner bio-one, UK) which were then placed in a 24 well cell culture plate. The material was left to completely set for 24 hours at 37°C in 5% CO<sub>2</sub> incubator.

#### **2.2.1.4 Culturing HDPCs with pulp capping materials**

HDPCs with density of  $1 \times 10^5$  cells/well were seeded in 24 well cell culture plates containing 2 ml of  $\alpha$ -MEM medium (Lonza, Switzerland) and allowed to attach overnight. The permeable membranes in the Transwell® inserts prevented direct physical contact between cells and the specimens while allowing for soluble components from the specimens to reach the cells. Each sample was repeated in triplicate. The prepared samples, that had been left to set for 24 hours, were placed into the 24 well cell culture plates (Greiner bio-one, UK) containing HDPCs as shown in Figure 5. Cells were incubated for different time periods (12 and 24 hours). HDPCs cultured without pulp capping material served as controls.

## Cell culture setup with MTA & propolis

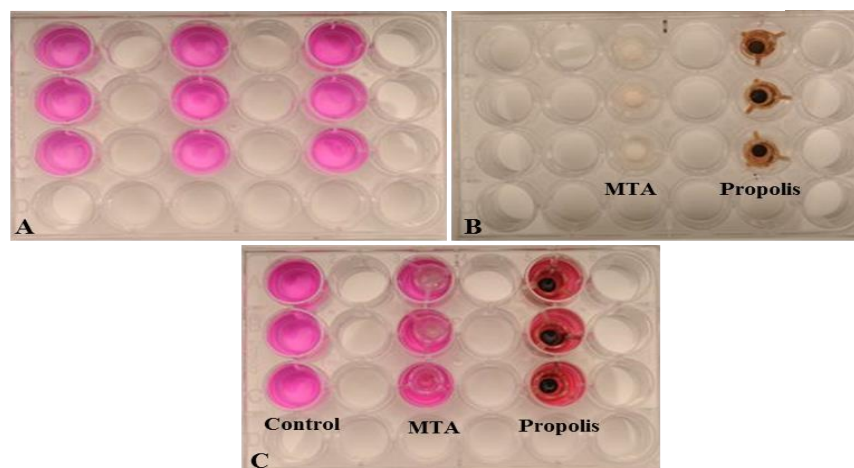


Figure 5. Cell culture setup with MTA & propolis.

A) 24 well cell culture plate containing cultured HDPCs overnight. B) Transwell<sup>®</sup> inserts containing MTA in white colour and propolis in black at the bottom of each Transwell<sup>®</sup> inserts. C) MTA and propolis in cultured cell medium after incubation (24 hours).

### 2.2.2 Optimisation of propolis preparation

Initial experiments indicated that cells incubated with propolis, demonstrated reduced HDPCs viability in comparison to the untreated controls or cells treated with MTA. This may have been related to the very high concentration of the used propolis mixture. This hypothesis was very likely to be true given the significant change in the colour of the culture medium once the Transwell<sup>®</sup> inserts containing propolis was inserted in the 24 well cell culture plate. Thus, the concentration of propolis used for later experiments was reduced to 0.8 g/ml.

### 2.2.3 Effect of Transwell<sup>®</sup> inserts on the viability of HDPCs

HDPCs ( $1 \times 10^5$ /well) were cultured in 24 well cell culture plates overnight in two different conditions (n=3). The first three wells contained HDPCs that were cultured without Transwell<sup>®</sup> inserts and the second three wells contained HDPCs that were

cultured with the presence of Transwell<sup>®</sup> inserts. The number of viable HDPCs with and without Transwell<sup>®</sup> inserts in the monolayer was calculated. The number of detached/floating cells in the culture medium was calculated and the viability percentage was calculated for both groups according to Materials and Methods section 2.1.5.

#### **2.2.4 Measuring pH of cultured media with Dycal**

pH was measured for 1× PBS, HDPC culture in 2 ml 1× PBS after 12 hours incubation period with or without Dycal treatment, also  $\alpha$ -MEM cell culture medium, HDPC culture in 2 ml of  $\alpha$ -MEM after 12 hours incubation period with or without Dycal treatment were measured.

The pH of the above six conditions was measured using a pH meter (Orion, U.S.A). The pH meter was calibrated prior to use. The calibration was accomplished using buffer solutions with different pH values. The first calibration was done using citrate buffer (ACROS, U.S.A) pH 4, the second pH reading was adjusted by using phosphate buffer (ACROS, U.S.A) pH 7 and the last reading for calibration was done using carbonate buffer (ACROS, U.S.A) pH 10. The pH electrode was cleaned thoroughly with distilled water and left to dry between consecutive readings. Each measurement was repeated twice for samples in triplicate within each group and an overall mean pH reading and standard deviation was calculated for each condition.

## **2.2.5 Measuring the amount of released MTA and propolis**

### **2.2.5.1 Spectrophotometric determination of the amount of released $\text{Ca}^{+2}$ from pulp capping materials**

A colorimetric assay, the Arsenazo III, is a quantitative method used for measuring the amount of calcium ions in liquid substrates. Arsenazo III, a complexometric reagent used in this assay, reacts with calcium in a solution to form a bluish-purple complex. The intensity of colour developed is proportional to the calcium concentration and can be determined spectrophotometrically according to Beer-Lambert law. This method is a highly sensitive technique for solutions containing  $\text{Ca}^{+2}$  concentrations ranging between 12.4–49.4  $\mu\text{M/l}$  (Attin *et al.*, 2005).

In the present study, preparation of the reagent solution for measuring  $\text{Ca}^{+2}$  concentrations was performed according to the calcium Arsenazo III method previously described by Smith and Bauer (Smith Jr and Bauer, 1979, Attin *et al.*, 2005, Sayed, 2013). Reagent solution used in this assay was prepared from 100 mM/l imidazole buffer (pH 6.5) and 0.12 mM/l Arsenazo III (Biocon Diagnostik, Vöhl/Marienhagen, Germany). A dilution series was prepared in distilled water, ranging from 0-50  $\mu\text{M}$   $\text{CaCl}_2$  (Fisher Scientific, UK). Additionally, a dilution series was prepared in  $\alpha$ -MEM culture medium, ranging from 0-4  $\mu\text{M}$   $\text{CaCl}_2$ . The prepared dilutions were mixed with Arsenazo III reagent solution in 1:1 ratio. A triplicate of 300  $\mu\text{l}$  from each mixture was pipetted into wells of a 96 well culture plate in flat microplate reader at 820 nm (Corning, USA).

The intensity of the colour change was colorimetrically assessed in the 96 well culture plates using a spectrophotometer at wavelength of 820 nm (Dynex Technologies, UK). The light absorbance readings from the dilution series made up from distilled water and

CaCl<sub>2</sub> were used to construct a standard calibration curve that was used to extrapolate the unknown concentration of calcium in solutions, such as culture medium with various pulp capping materials.

#### **2.2.5.2 Freeze drying method**

A 2 g/ml mixture of MTA was made by mixing powder with distilled water in a sterile 5 ml syringe. Similarly, a 0.8 g/ml propolis mixture was prepared from propolis powder and 70% ethyl alcohol. A 200 µl of each material (n=3, per group) was injected into the bottom of the Transwell<sup>®</sup> inserts which were then placed in a 24 well cell culture plate. The material was left to completely set for 24 hours at 37°C in 5% CO<sub>2</sub> incubator. Transwell<sup>®</sup> inserts containing fully set pulp capping material as well as empty Transwell<sup>®</sup> inserts (n=3) were weighed ( $W_{t_{dry0}}$ ) using a calibrated digital balance (A&D weighing, USA).

HDPCs with the density of  $1 \times 10^4$ /well were seeded in 24 well cell culture plates in 2 ml of  $\alpha$ -MEM incubated for overnight attachment of the cells. Transwell<sup>®</sup> inserts containing pulp capping materials as well as empty (control) Transwell<sup>®</sup> inserts were inserted in the well cell culture plates. The incubated Transwell<sup>®</sup> inserts were retrieved and weighed after 24 hours incubation period ( $W_{t_{24}}$ ). All Transwell<sup>®</sup> inserts were then immersed in liquid nitrogen for 5 seconds. The frozen samples were transferred quickly into the chamber of the freeze-dryer apparatus (ChristAlpha 2–4 L.S.C, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). The freeze drying cycle lasted for 24 hours under  $6 \times 10^{-6}$  Torr. As the sublimation of the frozen solution occurred, the temperature of the precursor was increased up to 10°C. The temperature was then progressively increased to 50°C while maintaining vacuum. The freeze drying cycle was deemed successful when no variation of the pressure in the chamber was recorded. The



frozen-dried Transwell<sup>®</sup> inserts were weighed ( $Wt_{freeze\ dry}$ ). The weight of the released material from MTA and propolis was calculated according to the equation below:

$$Wt_{released} = Wt_{dry0} - Wt_{freeze\ dry}$$

Where:  $Wt_{dry0}$  is the initial weight of Transwell<sup>®</sup> inserts and pulp capping material before incubation, and  $Wt_{freeze\ dry}$  is the weight of the freeze dried Transwell<sup>®</sup> inserts with the pulp capping material after 24 hours incubation time

### **2.2.6 Photography of the cells in monolayer**

A record of the morphology in culture of the confluent cells in 24-well cell culture plate with Transwell<sup>®</sup> inserts containing pulp capping material was obtained with a Sony T9 camera under phase contrast Olympus microscopes (ULWCD 0.30).

## **2.3 Extraction of nucleic acids**

### **2.3.1 RNA extraction using Tri reagent**

TRI<sup>®</sup> reagent is a solution which combines phenol and guanidine thiocyanate in a monophasic solution to rapidly inhibit RNase activity. The TRI<sup>®</sup> reagent performs by disturbing the cell membrane and keeping the ribonucleic acid in the nucleus intact. Biological samples are homogenised or lysed in TRI<sup>®</sup> reagent solution. Homogenated solution is then separated into a clear aqueous phase on the top and organic phases by adding bromochloropropane (BCP) and centrifuging at  $1200 \times G$  for 5-10 minutes. RNA partitions to the aqueous phase, DNA to the interphase, and proteins to the organic phase. RNA can be precipitated from the aqueous phase with isopropanol, and then washed with ethanol and solubilised thereafter (Rio *et al.*, 2010).

TRI reagent<sup>®</sup> (Ribopure kit Ambion, US) was used to extract RNA from freshly harvested HDPC pellets or HDPC pellets stored in RNALater<sup>®</sup> (Lifetechnologies,UK) at -20°C in a freezer. 1 ml of TRI<sup>®</sup> reagent was added to each HDPC pellet and resuspended by mixing using a pipette. Samples were incubated for 5 minutes at room temperature and transferred into Eppendorf<sup>®</sup> tubes after the incubation period. 100 µl of 1-Bromo 3-Chloropropane (BCP) (Sigma Aldrich, UK) was added to each Eppendorf<sup>®</sup> tube and vortexed for 15 seconds (Scientific Industries, USA). The tubes were incubated at room temperature for 5 minutes and then centrifuged at 12000 × G for 10 minutes at 4°C (5417R, Eppendorf<sup>®</sup>, Germany). The three phases were visible after centrifugation, an uppermost transparent aqueous phase, a thin white layer in the middle and a lower pink chloroform layer. The aqueous layer was carefully transferred into a clean Eppendorf<sup>®</sup> tube without disturbing the other layers and then 200 µl of 100% ethanol was added and the mixture was immediately vortexed for 5 seconds. A filter cartridge (supplied by manufacturer) was placed over each tube and the solution was passed through it and then centrifuged at 1200 × G for 30 seconds at room temperature. The flow was discarded and the filter cartridge collection was returned to the previously used tube. 500 µl of the wash solution (Ambion Ribopure kit) was added to the filter tube and centrifuged twice again for 30 seconds at room temperature, the process was performed twice. After the second wash filter tube was further centrifuged to remove any trace of the solution. The filter was transferred into a new Eppendorf<sup>®</sup> tube and 100 µl of elution buffer was added and centrifuged for 30 seconds, finally the solution containing eluted RNA was stored at -20°C.

### **2.3.2 DNA extraction using EZ-DNA reagent**

EZ-DNA<sup>®</sup> is a non-organic reagent that is used for quick isolation of genomic DNA from samples of human, animal, plant, yeast, bacterial and viral origins. EZ-DNA<sup>®</sup> has

no phenol and is an improved version of Trizol DNA extraction (Chomczynski, 1993). The technique involves disruption of cells by lysing solution that hydrolyses RNA and permits selective precipitation of DNA from a cell lysate with ethanol. Following an ethanol wash, DNA is solubilised in 8 mM NaOH. The benefit of this method is recovery of 70-100% of DNA and no purification is required. The isolated DNA can be used for various molecular biology and biotechnology applications.

HDPCs were grown in cell culture as described in Material and Methods section 2.1.2.2 and the cell pellets were harvested. 500 µl of EZ-DNA<sup>®</sup>, genomic DNA isolation reagent (Gene flow, Israel) was added immediately to fresh cell pellets and re-suspended several times by mixing using a pipette. The samples were then centrifuged (5417R, Eppendorf<sup>®</sup>, Germany) at 10000 × G at 18°C for 10 minutes. 500 µl of absolute ethanol was added to the tube and mixed by inverting the tube several times, until a homogenous solution was achieved and the DNA was visible. Samples were centrifuged for another 5000 × G at 18°C for 5 minutes. The DNA pellet was washed twice with 100 µl of 95% ethanol and centrifuged at 1000 × G for 1 minute at 18°C to remove the contaminants. After the second wash, the tube was inverted on clean tissue paper to air-dry the DNA pellet for 5 minutes. DNA was dissolved the in 200 µl of 8 Mm NaOH and then stored at -20°C.

### **2.3.3 Quantification of nucleic acid by NanoDrop**

In order to obtain nucleic acid concentration values from very small volumes, DNA and RNA were quantified using the NanoDrop spectrophotometer (Thermo scientific, UK). After initial calibration using distilled water, 1.2 µl of each sample was pipetted over the platform and the lid was closed. Absorbance values taken at 260 nm and 280 nm were used to determine DNA and RNA concentrations and purity. A sample with

260/280 ratio between 1.8 and 2.1 can be regarded as a pure RNA sample with high integrity (Brown, 2000). The DNA and RNA concentration were obtained directly from the NanoDrop software calculations.

#### **2.3.4 Evaluation of RNA integrity and yield upon storing of HDPCs in RNAlater<sup>®</sup>**

RNAlater<sup>®</sup> (lifetechnologies, UK) is an aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilise and protect cellular RNA. RNAlater<sup>®</sup> solution minimizes the need to immediately process samples or to freeze samples in liquid nitrogen for later processing. Samples could be harvested and submerged in RNAlater<sup>®</sup> solution for storage without jeopardizing the quality or quantity of RNA obtained after subsequent RNA isolation.

HDPCs with density of  $1 \times 10^5$  cells/well were seeded in 6 well cell culture plates containing 2 ml of  $\alpha$ -MEM medium Lonza (Switzerland) and incubated for 48 hours at 37°C, 5% CO<sub>2</sub> in 100% humidity. Samples were prepared in triplicate. HDPCs from passage 2, 6, 10 and 12 were harvested. The HDPCs within the harvested cell pellet were counted. In one set of triplicates, RNA was extracted immediately while in the other set of triplicates, RNA was extracted from HDPCs that were stored for 10 days in 200  $\mu$ l of RNAlater<sup>®</sup>. The concentration and 260/280 ratio was measured for all samples using the NanoDrop spectrophotometer (ND 1000, Thermo Scientific, UK). The RNA content per cell was calculated from the overall RNA concentration divided by cell number. A similar experiment was repeated in triplicate for HDPCs from passage 2 but obtained from four different donors.

### **2.3.5 DNase treatment**

Dioxyribonuclease I (DNase I) is non-specific endonuclease that performs on single or double stranded DNA molecules. The enzymatic process is activated at 37°C for 10 minutes. EDTA suppresses DNase I activity by chelating the salts required for the enzymatic digestion.

Any trace of genomic DNA after RNA purification was removed using the DNase I kit (Invitrogen, Foster City CA). The mixture reaction comprised of 1 µl of DNase I enzyme, 1 µl of 10× DNase buffer and required amount of DNase-RNase free water for a total reaction volume of 10 µl. The mixture was used to treat RNA aliquots containing 1 µg RNA.

## **2.4 Agarose gel electrophoresis for nucleic acid and PCR product**

Agarose gel electrophoresis is used for separation of nucleic acids based on their molecular size. Agarose is a natural polysaccharide obtained from agar, which upon setting, forms a gelatine macroscopic matrix that performs as a sieve in separation of nucleic acid based on their size under electric field. Combination of fluorescent dye (EtBr) in the gel assists in visualisation of separated nucleic acids under UV light (Brown, 2000).

Nucleic acids were analysed by agarose gel electrophoresis. The products were detected on gels of a specified percentage of molecular grade agarose (0.8% for DNA, 1.6% for PCR and 1.2% for RNA) (Bio-Lab, USA) containing 0.25 µg/ml<sup>-1</sup> ethidium bromide (Promega, USA). The agarose gel was formed by dissolving the required mass of agarose powder in 100 ml of 1 × Tris acetate /EDTA buffer (TAE buffer, Sigma, Poole). The gel was heated in the microwave for 2-3 minutes until completely dissolved. The

agarose gel was poured into a gel tray, a comb inserted and allowed to cool for 1 hour before electrophoresis. After setting of the gel, the comb was removed and the tray was placed in the gel tank filled with approximately 500 ml of 1× TAE buffer. 10 µl of each samples were loaded onto the wells. The electrophoresis was performed for 35 minutes at 86 volt continuously with 20 minutes for 100 volt. After the completion of the run the gel was viewed under transilluminator U.V light (SYNGENE, UK).

## **2.5 Polymerization chain reaction (PCR)**

PCR is a biomedical technology that is used to amplify a single copy of DNA sequence, generating millions of copies of the particular sequence. This procedure is carried out in three different steps; denaturation, annealing and extension. PCR phase consist of 30-40 cycles. Initially the double stranded DNA is denatured at high temperature (95°C) to provide two single stranded DNAs, into which oligonucleotide primers or custom designed primers for the genes of interest bind to the target sequence at 40-60°C. The annealed oligonucleotides act as primers for DNA synthesis. Finally the DNA strand is extended by DNA polymerase I at 72°C using deoxyribonucleotide triphosphate (dNTP's).

### **2.5.1 Amplification of cDNA using reverse transcriptase polymerase chain reaction (RT-PCR)**

#### **2.5.1.1 Reverse transcription (RT) of mRNA**

Reverse transcription (RT) is a method that utilises reverse transcription enzyme to transcribe the RNA substrate into complementary DNA (cDNA). Reverse transcriptase, similar to DNA polymerase, requires DNA primer to initiate its function. An ideal primer for reverse transcription of mRNA is poly (T) oligonucleotide (dt). RNA can be

transcribed into cDNA from 5'-3' direction. The newly synthesised cDNA is amplified using traditional PCR (Allen *et al.*, 2008, Brown, 2010).

Conveniently, the master mix is composed of, the RNA template, the dNTPs, buffers, taq polymerase, reverse transcriptase and oligonucleotide primers. The reaction is reheated to 37°C, which allows RT to function and permits the production of cDNA that is annealed to the primer. Following the first synthesis, normal PCR is carried out to amplify the cDNA and thereby, quantitatively detects gene expression through the creation of complementary DNA (cDNA) transcripts from mRNA (Walker and Rapley, 2000).

In this study, first strand cDNA was synthesised from 1µg of mRNA using high-capacity cDNA reverse transcription kit (Ambion, Applied Biosystems, U.S.A). The RT master mix was prepared from 2 µl of 10 × RT buffer, 4 µl of dNTP mix, 1 µl of RNase inhibitor and 1 µl of reverse transcription enzyme. Eight µl of the prepared master mix was added to RNA volume which contained 1µg of mRNA. DNase free water was added to make a final mixture of 20 µl. The mixture was then briefly centrifuged at 1200 × G for 30 seconds, to spin down the content and eliminate air bubbles. It was incubated at 37°C for 60 minutes followed by incubation at 95°C for 5 minutes then at 4°C until collection. Temperature cycling was performed in an automated thermal cycler (Version 9, PTC-100, Peltier, Bio-Rad, USA). cDNA samples were stored at -20°C until time of use.

#### **2.5.1.2 Amplification of cDNA using polymerase chain reaction (PCR)**

Amplification of the genes of interest was carried out using gene specific primers (see Tables 1 and 3. The polymerase chain reaction was performed using Go-Taq Green Taq polymerase (Promega, U.S.A). 1µl of forward and reverse primers (5µM) and 5 µl of

template were added to 13  $\mu\text{l}$  of SensiMix master mix (Quantace, UK). The master mix consisted of SensiMix dt buffer, 1 $\times$  SYBR-Green solution and  $\text{MgCl}_2$  (1.2mM) finally 6  $\mu\text{l}$  PCR grade water were added to achieve a total volume of 25  $\mu\text{l}$  final mix. Gene amplification process was performed in a Progene thermal cycler (Techne) machine with the following conditions, denaturing at 30 cycles of 94°C for 30 seconds, annealing at 58°C for 1 minute varying according to  $T_m$  of the primers and extension at 72°C for 40 seconds, a final extension of 72°C for 5 minutes. The PCR product was analysed on 1.2 % agarose gel as described in Materials and methods section 2.4. This process was used only to investigate the expression of *GAPDH* gene in HDPCs (P2 and 6) from two different donors.

### **2.5.2 Designing primers for PCR**

A primer is an oligonucleotide used for amplification of the gene of interest. The criteria to consider regarding primer selection are; the primer's melting temperature ( $T_m$ ), specificity and efficiency of amplification, adequacy of GC content, and location of the 3' end of the primer, homology among the primers, content and primer product length as well as the location of primer binding on target template (Dieffenbach *et al.*, 1993, Brown, 2010).

The SYBR-Green<sup>®</sup> primers used in this study are shown in Table 2. The selected primers were synthesized using previously published primer sequences as mentioned in Table 1. Each primer was further evaluated for its specificity for the gene of interest using a Blast search on the NCBI web site ([www.http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)). All the SYBR-Green<sup>®</sup> primers were purchased from Invitrogen Custom Primers (Lifetechnologies, UK) as shown in (Table 2).



Table 2 A list of the forward and reverse primers used for SYBR-Green® study.

Primers	Sequence	Amplicon size(bp)	Tm(°C)
BCN1-F(Bose, 2010)	5' GGCTGAGAGACTGGATCAGG3'	190	64°C
BCN1-R(Bose,2010)	5' CTGTCCACTGTGCCAGATGT3'		62°C
BID-F(Bose, 2010)	5'- TCGCGTAAGGGTTCGCCACT-3'	383	66°C
BID-R(Bose, 2010)	5'- AGGCCCGGAGGGATGCTACG-3'		68°C
TERC-F(Westin et al., 2007)	5'TCTAACCTAACTGAGAAGGGCGTAG-3'	126	52.9°C
TERC-R(Westin et al., 2007)	5'-GTTTGCTCTAGAATGAACGGTGGAAAG-3'		54.4°C
TERT-F(Oh et al., 2004)	5'-GACATGGAGAACAAGCTGTTTGC-3'	185	50.2°C
TERT- R(Oh et al., 2004)	5'-ACAGGGAAGTTCACCACTGTC-3'		49.2°C
GAPDH-F	5'-GTCAGTGGTGGACCTGACCT-3'	400	50.75°C
GAPDH-R	5'-TGAGGAGGGGAGATTCAGTG-3'		48.7°C
DSPP-F (Fan et al.,2003)	5'- AATGGGACTAAGGAAGCTG -3'	814	56°C
DSPP-R (Fan et al.,2003)	5'- AAGAAGCATCTCCTCGGC-3'		56°C
BMP-2-F(Hidefumi et al.2010)	5'-TCCACTAATCATGCCATTGTTTCAGA-3'	74	49.3°C
BMP-2-R (Hidefumi et al.2010)	5'-GGGACACAGCATGCCTTAGGA-3'		51.2°C

The gene ID of genes of interest was obtained from the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The corresponding Taq-Man® primers were purchased from Primerdesign, UK. The sequence Taq-Man® primers used to investigate genes of interest in this study were provided by the vendor company and are shown in Table 3. The primer sequence for *GAPDH*, *WYHAZ* and  $\beta$ -*actin* housekeeping genes was not provided as they are reference genes and considered as proprietary information.

Table 3 A list of the forward and reverse primers used for Taq-Man® study.

Primers	Sequence	Amplicon size(bp)	Tm(°C)
TERC F	5'-TTTTGTCTAACCCCTAACTGACAAG-3'	73	55.3
TERC R	5'-TGAAAGTCAGCGAGAAAAACAG-3'		56
TERT F	5'-CATCCTCTCCACGCTGCTC-3'	113	58
TERT R	5'-CCAACAAGAAATCATCCACCAAAA-3'		56.5
DSPP-F	5'-TCAAAGAAAGTGGTGTCCCTGGT-3'	147	58.2
DSPP-R	5'-CTTCGTTTGCTAATGTGGAATATGT-3'		57.4
BMP-2-F	5'-GGGCATCCTCTCCACAAAAG-3'	109	56.5
BMP-2-R	5'-CCACGTCACTGAAGTCCAC-3'		57.9
RUNX-2-F	5'-TTCTCCCCTTTTCCCCTGA-3'	126	56.1
RUNX-2-R	5'-CAAACGCAATCATCTATACCAT-3'		56.5
OCN-F	5'-GGCACCCCTTCTTCTCTTC-3'	100	56.2
OCN-R	5'-TTCTGGAGTTTATTTGGGAGCA-3'		56.1
ALP-F	5'-CTTGGGCAGGCAGAGAGTA-3'	148	56.4
ALP-R	5'-AGTGGGAGGGTCAGGAGAT-3'		56.5
DMP-1-F	5'-AAGACAGTGCCCAAGATACCA-3'	83	57.2
DMP-1-R	5'-GTCACCTCCCTCAGGCTTG-3'		57.4
BCN-F	5'-CTGGCACTAGAGGAGGAGAG-3'	99	56.3
BCN-R	5'-AGCCTCAGCCTGGACCTT-3'		57.5

### 2.5.3 Selection of housekeeping genes

Housekeeping genes are genes that are necessary for the maintenance of basic cellular function. They are constitutively expressed in all cells of an organism under normal and patho-physiological conditions. Some housekeeping genes (such as *GAPDH*,  $\beta$ -*actin* and *YWHAZ*) are expressed at relatively constant levels in the majority of non-pathological situations (Barber *et al.*, 2005, Greer *et al.*, 2010, Ragni *et al.*, 2013). Conversely, it has been reported that the levels of expression of housekeeping gene may differ between tissues (VanGuilder *et al.*, 2008, Gasparic *et al.*, 2010).

In order to determine which is the most reliable housekeeping gene to use as internal control for HDPCs, the expression of *GAPDH*, *β-actin*, *TERC* and *YWHAZ* in HDPCs, HDPCs treated with MTA or propolis was investigated. Taq-Man<sup>®</sup> qRT-PCR was carried out using the Bio-Rad lightcycler LC 480 (Roche, UK) according to the instruction described in Materials and Methods section 2.5.4.2.

#### **2.5.4 Quantitative real time PCR (qRT-PCR)**

Real-time PCR (RT-PCR) refers to a specific feature of the PCR machine and associated software that enables users to monitor the progress of amplification curves within each reaction. Quantitative real time PCR (qRT-PCR) refers to continuous collection of fluorescent signals emitted by fluorescent probes attached to amplified DNA sequence and subsequently convert them to numerical values for a particular sample (Walker and Rapley, 2000, Dorak, 2007, Brown, 2010). SYBR-Green<sup>®</sup> and Taq-Man<sup>®</sup> are among the widely used assays for qRT-PCR studies and were applied in this work.

##### **2.5.4.1 SYBR Green qRT-PCR**

SYBR Green<sup>®</sup> is an asymmetrical cyanine dye that binds to double stranded DNA sequences and forms a DNA-Dye complex that absorbs blue light and emits green fluorescent light (Zipper *et al.*, 2004). Binding of the dye to the primer may be unspecific and thus, accurate design of the primers binding to the DNA sequence of interested should be warranted. The intensity of the fluorescence is proportional to the concentration of PCR product (Tajadini *et al.*, 2014, Ponchel *et al.*, 2003).

qRT-PCR was carried out using the Rotorgene machine (model 6000, Corbett life science, Australia) to determine expression of selected genes (*TERT*, *TERC*, *BID*, *BCN1*, *DSPP*, and *BMP-2*). *GAPDH* was used as internal control. 1 µl of each custom designed

primer (forward and reverse, 5 $\mu$ M) and 5  $\mu$ l of previously prepared cDNA was added to 13  $\mu$ l SensiMix master mix (Quantace, UK). The master mix contained; SensiMix dt buffer, 1 $\times$  SYBR-Green solution and MgCl<sub>2</sub> (1.2mM). 5  $\mu$ l PCR grade water were added to achieve a total volume of 25  $\mu$ l final mix. The contents were mixed thoroughly by a short spin in a microcentrifuge.

Each sample was studied in triplicate and no template control (NTC) for each master mix reaction was used. The latter is similar to the final master mix prepared as above with the only difference that 5  $\mu$ l of PCR grade water replaced cDNA. qRT-PCR. The programme comprised initial denaturation phase at 95 $^{\circ}$ C for 15 minutes, 40 cycles at; denaturation phase at 95 $^{\circ}$ C for 15 seconds, primer annealing phase at 60 $^{\circ}$ C for 30 seconds and extension phase at 72 $^{\circ}$ C for 20 seconds.

#### **2.5.4.2 Taq-Man<sup>®</sup> qRT-PCR**

Taq-Man<sup>®</sup> probes are hydrolysis probes that are designed to increase the specificity of quantitative PCR. Taq-Man<sup>®</sup> probe principle relies on the 5'-3' exonuclease activity of Taq polymerase to cleave a dual-labelled probe during hybridization to the complementary target sequence and fluorophore-based detection. This method is highly recommended due to its high specificity, efficiency and sensitivity. Since precise quantification was an absolute necessity, validation of the SYBR-Green<sup>®</sup> data was required by Taq-Man<sup>®</sup> assay.

Quantitative PCR was carried out using the Bio-Rad lightcycler LC 480 (Roche, UK) to investigate the expression of selected genes in HDPCs treated with MTA or propolis as well as control group (*DSPP*, *BMP-2*, *DMPI*, *ALP*, *RUNX2* and *OCN*). Each 20  $\mu$ l reaction was prepared from 10 $\mu$ l 2 $\times$  precision mastermix (Primerdesign, UK), 1  $\mu$ l of 300 nM primer/probe mix and 4  $\mu$ l of RNase/DNase free water. 5  $\mu$ l of cDNA were

then added to the final mixture and thoroughly mixed by a short spin in microcentrifuge. Each sample was studied in triplicate and no template control (NTC) for each master mix reaction was used. qRT-PCR programme consisted of enzyme activation phase at 95°C for 10 minutes, denaturation phase of 50 cycles at 95°C for 15 seconds and data collection phase for 1 minute at 60°C.

### 2.5.5 qRT- PCR data analysis

Data were analysed using ABI 7000 system software (Applied Biosystem). Relative expression levels of each gene were calculated using the  $2^{-\Delta\Delta Ct}$  method (Haimes *et al.*, 2006). Briefly, the cycle threshold (Ct) values for each target gene reaction were normalised to Ct value of housekeeping gene at each time point by subtracting the house keeping gene Ct from the target gene Ct giving  $\Delta Ct$ . This value was then normalised to the  $\Delta Ct$  of control sample which yielded  $\Delta\Delta Ct$ . The fold change in gene expression (relative expression) was then calculated by  $2^{-\Delta\Delta Ct}$  or  $2^{-\Delta Ct}$ . The same threshold level was used for the analysis of all the samples so they could be compared to one another. Data are shown as mean  $2^{-\Delta\Delta Ct}$  and standard error of the mean. The formulas used for calculations are shown below (VanGuilder *et al.*, 2008, Haimes and Kelley, 2010):

$\Delta Ct = Ct \text{ of gene of interest} - Ct \text{ of housekeeping gene}$

$2^{-\Delta Ct}$  was calculated to determine the level of expression of the gene of interest relative to the housekeeping gene.

$\Delta\Delta Ct = \Delta Ct \text{ of each sample} - \Delta Ct \text{ of the control sample}$

$2^{-\Delta\Delta Ct}$  was calculated to determine the level of expression of the gene of interest relative to the control.

$$\Delta Ct \text{ expression of } s.d = \sqrt{s.d \text{ gene of interest}^2} + \sqrt{s.d \text{ house keeping gene}^2}$$

## 2.6 Western blot

In order to evaluate the correlation between mRNA and protein levels, and to validate the *BMP-2* gene expression data for HDPCs incubated with MTA and propolis, western blot technique was used. Western blot is a widely used technique to detect specific proteins in individual cells or tissues. The Western blotting process relies upon three essential factors: firstly, accurate separation of protein by size using gel electrophoresis, secondly, the efficient transfer of separated proteins to a membrane; and finally specific detection of a target protein by appropriately designed antibodies. The detected target protein is visualised as a band on a blotting membrane, X-ray film, or an imaging system (Towbin *et al.*, 1979, Moore, 2009).

HDPCs (P2, 4, 6 and 12) with seeding density of  $1 \times 10^5$  cell/well were cultured using  $\alpha$ -MEM overnight. Cells received MTA, propolis or no treatment (n=3 per group) and were incubated for 24 hours. Protein isolation was performed as follows the cell culture plate was placed on ice and the cells were washed with ice-cold PBS. The sample buffer was prepared by adding 50  $\mu$ l of  $\beta$ -Mercaptoethanol to 950  $\mu$ l of 2 $\times$  sample buffer (lysis buffer) and was placed on ice (Laemmli sample buffer, Sigma UK). The PBS was drained from the plate, and then 0.5 ml of ice-cold lysis buffer was added to the cultured HDPCs. The adherent cells were scraped off the well cell culture plate using a cold plastic cell scraper, and the cell suspension was gently transferred into a pre-cooled microfuge tube. The samples were centrifuged at 12000  $\times$ G for 2 minutes at 4°C. The supernatant was gently aspirated and transferred into a fresh tube and placed on ice. The supernatant was spun down for 10 seconds and the samples were incubated on a pre-warmed heat block at 95°C for 3-5 minutes to completely denature the proteins. Running buffer was prepared for gel electrophoresis by adding 100 ml of running buffer to 900 ml of distilled water. 12% pre-cast SDS page gel (Mini-Protean<sup>®</sup> TGX Stain-free

Gels, Bio-Rad, UK) with 10 well comb used for electrophoresis. The running buffer was added to the middle of the gel ring until it was completely full including each well. 5 µl of protein marker ladder (10-250 KDa) and 45 µl of each protein samples were loaded into each lane. The gel was run at 100 Volt for 1.5 hours.

### **2.6.1 Blot transfer and blocking**

For blot transfer, the washing buffer was prepared by mixing 100 ml Tris buffer saline (TBS-10X) with 1 ml of viscous Tween 20 reagent and 900 ml of distilled water (New England Biolabs Ltd, UK). 5% milk or BSA solution was prepared with 5g of BSA powder added to pre-prepared 100 ml of TBS-T buffer. The solution was mixed thoroughly and filtered. After gel electrophoresis the gel was removed and rinsed with the running buffer. The nitrocellulose membrane 0.2 µm PVDF (Trans-blot R Turbo™ transfer pack, Bio-Rad, UK) was transferred to the blotting cassette. The right top corner of the membrane was marked to indicate the blot orientation and the protein side could be easily distinguished. The gel was then transferred to the blotting module.

For blotting, the cassette containing the membrane was placed in the Tans-Blot Turbo transfer system machine (Bio-RAD, UK), and incubated for 7-8 minutes. After the protein was transferred to the membrane, the membrane was transferred carefully to plastic dish on the shaker (IKA-VIBRAX-VXR, UK). The membrane was rinsed with distilled water and enough blocking solution added to cover the membrane; 25 ml/membrane. The membrane was incubated in the blocking solution for 1 hour at room temperature on the shaker with slow agitation. The blocking buffer was discarded and the membrane was washed with washing buffer (TBS-T) for 3 to 5 times for 5 minutes.

1µl of primary (rabbit polyclonal) anti-BMP2 and anti-βactin antibodies (Abcam,UK) were diluted as 1:1000 concentration with 1ml of TBS-T buffer with final concentration

of 1mg/ml. 2-3 ml of the prepared primary antibody solution was added to each membrane. 3  $\mu$ l of the primary antibody was added to 3 ml of the TBS-T and was mixed thoroughly. The membrane was folded in a 50 ml centrifuge tube and the primary Antibodies were added to the blotting side of the membrane. The 50 ml centrifuge tube was wrapped with Parafilm<sup>®</sup> to prevent evaporation. The membrane was incubated with the primary antibodies in the 50  $\mu$ l centrifuge tube on the roller overnight at room temperature. After overnight incubation, 5 washes for 5 minutes each with washing buffer (TBS-T) were completed. Secondary antibodies: mouse anti rabbit, BMP2 and  $\beta$ -actin antibody, were prepared by diluting 1 $\mu$ l of secondary antibody in 1 ml of TBS-T buffer with final concentration of 1mg/ml and stored on ice. The primary antibodies were discarded and the membrane was incubated in a new plastic dish for 1 hour with the secondary antibodies. After one hour incubation, the secondary Antibodies were discarded and continued with the 5 washes for 5 minutes for each membrane with washing buffer (TBS-T). The membrane was placed on a piece of tissue to dry.

### **2.6.2 Detection**

The detection solution (SuperSignal West Femto chemiluminescence substrate) (ThermoScientific, Uk) was prepared according to the manufacturer's instructions. The membrane was transferred on a piece of clean acetate cling film, and 1 ml of detection solution in drops was added over the membrane evenly. The membrane was incubated for 4-5 minutes at room temperature. After the incubation period, the membrane was covered with an acetate sheet and the membrane was immediately developed while it was on the acetate.



### **2.6.3 Western blot data analysis**

Western blot data was analysed using ChemiDoc MP imaging system (Bio-Rad, UK). Each protein band was detected and quantified by comparing the BMP-2 protein band to the protein marker ladder. The intensity of each specific protein band was measured using densitometry and the signal intensity was measured relative to the control sample.

## **2.7 Analysing telomere length by southern blot**

Southern blotting is a technique used for detection of a specific DNA sequence in DNA samples. It involves transferring electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridisation (Kimura *et al.*, 2010). Telomere length in genomic DNA could be evaluated using different selected methods including; TRF, STELLA, qRT-PCR, Q-FISH and flow-fish. Each method has its own advantages and disadvantages. Southern blot technique remains the gold standard technique for measuring absolute telomere length (Cawthon, 2009, Ruiz-Herrera *et al.*, 2009, Aubert *et al.*, 2012).

DNA was extracted from serially-passaged HDPCs with initial seeding density of  $1 \times 10^5$ . HDPCs received MTA or propolis treatment or no treatment (n=3). Genomic DNA was isolated from freshly harvested HDPCs as described in Materials and Methods section 2.3.2. Required volume of  $1 \mu\text{g}/\mu\text{l}$  genomic DNA and  $0.1 \mu\text{g}/\mu\text{l}$  control DNA from the kit was digested using restriction enzymes (HinfI and RsaI) (Roche, US). These enzymes cut the entire DNA into small fragments whereas the telomere DNA remained intact. A volume containing  $1 \mu\text{g}$  DNA,  $2 \mu\text{l}$  of  $10\times$  digestion buffer,  $1 \mu\text{l}$  of ( $40 \text{ U}/\mu\text{l}$ ) of HinfI or RsaI enzyme was mixed to produce a final volume of  $20 \mu\text{l}$ . The mixture was spun and then incubated at  $37^\circ\text{C}$  for 2 hours. Digested DNA samples were

electrophoresed in 0.80% agarose gel as described in Materials and Methods section 2.4 to separate DNA samples according to their size. Southern blot was carried out using telo TAGGG telomere length assay kit (Roche, US). The Southern blot hybridisation was performed following instructions provided by the manufacturer. Chemiluminescence signal on developed x-ray was scanned by ChemiDoc MP imaging system (BIO-RAD, UK) in order to determine changes of telomere length.

## **2.8 Statistical data analysis**

Statistical package for the social sciences (SPSS, version 19, IBM, USA) was used to perform One-Way Analysis of Variance (ANOVA) on the data obtained from cell viability assays and data from gene expression studies. Post-hoc analysis (LSD) was used to compare differences between different experimental groups wherever indicated. Level of significance was set at p-value of 0.05.

## Chapter 3: Results

### 3.1 Comparison of growth rate of HDPCs isolated by different methods

HDPCs were isolated successfully from four extracted upper and lower third molar teeth from four female patients aged between 22-28 years as described in Materials and Methods section 2.1.3. The average number of HDPCs isolated utilising the collagenase digest method was approximately  $6 \times 10^6$  cells after five days of incubation of processed dental pulp. The time to reach 80-90% confluence of HDPCs obtained by this method was 3-5 days. In contrast, the explant outgrowth isolation method yielded  $1.2 \times 10^6$  HDPCs after five days of incubation of processed dental pulp and the time to reach 80-90% confluence of these HDPCs was as high as 5-7 days.

The findings indicated that the collagenase digest method was superior to the explant outgrowth method yielding higher numbers of HDPCs with higher proliferation capacity as indicated by the shorter time to reach 80-90% confluence.

HDPCs from passage 2 were cultured separately with initial seeding density of  $1 \times 10^4/\text{cm}^2$ . After an incubation period of five days, both cell groups were studied using the light microscope. HDPCs isolated using the collagenase digest method reached higher number and density at the end of incubation time. Both studied HDPCs grew uniformly and appeared as fibroblast-like cells with triangular or stellate shape at the stage of examination, as shown in Figure 6.

The light microscopy study supported the findings of lower doubling time of HDPCs isolated using collagenase digest method. These findings may indicate that the

collagenase method is more effective in isolating viable cells from pulp tissue and it is less likely to affect the proliferation ability of the isolated HDPCs.

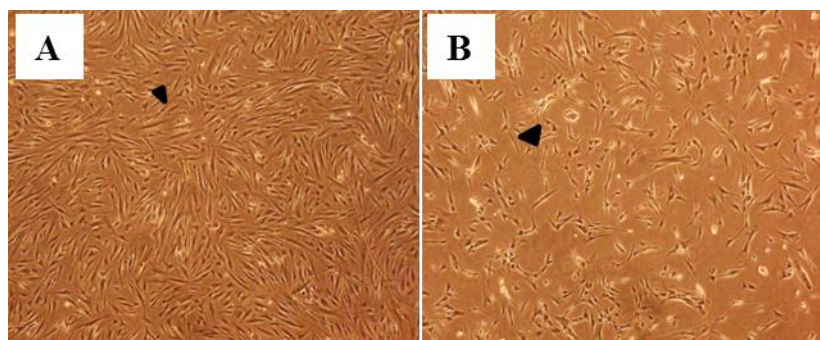


Figure 6. *In situ* light microscope image of cultured HDPCs at 5 days.

A) Higher density of HDPCs isolated by collagenase digest method in comparison to, B) HDPCs isolated using explant culture method. Arrows point to cell bodies of HDPCs which is comparable to fibroblast-like cells. Magnification: 100x.

### **3.1.1 The effect of isolation method on viability and doubling time of serially passaged HDPCs**

HDPCs isolated using both isolation techniques, were serially passaged (P2-P12). An initial seeding density of  $1 \times 10^4/\text{cm}^2$  was used to culture HDPCs. HDPC viability, doubling time and total cell number after five days incubation period were estimated for serially passaged HDPCs isolated by either collagenase digest or explant outgrowth methods as described in Materials and Methods section 2.1.5.

The total HDPC number isolated using the collagenase digest method was significantly higher in comparison to explant outgrowth methods in all passages except 11 and 12. This is attributed to the shorter doubling time exhibited by HDPCs isolated by collagenase method (7-12 hours) in comparison to explant outgrowth method (10-16 hours). Additionally, the percentage of viable HDPCs was higher upon the use of the collagenase method. However, the latter two findings were not applicable to HDPCs from passage 12 where the doubling time was shorter for HDPCs isolated by explant

method with higher viability percentage. Figure 7 summarises the findings of the comparison between the two isolation methods. For this study we were interested in obtaining a large number of HDPCs in a short period of time. Thus, HDPCs isolated with collagenase were used for all the subsequent studies.

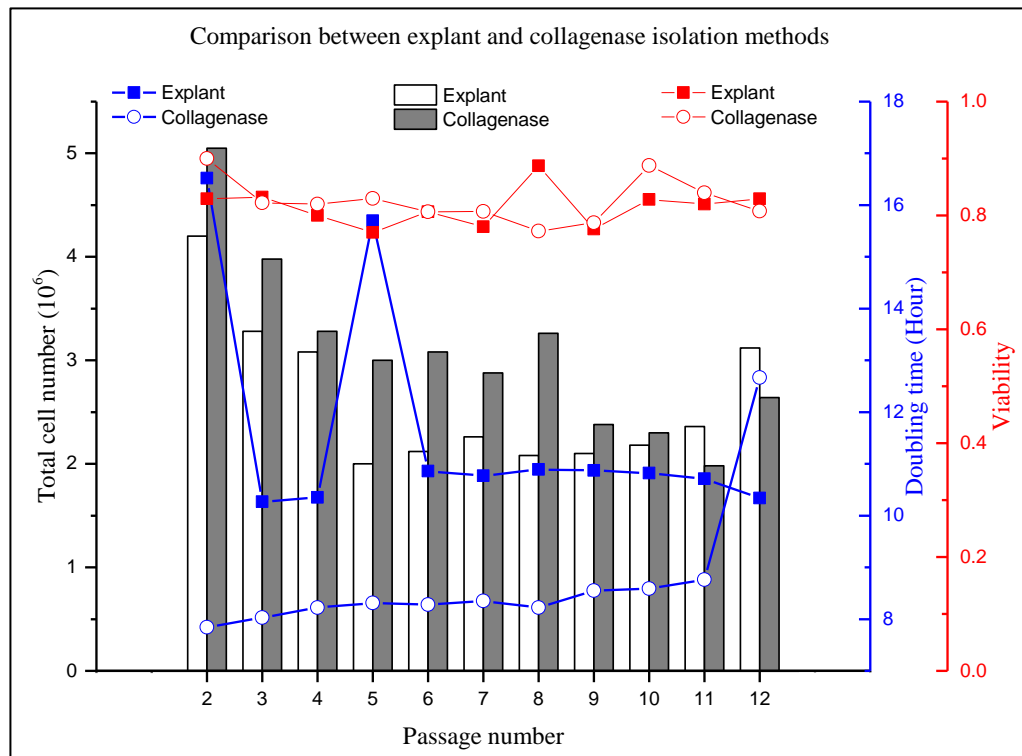


Figure 7. Comparison between explant and collagenase isolation methods. The difference in doubling time, total cell number and viability percentage is demonstrated for serially passaged HDPCs isolated using both methods.

### 3.2 The effect of pulp capping materials on HDPC viability

HDPCs isolated using the collagenase digest method were cultured with Dycal, MTA and propolis as described in Materials and Methods section 2.2.1. The cytotoxic effect of the studied materials on HDPCs was investigated by calculating the percentage of viable cells at different time points.

### 3.2.1 Effect of Dycal on the viability of HDPCs

In the light of contradicting findings regarding the effects of Dycal on cellular viability and proliferation, the effects of Dycal on viability of cultured HDPCs was evaluated utilising Trypan Blue assay as described in Materials and Methods section 2.1.5. The viability of HDPCs cultured with Transwell® inserts containing Dycal was compared with control HDPCs (n=3 per group). HDPCs were from passage 2 and 12 and incubated with Dycal for 5, 10, 24 or 48 hours as described in Materials and Methods section 2.2.1.1. At every time point, Dycal treated HDPCs from both passages had significantly lower number of attached (viable) cells in the monolayer in comparison to the control group. The cytotoxic effect of Dycal on HDPCs seemed to increase with time as the number of viable cells decreased, especially with HDPCs from passage 2. The trend was less consistent with HDPCs from passage 12. Significant difference between the number of viable HDPCs within the monolayer among the two groups ( $p < 0.05$ ) as shown in Figures (8 & 9).

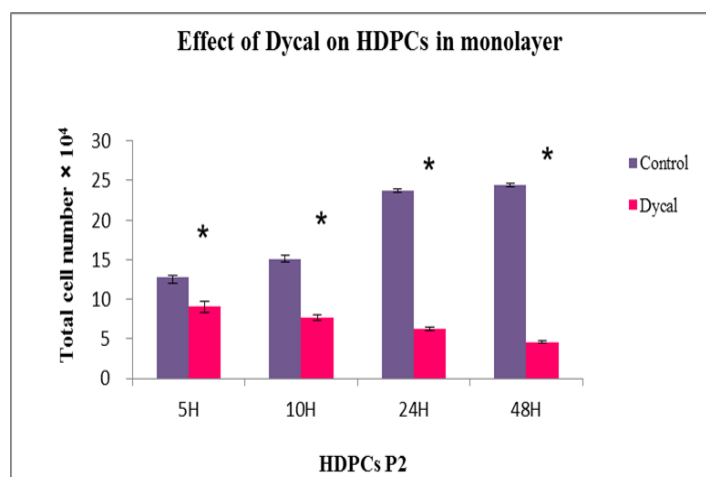


Figure 8. Effect of Dycal on total number of viable cells in monolayer (P2). Total number of viable HDPCs upon exposure to Dycal compared to control from passage 2 after incubation periods of 5, 10, 24 and 48 hours. Data represent mean  $\pm$  standard deviation (n=3),  $p < 0.05$  comparing Dycal to control at different time points.

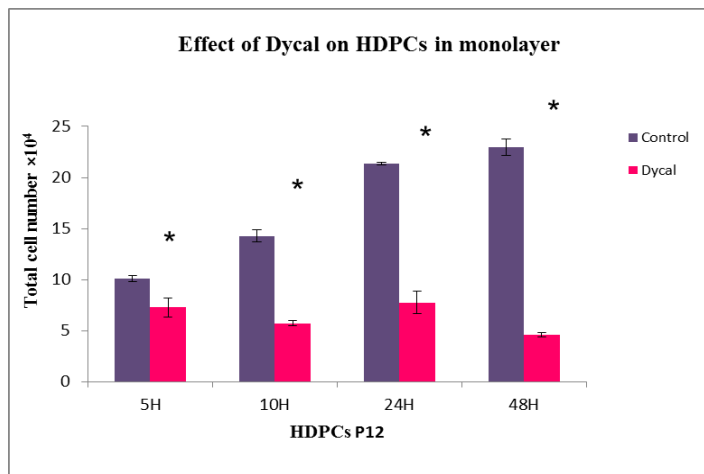


Figure 9. Effect of Dycal on total number of viable cells in monolayer (P12). Total number of viable HDPCs upon exposure to Dycal compared to control from passage 12 after incubation periods of 5, 10, 24 and 48 hours. Data represent mean  $\pm$  standard deviation (n=3).  $p < 0.05$  comparing Dycal to control at different time points.

Higher number of detached (dead) HDPCs was noticed in the culture medium upon culturing with Transwell<sup>®</sup> inserts containing Dycal in comparison to control HDPCs. Dycal caused time-dependent cell detachment in HDPCs from passage 2 and 12. One way ANOVA revealed that the statistically significant difference in two groups ( $p < 0.05$ ) as shown in Figures (10 & 11).

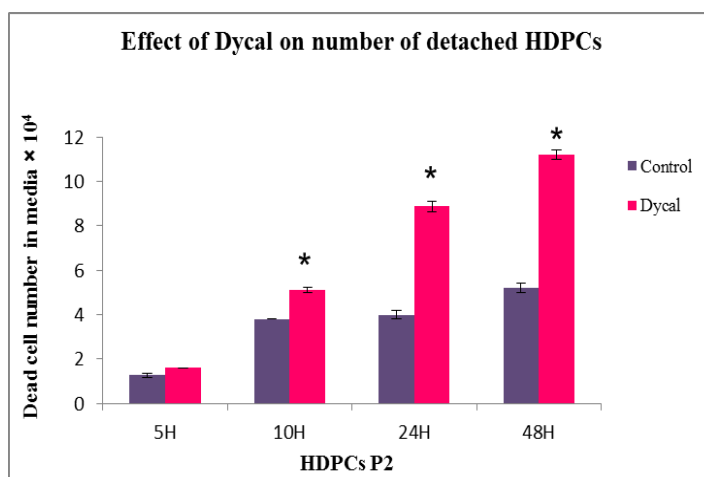


Figure 10. Effect of Dycal on detached/floating HDPCs in the culture medium (P2). Total number of detached/floating HDPCs in the culture medium, upon exposure to Dycal compared to control from passage 2 after incubation periods of 5, 10, 24 and 48 hours. Data represent mean  $\pm$  standard deviation (n=3).  $p < 0.05$  comparing Dycal to control at different time points.

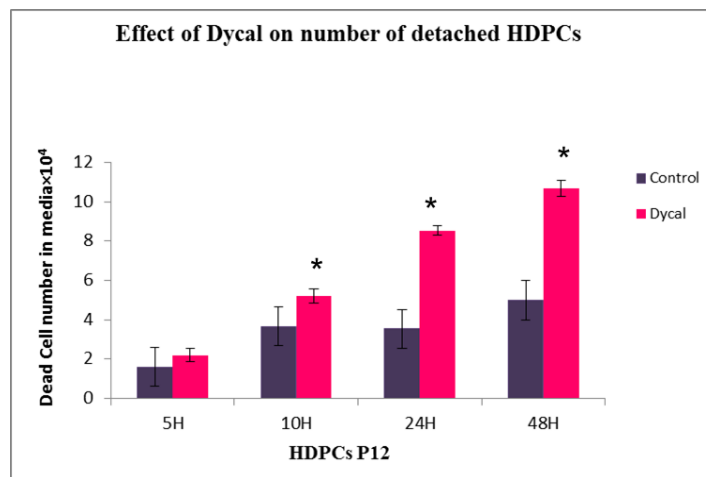


Figure 11. Effect of Dycal on detached/floating HDPCs in the culture medium (P12).

Total number of detached/floating HDPCs in the culture medium, upon exposure to Dycal compared to control from passage 12 after incubation periods of 5, 10, 24 and 48 hours. Data represent mean  $\pm$  standard deviation (n=3).  $p < 0.05$  comparing Dycal to control at different time points.

Overall, HDPCs from late passages had less viability percentage in comparison to those from early passages (P2). This may be attributed to the time-dependent loss of proliferation and growth potential as well as cellular ageing. Dycal treatment significantly reduced viability of HDPCs. Dycal-treated HDPCs from passage 2 had viability percentage throughout four time points equals to  $35 \pm 10$  % in comparison to  $69 \pm 16$  % for the control HDPCs (Figure 12). Furthermore, Dycal-treated HDPCs from passage 12 had viability percentage throughout four time points equals to  $27 \pm 7$  % in comparison to  $57 \pm 10$  % for the control HDPCs (Figure 13). These findings clearly demonstrate the cytotoxic effect of Dycal on HDPCs which can be attributed to the chemical cell injury induced by hydroxyl ions released from Dycal, changes of pH of culture medium induced by Dycal or cytotoxic effect of Transwell<sup>®</sup> inserts containing Dycal. Finally, data may suggest that HDPCs from late passages may be more susceptible to Dycal's cytotoxicity owing to their compromised growth and proliferation potential.



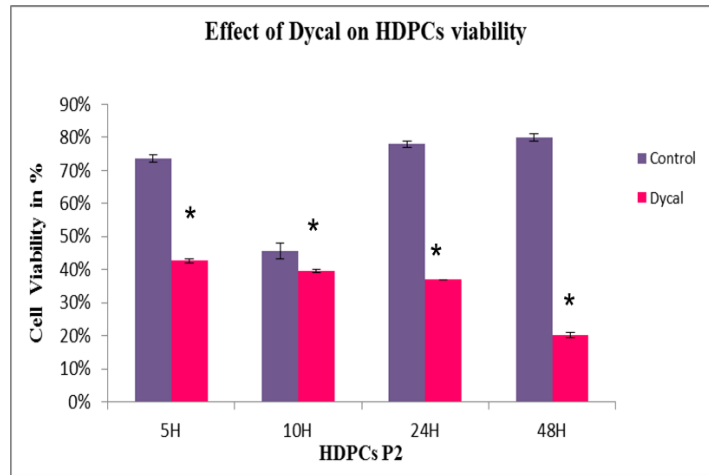


Figure 12. Effect of Dycal on viability of HDPCs (P2).

Mean percentage of viable HDPCs, upon exposure to Dycal compared to control from passage 2 after incubation periods of 5, 10, 24 and 48 hours. Data represent mean  $\pm$  standard deviation (n=3),  $p < 0.05$  comparing Dycal to control at different time points.

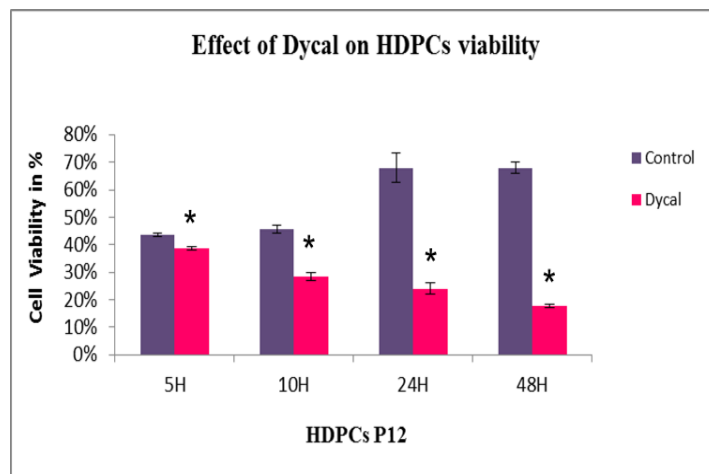


Figure 13. Effect of Dycal on viability of HDPCs (P12).

Mean percentage of viable HDPCs, upon exposure to Dycal compared to control from passage 12 after incubation periods of 5, 10, 24 and 48 hours. Data represent mean  $\pm$  standard deviation (n=3),  $p < 0.05$  comparing Dycal to control at different time points.

### **3.2.2 Effect of Transwell<sup>®</sup> insert on the viability of HDPCs**

Clear, sterile polystyrene Transwell<sup>®</sup> inserts with polycarbonate membrane are one of the most widely used tools to assess the effect of indirect contact of a particular material on the biological properties of various cells. The Transwell<sup>®</sup> insert is known to be highly biocompatible. However, there was no available data in the literature regarding the effect of these Transwell<sup>®</sup> inserts on HDPCs in particular. In this study, the potential confounding effect (positive or negative) of the Transwell<sup>®</sup> inserts on the viability of HDPCs was assessed. Additionally, if Transwell<sup>®</sup> inserts had no effect on HDPC viability, untreated/control cells could be cultured without using them and thereby the number/cost of Transwell<sup>®</sup> inserts required could be reduced. The viability of HDPCs cultured with Transwell<sup>®</sup> inserts was compared with control HDPCs (n=3 per group), the viability of HDPCs was estimated as described in Materials and Methods section 2.1.5. HDPCs were from passage 2 incubated with and without Transwell<sup>®</sup> inserts for 24 hours as described in Materials and Methods section 2.2.3.

HDPCs in monolayers that were cultured with empty Transwell<sup>®</sup> inserts for 24 hours had higher viable cell counts in comparison to HDPCs in monolayers cultured without Transwell<sup>®</sup> inserts (Figure 14). However, one-way analysis of variance (ANOVA) yielded a  $p > 0.05$  which indicated that the difference was not statistically significant.

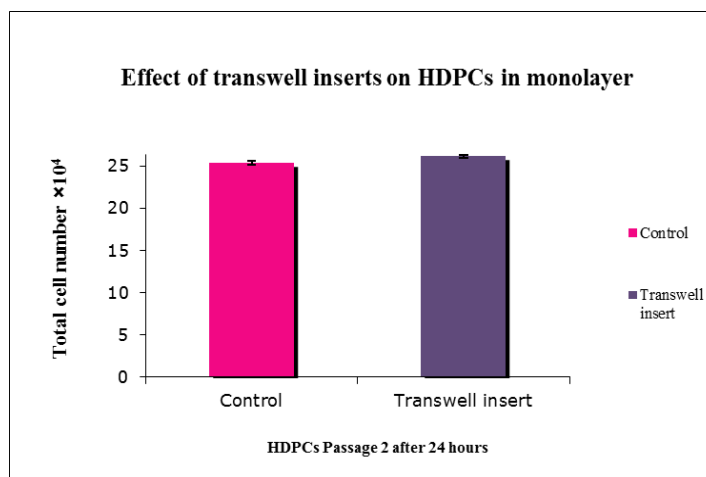


Figure 14. Effect of Transwell<sup>®</sup> inserts on total number of viable cells in monolayer (P2).

Total number of viable HDPCs, upon exposure to Transwell<sup>®</sup> inserts compared to control from passage 2 after incubation period of 24 hours. Data represent mean ± standard deviation (n=3).

The number of detached/floating cells in the culture medium was lower among HDPCs cultured with Transwell<sup>®</sup> inserts for 24 hours when compared to control (Figure 15). One way ANOVA revealed that the difference was not statistically significant ( $p > 0.05$ ).

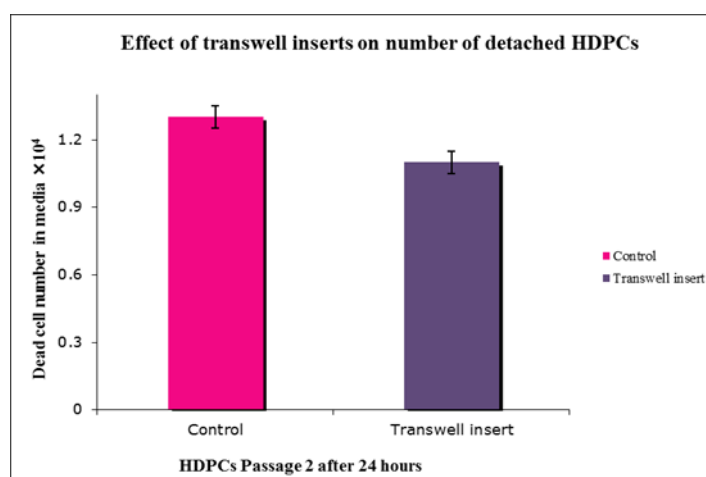


Figure 15. Effect of Transwell<sup>®</sup> inserts on detached/floating HDPCs in the culture medium (P2).

Total number of detached or floating HDPCs in the culture medium, upon exposure to Transwell<sup>®</sup> inserts compared to control from passage 2 after incubation period of 24 hours. Data represent mean ± standard deviation (n=3).

Overall, viability of HDPCs was similar with or without exposure to Transwell® inserts, 81% and 80%, respectively (Figure 16). Statistical analysis using (ANOVA) revealed no statistically significant differences between the percentages of viability of HDPCs when cultured with or without Transwell® inserts ( $p>0.05$ ). As a consequence, Transwell® inserts were only used with HDPCs cultured with MTA or propolis and were not used in the control wells.

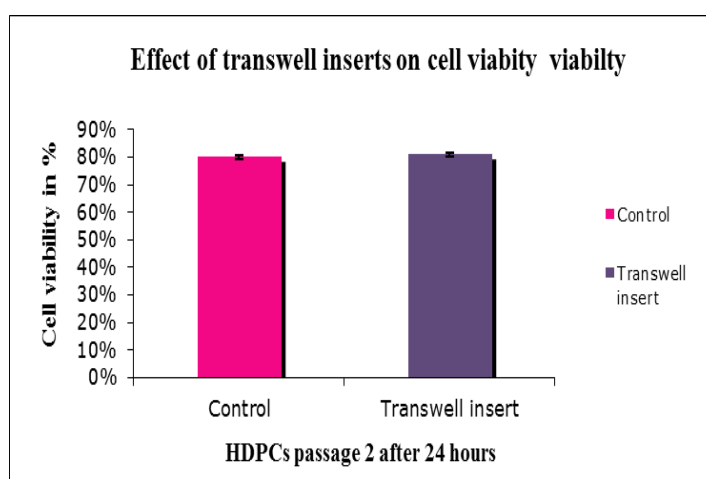


Figure 16. Effect of Transwell® inserts on viability of HDPCs (P2).

Mean percentage of viable HDPCs, from passage 2, upon exposure to Transwell® inserts upon exposure to Transwell® inserts compared to control from passage 2 after incubation period of 24 hours. Data represent mean  $\pm$  standard deviation (n=3).

### 3.2.3 Effect of Dycal on pH of cultured media

The data from section 3.2.2 suggested that cytotoxicity of Transwell® inserts can be ruled out as a contributing factor to reduced viability percentage of HDPCs exposed to Dycal. Thus, Dycal induced pH changes as a contributing factor to high cell death was evaluated. Such approach was indicated as Dycal induced yellowish discoloration of cultured medium of HDPCs which points toward possible acidic changes. pH changes of culture media were investigated as described in Materials and Methods section 2.2.4.

When HDPCs cultured in PBS (pH = 7), HDPCs culture had slightly acidic pH (6.78±0.012). Upon application of Transwell® insert containing Dycal, pH slightly increased to 6.84±0.01, but conditions were still acidic. Conditions were slightly alkaline when HDPCs were cultured in  $\alpha$ -MEM (pH = 7.77±0.012). However, with application of Transwell® insert containing Dycal, resulted in a slight reduction of the pH to 7.64±0.01 (Table 4). The data indicated that Dycal application utilising Transwell® inserts induced subtle pH changes which could contribute toward the increased cell death of HDPCs upon exposure to Dycal. However, the difference was not statistically significant in both PBS and  $\alpha$ -MEM containing HDPCs ( $p > 0.05$ ).

Table 4 Comparison of pH in different conditions.

Sample	Sample condition	pH reading(mean $\pm$ SD)
1	HDPCs in PBS only	6.78 $\pm$ 0.012
2	Dycal treated HDPCs in PBS	6.84 $\pm$ 0.01
3	HDPCs in $\alpha$ -MEM	7.77 $\pm$ 0.012
4	Dycal treated HDPCs in $\alpha$ -MEM	7.64 $\pm$ 0.01

### 3.2.4 Effect of MTA and propolis on viability of HDPCs

The studies performed on Dycal suggested a highly cytotoxic effect on HDPCs which could not be attributed to a single factor as shown in the Result section 3.2.1, 3.2.2 and 3.2.3. The biological effects, in terms of HDPC morphology and viability of a widely used alternative material such as MTA and the novel biological agent, propolis were investigated.

Light microscopy studies of HDPCs incubated with MTA and propolis revealed high biocompatibility of both materials. HDPCs grew uniformly on the bottom of the well cell culture plate, especially in the control group. Conversely, HDPCs aggregated in the vicinity of the Transwell® inserts where the highest concentration of MTA and propolis could be expected. None of the HDPC cultures exhibited a growth-inhibition zone which confirms the high biocompatibility of both materials. HDPCs incubated with MTA and propolis as well as control HDPCs appeared to have similar morphology which resembled normal fibroblast-like cells (Figure 17).

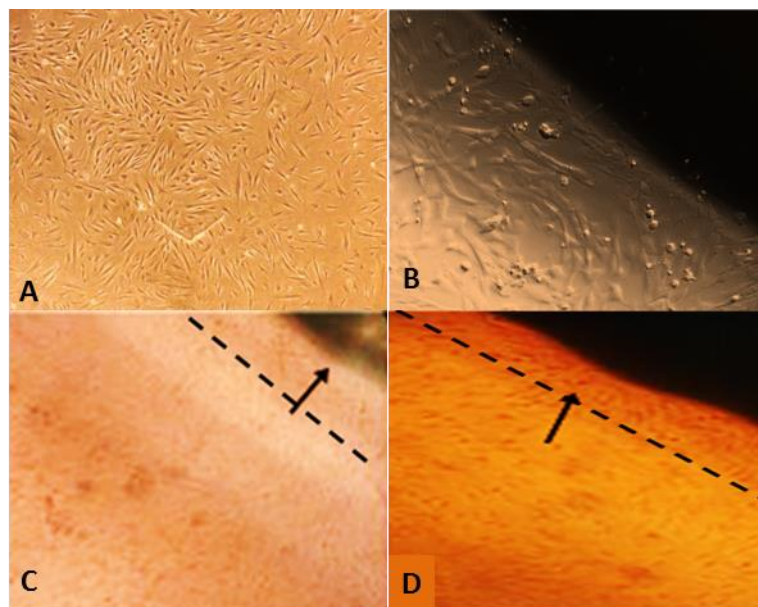


Figure 17. *In situ* light microscope image of cultured HDPCs treated with MTA and propolis.

A) After 24 hours incubation, B) with exposure to empty Transwell® inserts, C) with exposure to MTA and D) with exposure to propolis. Note the high cell density near the Transwell® inserts containing MTA (black area on top right corner) and propolis (black area on bottom right).

Cell viability is a major indication of the biocompatibility of the material. Additionally, the potential of pulp repair and hard bridge formation is heavily dependent on the viability of odontoblast or odontoblast-like cells. The viability percentage of HDPCs

from early passage (P2) was determined upon incubation with MTA or propolis for 12 and 24 hours as described in Materials and Methods section 2.1.5.

After 12 hours incubation, HDPCs incubated with propolis had the highest number of viable cells within the monolayer, followed by MTA and control groups. One-way ANOVA and post-hoc analysis utilising Fishers Least Significant Difference (LSD) test revealed no significant difference between the number of viable HDPCs within the monolayer in MTA and propolis groups ( $p > 0.05$ ) after 12 hours, whereas the latter groups had significantly higher number of viable cells in the monolayer than the control group ( $p < 0.05$ ). After 24 hours incubation, the propolis group had the lowest number of viable cells in the monolayer while the MTA group had the highest in the comparison to the control group. One-way ANOVA and post-hoc analysis utilising LSD test revealed significant difference between the number of viable HDPCs within the monolayer among the three groups ( $p < 0.05$ ) (Figure 18).

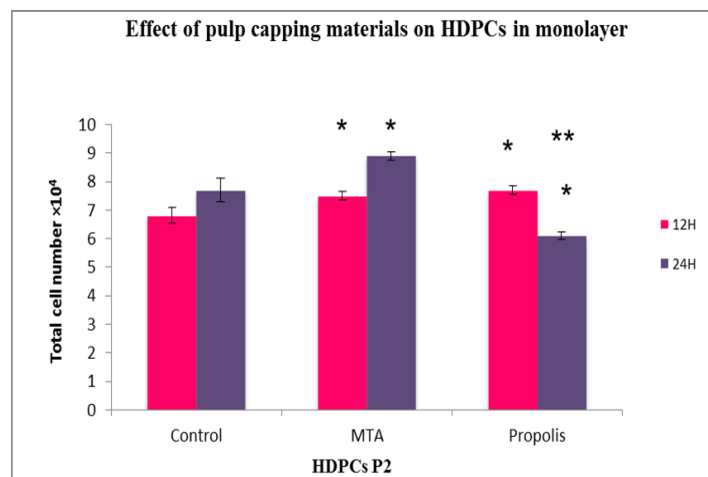


Figure 18. Effects of MTA and propolis on number of viable cells in monolayer. Total number of viable HDPCs upon exposure to MTA and propolis compared to control from passage 2 after incubation period of 12 and 24 hours. Data represent mean  $\pm$  standard deviation ( $n=3$ ), \* $p < 0.05$  among groups at 12 and 24 hours incubation in comparison to control, \*\* $p < 0.05$ , comparing MTA and propolis.

At incubation periods of 12 and 24 hours, higher numbers of floating/detached HDPCs in the media were noticed with propolis group in comparison to the other two groups (Figure 19). One-way ANOVA and post-hoc analysis utilising LSD test revealed no significant difference between the number of floating HDPCs in culture medium of MTA and control groups ( $p>0.05$ ), while the propolis group had a significantly higher number in comparison to the latter groups ( $p<0.05$ ).

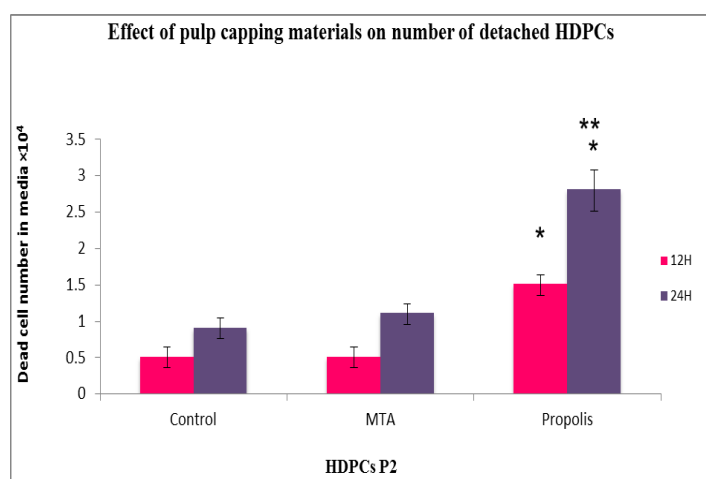


Figure 19. Effects of MTA and propolis on detached/floating HDPCs.

Total number of detached/floating HDPCs in the culture medium, upon exposure to MTA and propolis compared to control from passage 2 after incubation period of 12 and 24 hours. Data represent mean  $\pm$  standard deviation ( $n=3$ ),  $*p<0.05$  among groups at 12 and 24 hours incubation in comparison to control,  $**p<0.05$ , comparing MTA and propolis.

Overall, after 24 hours, MTA treatment resulted in the highest mean percentage of viable HDPCs ( $87.5\pm 0.007\%$ ), followed by control ( $85.98\pm 0.014\%$ ) and propolis  $79.0\pm 0.014\%$  groups. A similar trend was found after 12 hours incubation period as shown in Figure 20. Statistical analysis revealed that at after 12 hours incubation period, there was no significant difference between the viability percentage of the propolis and control groups ( $p>0.05$ ). However, the MTA group had a significantly higher viability percentage ( $p<0.05$ ). After 24 hours incubation period, MTA and control groups had



comparable viability percentage ( $p>0.05$ ); whereas propolis had significantly lower viability percentage in comparison to the latter two groups ( $p<0.05$ ).

The possible cytotoxic effect of propolis could be attributed to the high concentration of the material released in culture medium. Thus, the experiment was repeated with a similar amount of propolis in each Transwell<sup>®</sup> insert, but from a lower concentration mixture.

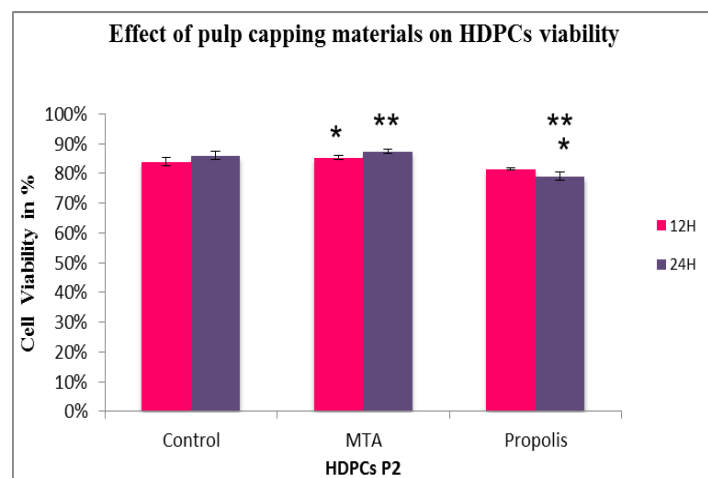


Figure 20. Effects of MTA and propolis on viability of HDPCs.

Mean percentage of viable HDPCs, from passage 2, upon exposure to MTA and propolis compared to control from passage 2 after incubation period of 12 and 24 hours. Data represent mean  $\pm$  standard deviation ( $n=3$ ), \* $p<0.05$  among MTA and control groups at 12 and among propolis and control at 24 hours, \*\* $p<0.05$  among propolis and MTA at 24 hours incubation.

### 3.2.5 Effect of lower concentration of propolis on viability of HDPCs

The use of Transwell<sup>®</sup> inserts containing propolis caused notable brownish discoloration of the culture medium. This suggested that there is a high concentration of the material released in the culture medium which may have been responsible for the higher cell death rates observed upon the exposure to propolis. This study was conducted to investigate the effects of a lower concentration of propolis (0.80 g/ml in

comparison to 2 g/ml) on HDPCs viability as described in Materials and Methods section 2.2.2. The cell viability was measured after 24 hours incubation period as described in Materials and Methods section 2.1.5.

After 24 hours incubation, the culture medium containing floating cells was aspirated. The HDPC count after trypsinisation was highest in the MTA group followed by propolis and control groups, respectively. However, there was no statistically significant difference between the three groups as indicated by one-way ANOVA and LSD post-hoc analysis ( $p > 0.05$ ) (Figure 21).

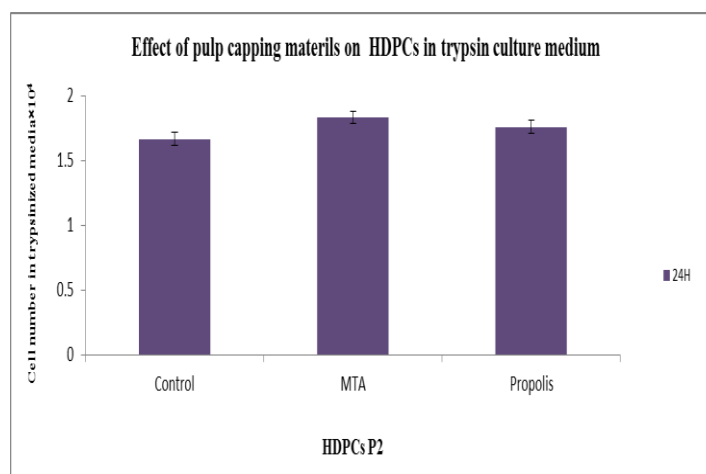


Figure 21. Effects of MTA and propolis on viability of HDPCs in trypsinised medium. Total number of dead/floating HDPCs in the supernatant, HDPCs, upon exposure to MTA and propolis compared to control from passage 2 after incubation period of 24 hours. Data represent mean  $\pm$  standard deviation ( $n=6$ ).

After 24 hours incubation, propolis and MTA resulted in a comparable number of viable HDPCs in the monolayer ( $p=0.05$ ). The number of viable HDPCs in the monolayer of the control group was significantly lower as indicated by one-way ANOVA and LSD post-hoc analysis ( $p < 0.05$ ) in comparison to MTA and propolis (Figure 22).

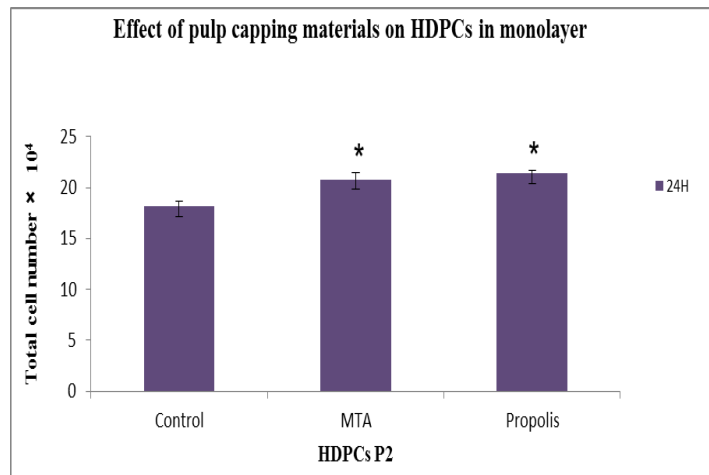


Figure 22. Effects of MTA and propolis on total number of viable cells in monolayer at 24 hours.

Total number of viable HDPCs, upon exposure to MTA and propolis compared to control from passage 2 after incubation period of 24 hours. Data represent mean ± standard deviation (n=6), \*p<0.05 among among groups at 24 hours incubation in comparison to control.

The number of floating/detached cells in the aspirated medium was determined. It was highest in MTA group and followed by the propolis group. The least number of floating HDPCs was found in the control group. The difference was statistically significant between the three studied groups (p<0.05) (Figure 23).

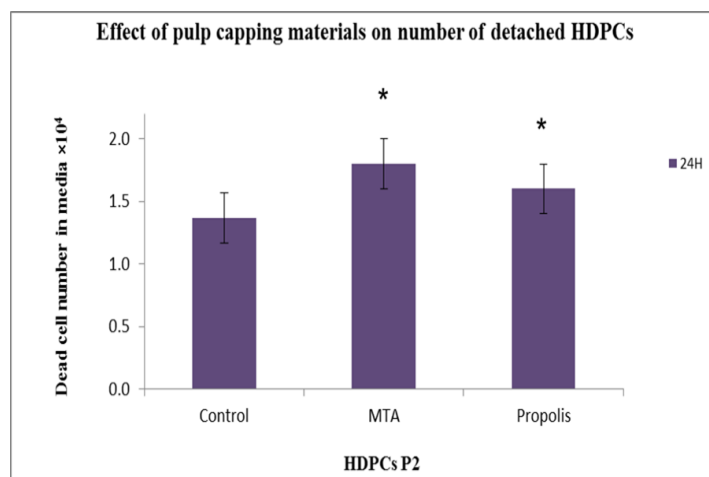


Figure 23. Effects of MTA and propolis on detached/floating HDPCs in culture medium at 24 hours.

Total number of detached/floating HDPCs in the culture medium, upon exposure to MTA and propolis compared to control from passage 2 after incubation period of 24 hours. Data represent mean ± standard deviation (n=6), \*p<0.05 among among groups at 24 hours incubation in comparison to control.

The viability percentage after 24 hours incubation was highest in propolis and MTA groups ( $92.69\pm 0.012\%$  and  $92.44\pm 0.016\%$ , respectively). There was no statistically significant difference between the two groups ( $p > 0.05$ ). The control group had the lowest mean viability percentage ( $88.02\pm 0.018\%$ ). Although the difference was not statistically significant, there was a tendency of the control group to have lower viability percentage in comparison to MTA and propolis groups as indicated by the p-values that were close to 0.05 (Figure 24).

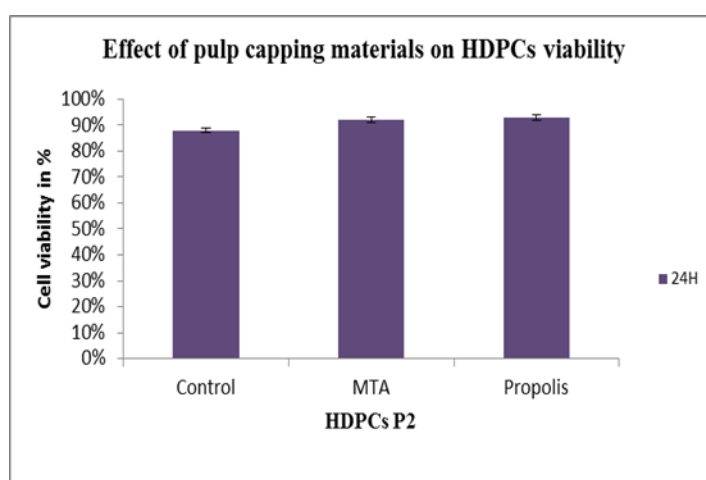


Figure 24. Effects of MTA and propolis on viability of HDPCs at 24 hours. Viability percentage of HDPCs, from passage 2, upon exposure to MTA and propolis compared to control from passage 2 after incubation period of 24 hours. Data represent mean  $\pm$  standard deviation (n=6).

The findings of this study suggested that MTA and propolis enhanced the proliferation and growth potential of HDPCs upon incubation for 24 hours as indicated by the higher total cell counts (viable and floating) when compared with control group as indicated by post-hoc LSD tests ( $p < 0.05$ ). Both materials had similar effect on the viability of HDPCs which was slightly higher than the control group. Furthermore, reduction of the concentration of propolis resulted in increased viability percentage of HDPCs after 24 hours incubation period. The use of 2 g/ml propolis concentration yielded  $79.0\pm 0.014\%$

viable HDPCs after 24 hours incubation period, whereas reduction of concentration to 0.8 g/ml resulted in significant improvement of the viability ( $92.69\pm 0.012\%$ ) as indicated by one-way ANOVA test ( $p < 0.05$ ).

### **3.2.6 Determination of the amount of released MTA and propolis in the culture medium**

The concentration of propolis seemed to have a significant effect on the viability of HDPCs as demonstrated in the Results section 3.2.5. Thus, it can be concluded that the amount of released pulp capping material may have fundamental effects on the viability of HDPCs. Propolis and MTA seemed to have a similar effect on the viability percentage of HDPCs. Despite the fact that a similar volume of both materials was used, this did not guarantee that the 'effective' amount available for HDPCs was similar. Thus, a series of studies was performed to determine the exact amount of released MTA and propolis in culture medium after 24 hours incubation.

#### **3.2.6.1 Spectrophotometric determination of released $\text{Ca}^{2+}$ in the medium**

$\text{Ca}^{2+}$  is the major constituent of the compounds within MTA as indicated in the data sheet of the used, commercially available product. Furthermore, an earlier pilot work investigated the elemental composition of propolis utilising Electron Dispersive Spectroscopy, revealing that propolis has a low percentage of  $\text{Ca}^{2+}$  within its chemical composition as shown, in Figure 25. Thus, the amount of released substance from MTA and propolis in cell culture can be measured indirectly by calculating the released  $\text{Ca}^{2+}$  in the culture medium. The feasibility of using a colorimetric assay for this purpose was investigated.

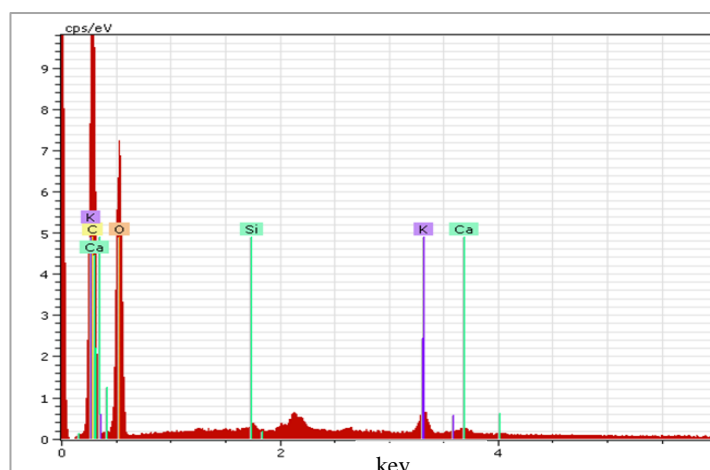


Figure 25. Electron Dispersive Spectroscopy of representative propolis sample. The spectroscopic data demonstrated presence of dominant carbon composition with traces of calcium, silicon and potassium.

Arsenazo III, a complexometric reagent used in this assay, reacts with calcium in a solution to form a bluish-purple complex. The concentration of calcium can be subsequently determined by means of spectrophotometry as discussed in Materials and Methods section 2.2.5.1. A calibration curve was constructed by performing the assay on a series of dilutions made up in distilled water with 0-50  $\mu\text{M}$   $\text{CaCl}_2$  (Figure 26).

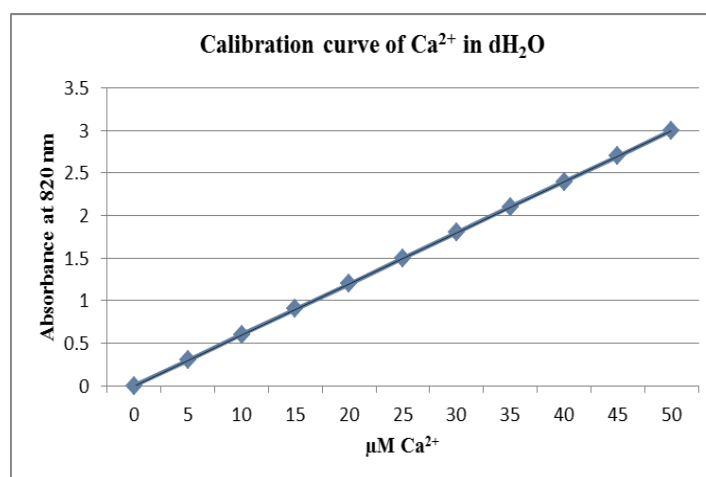


Figure 26. Calibration curve of  $\text{Ca}^{2+}$  ions in distilled water. Calibration curve showing linear relationship ( $R=1$ ) between absorbance at 820 nm and  $\text{CaCl}_2$  concentration in  $\text{dH}_2\text{O}$ .

When analysing the  $\text{Ca}^{2+}$  content of  $\alpha$ -MEM in culture medium, the absorbance was in excess of 1.8 which translates to approximately 27  $\mu\text{M}$  of  $\text{Ca}^{2+}$  according to the above calibration curve. Adding amounts of  $\text{CaCl}_2$  to achieve 0 to 3.5  $\mu\text{M}$   $\text{Ca}^{2+}$  concentrations in the culture medium caused a steady state in the absorbance readings as shown in Figure 27. These findings suggest that the tested  $\alpha$ -MEM culture medium has a substantial amount of  $\text{Ca}^{2+}$  which renders the assay invalid to assess the release of  $\text{Ca}^{2+}$  from MTA.

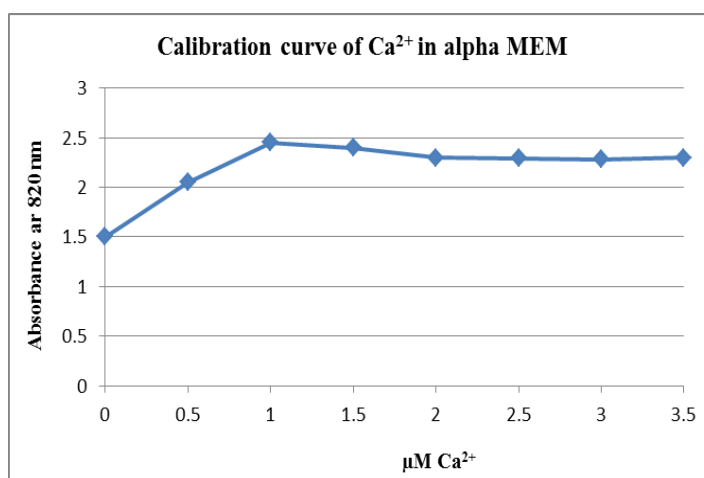


Figure 27. Concentration of  $\text{Ca}^{2+}$  ions in the culture medium.

Calibration curve showing nonlinear relationship ( $R=0.269$ ) between absorbance at 820 nm and  $\text{CaCl}_2$  concentration in alpha MEM.

### 3.2.6.2 Direct measurement of the amount of released MTA and propolis

The use of colorimetric assay was not suitable to measure the amount of the released materials into cultured medium, as shown in the previous section, due to a high level of existing calcium within the culture medium. Alternatively, a novel application of a commonly used experimental technique was investigated for this purpose. Freeze drying is a cryodesiccation method where dehydration of samples is achieved by freezing liquids within the material and subsequently extracting them in their solidified form via introducing negative pressure in a closed system. The initial hypothesis was that the weight of the Transwell<sup>®</sup> inserts and pulp capping material prior incubation would

decrease by the amount of released material into culture medium and increase by the amount of absorbed liquids (medium). If the absorbed medium could be eliminated, the exact amount of released pulp capping material could thereby be determined.

The amount of released MTA and propolis was subsequently determined by weighing the Transwell<sup>®</sup> inserts with and without the material at certain time points as discussed in Materials and Methods section 2.2.5.2. Primarily the empty Transwell<sup>®</sup> inserts with and without MTA and propolis were weighed prior to incubation and after freeze drying.  $W_t$  release of MTA and propolis materials was determined as described in Materials and Methods section 2.2.5.2.

The amount of MTA and propolis that was released from the Transwell<sup>®</sup> inserts into medium was 0.094 g and 0.089 g, respectively. Details of the weight of the Transwell<sup>®</sup> inserts prior to and after incubation and freeze drying are summarised in Table 5. There was no statistically significant difference between the amount of released MTA and propolis in the culture medium (p value >0.05). These findings may suggest that MTA and propolis have a similar advantageous impact on biological characteristics of the studied HDPCs that received a similar amount of material in the culture medium.

Table 5 Amount of released materials within the culture medium, units in grams.

<b>Sample</b>	<b>Wt<sub>dry0</sub></b>	<b>Wt 24</b>	<b>Wt<sub>freeze dry</sub></b>	<b>Wt release</b>
<b>Transwell<sup>®</sup> inserts</b>	0.594 ±0.003	0.623 ±0.002	0.597 ±0.004	-
<b>Transwell<sup>®</sup> inserts with MTA</b>	0.996 ±0.116	1.077 ±0.071	0.902 ±0.079	0.094 ±0.005
<b>Transwell<sup>®</sup> inserts with propolis</b>	0.754 ±0.039	0.791 ± 0.079	0.665 ±0.093	0.089 ±0.009

Wt<sub>dry0</sub>: Initial weight, Wt24: Weight after 24 hours incubation in medium, (wet), Wt<sub>freedry</sub>: weight after freeze dry and Wt release: weight of the released material in medium.



### **3.2.7 Effect of MTA and propolis on HDPC viability and proliferation in serial passage**

MTA and propolis may enhance HDPC proliferation and stimulate cell growth as indicated in the Result section 3.2.4. This study was performed on HDPCs from early passage number (P2). Investigation of the longevity of this effect in later passages was performed given the time-dependent loss of growth potential due to cell senescence. This is of high importance in the light of increased demand for cells with high proliferation potential for tissue engineering applications. The effects of MTA and propolis on viability of cultured HDPCs was evaluated utilising Trypan Blue assay as described in Materials and Methods section 2.1.5. The viability of HDPCs cultured with Transwell<sup>®</sup> inserts containing MTA and propolis was compared with control HDPCs (n=3 per group). HDPCs were from passage 2 to 12 and incubated with MTA and propolis for 12 and 24 hours as described in Materials and Methods section 2.2.2.

After incubation for 12 hours, MTA treatment was associated with the highest number of viable HDPCs in the monolayer among all passages. On the other hand, propolis treatment resulted in the lowest number of viable HDPCs among all passages ( $p < 0.05$ ). After incubation for 12 hours, MTA-treated and untreated HDPCs from passage 4 had the slightly higher number of viable cells in the monolayer when compared to passage 2. The numbers progressively decreased until passage 12. However, the number of viable cells in the monolayer as a result of propolis treatment increased from passage 2 to passage 6. A notable decrease was however noticed in the number of viable HDPCs at passage 12 (Figure 27). Among all passages, untreated HDPCs had the lowest number of detached/floating HDPCs in culture medium followed by MTA and propolis treatments. In all studied groups, the number of detached cells increased from passage 2 to passage 12 (Figure 28).

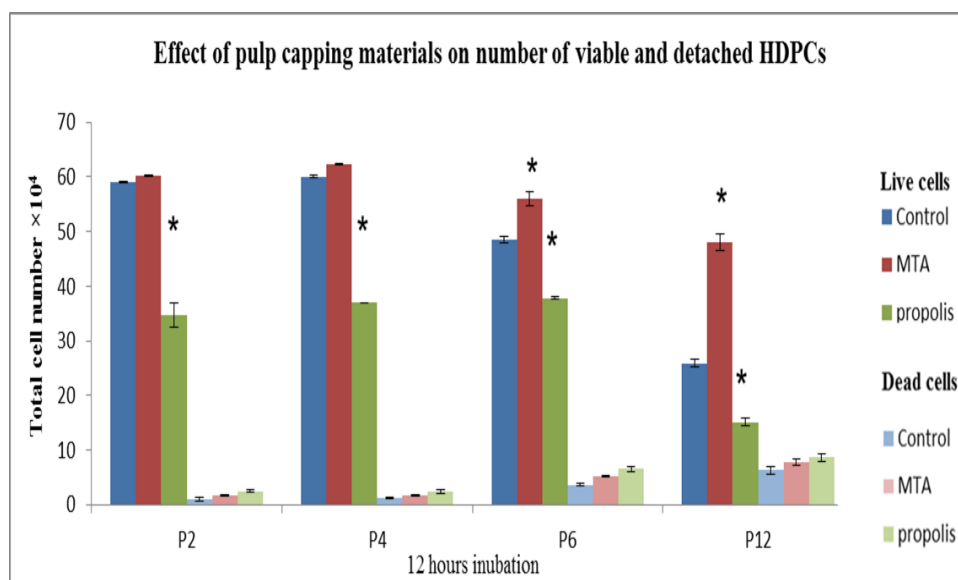


Figure 28. Effect of MTA and propolis on number of viable and dead HDPCs.

The effect of passage number on the number of viable and dead HDPCs (Passage 2, 4, 6 and 12) in MTA, propolis and control groups after incubation periods of 12 hours. Data represent mean  $\pm$  standard deviation (n=3). \*p<0.05 among MTA and propolis groups in each passage in comparison to control group.

After 24 hours of incubation, MTA treatment was associated with the highest number of viable HDPCs in the monolayer among all passages. On the other hand, propolis treatment resulted in the lowest number of viable HDPCs among all passages. MTA-treated and untreated HDPCs from passage 4 have the slightly higher number of viable cells in the monolayer when compared to passage 2. The numbers progressively decreased until passage 12. However, the number of viable cells in the monolayer as a result of propolis treatment progressively increased from passage 2 to passage 6. A notable decrease was however noticed in the number of viable HDPCs at passage 12. Among all passages, untreated HDPCs had the lowest number of detached/floating HDPCs in culture medium followed by MTA and propolis treatments. In all studied groups, the number of detached cells progressively increased from passage 2 to passage 12 (Figure 29).

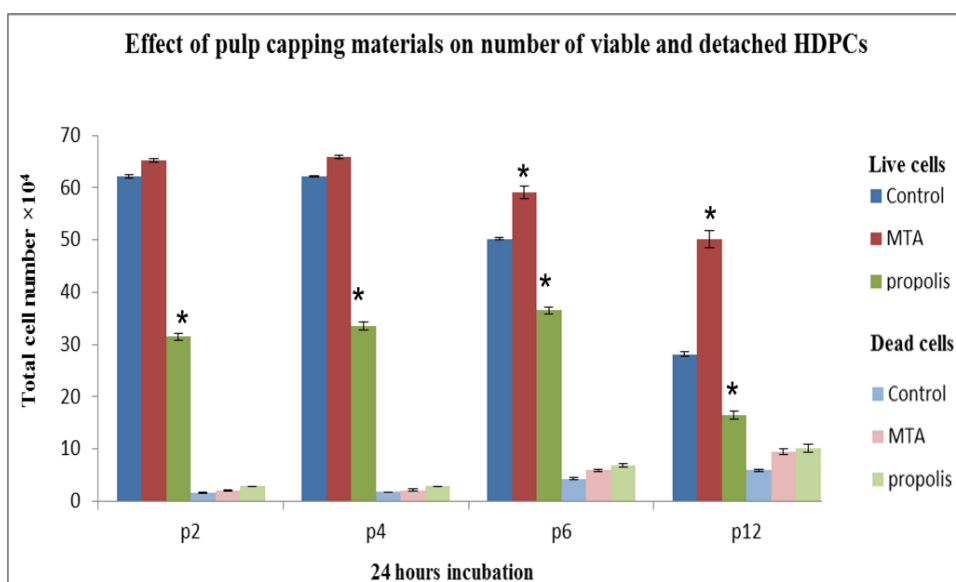


Figure 29. Effect of MTA and propolis on number of viable and dead HDPCs.

The effect of passage number on the number of viable and dead HDPCs (Passage 2, 4, 6 and 12) in MTA, propolis and control groups after incubation periods of 24 hours. Data represent mean  $\pm$  standard deviation (n=3). \*p<0.05 among MTA and propolis groups in each passage in comparison to control group.

MTA-treated and untreated HDPCs had comparable viability percentages that gradually decreased from passage 2 to 12. Propolis treatment resulted in slightly lower viability percentages in comparison to other groups in passage 2 and 4. It however exhibited an abrupt decrease in passage 6 and 12 (Figure 30). The viability percentage decreased progressively from passage 2 to 12. MTA-treated and untreated HDPCs had comparable viability percentages that gradually decreased from passage 2 to 12. Propolis treatment resulted in slightly lower viability percentages in comparison to other groups in passage 2 and 4, however an abrupt decrease in passage 6 and 12 was observed (Figure 30). Statistical analysis (ANOVA and LSD post-hoc tests) revealed that viability percentage has degraded in all experimental groups starting from passage 6 and onwards (p<0.05). In contrast, HDPCs from passage 2 and 4 had comparable viability percentage in all experimental groups (p>0.05) except propolis. These findings indicate that HDPCs from

later passages exhibit decreased proliferation potential and percentage of viability especially when exposed to propolis.

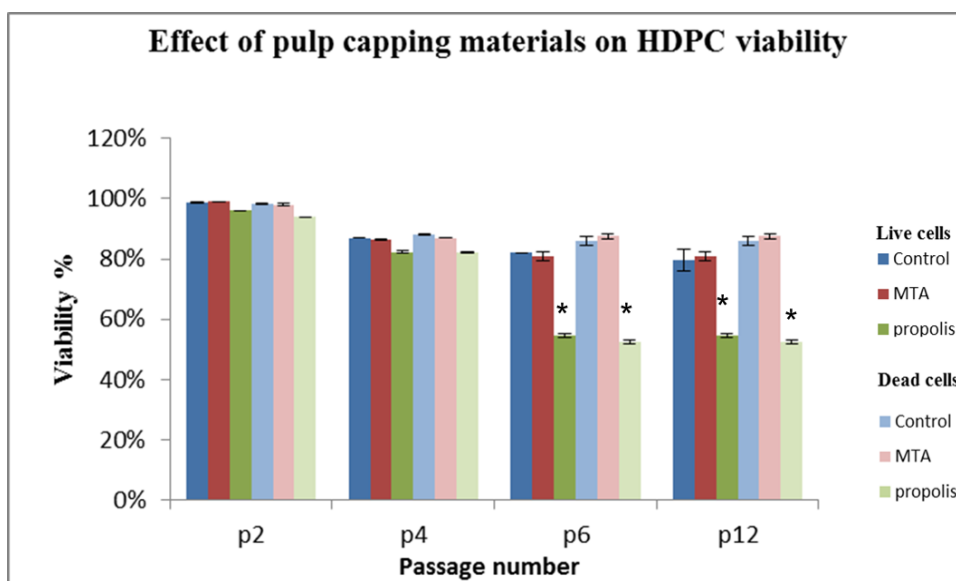


Figure 30. Effect of MTA and propolis on viability percentage of HDPCs.

The effect of passage number on the viability percentage of HDPCs (Passage 2, 4, 6 and 12) in MTA, propolis and control groups after incubation periods of 12 and 24 hours. Data represent mean  $\pm$  standard deviation (n=3), \*p<0.05 among propolis groups in each passage in comparison to control and MTA groups.

### 3.3 Preliminary gene expression studies

The genetic basis of the changes of biological characteristics of HDPC upon exposure to MTA and propolis were investigated utilising gene profile expression studies. Additionally, gene expression studies were used to determine the effect of MTA and propolis on the differentiation potential of HDPCs which has an essential effect on their function *in vitro*. Furthermore, gene expression studies were used to provide better understanding of the effect of MTA and propolis on the phenomenon of cell senescence by investigating the genes orchestrating this process.

### 3.3.1 Quantification of RNA using NanoDrop

Quantification of RNA samples is an essential step toward achieving identical cDNA amounts from different samples and thereby, valid comparison across different experimental groups in terms of qRT-PCR data. Additionally, the use of Nanodrop allows assessment of the quality of the extracted RNA.

Quantification of RNA concentration was performed utilising the NanoDrop following the protocol described in Materials and Methods section 2.3.3. Concentration of RNA from various samples and corresponding ratios of absorbance at 260 nm and 280 nm are presented in Table 5. The RNA concentrations from HDPCs (P2) which were incubated for 12 hours and received no treatment, MTA or propolis, were notably low. In addition, the corresponding 260/280 ratios were notably lower than what is considered as adequately pure RNA. This may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. Doubling the incubation time resulted in significantly higher concentrations of RNA, especially for untreated HDPCs followed by MTA and propolis groups. The purity of RNA was also superior as indicated by 260/280 ratios which were slightly lower than 2 as shown in Table 6.

It is noticeable that RNA extracted from both groups has relatively low concentration and suboptimal quality. This could be attributed to technical errors during the RNA extraction process. This, however, may not be the case as the exact same protocol yielded a better quality RNA from 24 hours sample in comparison to 12 hour sample. The qRT-PCR experiments were carried out using the RNA samples with highest concentration and best quality.

Table 6 Quantification of RNA from all experimental groups (P2).

<b>Incubation Hours</b>	<b>Name</b>	<b>Concentration ng/μl</b>	<b>260/280</b>
<b>12</b>	Control	4.5	1.56
	Control	6.7	1.26
	Control	10.1	1.39
	Control	5.9	1.47
	Control	7.9	1.54
	Control	8.78	1.45
	MTA	11.4	1.41
	MTA	15.3	1.29
	MTA	7.4	1.37
	MTA	7	1.6
	MTA	10	1.5
	MTA	9.71	1.43
	Propolis	6.1	1.36
	Propolis	11.6	1.42
	Propolis	4.1	1.37
	Propolis	6.7	1.77
	Propolis	7.6	1.27
	Propolis	9.8	1.87
<b>24</b>	Control	191.5	1.78
	Control	371.9	1.91
	Control	129.2	1.95
	Control	301.5	1.85
	Control	441.9	1.68
	Control	546.4	2.39
	MTA	614.8	1.53
	MTA	212.8	1.85
	MTA	130.3	1.71
	MTA	174.6	1.79
	MTA	75.6	1.79
	MTA	16.6	2.62
	Propolis	173.6	1.75
	Propolis	296.4	1.82
	Propolis	192.6	1.77
	Propolis	174.4	1.75
	Propolis	123.6	1.83
	Propolis	298.8	1.705

### 3.3.2 Analysis of *GAPDH* gene expression

*GAPDH* is widely used as a housekeeping gene in qRT-PCR studies owing to its constitutive expression in several cell types and under various experimental conditions. The expression of *GAPDH* was analysed to ensure constitutive expression in HDPCs from passage 2 and 6. RNA from two different donors was reverse-transcribed to produce cDNA as described in Materials and Methods section 2.5.1. The expression of *GAPDH* was determined using RT-PCR assay as described in Material and Methods section 2.5.1.2. and 2.4, respectively.

A number of positive control cDNA (see lanes 1-5 in Figure 31) were included to ensure that the *GAPDH* primer enzyme and the template were functional together in the RT-PCR reaction. In lane 1 and 5 is cDNA from HDPCs passage 2 in Figure 31 A or HDPCs passage 6 in Figure 31 B which was used as positive control (including all the PCR reaction mix) for the *GAPDH* gene expression, lanes 2 and 6 negative control (lacking Taq polymerase), lanes 3 and 7 were the negative control (lacking primers) used to aid detection of primer artefacts in the other reactions. Lanes 4 and 8 were the negative control (lacking template) to confirm that the expected bands were only obtained when the template was present. Figure 31 A shows that the control PCR product representing the expected 400 bp *GAPDH* band was generated. These results confirm that samples have been successfully reverse-transcribed and that *GAPDH* gene expression is seen in the lanes (1 and 5) for HDPCs passage 2. Figure 31 B shows a similar pattern for HDPCs with one sample showing expression of *GAPDH* as shown in lane 5, with the exception being in passages 6 in lane 1. In subsequent experiments the cDNA from the passages that were negative for *GAPDH* were not analysed for the expression of the other genes as it was concluded that these samples had not been successfully reverse-transcribed. The reasons for the failure to reverse-transcribe were

not investigated. HDPCs from passage 2 samples were used as control samples for detection of expression of all other studied genes.

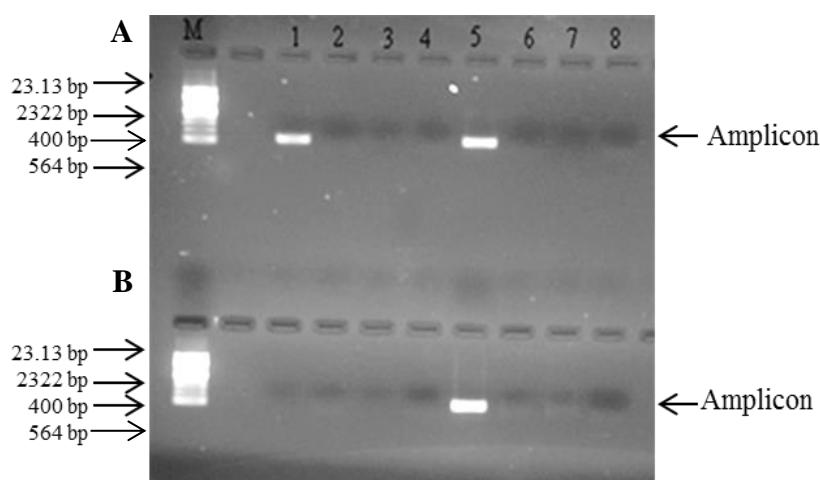


Figure 31. RT-PCR *GAPDH* expression of HDPCs.

cDNA A) PCR from RNA HDPCs P2 in lanes 1-8, B) PCR from RNA HDPCs P6 in lanes 1-8, M: lambda-HindIII marker, well-1/5:positive control,well-2/6:negative enzyme,well-3/7:negative primer, well-4/8:negative template Arrows indicates the lambda-HindIII marker size in bp.

### 3.3.3 Standard calibration curves for selected primers for SYBR-Green® assay

The quality, sensitivity and specificity of the SYBR-Green® primers was analysed by conducting a qRT-PCR experiment on a serially diluted cDNA obtained from HDPCs (P2).

Standard calibration curves for the seven genes selected for this study, housekeeping gene (*GAPDH*), telomerase associated genes (*TERC*, *TERT*), cell death mechanism genes (*BID*, *BCNI*), odontogenic genes (*DSPP* and *BMP-2*) were produced prior to qRT-PCR experiments. Serial 1 in 10 dilutions of the positive control cDNA samples from HDPCS passage 2 (in triplicate) were studied using qRT-PCR as described in Materials and Methods section 2.5.4.1 In Figure 32, the Ct values for each gene were plotted against the  $\log_{10}$  of relative cDNA concentration. The trend lines represent the



reaction efficiency for each assay. Calibration curves for all genes exhibited inverse linear relationship between Ct value and cDNA concentration which indicated high sensitivity and specificity of the used primers. The primers for *TERT*, *BID* and *DSPP* genes were exceptions as their expression was only detectable with the highest cDNA concentration and resulted in very high Ct values. In general, the majority of the primers were highly sensitive and could be reliably used to investigate the receptive gene expression profiles in HDPCs.

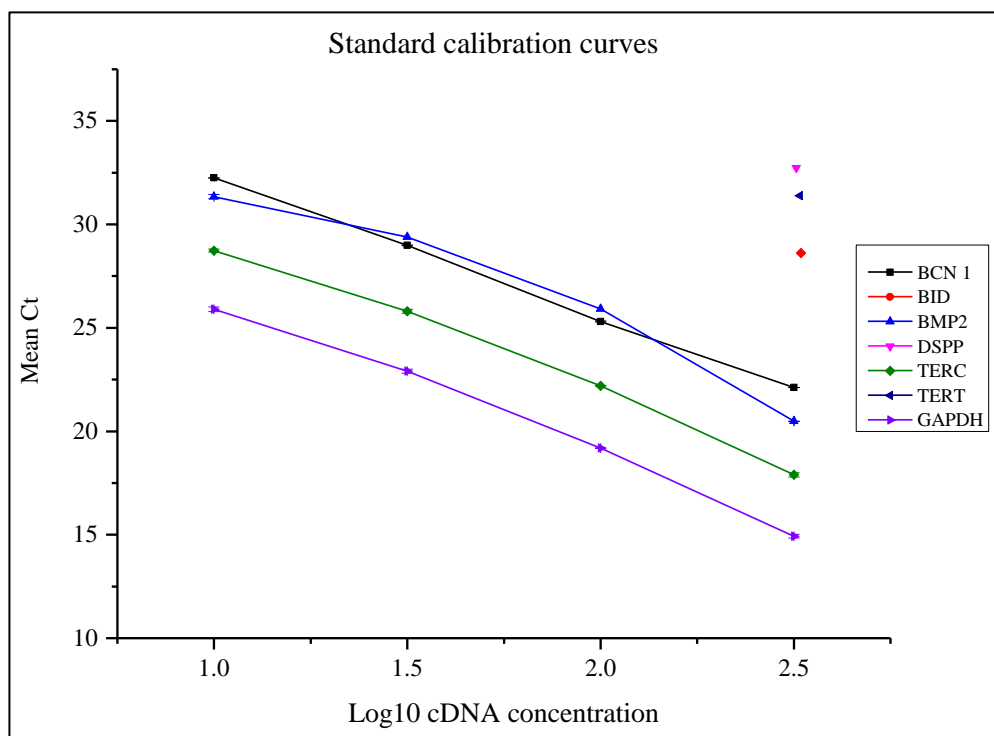


Figure 32. Calibration curve of different primers for SYBR-Green<sup>®</sup> assay. Reaction efficiency for SYBR-Green<sup>®</sup> assay. Different colour plots represent different calibration curve generated from each set of primers in 4 tenfold serial dilutions. The trend line Ct values are plotted against the log<sub>10</sub> cDNA. Data represent mean ± standard deviation (n=3).

### **3.3.4 Preliminary investigation of the expression of genes controlling cell death, differentiation and ageing using SYBR-Green<sup>®</sup> assay**

A preliminary study was conducted to check the efficiency of SYBR-Green<sup>®</sup> assay investigating gene expression profile for selected genes in HDPCs. RNA was extracted from HDPC pellet that was stored in RNAlater<sup>®</sup> for 10 days. cDNA was reverse transcribed as described in Materials and Methods section 2.5.1. Genes orchestrating important processes that affect the function of HDPCs (cell ageing, cell death and differentiation) were studied. The *GAPDH* gene was used as housekeeping gene as it has been shown to be constitutively expressed in HDPCs (Shi *et al.*, 2001, Kawai *et al.*, 2013).

#### **3.3.4.1 Determining Telomerase associated genes expression**

Cell ageing phenomenon is characterised by the reduction of the telomere length. Telomerase has a primary function to protect telomere from shortening and its expression is regulated by *TERT* and *TERC* genes being expressed simultaneously. To determine whether cell ageing is affected by the pulp capping materials, expression of telomerase associated genes (*TERT* and *TERC*) was investigated in triplicate samples of cDNA obtained from cultured HDPCs (P2) treated with MTA and propolis (12 and 24 hours). The expression profile of telomerase associated genes (*TERT* and *TERC*) was assessed based on quantitative analysis (SYBR-Green<sup>®</sup> qRT-PCR) of individual genes normalized to the *GAPDH* as described in Materials and Methods section 2.5.4.1. The change in  $\Delta\text{Ct}$  relative to the  $\Delta\text{Ct}$  for the control sample ( $\Delta\Delta\text{Ct}$ ) and standard deviations as described in Materials and Methods section 2.5.5 are shown in Table 7.

Table 7 The Ct,  $\Delta$ Ct,  $\Delta\Delta$ Ct values of *TERC* and *TERT* gene expressed in HDPCs from passage 2 treated with MTA and propolis (qRT-PCR).

Materials		<i>TERC</i>					<i>TERT</i>				
		Ct	Ct_ <i>GAPDH</i>	$\Delta$ Ct	$\Delta\Delta$ Ct	s.d	Ct	Ct_ <i>GAPDH</i>	$\Delta$ Ct	$\Delta\Delta$ Ct	s.d
Control	12 h	27.83	23.59	-4.25	0	3.23	20.15	23.59	2.73	0	0.98
	24 h	27.86	23.25	-4.61	0	1.35	15.53	23.25	1.92	-0.99	0.97
MTA	12 h	27.88	23.01	-4.87	-2.01	1.27	16.5	23.01	1.44	-0.99	0.7
	24 h	29.82	24.29	-3.08	-3.8	1.59	16.08	24.29	1.74	-0.68	0.79
Propolis	12 h	29.82	29.73	-1.75	-2.92	5.6	15.78	29.73	1.74	-0.81	1.47
	24 h	28.67	25.74	-2.93	-3.04	#DIV/0!	17.12	25.74	2.05	-0.51	1.87

A plot of  $2^{-\Delta\Delta ct}$  normalised to the value of control samples was obtained and is shown in Figure 33. The expression of the *TERT* gene in the control, MTA and propolis treated HDPCs was below the detectable level (data not shown). The results indicated that *TERC* expression is elevated in HDPCs treated with MTA in both 12 and 24 hours compared to the control and propolis treated HDPC, demonstrating an overall increasing of *TERC* expression with MTA treated HDPCs. However, significant up-regulation of *TERC* in MTA treated for 24 hours may indicate experimental artefacts. Overall, in the light of the aforementioned findings, the absence of detectable levels of *TERT* would suggest the telomere length would not be maintained in HDPCs and the pulp capping agents had no protective effect against telomere shortening.

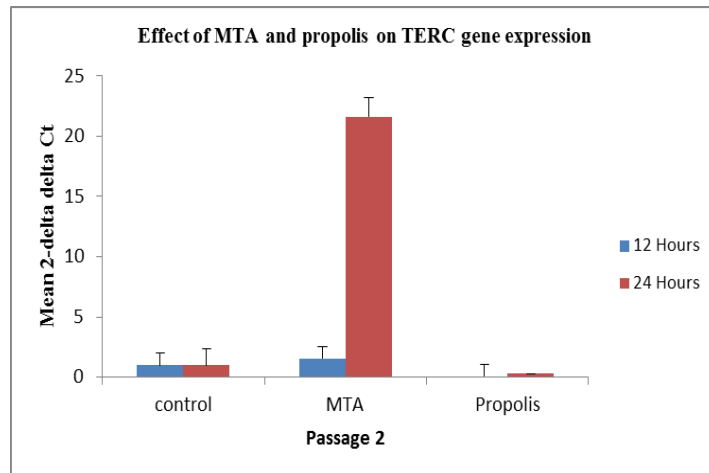


Figure 33. Expression profile of *TERC* gene.

Comparison of *TERC* gene expression in HDPCs treated with MTA, propolis and control for 12 and 24 hours from (P2). Data represents mean  $2^{-\Delta\Delta Ct} \pm$  standard deviation (n=3).

### 3.3.4.2 The effect of pulp capping materials on *BID* and *BCN1* gene expression of HDPCs

The effect of MTA and propolis on the mechanisms associated with programmed cell death was investigated. The apoptosis related gene *BID* and the autophagy related gene *BCN1* were analysed in HDPCs (P2) treated with MTA and propolis at two different time points (12 and 24 hours). The expression profile of (*BID* and *BCN1*) genes was assessed based on quantitative analysis (SYBR-Green<sup>®</sup> qRT-PCR) of individual genes normalized to the *GAPDH* as described in Materials and Methods section 2.5.4.1. The change in  $\Delta Ct$  relative to the  $\Delta Ct$  for the control sample ( $\Delta\Delta Ct$ ) and standard deviations as described in Materials and Methods section 2.5.5 are shown in Table 8.

Table 8 The Ct,  $\Delta$ Ct,  $\Delta\Delta$ Ct values of *BCNI* gene expressed in HDPCs from passage 2 treated with MTA and propolis (qRT-PCR).

Materials		<i>BCNI</i>				
		Ct	Ct_ <i>GAPDH</i>	$\Delta$ Ct	$\Delta\Delta$ Ct	s.d
Control	12 h	25.68	23.59	-2.09	0	3.38
	24 h	25.73	23.25	-2.48	-3.13	1.19
MTA	12 h	26.25	23.01	-3.24	-1.15	1
	24 h	29.91	24.29	-5.61	-0.68	3.21
Propolis	12 h	34.40	29.73	-4.66	-2.58	4.49
	24 h	32.53	25.74	-6.79	-4.31	1.39

A plot of  $2^{-\Delta\Delta Ct}$  normalised to the value of control samples is shown in Figure 34. The expression of the *BID* gene in the control, MTA and propolis treated HDPCs was below the detectable level (data not shown). The highest level of *BCNI* gene expression was noticed in propolis treated HDPCs. MTA treated HDPCs demonstrated up-regulation of *BCNI* gene but to a lesser extent. Expression of *BCNI* gene has exhibited time dependence in both MTA and propolis treated HDPCs. The up-regulation level was higher after 24 hours incubation in comparison to 12 hours in both groups, however, significant up-regulation was seen in propolis treated HDPCs after 24 hours. These findings may explain the reduced viability of HDPCs associated with propolis in comparison to MTA treatment. Propolis may have cytotoxic effect on the studied cells via induction of programmed cell death.

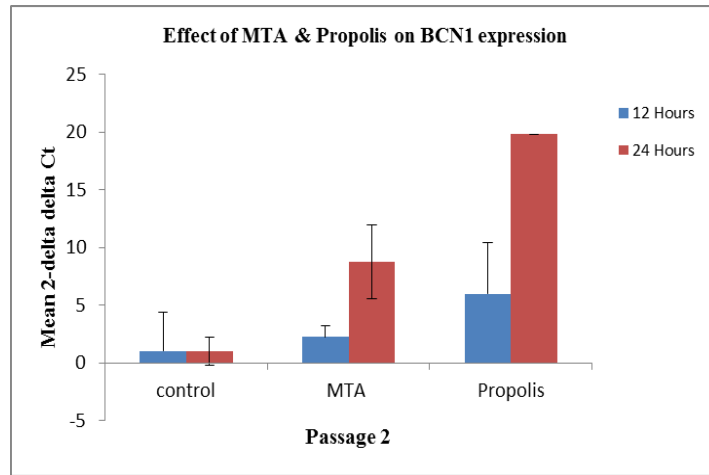


Figure 34. Expression profile of *BCN1* gene.

Comparison of *BCN1* gene expression in HDPCs treated with MTA, propolis and control for 12 and 24 hours from (P2). Data represents mean  $2^{-\Delta\Delta Ct} \pm$  standard deviation (n=3).

### 3.3.4.3 The effect of pulp capping materials on *BMP-2* and *DSPP* gene expression

The effect of pulp capping materials on odontogenic differentiation potential of HDPCs was investigated by studying the expression of (*BMP-2* and *DSPP* genes) in HDPCs (P2) treated with MTA and propolis at two different time points (12 and 24 hours). Gene expression patterns were assessed based on quantitative analysis of the selected genes normalized to *GAPDH* as described in the Materials and Methods section 2.5.4.1. The change in  $\Delta Ct$  relative to the  $\Delta Ct$  for the control sample ( $\Delta\Delta Ct$ ) and standard deviations as described in Materials and Methods section 2.5.5 are shown in Table 9.

Table 9 The Ct,  $\Delta$ Ct,  $\Delta\Delta$ Ct values of *BMP-2* gene expressed in HDPCs from passage 2 treated with MTA and propolis (qRT-PCR).

Materials		<i>BMP-2</i>				
		Ct	Ct_ <i>GAPDH</i>	$\Delta$ Ct	$\Delta\Delta$ Ct	s.d
Control	12 h	31.31	23.59	-7.72	0	3.83
	24 h	31.48	23.25	-8.24	0	1.54
MTA	12 h	31.16	23.01	-8.15	-0.43	1.68
	24 h	31.68	24.29	-7.38	0.85	1.58
Propolis	12 h	34.37	29.73	-4.64	3.09	3.67
	24 h	25.74	25.74	-1.87	-3.04	1.09

A plot of  $2^{-\Delta\Delta ct}$  normalised to the value of control samples was obtained and is shown in Figure 35. The expression of the *DSPP* gene in the control, MTA and propolis treated HDPCs was below the detectable level (data not shown). The highest level of *BMP-2* gene expression was noticed in propolis treated HDPCs after 24 hours. MTA treated HDPCs demonstrated up-regulation of *BMP-2* gene but to a lesser extent. Expression of *BMP-2* gene has exhibited time dependence in propolis but not MTA group. The up-regulation level was higher after 24 hours incubation for propolis treated HDPCS in comparison to 12 hours. Conversely, there was down-regulation of *BMP-2* after 24 hours in comparison to 12 hours incubation for MTA treated HDPCs. The lack of expression of a very specific dentinogenesis marker (*DSPP*) may indicate absence of odontogenic differentiation at the studied time points. The variable expression of *BMP-2* may indicate that propolis and to a lesser extent, MTA may be indicative of odontogenic or osteogenic differentiation potential of HDPCs. However there is a high

chance that these findings could be attributed to qRT-PCR artefact, technical errors or poor quality cDNA as indicated by the large standard deviations of mean  $\Delta\Delta\text{Ct}$  values.

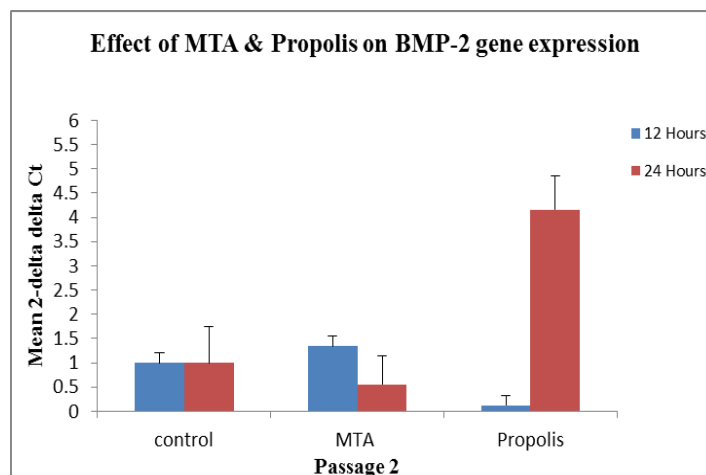


Figure 35. Expression profile of *BMP-2* gene.

Comparison of *BMP-2* gene expression in HDPCs treated with MTA, propolis and control for 12 and 24 hours from (P2). Data represents mean  $2^{-\Delta\Delta\text{Ct}} \pm$  standard deviation (n=3).

#### 3.3.4.4 The expression of telomerase associated gene in serially passaged HDPCs

Cells from later passages may exhibit different biological characteristics as a consequence of cell ageing. The expression profile of telomerase associated genes (*TERT* and *TERC*) was studied in triplicate samples of cDNA obtained from serially passaged HDPCs (P2, 4, 6 and 12) to investigate the cell ageing of the cultured HDPCs. The expression profile of *TERT* and *TERC* genes was assessed utilising SYBR-Green<sup>®</sup> qRT-PCR assay and individual genes were normalised to the *GAPDH* as described in Materials and Methods section 2.5.4.1. The change in  $\Delta\text{Ct}$  relative to the  $\Delta\text{Ct}$  for the control sample ( $\Delta\Delta\text{Ct}$ ) and standard deviations were calculated as described in Materials and Methods section 2.5.5.



A plot of  $2^{-\Delta\Delta Ct}$  normalised to the value of control samples was obtained and is shown in Figure 36. The expression of the *TERT* gene in HDPCs was below the detectable level (data not shown). The results indicated that *TERC* expression is down-regulated in almost all studied passages of HDPCs. However, up-regulation of *TERC* expression may be due to qRT-PCR artefact. Overall, in the light of the aforementioned findings, the expression of *TERC* alone would suggest the HDPCs from later passages may be susceptible to telomere length shortening. However, there is a high chance that these findings could be attributed to qRT-PCR artefact, technical errors or poor quality cDNA as indicated by the large standard deviations of mean  $\Delta\Delta Ct$  values.

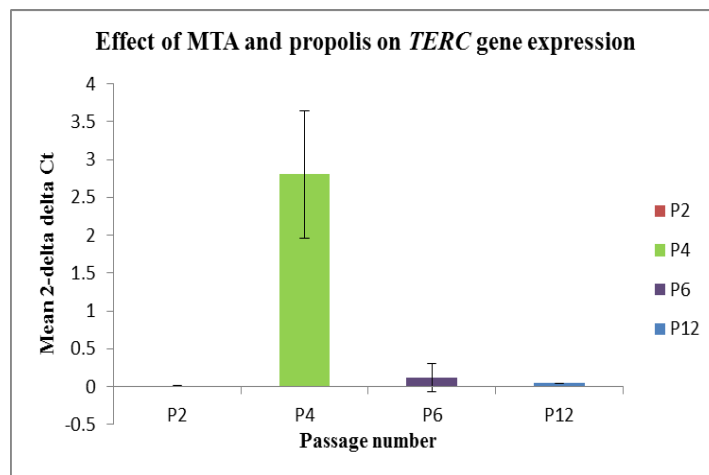


Figure 36. Expression profile of *TERC* gene.

Comparison of *TERC* gene expression in serially passaged HDPCs for 24 hours from (P2, 4, 6 and 12). Data represents mean  $2^{-\Delta\Delta Ct} \pm$  standard deviation (n=3).

### 3.3.5 Preliminary investigation of the expression of genes controlling cell death, ageing and differentiation using Taq-Man<sup>®</sup> assay

Gene expression data obtained from the SYBER-Green<sup>®</sup> qRT-PCR was inconsistent as indicated by the high standard deviations. Additionally, the expression of some genes was not detected while others had significant up-regulation which could be attributed to

low sensitivity of the used primers or other technical qRT-PCR artefacts. Thus, a qRT-PCR assay that is known for higher precision and specificity (Taq-Man<sup>®</sup>) was used to study the gene expression in samples mentioned in Results section 3.3.4. The assay was used to study the changes in the gene expression profile after 24 hours where most notable changes can be detected, as indicated by the data presented in Results section 3.3.4.

### **3.3.5.1 Determining Telomerase associated genes expression**

To determine whether cell ageing is affected by the pulp capping materials, expression of telomerase associated genes (*TERT* and *TERC*) was investigated in triplicate samples of cDNA obtained from cultured HDPCs (P2) treated with MTA and propolis for 24 hours. The expression profile of telomerase associated genes (*TERT* and *TERC*) was assessed based on quantitative analysis (Taq-Man<sup>®</sup> qRT-PCR) of individual genes normalized to the *GAPDH*. Telomerase associated gene expression was measured as described in Materials and Methods section 2.5.4.2 *GAPDH* gene was used as internal control. The change in  $\Delta\text{Ct}$  relative to the  $\Delta\text{Ct}$  for the control sample ( $\Delta\Delta\text{Ct}$ ) and standard deviations was calculated as described in Materials and Methods section 2.5.5.

A plot of  $2^{-\Delta\Delta\text{Ct}}$  normalised to the value of control samples was obtained and is shown in Figure 37. The expression of the *TERT* gene in the control, MTA and propolis treated HDPCs was below the detectable level (data not shown). The results indicated that *TERC* expression is elevated in HDPCs treated with propolis in 24 hours compared to the control and MTA treated HDPC. However, significant up-regulation of *TERC* in propolis treated for 24 hours may indicate experimental artefacts. Overall, in the light of the aforementioned findings, the absence of detectable levels of *TERT* would suggest the studied HDPCs are susceptible to telomere shortening. However, the same problem

was encountered as with the SYBR-Green<sup>®</sup> assay that the mean  $2^{-\Delta\Delta Ct}$  values were not consistent as indicated by the high values of standard deviation.

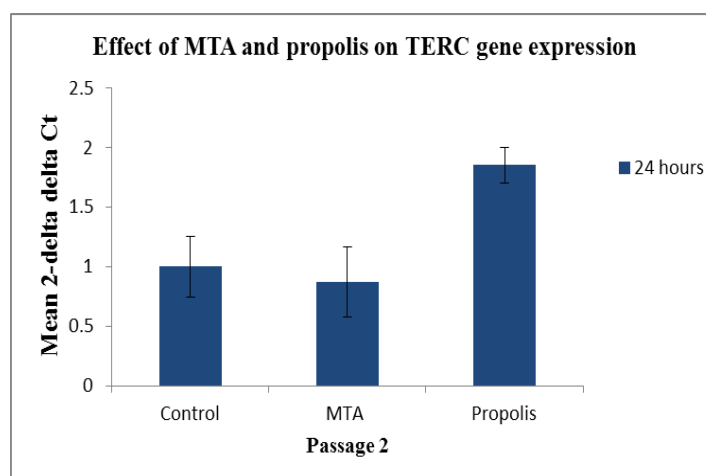


Figure 37. Expression profile of *TERC* gene.

Comparison of *TERC* gene expression in HDPCs treated with MTA, propolis and control for 24 hours from (P2). Data represents mean  $2^{-\Delta\Delta Ct} \pm$  standard deviation (n=3).

### 3.3.5.2 The effect of pulp capping materials on *BID* and *BCN1* gene expression of HDPCs

The apoptosis related gene *BID* and the autophagy related gene *BCN1* were analysed in HDPCs (P2) treated with MTA and propolis at 24 hours utilising Taq-Man<sup>®</sup> qRT-PCR assay and *GAPDH* gene was used as internal control as described in the Materials and Methods section 2.5.4.2. The change in  $\Delta Ct$  relative to the  $\Delta Ct$  for the control sample ( $\Delta\Delta Ct$ ) and standard deviations was calculated as described in Materials and Methods section 2.5.5

A plot of  $2^{-\Delta\Delta Ct}$  normalised to the value of control samples is shown in Figure 38. The expression of the *BID* gene in the control, MTA and propolis treated HDPCs was below the detectable level (data not shown). The expression profile of *BCN1* was comparable

among the three studied groups which was significantly different from the trend noticed in SYBR-Green® data. Furthermore, the same problem encountered with SYBR-Green® assay arose, that the mean  $2^{-\Delta\Delta Ct}$  values were not consistent, as indicated by the high values of standard deviation.

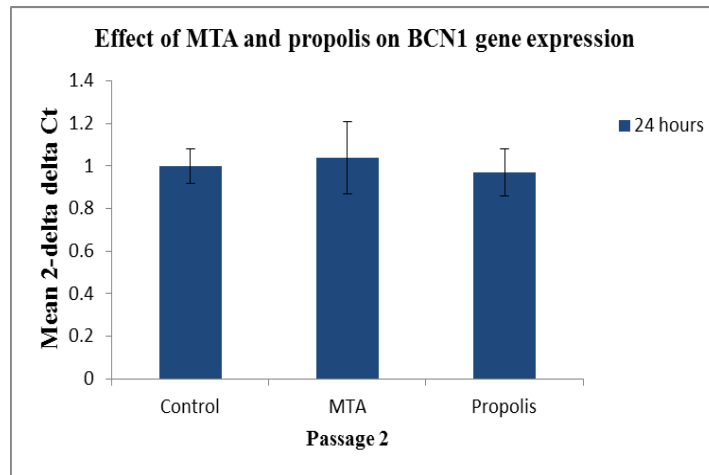


Figure 38. Expression profile of *BCN1* gene.

Comparison of *BCN1* gene expression in HDPCs treated with MTA, propolis and control for 24 hours from (P2). Data represents mean  $2^{-\Delta\Delta Ct} \pm$  standard deviation (n=3).

### 3.3.5.3 The effect of pulp capping materials on *BMP-2* and *DSPP* gene expression

The effect of pulp capping materials on odontogenic differentiation potential of HDPCs was investigated. The expression of (*BMP-2* and *DSPP* genes) in HDPCs (P2) treated with MTA and propolis at 24 hours was studied utilising Taq-Man® qRT-PCR assay and *GAPDH* gene was used as internal control as described in the Materials and Methods section 2.5.4.2. The change in  $\Delta Ct$  relative to the  $\Delta Ct$  for the control sample ( $\Delta\Delta Ct$ ) and standard deviations was calculated as described in Materials and Methods section 2.5.5.

A plot of  $2^{-\Delta\Delta Ct}$  normalised to the value of control samples was obtained and is shown in Figure 39. The expression of the *DSPP* gene in the control, MTA and propolis treated HDPCs was below the detectable level (data not shown). The highest level of *BMP-2* gene expression was noticed in propolis treated HDPCs after 24 hours. MTA treated HDPCs demonstrated up-regulation of *BMP-2* gene but to a lesser extent. The lack of expression of a very specific dentinogenesis marker (*DSPP*) may indicate absence of odontogenic differentiation at the studied time points. However, the variable expression of *BMP-2* may indicate that propolis, and to a lesser extent, MTA may be indicative of odontogenic or osteogenic differentiation potential of HDPCs. However, the change in gene expression profile is significantly different when compared to that obtained for similar samples using SYBR-Green<sup>®</sup> assay which adds to the uncertainty around these findings.

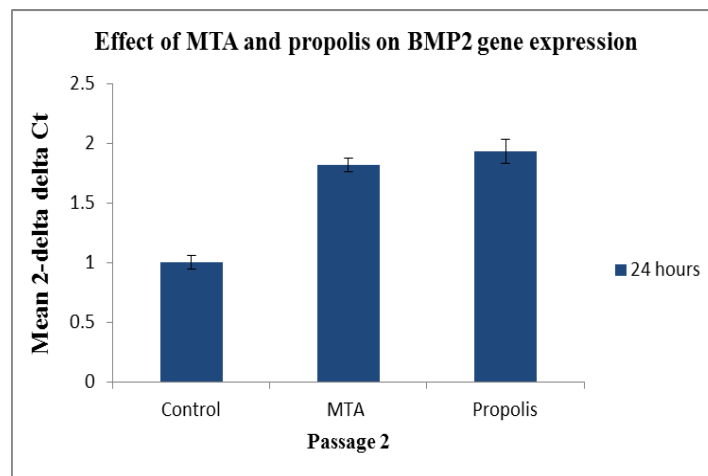


Figure 39. Expression profile of *BMP-2* gene.

Comparison of *BMP-2* gene expression in HDPCs treated with MTA, propolis and control for 24 hours from (P2). Data represents mean  $2^{-\Delta\Delta Ct} \pm$  standard deviation (n=3).

### 3.3.5.4 The expression of telomerase associated genes in serially passaged HDPCs

The expression profile of telomerase associated genes (*TERT* and *TERC*) was studied in triplicate samples of cDNA obtained from serially passaged HDPCs (P2, 4, 6 and 12) to investigate the cell ageing of the cultured HDPCs. The expression profile of *TERT* and *TERC* genes was assessed utilising Taq-Man<sup>®</sup> qRT-PCR assay and individual genes were normalised to the *GAPDH* as described in Materials and Methods section 2.5.4.2. The change in  $\Delta\text{Ct}$  relative to the  $\Delta\text{Ct}$  for the control sample ( $\Delta\Delta\text{Ct}$ ) and standard deviations was calculated as described in Materials and Methods section 2.5.5.

A plot of  $2^{-\Delta\Delta\text{Ct}}$  normalised to the value of control samples was obtained and is shown in Figure 40. The expression of the *TERT* gene in HDPCs was below the detectable level (data not shown). The results indicated that *TERC* expression is down-regulated in all the passages of HDPCs. Overall, the absence of detectable levels of *TERT* and *TERC* would suggest that the studied HDPCs are susceptible to telomere length shortening and ageing.

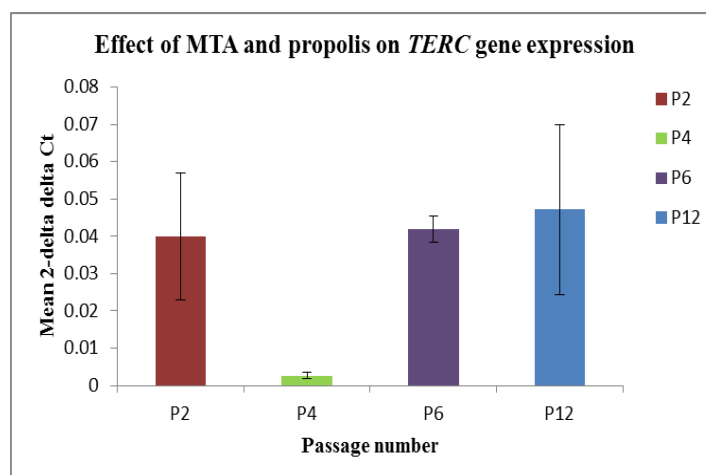


Figure 40. Expression profile of *TERC* gene.

Comparison of *TERC* gene expression in serially passaged HDPCs for 24 hours from (P2, 4, 6 and 12). Data represents mean  $2^{-\Delta\Delta\text{Ct}} \pm$  standard deviation (n=3).

### **3.4 Analysing RNA integrity upon immediate or delayed RNA extraction**

The large variability, lack of consistency and poor interpretability of results from gene expression studies despite repeating the experiment several times utilising two different assays, indicated that the problem could be attributed to poor integrity of RNA samples. This was the first explanation considered as the studied RNA samples had low concentration and suboptimal quality. Poor RNA extraction technique was ruled out as a cause for such findings as the quality and concentration of RNA was improved upon the use of exactly the same technique with two different samples (HDPCs incubated for 12 and 24 hours).

Storage of HDPC pellets for 10 days prior to RNA extraction was studied as a possible causative factor for poor integrity of RNA and thereby, unreproducible qRT-PCR results. However, both samples were less than ideal which raised concerns regarding adverse effect on RNA integrity as a result of storage of HDPCs in RNAlater<sup>®</sup> prior to RNA extraction. Thus, a series of experiments was performed to analyse the effect of storing HDPC pellet in RNAlater<sup>®</sup> prior to RNA extraction.

#### **3.4.1 Comparison of RNA yield and integrity with immediate and delayed RNA extraction**

RNA integrity is critical in relation to qRT-PCR performance. This study was conducted to assess the effect of storing HDPC in RNAlater<sup>®</sup> on RNA integrity. RNA was extracted and quantified immediately after harvesting the HDPCs or following storage of cell pellets in RNAlater<sup>®</sup> for 10 days as described in Materials and Methods section 2.3.4.

Immediate extraction of RNA from freshly harvested HDPC yielded concentrations ranging between 5.7-19.3  $\mu\text{g}/200\mu\text{l}$ . However, delaying RNA extraction for 10 days while HDPCs are stored in RNALater<sup>®</sup> resulted in RNA concentrations ranged between 1.8-6.2  $\mu\text{g}/200\mu\text{l}$ . For similar passage number, immediate extraction resulted in a significantly higher RNA concentration when compared to the delayed counterpart ( $p<0.05$ ). Similarly, the purity of RNA was higher whenever the RNA extraction was performed immediately after harvesting HDPC as indicated by the 260/280 ratios. Table 10 summarises the details of RNA concentrations and 260/280 ratios.

Table 10 Quantitative analysis of RNA content per cell of HDPCs from different passages and following immediate or delayed RNA extraction procedures.

RNA	Mean Total Cell number $\times 10^5$	Mean RNA ng/200 $\mu\text{l}$	260/280
P2 fresh	5.4	5733 $\pm$ 498.8	1.76
P2 RNALater <sup>®</sup>	5.4	3340 $\pm$ 179.6	1.55
P6 fresh	2.3	19267 $\pm$ 193.4	1.62
P2 RNALater <sup>®</sup>	3.6	5020 $\pm$ 99.3	1.47
P10 fresh	3.0	10007 $\pm$ 8396.3	2
P10 RNALater <sup>®</sup>	2.9	6167 $\pm$ 910.4	1.67
P12 fresh	2.2	5435 $\pm$ 900.1	1.98
P12 RNALater <sup>®</sup>	2.3	1800 $\pm$ 690.7	1.64

The comparison based on the RNA concentration may be not valid owing to the difference in total cell number harvested from the culture. For instance, the cultured HDPCs from passage 2 may had a high number of dead cells and the concentration may be not indicative on the effect of cell storage on the integrity and the yield of RNA. Thus, the average amount of RNA per cell was calculated by dividing the total amount of RNA by the total numbers of cells in 200  $\mu\text{l}$ .



The average RNA yield per cell was estimated to be 8-34 pg when RNA extraction was performed immediately after harvesting HDPCs. By contrast, the average RNA yield per cell was as low as 1-21 pg in delay extraction. The average amount of RNA yield from immediate RNA extraction is more in accordance with the expected average RNA within mammalian cells which has been estimated between 10-30 pg (Tuffaha, 2008). Additionally, HDPCs from later passages had a higher amount of RNA per cell when compared to those from early passages. Results indicated significantly higher RNA amounts per cell when RNA was extracted immediately from fresh HDPC pellet especially in HDPCS from passage 6, 10 and 12 in comparison to the later (Figure 41).

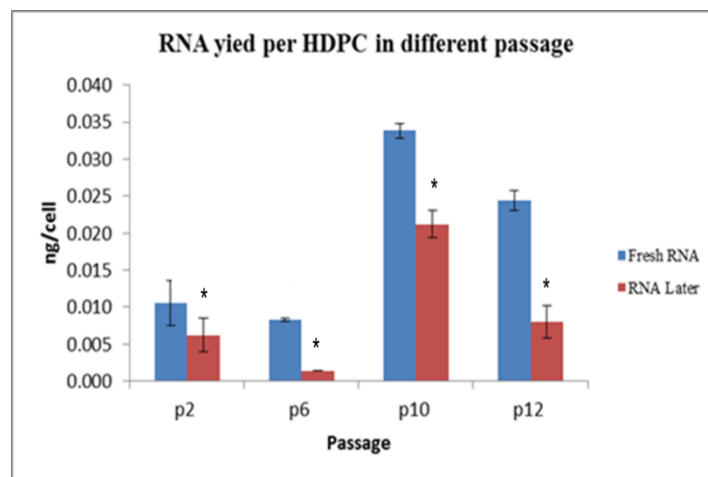


Figure 41. Effect of HDPC pellet storage on RNA yield per cell. Comparison of RNA content per cell in (P2, 6, 10 and 12) between immediate RNA extraction and delayed extraction (HDPCs stored in RNAlater<sup>®</sup> for 10 days). Data represent mean  $\pm$  standard deviation, \* $p < 0.05$  comparing the RNALater to fresh RNA groups at different passage.

The above study was performed on HDPCs taken from one a single donor. A study comparing the effect of delayed versus immediate RNA extraction was performed on HDPCs (P2) harvested from four different teeth obtained from different donors as described in Materials and Methods section 2.1.2. This was performed to investigate

whether storing HDPCs in RNALater<sup>®</sup> would have the same adverse effect on extracted RNA.

Immediate extraction of RNA from freshly harvested HDPC yielded concentrations ranging between 7.8-15.1 µg/200µl. However, delaying RNA extraction for 10 days while HDPCs are stored in RNALater<sup>®</sup> resulted in RNA concentrations ranged between 1.2-2.8 µg/200µl. Within each donor, the concentration of RNA was higher with immediate extraction in comparison to delayed counterparts. Similarly, the purity of RNA was higher whenever the RNA extraction was performed immediately after harvesting HDPC as indicated by the 260/280 ratios. Table 11 summarises the details of RNA concentrations and 260/280 ratios.

Table 11 Quantitative analysis of RNA content per cell of HDPCs from four different donors, following immediate or delayed RNA extraction procedures.

RNA	Mean Total Cell number×10 <sup>5</sup>	Mean RNA ng/200µl	260/280
Donor A fresh	8.4	15056±5726.8	1.75
Donor A RNALater <sup>®</sup>	9.4	1876±875.2	1.38
Donor B fresh	6.5	7791±608.2	1.5
Donor B RNALater <sup>®</sup>	12.3	1229±119.6	1.36
Donor C fresh	8.7	12229±6688.1	1.47
Donor C RNALater <sup>®</sup>	9.4	2807±1345.5	1.30
Donor D fresh	7.2	11484±6399.1	1.46
Donor D RNALater <sup>®</sup>	10.2	2040±542.1	1.29

The average RNA yield per cell was estimated to be 14-18 pg when RNA extraction was performed immediately after HDPC harvesting. By contrast, the average RNA yield per cells was as low as 1-3 pg after delayed extraction. The difference between RNA yields in each donor was significantly different upon using delayed or immediate RNA extraction techniques (p<0.05). The average amount of RNA yield from immediate

RNA extraction is more in accordance with the expected average RNA within mammalian cell which has been estimated between 10-30 pg (Figure 42). These findings confirmed that storing HDPC pellet in RNAlater<sup>®</sup> may consistently have an adverse effect on the RNA yield and integrity as it was observed in HDPCs from different passages and different donors.

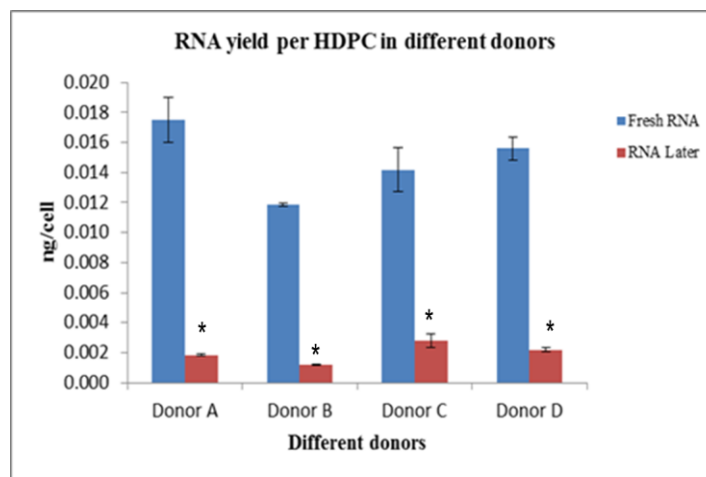


Figure 42. Effect of HDPC pellet storage on RNA yield per cell.

Comparison of RNA content per cell in donor (A, B, C and D) between immediate RNA extraction and delayed extraction (HDPCs stored in RNAlater<sup>®</sup> for 10 days). Data represent mean  $\pm$  standard deviation, \* $p < 0.05$  comparing the RNAlater to fresh RNA groups at different passage.

### 3.4.2 Assessment of the effect of immediate and delayed extraction on RNA integrity using agarose gel

Inferior integrity was noticed for RNA samples extracted from HDPC pellets that were stored in RNAlater<sup>®</sup> as shown in Results section 3.4.1. The integrity of RNA was determined based on the 260/280 ratios obtained from Nanodrop readings. These readings, however, may not be a true reflection of the RNA integrity. Thus, this study was performed to determine RNA integrity utilising agarose gel. Ethidium bromide-staining pattern of intact RNA is detected as clearly defined 28S and 18S RNA bands.

The studied RNA samples were obtained from fresh or stored HDPCs (in RNAlater<sup>®</sup>) from passage 2,6,10 and 12. Aliquots (10µl) of the extracted RNA from both groups (n=3 per group) were run on an agarose gel stained with ethidium bromide as described in Materials and Methods section 2.4.

Immediate extraction of RNA from HDPC pellet yielded highly intact RNA as indicated by the sharp, clear 28S and 18S bands. These findings were applicable to all RNA samples extracted from different passages. Conversely, storage of HDPCs pellet in RNAlater<sup>®</sup> for 10 days, this seemed to undermine the integrity of RNA. This was indicated by; the lack of distinct 28S and 26S bands, RNA smearing and accumulation of degraded RNA along lane 1,2,3 and 11 (Figure 43).

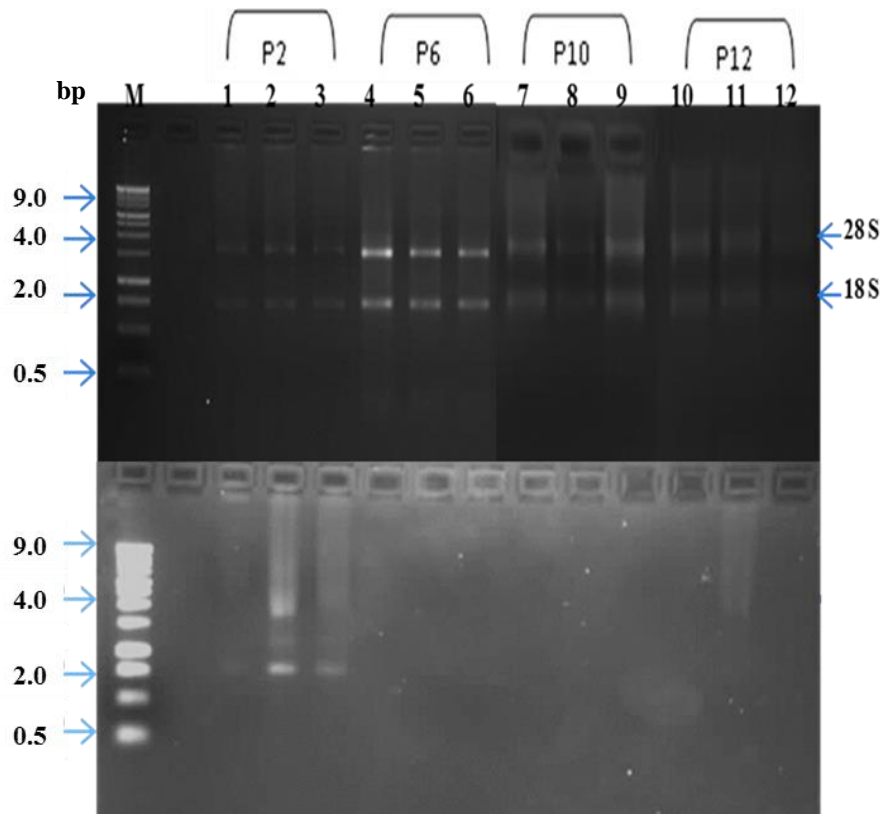


Figure 43. Assessment of RNA integrity upon immediate and delayed extraction using agarose gel for single donor.

Comparative agarose gel following immediate RNA extraction from HDPCs (Top) and HDPC pellet stored in RNAlater<sup>®</sup> (Bottom). Data is shown for HDPCs from passage 2, 6, 10 and 12 (n=3 per passage). Lane, M: 100bp marker. Arrows indicates the RNA band size in bp.

The effect of delayed and immediate RNA extraction on the integrity of RNA was also assessed for HDPCs (P2) obtained from different donors. Aliquots (10 $\mu$ l) of the extracted RNA from four different donors (n=3 per donor) were run on agarose gel stained with ethidium bromide as described in Materials and Methods section 2.4.

Immediate extraction of RNA from HDPCs (P 2) from four different donors yielded intact RNA as indicated by the sharp, clear 28S and 18S bands. However, the RNA was possibly contaminated with genomic DNA as indicated by the smearing above 28S band. Similar to data obtained from single donor storage of cell pellet in RNAlater<sup>®</sup> for 10 days seemed to undermine the integrity of RNA as indicated by the absence of 28S and 18S bands on the agarose gel for all donors (Figure 44).

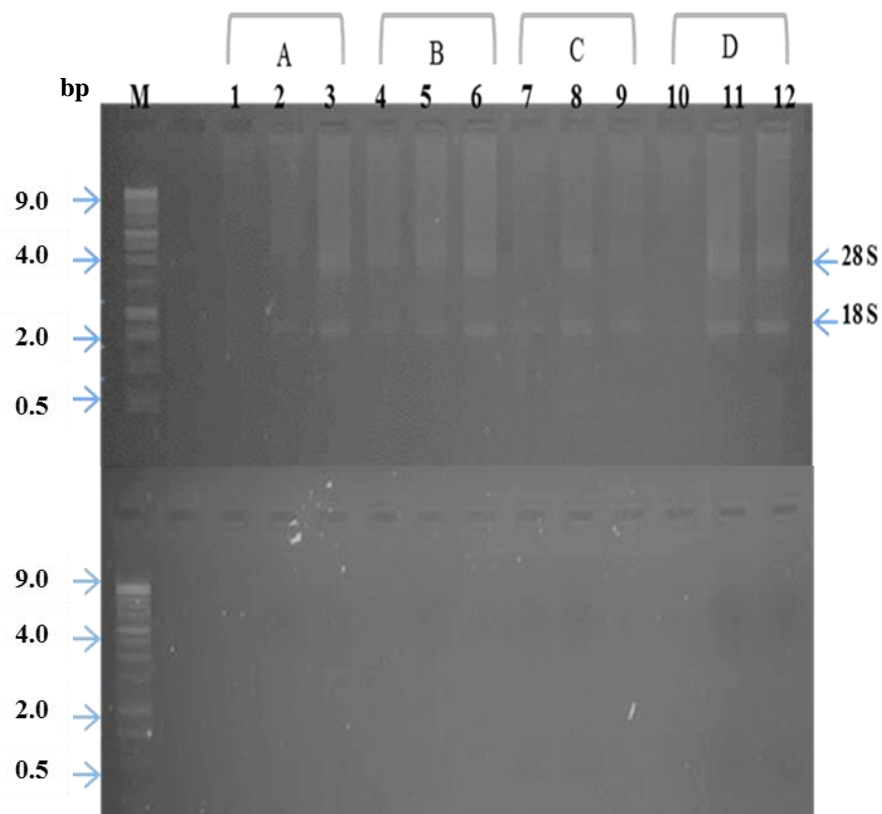


Figure 44. Assessment of RNA integrity upon immediate and delayed extraction using agarose gel for different donors.

Comparative agarose gel following immediate RNA extraction from HDPCs (Top) and HDPC pellet stored in RNAlater<sup>®</sup> (Bottom). Data is shown for HDPCs (P2) from four different donors (n=3 per donor). Arrows indicates the RNA band size in bp.

### 3.5 Optimised gene expression studies

The findings presented in the Results sections 3.4.1 and 3.4.2 indicated that storage of HDPCs in RNAlater® may have a detrimental effect on the RNA yield and integrity. This could possibly account for the inconsistency encountered with the qRT-PCR data presented in Results section 3.3. As a consequence, qRT-PCR work has been repeated for HDPC samples where RNA was extracted immediately after cell harvesting.

#### 3.5.1 Comparison of the efficiency and precision of Taq-Man® and SYBR-Green® assays

SYBR-Green® and Taq-Man® assays were used to analyse the expression profile of certain genes in the HDPCs. Owing to the less than ideal RNA that was used in these experiments, the resultant data was inconsistent, irreproducible and of limited analytical value as was described in Results section (3.3). In addition, the poor quality data rendered identification of the superior assay among the two used, impossible. Thus, the current study was conducted to investigate the sensitivity, reproducibility and specificity using two different assays (Taq-Man® and SYBR-Green®).

The expression of the *GAPDH* gene was investigated in serially diluted cDNA obtained from HDPCs (P4) using both qRT-PCR assays (n=3 per group). Ct values from both assays were plotted against their respective cDNA concentration as shown in Figure 45. The results indicated that Taq-Man® assay was more sensitive in comparison to SYBR-Green®. This was clearly demonstrated as the Taq-Man® assay detected various cDNA concentrations earlier (lower Ct values) than SYBR-Green® assay. In addition, Taq-Man® assay was more precise as indicated by the low disparity of Ct values within each triplicate. The reaction efficiency of both assays was determined by fitting a linear function to Ct values plotted against the  $\log_{10}$  cDNA relative concentration.  $R^2$  values

and the slope of both lines were closely related which may indicate that the efficiency was similar for both assays. Consequently, Taq-Man<sup>®</sup> assays were used for all subsequent gene expression experiments in an attempt to produce a highly consistent and reproducible q-RT-PCR data.

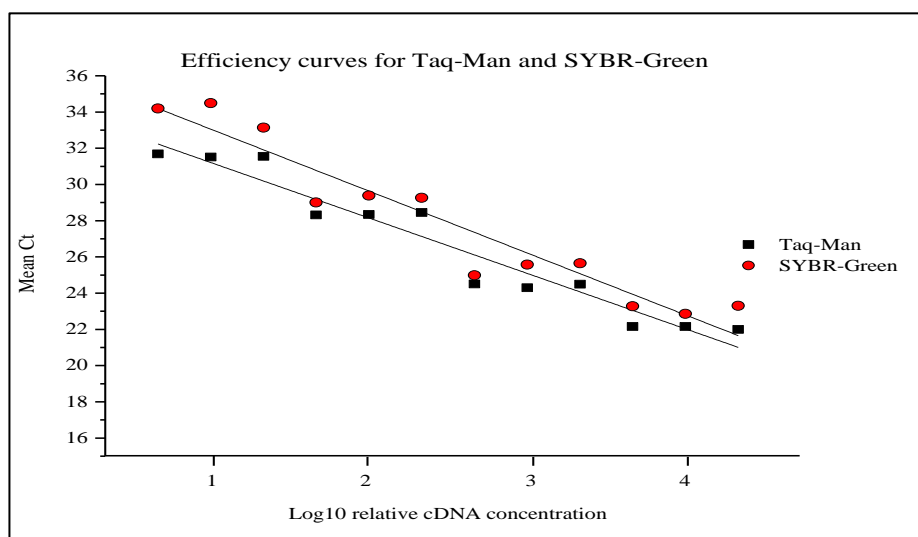


Figure 45. Calibration curve for Taq-Man<sup>®</sup> and SYBR-Green<sup>®</sup> assay.

Comparison of efficiency of SYBR-Green<sup>®</sup> and Taq-Man<sup>®</sup> assays using *GAPDH* primer in HDPCs (P4). The bottom trend line ( $R^2=0.94$ , slope=1.0006) fitted to Ct values obtained by Taq-Man<sup>®</sup> assay. The top trend line ( $R^2=0.91$ , slope=1.14) fitted to Ct values obtained by SYBR-Green assay.

### 3.5.2 Selection of housekeeping genes

Several housekeeping genes that are constitutively expressed can be used as an internal control for qRT-PCR assays. The aim of this study was to validate and select the most constitutively expressed housekeeping gene for HDPCs treated with MTA or propolis in addition to a control group. All HDPCs were from passage 2 and were incubated for 24 hours treated with MTA and propolis. For this purpose, expression of four genes *GAPDH*, *TERC*,  *$\beta$ -actin* and *WYHAZ* was investigated under various conditions as described in Materials and Methods section 2.5.3.

*TERC* gene exhibited the least Ct variation with different conditions as indicated by ANOVA ( $p=0.269$ ). *GAPDH* was the second most constitutively expressed gene as indicated by Brown-Forsythe test ( $p=0.006$ ). Ct values for  $\beta$ -actin and *WYHAZ* varied significantly with various conditions,  $p=0.00$  and  $0.01$ , respectively (Figure 46). According to the results *TERC* was constitutively expressed in HDPCs in various conditions in comparison to the other genes, therefore these two genes were used as housekeeping genes for all subsequent gene expression studies. *TERC* gene, however, was used as control when data analysis was performed.

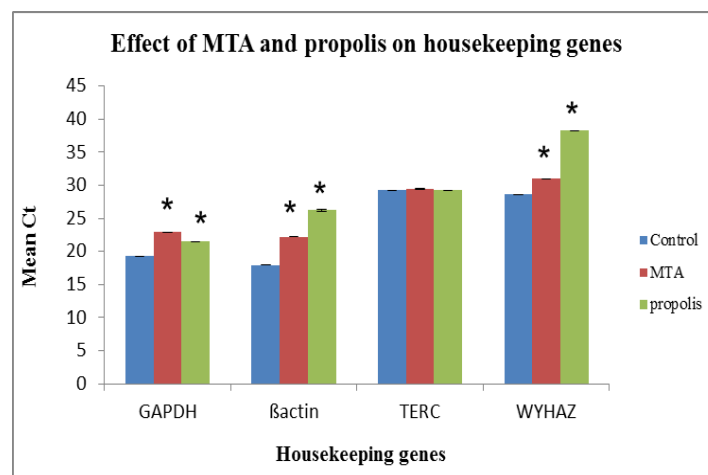


Figure 46. Assessment of potential housekeeping genes.

Changes of Ct values of different genes in HDPCs exposed to MTA, propolis and control after 24 hours of incubation. Data represent mean  $\pm$  standard deviation ( $n=3$ ),  $*p<0.05$  among MTA and propolis groups in comparison to control for the same gene.

### 3.5.3 Effect of pulp capping materials on expression profile of various genes

The genetic basis of the changes of biological characteristics of HDPCS upon exposure to MTA and propolis were investigated utilising gene profile expression studies using Taq-Man qRT-PCR assay following immediate RNA extraction procedure. Gene expression studies were used to determine the effect of MTA and propolis on the



proliferation and differentiation potential of HDPCs which has an essential effect on their function *in vivo*. Furthermore, gene expression studies were used to provide better understanding of the effect of MTA and propolis on the phenomenon of cell ageing by investigating the genes orchestrating this process.

### **3.5.3.1 The effect of pulp capping materials on genes controlling programmed cell death**

Treatment of HDPCs with propolis resulted in reduced cell viability while MTA seemed to increase the proliferation capacity and the number of viable cells as described in the results section 3.2.4. The apoptosis related gene *BID* and the autophagy related gene *BCN1* were analysed in serially passaged HDPCs to investigate whether the noticed effect of MTA and propolis on cell viability can be related to the expression of these genes. Taq-Man<sup>®</sup> qRT-PCR assay was used to analyse the *BID* and *BCN1* expression profile in serially passaged HDPCs incubated with MTA and propolis for 24 hours. Gene expression patterns were assessed based on qualitative analysis of individual genes normalized the *TERC* using Taq-Man<sup>®</sup> assay as described in Materials and Methods section 2.5.4.2. The  $\Delta C_t$  relative to the  $C_t$  for the control sample and standard deviations were calculated for all experimental groups as described in Materials and Methods section 2.5.5

A plot of  $2^{-\Delta C_t}$  normalised to the value of housekeeping gene is shown in Figure 47. The expression of the *BID* gene in the control, MTA and propolis treated HDPCs was below the detectable level (data not shown). In all studied groups, the expression of *BCN1* gene seemed to be the highest in HDPCs from passage 4 and gradually decrease in later passages. The expression of *BCN1* was the highest in the propolis group when

compared to similar passage number in control and MTA groups. However the difference was more pronounced in passage 6 and 12 ( $p < 0.05$ ).

Incubation of HDPCs from various passages seemed to induce cell death by activating the gene regulating the autophagy. These findings may also suggest that this effect is more pronounced on HDPCs from later passages where cells naturally lose differentiation and growth potential. Thus, the presented findings may account for the high cell death noticed in the propolis group at later passages when compared to MTA and control groups.

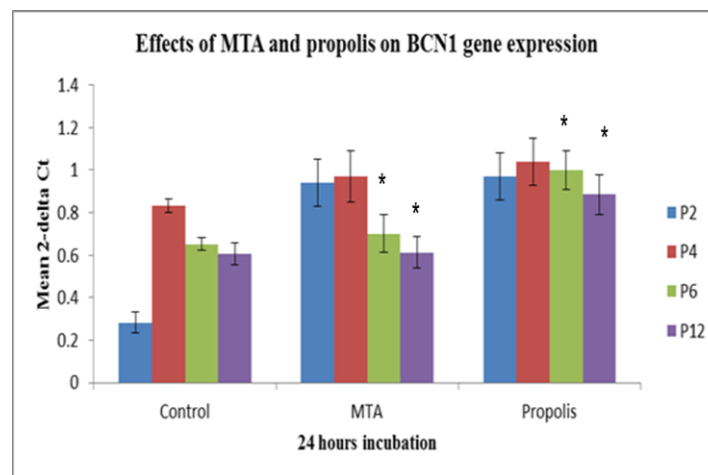


Figure 47. Expression profile of *BCN1* gene.

Comparison of *BCN1* gene expression in HDPCs treated with MTA, propolis and control after 24 hours of incubation from (P2, 4, 6 and 12). Data represents mean  $2^{-\Delta ct} \pm$  standard deviation ( $n=3$ ),  $*p < 0.05$  among MTA and propolis groups in comparison to control in each passage.

### 3.5.3.2 The effect of pulp capping materials on differentiation potential of HDPCs

The primary aim of pulp capping is to enhance hard bridge formation to stimulate the healing of the injured dental pulp. The formation and features of the hard tissue bridge are directly influenced by the differentiation of mesenchymal HDPCs that can differentiate to osteoblast- or odontoblast-like cells. The effect of MTA and propolis on

the differentiation potential of HDPCs was thereby determined by investigating the expression profile of the genes regulating the osteogenesis and odontogenesis processes.

### **3.5.3.2.1 The effect of MTA and propolis on the expression profile of odontogenic genes**

The effect of MTA and propolis on the odontogenic differentiation potential was investigated by evaluating (*DSPP*, *DMP-1* and *BMP-2*) genes. Taq-Man<sup>®</sup> qRT-PCR assay was used to analyse the expression profile of these genes in serially passaged HDPCs incubated with MTA and propolis for 24 hours. Gene expression patterns were assessed based on qualitative analysis of individual genes normalized the *TERC* using Taq-Man<sup>®</sup> assay as described in Materials and Methods section 2.5.4.2. The  $\Delta C_t$  relative to the  $C_t$  for the control sample and standard deviations were calculated for all experimental groups as described in Materials and Methods section 2.5.5.

Expression of specific odontogenesis markers (*DSPP* and *DMP-1*) was below the detectable level in all experimental groups and passages (data not shown). The undetectable expression of *DSPP* and *DMP-1* gene in HDPCs was surprising as they are highly specific odontogenesis markers and they are necessary for *de novo* dentine formation by HDPCs. In order to rule out the possibility of technical error (e.g. malfunction in the used PCR primer) leading to such findings, we investigated the *DSPP* gene expression in a positive control (human brain cells) that are known to express *DSPP* gene using similar primers and PCR technique (Primer design, UK).

The expression of *DSPP* gene was successfully detected upon studying various cDNA dilutions using Taq-Man<sup>®</sup> qRT-PCR assay performed with the same primers used with samples from HDPCs. The calibration curve was linear which indicated 100% priming efficiency as shown in Figure 48. These findings appear to confirm that the absence of

*DSPP* gene expression in HDPCs in the current study is due to the undetectable expression of *DSPP* rather than a technical failure.

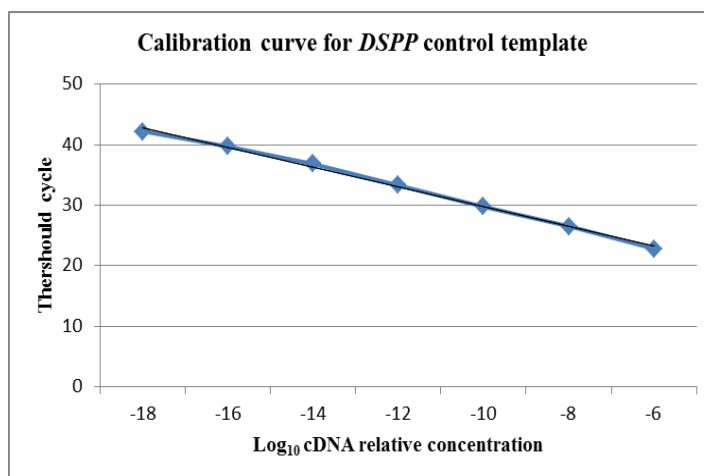


Figure 48. Calibration curve for *DSPP* gene.

Reaction efficiency for *DSPP* serially diluted cDNA from universal cells. The trend line fitted to Ct values are plotted against the log<sub>10</sub> cDNA concentration is also shown. The value of  $R^2=0.9963$  and the slope=  $-3.278$  closely matched to those provided by the supplier of the *DSPP* primers (PrimerDesign, UK).

*BMP-2* gene is a non-specific marker that can be expressed in odontogenesis or osteogenesis. The highest level of *BMP-2* gene expression was detected in propolis treated HDPCs from passage 4. MTA treated HDPCs demonstrated up-regulation of *BMP-2* gene in passage 4 and to a lesser extent in passage 6 (Figure 49). These findings may suggest that the level of *BMP-2* gene expression in HDPCs may be positively affected by MTA and propolis in early passages. However the undetectable level of expression of the two specific odontogenesis markers (*DSPP* and *DMP-1*) precluded the conclusion that MTA or propolis HDPCs stimulated odontogenic differentiation in the studied HDPCs.

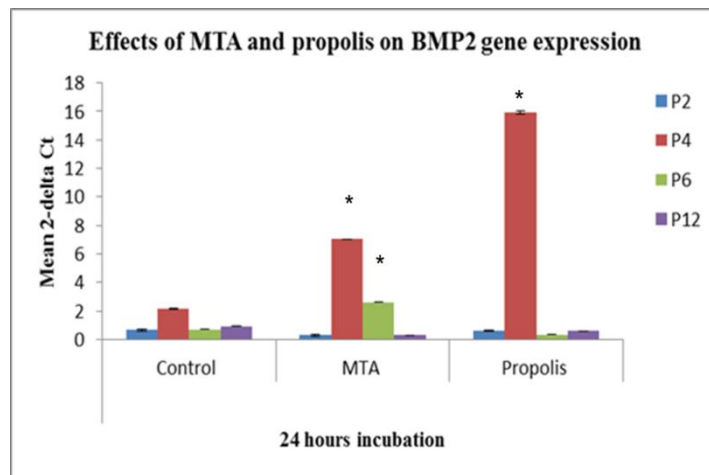


Figure 49. Expression profile of *BMP-2* gene.

Comparison of *BMP-2* gene expression in HDPCs treated with MTA, propolis and control after 24 hours of incubation from (P2, 4, 6 and 12). Data represents mean  $2^{-\Delta ct} \pm$  standard deviation (n=3), \*p<0.05 among MTA and propolis groups in comparison to control in each passage.

### 3.5.3.2.2 Proteomic analysis of BMP-2

Western blot was used to evaluate cellular BMP-2 production in HDPCs from different passages that received MTA or propolis treatment, as described in Materials and Methods section 2.6. The aim of the present study was to validate the qRT-PCR finding at the protein level.

Cellular production of BMP-2 protein was detected in MTA treated HDPCs in all studied passages as indicated by the well-demarcated 45 KDa bands (Figures 50). The expression of BMP-2 protein seemed to be higher in MTA treated HDPCs in comparison to control HDPCs. This effect was more pronounced in passage 2, 4 and 6 as indicated by the higher density of the 45 KDa bands for MTA groups in comparison to control HDPCs from similar passage number. However, expression of BMP-2 protein was slightly lower in MTA group in comparison to control in passage 12 (Figure 50).

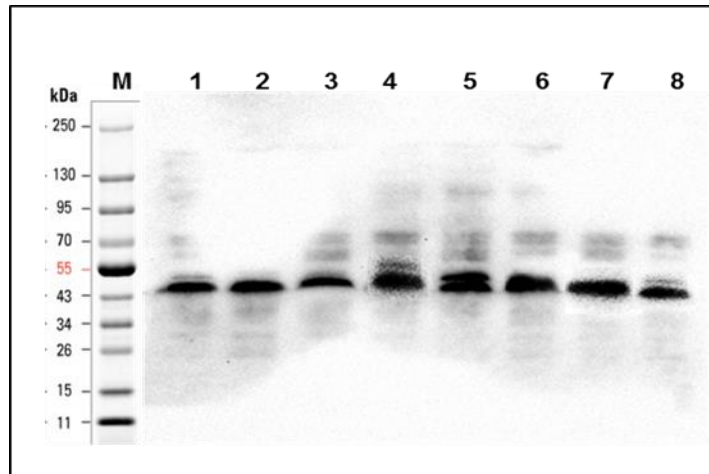


Figure 50. Western blot analysis of HDPCs treated with MTA in comparison to control. Protein ladder marker (lane M); passage 2 control (lane1), passage 2 MTA treated (lane2), passage 4 control (lane 3), passage 4 MTA treated (lane 4), passage 6 control (lane 5), passage 6 MTA treated (lane 6), passage 12 control (lane 7) and passage 12 MTA treated (lane 8). BMP-2 was detected at 45KDa.

The expression of BMP-2 protein was detected in propolis treated HDPCs in all studied passages as indicated by the well-demarcated 45 KDa bands (Figures 51). The expression of BMP-2 protein seemed to be higher in propolis treated HDPCs in comparison to control HDPCs. This effect was more pronounced in passage 4 and 6 as indicated by the higher density of the 45 KDa bands for propolis groups in comparison to control HDPCs from similar passage number. However, expression of BMP-2 protein was slightly lower in the propolis group in comparison to control in passage 2 and 12 (Figure 51).

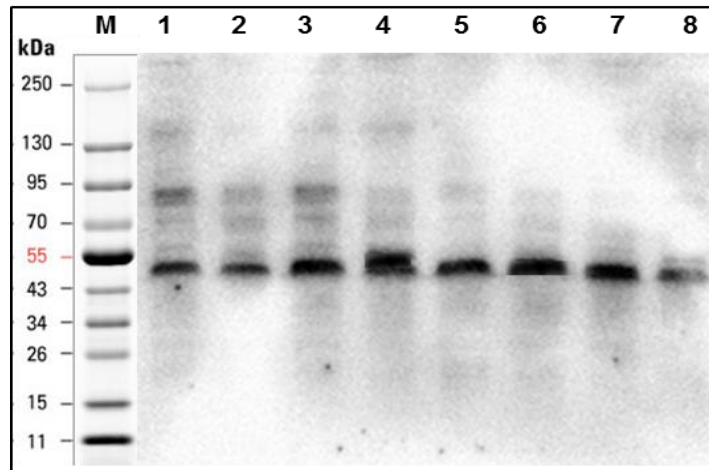


Figure 51. Western blot analysis of HDPCs treated with propolis in comparison to control.

Protein ladder marker (lane M); passage 2 control (lane1), passage 2 propolis treated (lane2), passage 4 control (lane 3), passage 4 propolis treated (lane 4), passage 6 control (lane 5), passage 6 propolis treated (lane 6), passage 12 control (lane 7) and passage 12 propolis treated (lane 8). BMP-2 was detected at 45KDa.

The current proteomic analysis revealed that propolis and MTA can increase synthesis of cellular BMP-2 in passage in early to intermediate passages (P4 and 6). BMP-2 up-regulation was significantly higher in MTA and propolis groups when compared to control HDPCs in these passages ( $p < 0.05$ ) as shown in Figure 52. This effect was in agreement with the changes of gene expression profile of *BMP-2* upon MTA and propolis treatment, where *BMP-2* gene was up-regulated in passage 4 and 6 in the MTA group and in passage 4 only in propolis group.

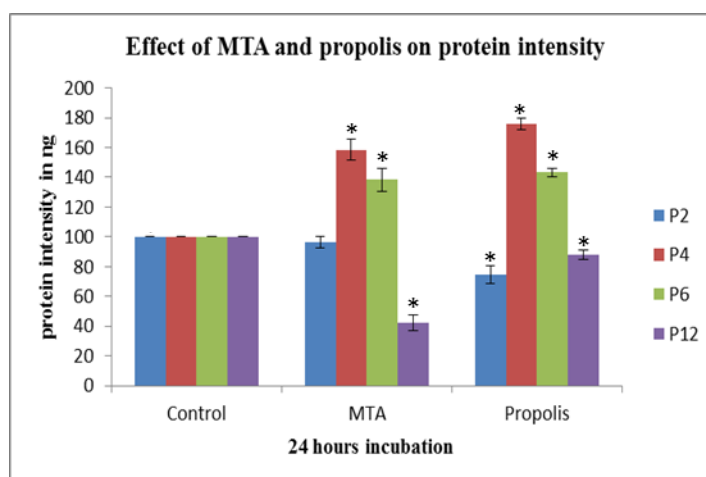


Figure 52. Densitometric analysis of BMP-2 protein expression.

The expression of BMP-2 protein in HDPCs treated with MTA & propolis in comparison to control. Mean BMP-2 intensity and  $\pm$  standard deviation of various groups and passage 2, 4, 6 and 12 (n=3), \*p<0.05 among MTA and propolis groups in comparison to control in each passage.

### 3.5.3.3 The effect of pulp capping materials on osteogenic differentiation potential

The present study revealed that the studied HDPCs may exhibit osteogenic differentiation potential as indicated by the undetectable expression of specific odontogenic markers (*DSPP* and *DMP-1*) as well as up-regulation of the non-specific *BMP-2* gene as described in Results section 3.5.3.2.1. The effect of MTA and propolis on the osteogenic differentiation potential was further investigated by evaluating, *ALP*, *RUNX-2* and *OCN* genes in HDPCs after 24 hours incubation period. Gene expression patterns were assessed based on qualitative analysis of individual genes normalized the *TERC* using Taq-Man<sup>®</sup> assay as described in Materials and Methods section 2.5.4.2. The  $\Delta$ Ct relative to the Ct for the control sample and standard deviations were calculated for all experimental groups as described in Materials and Methods section 2.5.5.



*RUNX-2* and *BMP-2* also regulate the expression of *ALP* gene that has a major function during the early mineralisation stage in osteogenesis. The expression of *ALP* gene was investigated in HDPCs from passage 2, 4, 6 and 12, treated with MTA or propolis for 24 hours. A plot of  $2^{-\Delta ct}$  normalised to the value of housekeeping gene is shown in Figure 53. The highest level of *ALP* gene expression was noticed in propolis treated HDPCs from passages 2 and 12. *ALP* gene expression in propolis treated HDPCs were significantly lower in passage 4 but still higher than the control group and below the detectable levels in passage 6 ( $p < 0.05$ ). In the MTA groups, HDPCs from passage 2, 4 and 6 exhibited progressive up-regulation of *ALP* gene. The level of expression abruptly declined in passage 12 (Figure 53). These findings suggest that the level of *ALP* gene expression in HDPCs may be affected by propolis. However, it seemed that the effects of MTA and propolis are dependent on the passage history of the HDPCs.

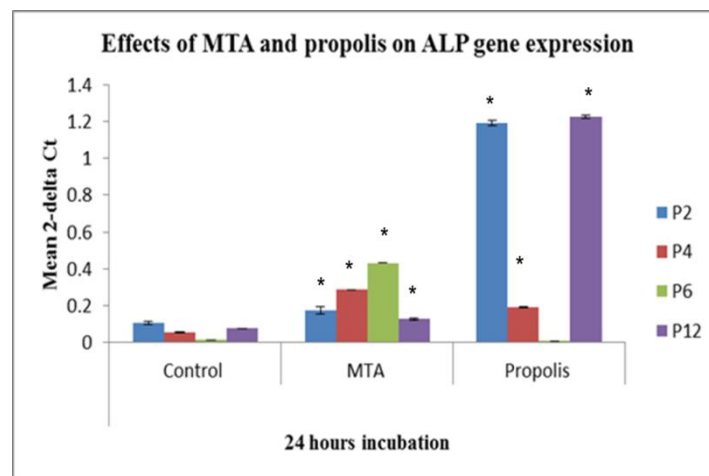


Figure 53. Expression profile of *ALP* gene.

Comparison of *RUNX-2* gene expression in HDPCs treated with MTA, propolis and control after 24 hours of incubation from (P2, 4, 6 and 12). Data represents mean  $2^{-\Delta ct} \pm$  standard deviation ( $n=3$ ),  $*p < 0.05$  among MTA and propolis groups in comparison to control in each passage.

A plot of  $2^{-\Delta ct}$  normalised to the value of housekeeping gene is shown in Figure 54. Expression of the early osteogenic marker, *RUNX-2*, was investigated in HDPCs from

passage 2, 4, 6 and 12 treated with MTA and propolis. The highest level of *RUNX-2* gene expression was seen in propolis treated HDPCs from passage 2 and followed by passage 4 and the least was seen in passage 12. HDPCs from passage 6 had a level of expression significantly lower than all passages and similar to the control group ( $p < 0.05$ ). MTA treated HDPCs demonstrated up-regulation of *RUNX-2* in passage 2 and further up-regulated in passage 4. In passage 6, there was an abrupt decrease in the expression level of *RUNX-2*. However, it remained higher than propolis and control groups. In passage 12, there was a moderate up-regulation that was comparable to the propolis group and higher than the controls (Figure 54).

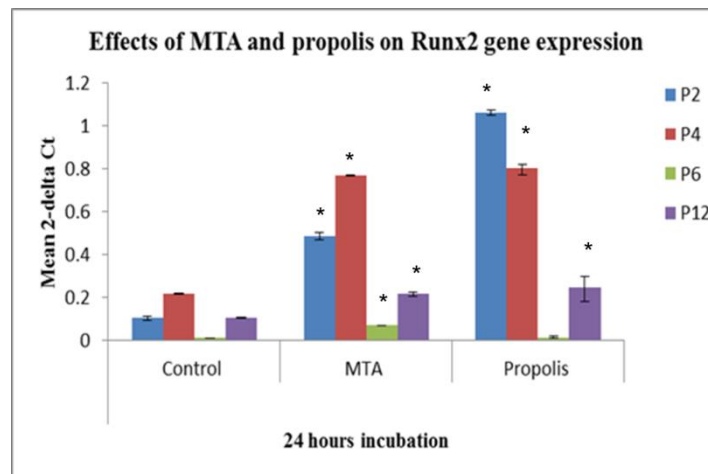


Figure 54. Expression profile of *RUNX-2* gene.

Comparison of *RUNX-2* gene expression in HDPCs treated with MTA, propolis and control after 24 hours of incubation from (P2, 4, 6 and 12). Data represents mean  $2^{-\Delta Ct} \pm$  standard deviation ( $n=3$ ),  $*p < 0.05$  among MTA and propolis groups in comparison to control in each passage.

*RUNX-2* is essential for the differentiation of HDPCs as it is a key mediator that initiates the transcription and subsequent translation of many other genes responsible for extracellular matrix deposition during the early phase of osteogenic differentiation. Thus, the present findings indicated that propolis, and to a lesser extent MTA, may up-regulate the expression of *RUNX-2* gene and thereby stimulate HDPC differentiation

toward osteoblast-like cells. However, the effect of such materials is passage dependent as it was more pronounced in early passages (P2 and 4).

*OCN* is an essential extracellular protein that is secreted to prepare for bone formation. The function of *OCN* is largely controlled by signalling from *RUNX-2*. The expression of *OCN* gene was investigated in HDPCs from passage 2, 4, 6 and 12, treated with MTA or propolis for 24 hours. A plot of  $2^{-\Delta Ct}$  normalised to the value of housekeeping gene is shown in Figure 55. The highest level of *OCN* gene expression was detected in MTA treated HDPCs from passage 6 with a slight up-regulation in the rest of the passages. Propolis treated HDPCs from passage 2 had a level of expression significantly higher than all other passages and also in comparison to the MTA and control groups in the same passage as shown in Figure 55.

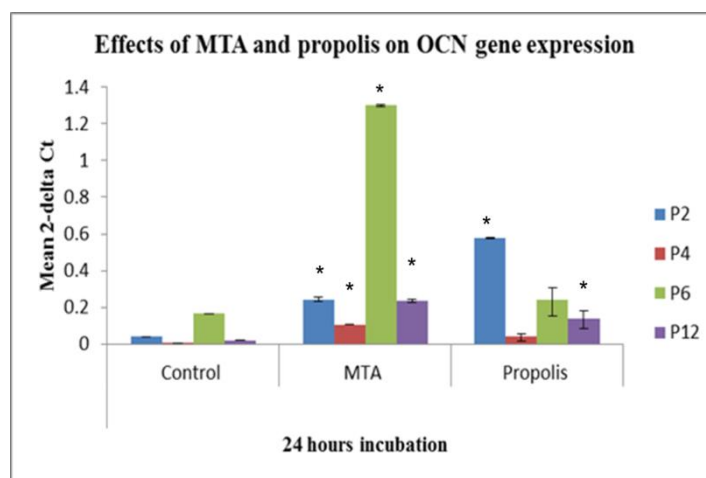


Figure 55. Expression profile of *OCN* gene.

Comparison of *OCN* gene expression in HDPCs treated with MTA, propolis and control after 24 hours of incubation from (P2, 4, 6 and 12). Data represents mean  $2^{-\Delta Ct} \pm$  standard deviation (n=3), \*p<0.05 among MTA and propolis groups in comparison to control in each passage.

These findings may indicate that MTA enhanced the response of HDPCs in intermediate passages to *RUNX-2* signalling via up-regulating *OCN*. The same effect was also noticed with propolis treated HDPCs but from earlier passages. The present

findings may indicate that MTA and, to a lesser extent, propolis may enhance the osteogenic differentiation potential of HDPCs but in different passages.

Overall, the present study demonstrated that the studied HDPCs had a natural tendency to undergo osteogenic differentiation. This was indicated by the absence of expression of specific odontogenic differentiation markers (*DSPP* and *DMP-1*) coupled with mild up-regulation of *BMP-2* and *RUNX-2* genes during early passages as well as *OCN* and *ALP* in later passages.

MTA and propolis clearly enhanced the osteogenic differentiation potential of HDPCs in comparison to control. This effect was largely affected by the passage number as the most notable up-regulation of osteogenic markers was in passage 4 and 6. This was evident from the significant up-regulation of *BMP-2*, *RUNX-2* and *ALP* genes during early passages. In later passages, both materials induced the expression of *OCN* and *ALP* genes while down-regulated *BMP-2* and *RUNX-2* genes. The gene expression profile of the studied genes was largely compatible with the sequence of genetic changes involved in the osteogenesis process where *RUNX-2*, *BMP-2* and *ALP* initiate the process and then downregulated in order to signal for genes orchestrating advanced stages of osteogenesis. A comparative graph of gene expression profile in MTA, propolis and control group in several passages is presented in Figure 56.

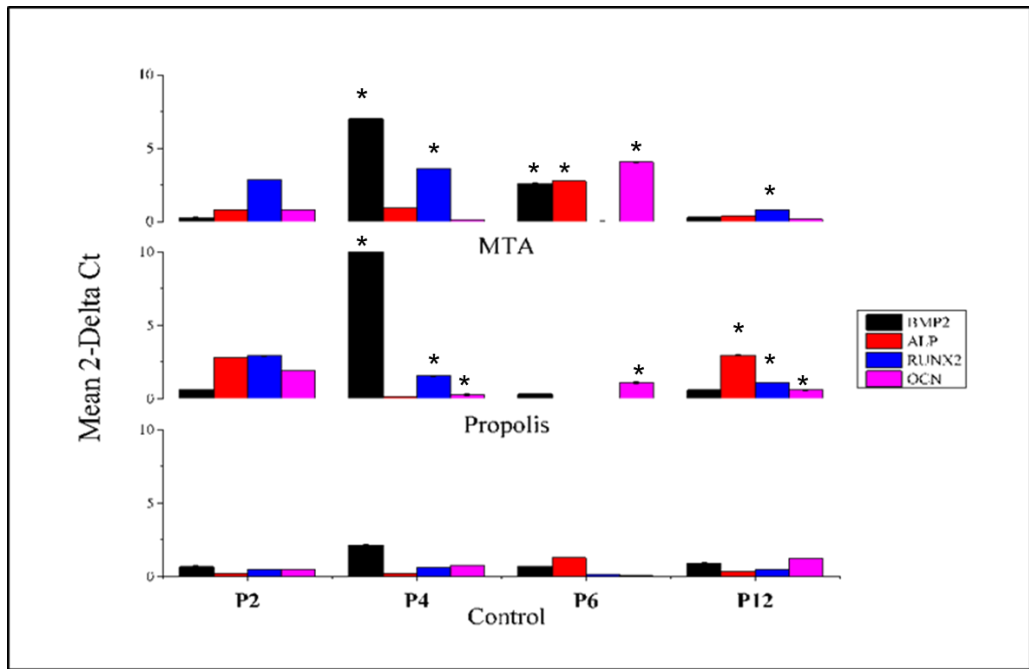


Figure 56. Comparative gene expression profile.

Expression of *BMP-2*, *RUNX-2*, *OCN* and *ALP* genes in HDPCs treated with MTA, propolis and control after 24 hours of incubation from (P2, 4, 6 and 12). Data represents mean  $2^{-\Delta Ct} \pm$  standard deviation. \*  $p < 0.05$  among MTA and propolis groups in comparison to passage 2 in each passage.

### 3.6 Cell ageing in serially passaged HDPCs

Cellular ageing or senescence describes a change in the state of the cell. Ageing cells exhibit low proliferation potential. A particular type of cell ageing that can be encountered *in vitro* upon serially passaging cells known as replicative senescence where the cells may cease to divide once they have gone through a certain number of cell divisions or once they reach what is known as Hayflick limit. This phenomenon may have a fundamental effect on the cells grown *in vitro* for purposes of tissue engineering treatments where they may exhibit suboptimal level of growth, proliferation and differentiation potential. The molecular basis of such phenomenon is very complicated but telomere length shortening and oxidative stress are among of the most widely reported events contributing to it. The loss of proliferation and growth potential

of HDPCs as a function of passage number as identified in the result of the present study (section 3.2.7) may be attributed to this phenomenon. Furthermore, the higher proliferation rate of HDPCs upon exposure to MTA indicated that the cells may have gone through a higher number of cell divisions and possibly sustained higher degree of cell ageing. The antioxidant effect of the novel, biological agent, and propolis has been widely cited and may have a protective role against telomere shortening. Thus, the phenomenon of replicative senescence was investigated serially passaged HDPCs that were cultured with MTA or propolis and compared to control cells (Hayflick, 1974, Huang *et al.*, 2006).

The level of cell ageing can be indirectly extrapolated from studying the expression profile of genes encoding for telomerase (*TERC* and *TERT*) that when up-regulated, it reduced telomere length shortening. This method was used in the present study (Section 3.3.6) and the findings indicated that HDPCs from all groups did not express *TERT* which may suggest that the studied cells will be susceptible to cell ageing but to unknown extent. Relative telomere length shortening can also be estimated using specific telomere primers. Previous study has suggested telomere length shortening in serially passaged HDPCs (Rezapour, 2008). In the present study, the exact extent of telomere length shortening was assessed in control, MTA or propolis-treated HDPCs using Southern blot hybridisation technique.

### **3.6.1 The effect of MTA and propolis on the extent of telomere length shortening**

In the present study, the extent of telomere length shortening in serially passaged HDPCs in culture was determined as described in Materials and Methods section 2.7. Telomere length of control, MTA and propolis-treated HDPCs after 24 hours incubation period were investigated. A telomere probe and Southern blot DNA hybridization technique was used to determine mean TRF length of HDPCs at passage 2, 4, 6, 10 and 12.

Telomeric DNA was detected in HDPCs from all studied passages. Chemiluminescent detection of TRF indicated telomere length shortening as a function of passage number as indicated by the progressive decrease in the TRF length from passage 2 to 12. The mean TRF lengths for HDPCs in serial passage (P2, 4, 6, 10 and 12) were as follows: P2=14.9 kb, P4=14.1kb, P6=13.3 kb, P10=12.6 kb, and P12=11.5 kb (Figure 57). One-way ANOVA and LSD post-hoc analysis revealed statistically significant difference ( $P<0.05$ ) in the mean TRF of consecutive passages. These findings indicated that serial passage may cause replicative senescence in the studied HDPCs as a result of telomere length shortening.

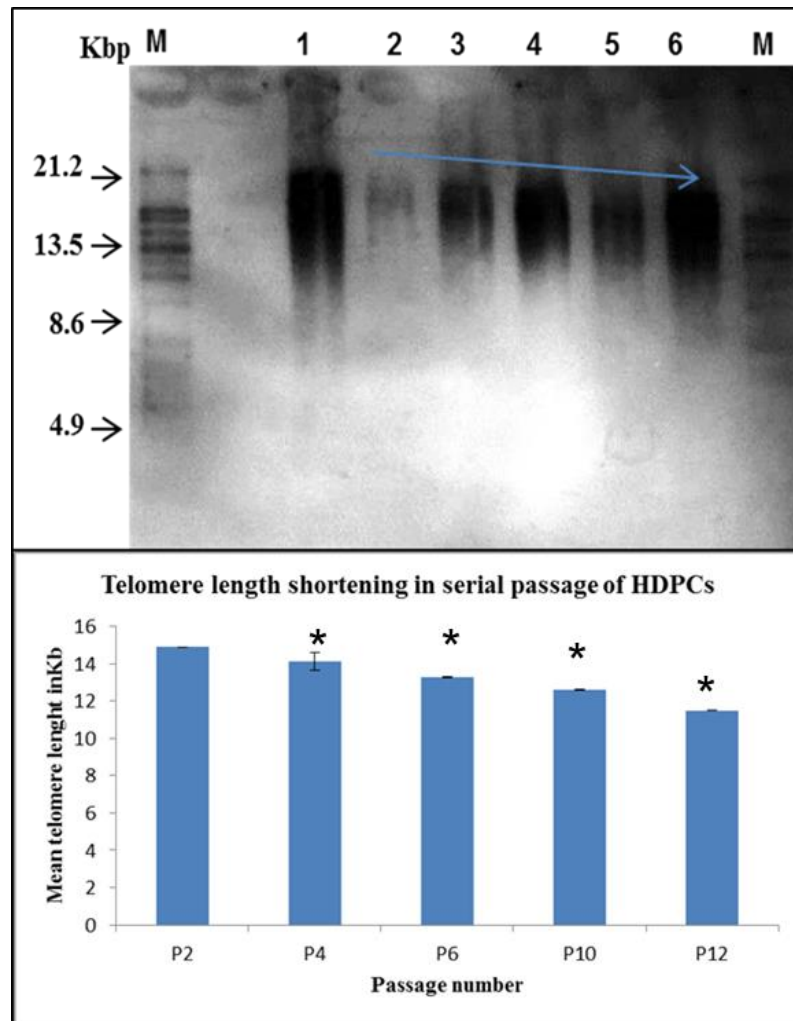


Figure 57. TRF measurements for serially passaged HDPCs.

(Top) Chemiluminescent detection of TRF for digested immortalised cell line (internal positive control, lane 1), HDPCs passage 2 (lane 2), passage 4 (lane 3), passage 6 (lane 4), passage 10 (lane 5) and passage 12 (lane 6). Lanes labelled M represent DIG marker. Arrow indicates telomere length reduction with serial passages. Bottom: Densitometric scanning analysis of blotted TRFs of serially passaged HDPCs. Data represent mean  $\pm$  standard deviation (n=3), \* p<0.05 among in each passage in comparison to passage 2.



The telomere length appeared to be highest in passage 2 and similar among control, MTA and propolis treated groups. Chemiluminescent detection of TRF revealed that MTA and control groups demonstrated gradual decrease mean TRF length as a function of passage number. However, propolis treated HDPCs had an abrupt decrease (90% loss) of TRF length after passage 2. Densitometry analysis of the blotted TRFs indicated that mean TRF length values decreased with serial passages in controls and with propolis and MTA treatments. The highest reduction in telomere length was detected in the propolis treated group, followed by MTA treated HDPCs in comparison to the control group (Figure 58). The difference between MTA and control groups was however not statistically significant for the respective passage number. There was, however, significantly higher telomere length shortening in the propolis group in comparison to MTA and control ( $p < 0.05$ ).

The current findings confirmed that HDPCs can exhibit telomere length shortening when serially passaged. Additionally, the positive effect of MTA on the proliferation capacity of HDPCs did not render them more susceptible to replicative senescence than control cells. HDPCs treated with propolis exhibited the highest telomere length shortening among all other groups. The hypothesised telomere protective role of propolis could not be identified in the current study. Finally, cell ageing may account for the loss of proliferation capacity and high rate of cell death observed with in HDPCs from later passages treated with propolis.

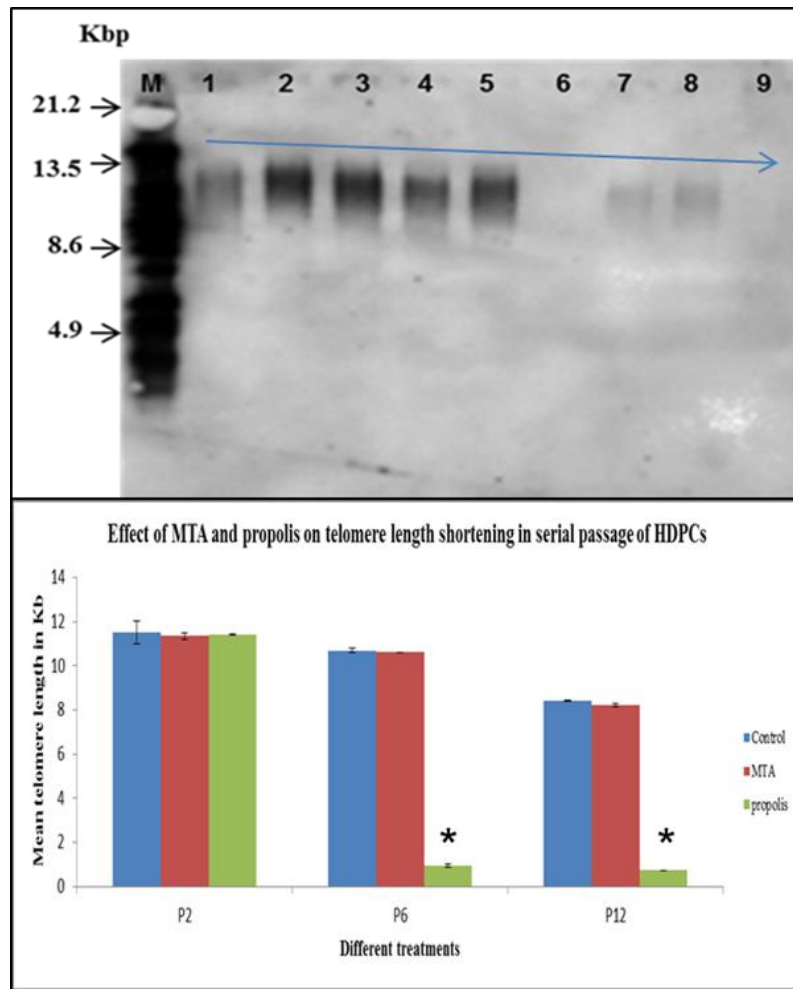


Figure 58. TRF measurements for serially passaged HDPCs with pulp capping agents.

(Top) Chemiluminescent detection of TRF for HDPCs passage 2 (Control, lane1), HDPCs treated with MTA passage 2 (lane 2), HDPCs treated with propolis passage 2 (lane 3), HDPCs passage 6 (Control, lane 4), HDPCs treated with MTA passage 6 (lane 5), HDPCs treated with propolis passage 6 (lane 6), HDPCs passage 12 (Control, lane7), HDPCs treated with MTA passage 12 (lane 8), HDPCs treated with propolis passage12 (lane9), Lanes labelled M represent DIG marker. Arrow indicates telomere length reduction with serial passages. Bottom: Densitometric scanning analysis of blotted TRFs of serially passaged HDPCs. Data represent mean  $\pm$  standard deviation (n=3), \*  $p < 0.05$  among propolis groups in comparison to control and MTA in each passage.

### **3.7 Investigating a strategy to delay cell ageing**

Cellular ageing is highly linked to the ageing of the organism and implicated in disease of ageing. Biogerontology is the discipline of science that is concerned with studying ageing mechanisms and identifying anti-ageing therapeutic approaches. Such approaches are of great importance in the light of emergence of tissue engineering where there is no place for ageing cells that may compromise the survival of the bioengineered tissue, owing to their degraded function. The present study revealed loss of proliferation capacity among serially passaged HDPCs. It is very likely that this may be attributed to the replicative ageing phenomenon, given the results indicated significant telomere shortening in HDPCs from later passages. The attrition in function and telomere length was more prevalent in HDPCs treated with propolis. The latter agent has been successfully used in pulp capping procedures in clinical settings. However, its effect on the function of pulp cell and pulp degeneration has not been elucidated. For these reasons, developing novel anti-ageing modalities may be required.

#### **3.7.1 DNA end replication problems**

DNA replication is an essential step in the transfer of genetic material to the successor cells from a dividing precursor. During DNA replication, the replication fork is comprised of two asymmetric parental strands. One DNA daughter strand is synthesised continuously in 5' to 3' direction and referred to as the leading strand. The second DNA daughter strand is also synthesised in 5' to 3' but in the opposite direction. This is carried out in a discontinuous manner, and is referred to as the lagging strand. Synthesis of the lagging strand involves adding nucleotides to 3' end of the newly forming DNA strand via DNA polymerase III to form Okazaki fragments. These are later connected to each other by DNA polymerase I. The 3' end of a linear chromosome will not be

synthesized completely as there is no mechanism to add a primer on the chromosome end. This will lead to loss of telomeric sequences and will result in chromosome shortening as a lack of ability to replicate the end of the chromosome would result in progressive shortening during each replication cycle. This is one of the principle causes of cellular senescence, where, after a certain number of cell divisions, telomeres reach a critically short length resulting in arrest of cell division (Stryer, 1995b, Granger *et al.*, 2002).

### **3.7.2 A strategy for counteracting the end replication problem**

Novel specific oligonucleotide primers were designed to prime synthesis to the telomeres on the DNA strand at the end of the chromosome in order to overcome the end replication problem of DNA. This approach was used to ameliorate the effect of replicative ageing on HDPCs viability and proliferation capacity.

The oligonucleotide primers were designed to prime toward the 3' end of replicating parental DNA strand. Both oligonucleotide primers were 12 base pair in length; "Telome 3" (forward primer: 5'-TAA-CCC-TAA-CCC- 3') and "Telome 5" (reverse primer: 3'-TTA-GGG-TTA-GGG-5') were synthesised and supplied in 100 µM/l solution (Eurogentec, UK). An illustration of the DNA replication fork demonstrating the leading and lagging strands is presented in figure 59. The "Telome 5" oligonucleotide primer was designed to prime synthesis in the same direction as the leading strand. However, it is unlikely that this would contribute to DNA replication as the primer would be expected be removed by the exonuclease subunit of DNA polymerase III synthesising the leading strand. On the other hand, the "Telome 3" oligonucleotide primer can theoretically prime toward the 3' end of the replicating DNA strand allowing the initiation of lagging strand synthesis much nearer to the 3' end. The

priming location and mechanism of action of “Telome 5” and “Telome 3” are illustrated in Figure 59.

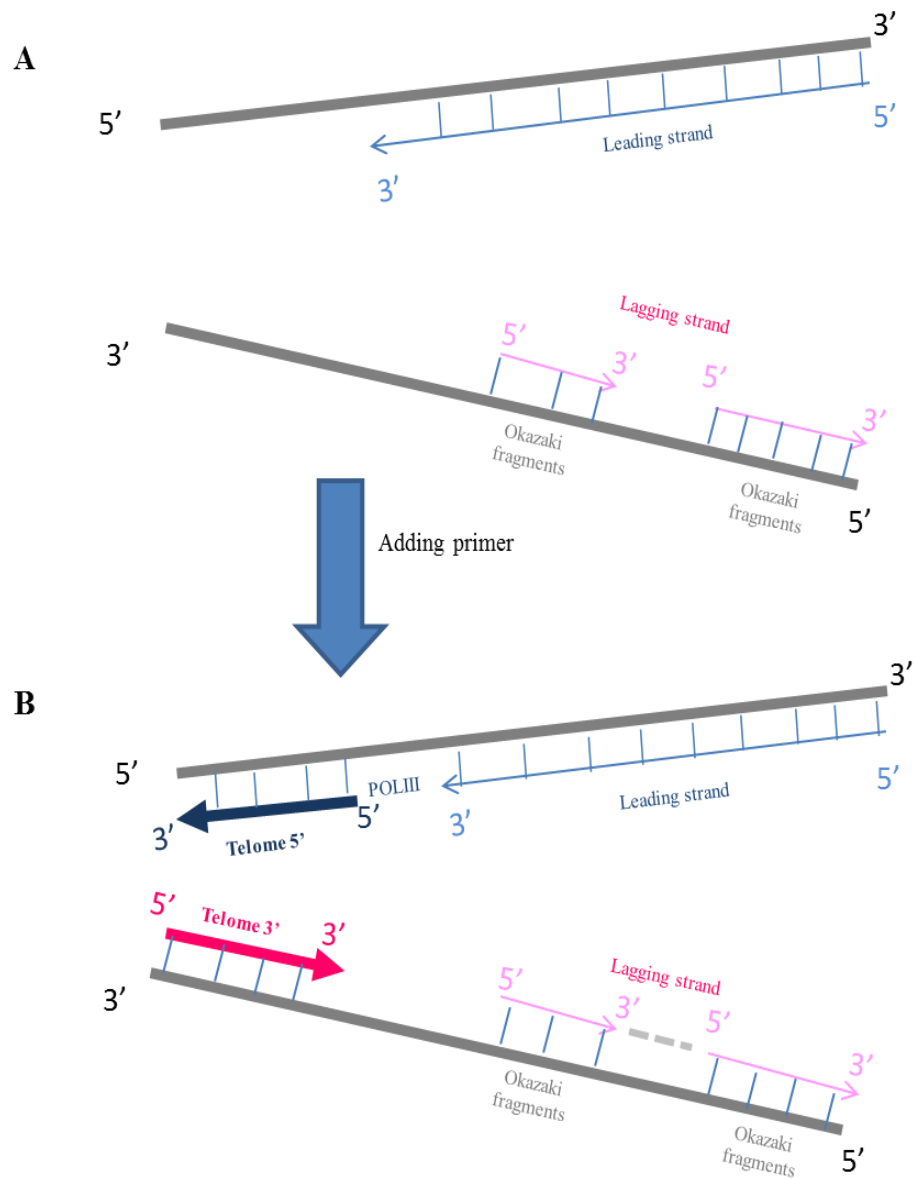


Figure 59. Illustration of the proposed mechanism of action of “Telome 3” and “Telome 5” primers.

A) DNA replication fork illustrating the leading strand (pale blue arrow) and lagging strand (pink arrows). B) The potential locations for priming by “Telome 5” (Dark Blue arrow) and “Telome 3” (Red Arrow).

### 3.7.3 Effect of telomere specific primers on viability of HDPCs

The hypothetical telomere-protective effect of the custom “Telome 3” oligonucleotide primer was investigated compared to an antisense control. The HDPCs were cultured in the presence of “Telome 3” oligonucleotide primer for up to 12 passages to evaluate the effect of these primers on the length of telomere in later passage.

Oligonucleotide primers (“Telome 5” and “Telome 3”) in sufficient quantity were added to the culture medium containing HDPCs. The number of the seeded HDPCs was  $1 \times 10^5$  and thus, the number of telomeres in such culture was estimated to be  $9.2 \times 10^6$  (46 chromosome  $\times$  2 telomere/each  $\times 10^5$  cells). The number of primers in 1  $\mu$ l (100  $\mu$ M) was estimated to be  $6 \times 10^{13}$  primers (Avogadro constant) which were in excess of the number of telomeres in the culture even after several cell replication cycles in 48 hours (the incubation period).

HDPCs with a density of  $1 \times 10^5$  cells per well were seeded in 12 well cell culture plates in  $\alpha$ -MEM medium and incubated overnight to allow for initial attachment. HDPCs were studied in four different groups; control HDPCs ‘C’, HDPCs incubated with forward oligonucleotide primer “Telome 5”, HDPCs incubated with reverse oligonucleotide primer “Telome 3” or HDPCs treated with combination of forward and reverse oligonucleotide primers “Telome 5” + “Telome 3”. Samples were studied in triplicates.

After initial cell attachment, culture medium was aspirated from each well cell culture plate, 1  $\mu$ l of oligonucleotide primers “Telome 5” or “Telome 3” were added to each well cell culture plate. This did not apply for the control group which received culture medium only and for HDPCs treated with combination of forward and reverse oligonucleotide primers “Telome 5”+“Telome 3” which received 1  $\mu$ l of each

oligonucleotide primer. After the required incubation period, HDPCs viability was assessed following Material and Methods section 2.1.5.

Treatment of HDPCs with the oligonucleotide primers (“Telome 5” and “Telome 3”) also had a significant effect on the number of viable HDPCs. This effect was more pronounced from passage 6 onward. In control and “Telome 5” treated groups, the number of viable HDPCs seemed to gradually decrease as a function of passage number. Remarkably, the number of viable HDPCs treated with “Telome 3” gradually increased between passage 2 and 5 and then abruptly increased at passage 7 where it reached a plateau. The number of viable HDPCs treated with “Telome 3” was significantly higher than any other group between passages 6 and 12. However, “Telome 5” or combination of both oligonucleotide primers, significantly reduced the number of viable HDPCs in comparison to control (Figure 60).

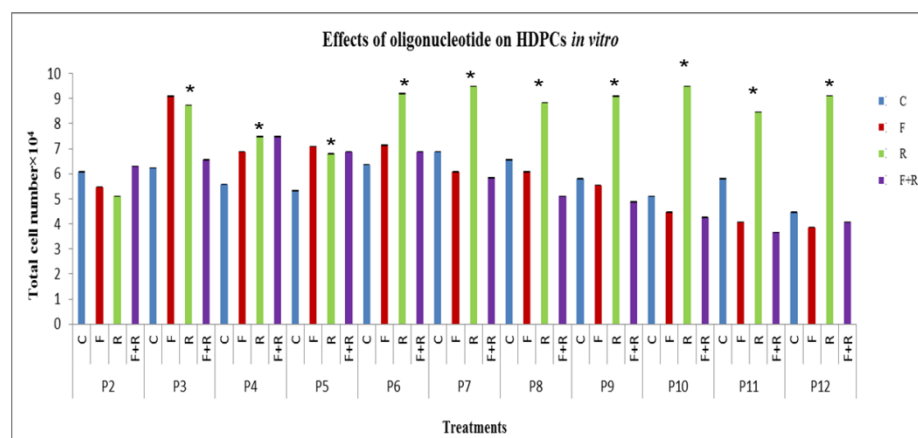


Figure 60. Total number of viable HDPCs cultured with and without primers from passage 2 to 12.

The mean number of viable cells is presented for HDPCs treated with “Telome 3”=R, “Telome 5”=F, “Telome 5” and “Telome 3”= F+R or control = C groups. HDPCs were serially passaged from passage 2 to 12. Viability was assessed after 48 hours incubation period. Data represent mean  $\pm$  standard deviation (n=3), \* p<0.05 among all “Telome 3” in comparison to control groups in each passage.

Treatment of HDPCs with the oligonucleotide primers (“Telome 5” and “Telome 3”) also had a significant effect on the percentage viability of HDPCs. In the control,

“Telome 5”, and “Telome 3” groups, the percentage viability of the HDPCs appeared to gradually decrease as a function of passage number. However, the decline of mean percentage viability was more pronounced in “Telome 5” and in combination of both “Telome 5”+“Telome 3” treated groups in comparison to the control group, especially from passage 6 and onward. However, HDPCs treated with “Telome 3” exhibited consistent percentage viability throughout all the passages that was significantly higher than all other groups, especially after passage 6 (Figure 61).

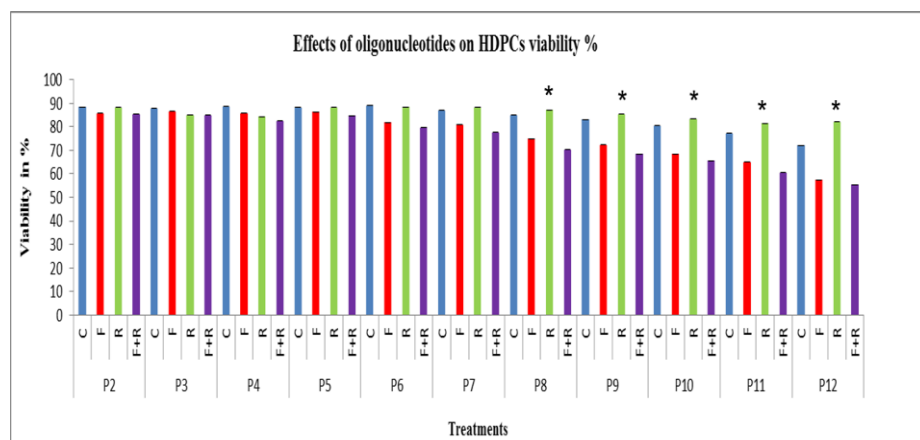


Figure 61. Viability percentage of HDPCs cultured with and without primers from passage 2 to 12.

The mean number of viable cells is presented for HDPCs treated with “Telome 3”=R, “Telome 5”=F, “Telome 5” and “Telome 3”= F+R or control = C groups. HDPCs were serially passaged from passage 2 to 12. Viability was assessed after 48 hours incubation period. Data represent mean  $\pm$  standard deviation (n=3). \*  $p < 0.05$  among all “Telome 3” in comparison to control groups in each passage.

The experiment was repeated to confirm that the effect of oligonucleotide primer “Telome 3” on the viability of HDPCs was reproducible. The treatment with combination of the both oligonucleotide primers “Telome 3”+ “Telome 5” was not used in this experiment as it has been shown to give a result similar to treatment with “Telome 5” alone. The findings of this experiment were consistent with the original results. The number of viable HDPCs in control and “Telome 5”, treated groups decreased significantly as a function of passage number, whereas treatment with



“Telome 3” resulted in a high number of viable HDPCs consistent throughout all passages. Treatment with “Telome 3” groups exhibited the highest percentage viability. There was significant difference between the three groups in the percentage viability from passage 6 to 12 (Figures 62 and 63).

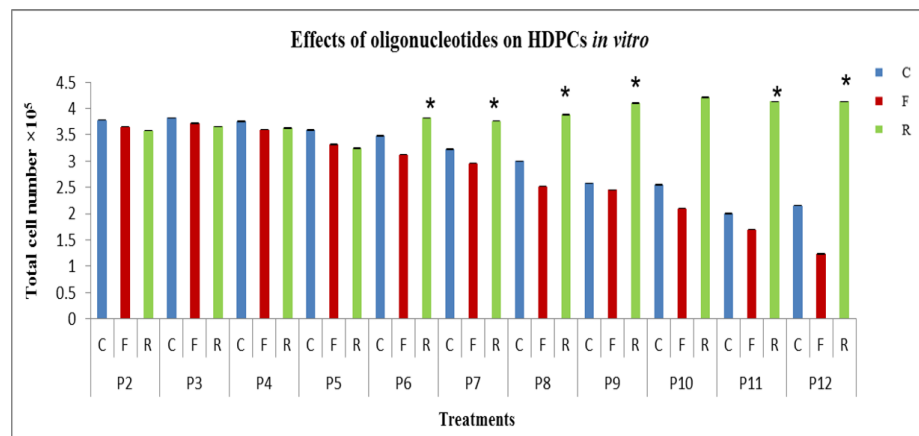


Figure 62. Total number of viable HDPCs cultured with and without primers from passage 2 to 12.

The mean number of viable cells is presented for HDPCs treated with “Telome 3”=R, “Telome 5”=F, or control = C groups. HDPCs were serially passaged from passage 2 to 12. Viability was assessed after 48 hours incubation period. Data represent mean ± standard deviation (n=3),\* p<0.05 among all “Telome 3” in comparison to control groups in each passage.

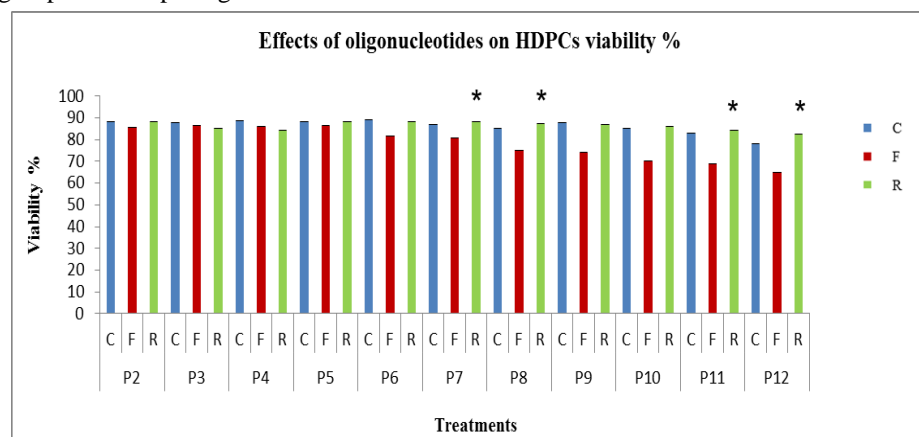


Figure 63. Viability percentage of HDPCs cultured with and without primers from passage 2 to 12.

The mean number of viable cells is presented for HDPCs treated Telome 3”=R, “Telome 5”=F, or control = C groups. HDPCs were serially passaged from passage 2 to 12. Viability was assessed after 48 hours incubation period. Data represent mean ± standard deviation (n=3),\* p<0.05 among all “Telome 3” in comparison to control groups in each passage.

These findings indicated that the use of oligonucleotide primers “Telome 3” which was designed to synthesise the 3’end of the DNA strand had a significant effect on HDPC proliferation and viability. However, “Telome 5” oligonucleotide primer may interfere with leading strand synthesis. The “Telome 3” oligonucleotide primer appeared to prevent reduction in viability and proliferation of the serially passaged HDPCs. The effect was highly significant when compared to control HDPCs and other primers. The effect of “Telome 3” oligonucleotide primer was more pronounced at passage 6 when other HDPC groups started to lose the ability to proliferate which may indicate that “Telome 3” oligonucleotide primer acts by priming the DNA strand synthesis to overcome the end replication problems.

#### **3.7.4 Effect of Telome 3’ on telomere length in HDPCs**

In an attempt to establish whether the positive effect of “Telome 3” primer on viability and proliferation of HDPCs was attributed to the ability to prime DNA synthesis toward the 3’ ends of replicating chromosome, a study was designed to determine whether the exogenous oligonucleotide primer “Telome 3” would influence the rate of telomere shortening. The extent of telomere shortening was determined by DNA Southern Blot hybridization following Materials and Methods section 2.9. To quantify the intensity of telomere from passage 2 and 12 following treatment with “Telome 3”, densitometric analysis was used.

Figure 65 demonstrates the densitometric scan of Southern Blot of telomeric DNA in HDPCs from passage 2 and 12 treated with oligonucleotide primer “Telome 3”. The densitometric analysis revealed that treatment with “Telome 3” led to maintenance of the telomere length in serially passaged HDPCs. TRF length was estimated to be 13.2 Kb in HDPCs from passage 2. Serially passaging the same cells and incorporating

“Telome 3” oligonucleotide primer, clearly indicated that telomere length was maintained at passage 12 (13.4 Kb) (Figure 64).

These findings indicated that the application of the novel exogenous telomere specific primer may prevent telomere length shortening and prevent cell ageing. These findings may also account for the improved biological characteristics in terms of proliferation and growth capacity as well as cell viability in the studied HDPCs. However, the effect of “Telome 3” oligonucleotide primer on telomere shortening needs to be assessed more thoroughly. In addition, telomere length degradation/maintenance at different passages and using different methods should be analysed to confirm such effect.

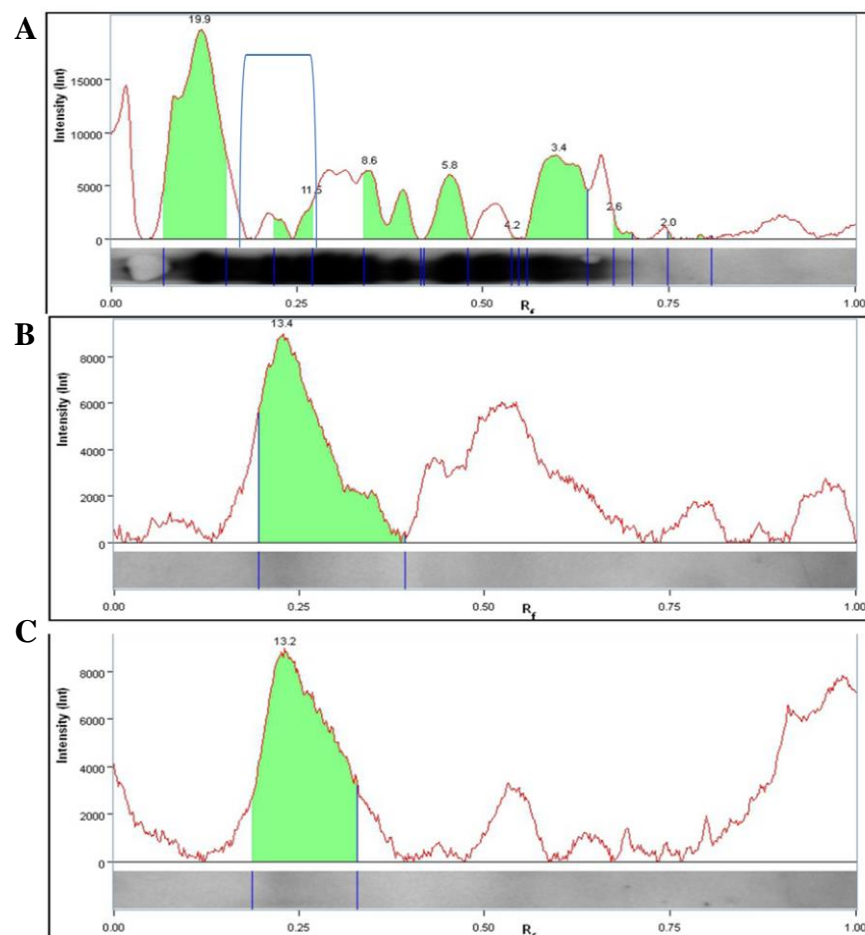


Figure 64. Densitometric analysis of Telomere length in HDPCs in passage 2 and 12.

A: DIG molecular marker. B: intensity of telomere length for HDPCs passage 2. C: intensity of telomere length for HDPCs passage 12 treated with forward primer “Telome 3”.

## Chapter 4: Discussion, conclusions and future work

### 4.1 Discussion

The properties of the currently used pulp capping materials have been widely investigated in clinical studies which concentrate on the amelioration of symptoms and maintenance of pulp viability. However, the effect of such treatments on the *in situ* tissue repair and cell characteristics is not as thoroughly investigated. There is an increasing clinical demand for development of alternative therapies that stimulate repair processes, especially for the injured/diseased human dental pulp tissues (Demarco *et al.*, 2011). Pulp tissue engineering provides a promising approach to meet both the current and future clinical demands, via dental pulp tissue engineering based therapies. This follows trends in basic science research, in which stem/stromal cells are cultured on biomaterials (scaffolds) in order to commence functional tissue formation (Demarco *et al.*, 2011). Indeed, an *in vivo* study by Prescott *et al.* demonstrated regeneration of pulp-like tissue within mechanically injured rat dental pulps following the use of HDPCs seeded on collagen scaffold and treated with DMP-1 growth factor (Prescott *et al.*, 2008).

Vital pulpal therapy, including pulp capping, is a widely adopted treatment approach and aims to encourage the repair of damaged pulpal tissue. It also aims to prevent further pulpal injury caused by various stimuli including chemical, mechanical, thermal and most importantly bacterial (Masuda-Murakami *et al.*, 2009). Pulp capping stimulates formation of the three types of dentine, including tertiary, reactionary and reparative dentine (Paranjpe *et al.*, 2010). The criteria for assessment of efficacy of pulp capping materials may widely vary (Min *et al.*, 2009). Complete or partial dentine

bridge formation and stimulation of odontogenesis are among the most important criteria (Parolia *et al.*, 2010).

Postnatal stem cells have been identified and isolated from various tissues, including dental pulp, bone marrow, skin, neural tissue, adipose tissue, umbilical cord and cornea (McKay, 1997, Pittenger *et al.*, 1999, Gronthos *et al.*, 2000, Toma *et al.*, 2001, Zuk *et al.*, 2001, Pierdomenico *et al.*, 2005, Pal *et al.*, 2009, Nekanti *et al.*, 2010, Kasim *et al.*, 2012). Karamzadeh *et al.* demonstrated high proliferation and multi-lineage differentiation capacity of human pulp-derived stem cells (HDPCs) from permanent teeth compared to the other source of adult stem cells. The authors stated that availability, plasticity and high differentiation capacity of HDPCs enable them to be a good candidate in tissue engineering and regenerative medicine (Karamzadeh *et al.*, 2011).

We have used dental pulp tissue from human healthy third molars as a source of mesenchymal cell or the so called HDPCs. As the third molar is the last tooth to erupt in the oral cavity, HDPCs from this tooth are considered to be more proliferative and undifferentiated (Gronthos *et al.*, 2000). The use of third molars as a source for HDPCs for tissue engineering purposes was further recommended by Atari *et al.* who suggested that dental pulp stem cells that were derived from third molars from different donors, possess multi-lineage differentiation potential (Atari *et al.*, 2012).

The isolation of HDPCs from pulp tissue is well established and has been performed in previous studies using either explant out growth or collagenase digestion techniques (About *et al.*, 2000, Pierdomenico *et al.*, 2005, Huang *et al.*, 2006, Spath *et al.*, 2010, Vishwanath *et al.*, 2013, Wang *et al.*, 2014). We have compared the viability and doubling time of HDPCs upon isolating them using explant outgrowth or collagenase

digest techniques. Our findings demonstrated superior viability, higher number and shorter doubling time of HDPCs isolated using collagenase digest method in comparison to explants outgrowth method. However, both techniques resulted in isolation of cells with similar morphology, thin fibroblast-like cells with triangular or stellate shape as shown in Figure 6. We have shown that collagenase method was more effective in isolating HDPCs and had no notable adverse effect on the proliferation and growth of the isolated HDPCs, as indicated by the shorter doubling time in comparison to explant outgrowth method as shown in Figure 7. Similar findings were reported by Huang *et al.* who concluded that the growth and proliferation rate of HDPCs isolated using the collagenase digest method was significantly higher than that for HDPCs isolated using explant outgrowth technique (Huang *et al.*, 2006). However, a study by Souza *et al.* comparing both methods suggested that the use of explant outgrowth method yielded higher number of HDPCs initially but after 14 days, HDPCs isolated using collagenase method demonstrated significantly higher proliferation rate (Souza *et al.*, 2010).

Our results indicate higher number, viability and proliferation rate of HDPCs when collagenase method was used for isolation in comparison to explant outgrowth technique as shown in Figure 7. Additionally, there is some evidence suggesting that such methods yield a population of cells with similar age (Gronthos *et al.*, 2000). Furthermore, Huang *et al.* demonstrated that the use of collagenase method is very likely able to isolate mesenchymal progenitor cells with high odontogenic differentiation capacity (Huang *et al.*, 2006). Karamzadeh *et al.* suggested that HDPCs isolated using collagenase digest had higher mineralisation capacity (Karamzadeh *et al.*, 2011). For these reasons, the present study only used HDPCs isolated using collagenase method in the subsequent experimental work.

One of the important aims of the current study was to investigate the effect of various pulp capping materials (Dycal, MTA and propolis) on the biological characteristics of HDPCs. In the clinical setting pulp capping materials are mixed according to manufacturer's instructions and applied directly to the exposed pulp. However, in *in vitro* studies, a certain delivery mechanism should be exploited to ensure consistent release of the material into the tissue culture. Microscopy cover-slips coated with Dycal were initially used as a method of direct contact between HDPCs and the material. The direct contact between pulp capping materials and cultured HDPCs caused high levels of cytotoxicity, as indicated by significant cell necrosis, which rendered this technique ineffective for the purpose of the study.

Further evaluation was carried out to expose HDPCs to pulp capping materials indirectly using Transwell<sup>®</sup> inserts. The effect of empty Transwell<sup>®</sup> inserts on the viability of HDPCs was investigated in order to rule out any positive or negative effect of the insert material on the viability or proliferation rate of the studied HDPCs. According to the current study, viability of HDPCs (passage 2) which were incubated with Transwell<sup>®</sup> inserts for 24 hours had a similar number of viable cells in the monolayer comparable to HDPCs unexposed to Transwell<sup>®</sup> inserts; 81% and 80%, respectively as shown in Figure 16. As a consequence, Transwell<sup>®</sup> inserts were only used with HDPCs exposed to pulp capping materials.

Ca(OH)<sub>2</sub> has been identified as the gold standard pulp capping material for several decades. However, alternative materials such as MTA and propolis, have been introduced more recently in an attempt to counteract the limitations of Ca(OH)<sub>2</sub> (Cox *et al.*, 1998, Mente *et al.*, 2014). Our aim was to compare the effect of Dycal, MTA and propolis on the biological properties of HDPCs.

We have revealed that Dycal caused exceptionally high rates of cell death and reduced the proliferation capacity of the studied HDPCs. This effect was consistent despite the use of HDPCs from different passage numbers as shown in Figures (8-13). These results were similar to a published *in vitro* study on rat dental pulp cells where Dycal induced significant cytotoxic effects within 72 hours of incubation (Yasuda *et al.*, 2008). Additionally, Min *et al.* reported that Dycal induced strong cytotoxic effect on HDPCs (Min *et al.*, 2007). This effect was widely reported in the literature and was attributed to the chemical injury-induced necrosis of pulp cells. The release of high concentrations of hydroxyl ions caused elevation of pH values to an extent which interfered with biological functions of the cells and induced necrosis (Ford, 1980, Schroder, 1985, Safavi and Nichols, 1994, Al-Shaher *et al.*, 2004).

Dycal is expected to increase the pH significantly. However, Dycal induced notable yellowish discolouration of the culture medium after 24 hours incubation which could be an indication of acidic changes in the culture medium. We have investigated pH changes associated with Dycal application in the culture medium. HDPCs were separately cultured using either  $\alpha$ -MEM or PBS, exposed to Dycal and incubated overnight. Our results demonstrated that pH of PBS was 7.0, however when HDPCs were cultured in PBS the pH dropped to 6.78. This could be attributed to the fact that HDPCs cultured in PBS are very likely to undergo apoptosis as a result of glucose deprivation. This in turn can cause release of acidic molecule such as intracellular uric acid and thereby explain the decline in pH. Application of Dycal increased the pH slightly which may rule out the possibility of Dycal causing a drop in pH. The initial pH of culture medium was 7.2 which increased to 7.77 upon culturing HDPCs. Application of Dycal caused slight decrease in pH which remained in the zone of alkalinity (7.64). The subtle changes in pH of the culture medium may have a role in the cell death



noticed in the current study. However, this role may be limited and other factors could be involved but were not investigated further. Furthermore, the effect of Dycal on other aspects of HDPCs (differentiation and ageing) was not investigated in the present study as it was impossible to obtain an adequate number of viable HDPCs for gene expression studies after treatment with Dycal as shown in Table 4.

MTA and propolis are alternative pulp capping material that have been shown to provide greater sealing ability, less pulpal inflammation, higher biocompatibility and have superior dentine bridge formation in contrast to  $\text{Ca}(\text{OH})_2$  (Nair *et al.*, 2008, Parolia *et al.*, 2010). We have investigated the effects of both pulp capping materials on biological characteristics of HDPCs. HDPCs from various passages were cultured with MTA and propolis loaded in Transwell<sup>®</sup> inserts for 12 and 24 hours. Control HDPCs grew uniformly in the bottom of well cell culture plate. However, HDPCs were more confluent in the vicinity of the Transwell<sup>®</sup> inserts containing MTA and propolis which may indicate that the pulp capping materials can stimulate proliferation of HDPCs. Additionally, the morphological appearance of HDPCs was not affected by the pulp capping materials and HDPCs appeared as thin, fibroblast-like cells in MTA, propolis and control groups as shown in Figure 17. An *in vitro* study reported similar findings and concluded that MTA did not induce morphological changes in dental pulp cells (Moghaddame-Jafari *et al.*, 2005).

The effect of MTA on the proliferation potential and viability of HDPCs was then investigated. MTA treated HDPCs exhibited greater viability and higher proliferation rate in comparison to control HDPCs, especially after 12 hours incubation period as shown in Figures 18 and 20. This may be attributed to the uptake of calcium released from MTA which might trigger an intracellular cascade of events to stimulate proliferation of HDPCs. Similar findings were reported in several publications where

MTA was found to exhibit high biocompatibility, increase pulp cell proliferation and decrease apoptosis rate (Moghaddame-Jafari *et al.*, 2005, Yasuda *et al.*, 2008). The low cytotoxicity of MTA to dental pulp cells and the proliferation induction capacity may account for the regeneration observed in the dentine-pulp complex when MTA is used for direct pulp capping and may explain the superior clinical performance of MTA in comparison to Dycal (Moghaddame-Jafari *et al.*, 2005, Hilton *et al.*, 2013).

We have investigated the effect of propolis on the proliferation potential and viability of HDPCs. Propolis treated HDPCs exhibited higher proliferation rate and similar viability in comparison to control HDPCs after 12 hours incubation period as shown in Figure 18. The viability percentage dropped significantly after 24 hours incubation period and was significantly lower than the control group as shown in Figure 20. The possible cytotoxic effects of propolis could be attributed to the high concentration of the material released in culture medium. A study by Al-Shaher *et al.* reported an inverse relationship between the viability of dental pulp cells and the concentration of propolis (Al-Shaher *et al.*, 2004). A study by Jahromi *et al.* evaluated the effect of propolis on fibroblast cells from healthy third molars and suggested that 100 mg/ml of propolis increased viability of these cells (Jahromi *et al.*, 2014). The concentration of the propolis that we used was twenty fold times higher (2gm/ml) than the one used in the Jahromi *et al.* study. We therefore, repeated the experiment using lower concentration of propolis (0.80 g/ml). After 24 hours, the reduction of propolis concentration resulted in higher proliferation rate and viability percentage when compared to the control group as shown in Figures 22 and 24.

We have also compared the effect of MTA and propolis on viability and proliferation capacity of HDPCs. MTA was significantly less cytotoxic to HDPCs when compared to high concentration propolis in culture. In addition, treatment with MTA enhanced

proliferation of HDPCs in culture after 24 hours. However, upon reduction of propolis concentration, there was no significant difference between the two groups in terms of viability or proliferation rate after 24 hours incubation period as shown in Figures 22 and 24. The present findings were in agreement with a clinical study that reported comparable anti-inflammatory properties and hard tissue bridge formation, when using propolis or MTA in comparison to Dycal, in mechanically injured dental pulps in humans (Parolia *et al.*, 2010).

Our results indicate that the amount of released pulp capping material may have an effect on the viability of HDPCs (section when we reduced propolis). Additionally, propolis (0.80 g/ml) and MTA (2 g/ml) seemed to have a similar effect on the viability percentage of HDPCs as shown in Figure 24. Each Transwell<sup>®</sup> insert contained 200 µg of the MTA or 80 µg of propolis. Despite the fact that similar volume and known mass of both materials was used, this did not guarantee that the ‘effective’ amount available for HDPCs was similar. Thus, a series of studies was performed to determine the exact amount of released MTA and propolis in culture medium after 24 hours incubation.

We have investigated the feasibility of using colorimetric assay to measure the amount of released pulp capping material in culture medium. This technique is widely used to determine the Ca<sup>2+</sup> content in liquids (Attin *et al.*, 2005). Ca<sup>2+</sup> is a major constituent of MTA and is present in propolis chemical composition. Thus, the amount of released Ca<sup>2+</sup> in the culture medium may indirectly indicate the amount of released pulp capping material. However, this technique was not feasible as the initial calcium concentration (1.8 mM/l) of the culture medium was high. Addition of different amounts of CaCl<sub>2</sub> to the culture medium resulted in similar light absorbance values which negated the use of this method as shown in Figure 27.

A freeze drying technique was used to directly measure the amount of released MTA or propolis into culture medium. The initial hypothesis was that the weight of the Transwell<sup>®</sup> inserts and pulp capping material prior to incubation would decrease by the amount of released material into culture medium and increase by the amount of absorbed liquids (medium). If the absorbed medium could be eliminated, the exact amount of released pulp capping material could thereby be determined. The amount of MTA and propolis that was released from the Transwell<sup>®</sup> inserts into culture medium was 0.094 g and 0.089 g, respectively. These findings may suggest that MTA and propolis have a similar advantageous impact on biological characteristics of the studied HDPCs that received a comparable amount of material in the culture medium as shown in Table 5.

The effect of pulp capping material was further investigated on HDPCs in serial passage. According to the results of current study, MTA treated and untreated HDPCs had comparable viability percentages that gradually decreased from passage 2 to 12 as shown in Figure 30. Propolis treatment resulted in slightly lower viability percentages in comparison to MTA and propolis treated HDPCs in early passages. However, the reduction of viability was significantly higher in later passages in the propolis groups with regard to control HDPCs as shown in Figure 30. Loss of growth and proliferation potential with later passages is a widely recognised phenomenon with pulp progenitor cells (Huang *et al.*, 2006). It might also be proposed that propolis and to a lesser extent MTA, may have time dependent toxic effects on HDPCs. Yoshino *et al.* reported a similar effect with MTA sealers on HDPCs, and suggested that MTA may have slight cytotoxic effect when incubated with HDPCs from later passages (Yoshino *et al.*, 2013).

In order to achieve a better understanding of the findings from cell viability and proliferation assays the effect of MTA and propolis on expression profile of various

genes was assessed in the current study. RNA profiling techniques such as qRT-PCR require generation of a cDNA copy of an RNA sample through a reverse transcription reaction. In order to process all the RNA samples at the same time, HDPC pellets were stored in RNALater<sup>®</sup> for 10 days from harvesting. RNA yield and integrity was very marginal for HDPCs stored in RNALater<sup>®</sup> but not to that extent negating their use for qRT-PCR. This decision was made on the grounds that other studies reported insignificant effect as shown in Table 6.

SYBR-Green<sup>®</sup> and Taq-Man<sup>®</sup> are among the widely used qRT-PCR assays. Taq-Man<sup>®</sup> assay increases the specificity of real time PCR and does not require optimisation but it requires a specific fluorogenic probe to detect PCR products. Taq-Man<sup>®</sup> also prevents cross-over amplification. On the other hand, SYBR-Green<sup>®</sup> assay is less expensive and does not require the design of specific internal probes. However, it is time consuming to perform and optimise. It also suffers from the drawback of generating large numbers of amplicons which can contaminate other PCR reactions (Cao and Shockey, 2012).

We have investigated the effect of MTA and propolis on the expression profiles of genes controlling ageing, cell death and differentiation in HDPCs using SYBR-Green<sup>®</sup> assay. *GAPDH* gene was chosen as the housekeeping genes for all qRT-PCR SYBR-Green<sup>®</sup> experiments as its expression was not affected by various treatment conditions in comparison to other studied housekeeping genes (*β-actin* and *YWHAZ*) as shown in Figure 31, which was in agreement with other studies conducted qRT-PCR work on HDPCs (McLachlan *et al.*, 2005, VanGuilder *et al.*, 2008, Paranjpe *et al.*, 2010, Eid *et al.*, 2013).

SYBR-Green<sup>®</sup> gene expression results of the current study were vastly variable, lacked consistency, were unreproducible and uninterpretable as shown in Figures (33-36). The

first reason considered was technical errors or poor sensitivity of the assay as shown in the result section 3.3.4. Thus, the effect of MTA and propolis on the expression profile of the same studied genes was repeated using the higher precision and specificity Taq-Man<sup>®</sup> assay. However, the results were not different from those obtained by SYBR-Green<sup>®</sup> assay as shown in Figures 37, 38 and 39. The same problem remained despite repeating the experiment several times and using HDPCs from different passages as shown in Figure 40, at that stage we hypothesised that this problem could be attributed to poor integrity of RNA samples, as the studied RNA samples had low concentration and suboptimal quality which can be a result of performing the RNA extraction after 10 days of storing the cell pellet in RNALater<sup>®</sup>. Poor RNA extraction technique was ruled out as a cause for such findings as the quality and concentration of RNA was improved upon the use of exactly the same technique with two different samples (HDPCs incubated for 12 and 24 hours) as shown in Table 6.

The effect of immediate versus delayed (storage of HDPCs in RNALater<sup>®</sup> for 10 days) RNA extraction on the RNA yield and integrity was investigated as shown in Figure 41. Additionally, to ensure accuracy and generalisability of the findings, the effect of cell storage in RNALater<sup>®</sup> on RNA integrity and yield was investigated in HDPCs from four different donors as shown in Figure 42. According to the present study, for all the donors, RNA yield was significantly higher when extraction, was performed immediately after harvesting HDPCs in comparison to delayed extraction following storage period of cell pellet in RNALater<sup>®</sup>. This was indicated by the higher total concentration of the extracted RNA and the higher, calculated RNA content per HDPC as shown in Figures 41 and 42. Additionally, RNA integrity was significantly better when RNA extraction was performed immediately after harvesting HDPCs in comparison to delayed extraction as indicated by the superior 260/280 ratios as shown

in Tables 10 and 11, as well as electrophoresis gel findings as shown in Figures 43 and 44. Our findings strongly suggested that storing HDPC pellets in RNAlater<sup>®</sup> may consistently have an adverse effect on the RNA yield and integrity as it was observed in HDPCs from different passages and different donors as shown in the result section 3.4. Our findings cast doubt on the manufacturer's recommendation and other studies which reported that storing cells in RNAlater<sup>®</sup> had no notable effect on RNA yield or integrity (Gorokhova, 2005, lifetechnologies, 2015). Additionally, a study compared storage of cells in RNALater<sup>®</sup> and resulted in significant RNA degradation in comparison to storage in liquid nitrogen (Olsvik *et al.*, 2007). Our result indicated that storage of HDPC pellet in RNAlater<sup>®</sup> may have detrimental effect on the RNA yield and integrity. This could possibly account for the inconsistency encountered with the qRT-PCR data.

Based on our results comparing RNA integrity following immediate and delayed RNA extraction, the decision was made to repeat q-RT-PCR studies using RNA samples extracted immediately after cell harvesting in an attempt to improve the quality of the qRT-PCR data. We also investigated the sensitivity, reproducibility and specificity of two different qRT-PCR assays (Taq-Man<sup>®</sup> and SYBR-Green<sup>®</sup>) on immediately extracted RNA from freshly harvested HDPCs. Both assays were used to study expression profile of *GAPDH* gene in serially diluted cDNA. Our results suggested that Taq-Man<sup>®</sup> assay was more sensitive and precise in comparison to SYBR-Green<sup>®</sup>. As a consequence, in the current study, Taq-Man<sup>®</sup> assay was used for later gene expression experiments as shown in Figure 45.

This study further investigated several housekeeping genes in order to select the most reliable housekeeping gene. Our results demonstrated that that *TERC* and *GAPDH* were constitutively expressed in HDPCs in various conditions in comparison to the other genes (*β-actin* and *YWHAZ*), therefore these two genes were used as housekeeping

genes for all later gene expression studies. *TERC* gene, however, was used as housekeeping gene for Taq-Man qRT-PCR assay when data analysis was performed as shown in Figure 46. *TERC* was also found to be constitutively expressed in Wilms' tumor and in normal kidneys (Dome *et al.*, 2005).

According to our results, treatment of HDPCs with propolis resulted in reduced cell viability while MTA seemed to increase the proliferation capacity and the number of viable cells as shown in Figures 28 and 29. Such effects may be attributed to the material's potential to induce genes controlling programmed cell death mechanisms. Autophagy is known as cytoplasmic type 2 programmed cell death. It involves, generally, a catabolic process of the cytoplasmic contents of the cells, which are trapped in double membrane vacuoles known as the autophagosome. These are responsible for degradation of intracellular components through lysosomes. During nutrient starvation, autophagy normally functions as a survival mechanism in starving cells to produce energy for survival. However, if the extent of autophagy increases it can lead to cell death and is therefore considered to be a mechanism of controlled cell death. However, the mechanism by which autophagy results in cell death is not fully understood (Debnath *et al.*, 2005). One autophagy gene, known as *BCN1*, is responsible for formation of the autophagosome (Mizushima, 2007). Furthermore, *BID* is involved in the extrinsic apoptotic pathway and is a member of the *BCL-2* family of cell death regulators. It is a mediator of mitochondrial damage induced by caspase-8 (CASP8). In early severe sepsis in peripheral human blood, gene expression pattern with induction of the pro-apoptotic Bcl-2 family members Bim and Bid, down-regulation of *BCN1* protein was observed (Weber *et al.*, 2008).

Taq-Man<sup>®</sup> assay was used to evaluate the effect of MTA and propolis on the expression profile of apoptosis related gene *BID* and the autophagy related gene *BCN1* in serially



passaged HDPCs. Our results indicate that the expression of the *BID* gene in the control, MTA and propolis treated HDPCs were below the detectable level. The expression of *BCN1* was the highest in the propolis group when compared to similar passage number in control and MTA group. Further, the difference was more pronounced in passage 6 and 12. The current findings indicated that HDPCs from various passages seemed to induce cell death by activating the gene regulating the autophagy as the passage number increased as cells naturally lost differentiation and growth potential. Additionally, propolis exhibited a potent effect on the induction of programme cell death especially at later passages when compared to MTA and control groups as shown in Figure 47.

HDPC's have odonto/osteogenic differentiation potential. They can differentiate to osteoblast-like or odontoblast-like cells which, in turn, determine the nature and structure of the hard bridge barrier formed during repair process. Various factors can affect the differentiation route of HDPCs include the cytokines, donors' age, passage number, cell injury and presence of chemical substrate such as pulp capping materials (Yan *et al.*, 2011, Wang *et al.*, 2013). The bone morphogenetic proteins (*BMP-2*) are mainly functional in bone but they have also been shown to be expressed in dental pulp and may induce dentine bridge formation (Yasuda *et al.*, 2008). The use of adult stem cells for cell therapy with *BMP-2* for dentine regeneration has been demonstrated in an *in vitro* study on HDPCs implanted into immunodeficient mice using a scaffold (Iohara *et al.*, 2006). Several proteins are responsible for mineralisation of dentine including the dentine sialophosphoprotein (*DSPP*) and dentine matrix phosphoprotein-1 (*DMP-1*). Both are non-collagenous, extracellular matrix proteins responsible for dentine mineralisation and synthesised by human odontoblasts (Paranjpe *et al.*, 2010, Suzuki *et al.*, 2012, Wang *et al.*, 2014). Non-collagenous proteins are secreted at the mineralization stage which constitutes specific markers for dentine and can be

found also in brain cells (Narayanan *et al.*, 2001, Goldberg *et al.*, 2011, Couve *et al.*, 2013). Furthermore, a high expression of *DMP-1* has been observed in early-differentiated odontoblasts forming primary dentine, while a gradual down-regulation of *DMP-1* expression is observed in mature odontoblasts forming secondary dentine in transgenic mice (Balic and Mina, 2011). *DMP-1* is expressed by the ectomesenchymal derived odontoblast cells of the tooth. It is suggested that deficiency of the protein is a causative factor in dentinogenesis imperfecta (Aikawa *et al.*, 2006).

The effect of MTA and propolis on HDPC's odontogenic differentiation ability was assessed using qRT-PCR to investigate expression profile of odontogenesis markers (*BMP-2*, *DSPP* and *DMP-1*) in serially passaged HDPCs in the current study. The qRT-PCR data that we have obtained demonstrated that the expression of *DSPP* and *DMP-1* were below the detectable level in all studied groups from different passages. Undetectable expression of *DSPP* and *DMP-1* gene in HDPCs was surprising as they are highly specific odontogenesis markers and they are necessary for *de novo* dentine formation by HDPCs. In order to rule out the possibility of technical error (e.g. malfunction in the used PCR primer) leading to these findings, the current study further investigated the *DSPP* gene expression in a positive control human brain cells that are known to express *DSPP* gene using similar primers and PCR technique. In the current study, qRT-PCR was performed on cDNA from cells with high expression of *DSPP*. Our results, demonstrated that qRT-PCR performed on this positive control confirmed abundant *DSPP* gene expression. This may indicate that the expression of *DSPP* gene in the current study on HDPCs was below the detectable level and may rule out the possibility of technical errors as shown in Figure 48. Undetectable *DSPP* and *DMP-1* expression may also indicate that the isolated HDPCs are comprised of mixed population of cells rather than purely odontoblast progenitor cells (Huang *et al.*, 2006).

Our findings are disagreement with previous studies that have demonstrated *DSPP* gene expression in HDPCs treated with MTA and enamel matrix derivative (Min *et al.*, 2009). Additionally, previous studies have shown that *DSPP* gene expression was up-regulated not only in the odontoblasts forming primary dentine but also in odontoblast-like cells forming reparative dentine (Hwang *et al.*, 2008). A study has demonstrated that expression of *DMP-1* gene in embryonic mesenchymal cells stimulated cells to differentiate into odontoblast like cells (Narayanan *et al.*, 2001).

Our results have demonstrated significant up-regulation of *BMP-2* transcription factor in propolis and to a lesser extent MTA treated HDPCs from passage 4. In the light of undetectable expression of other specific odontogenic differentiation markers (*DSPP* and *DMP-1*), it cannot be extrapolated that up-regulation of *BMP-2* was an indication of odontogenic differentiation of the studied HDPCs. Furthermore, the expression of *BMP-2* in propolis group was very high as shown in Figure 49, further analysis of the *BMP-2* at protein level was carried out to confirm qRT-PCR findings. Western blot analysis revealed high correlation with qRT-PCR data where the greatest amount of *BMP-2* protein expression was identified in propolis group (P4 and 6) followed by MTA group in the same passages as shown in Figure 52. *BMP-2* expression profile in the current study was in agreement with a study by Maeda *et al.* who reported that MTA stimulated *BMP-2* gene expression above the baseline levels (Maeda *et al.*, 2010).

The nature of the hard tissue bridge that forms over following pulp capping is not fully recognised. Histological studies revealed that the hard tissue may have multiple perforations and can be described as dentine-like, bone-like, and as a reparative dentine bridge (Andelin *et al.*, 2003). A study reported up-regulation of odontogenic and osteogenic markers (*RUNX-2*, *OCN*, *DSPP* and *DSP*) in apical papilla stem cells treated with MTA which may indicate that MTA may stimulate odonto/osteoblastic

differentiation (Yan *et al.*, 2014). However, in injured dental pulps, HDPCs may preferentially differentiate toward osteoblast-like cells (Wang *et al.*, 2013). Additionally, the physical and chemical properties of dentine collagen are similar to collagen within bone and both are especially adapted for binding matrix proteins and mineral deposition (Butler, 1991). This in turn, may suggest that the deposition of bone-like tissue over mechanically exposed pulps is possible. In the light of undetectable expression of odontogenesis markers, the present study investigated the effect of MTA and propolis on the osteogenic differentiation potential by evaluating the expression profile of *ALP*, *RUNX-2* and *OCN* genes in HDPCs after 24 hours as shown in Figures 53, 54 and 55.

Alkaline phosphatase (*ALP*) is another pre-osteoblastic key marker that is abundantly expressed at early stages of osteogenic differentiation and is responsible for bone mineralization (Bakopoulou *et al.*, 2011). *ALP* is expressed in low level by MSCs, gradually increasing through the first phase of differentiation, then decreasing in expression through the second phase of differentiation (Hoemann *et al.*, 2009). Matrix mineralization by up-regulation of *ALP* was reported in previous studies in human dermal fibroblast cells (Qian and Bhatnagar, 1996).

*RUNX-2* is an essential transcription factor that regulates bone and tooth development by regulating osteoblast and odontoblast differentiation. However, the function of *RUNX-2* in late stage odontoblast differentiation is not clear (Li *et al.*, 2011). *RUNX-2* is a key mediator in the early phase of osteogenic differentiation as it initiates the transcription and subsequent translation of many other genes responsible for extracellular matrix deposition, such as bone osteocalcin (*OCN*) that are expressed during osteoblast differentiation (Otto *et al.*, 2003, Bennett *et al.*, 2005). Furthermore, *OCN* is a bone-specific extracellular matrix protein synthesized by

osteoblasts/osteocytes and is a marker for osteoblast maturation during late stages of osteogenesis (Bronckers *et al.*, 1989, Nakamura *et al.*, 2009).

The current study demonstrated that MTA, and, to a lesser extent, propolis stimulated osteogenic differentiation of HDPCs. The level of expression of *BMP-2*, *ALP* and *RUNX-2*, genes was significantly higher in HDPCs treated with MTA and propolis in comparison to control. However, this effect was largely influenced by the passage number as the up-regulation of the studied genes was highest in HDPCs from early passages (P2-6) as shown in Figure 56. The current findings may be attributed to the loss of differentiation capacity in cells from later passages which are a widely reported phenomenon in mesenchymal progenitor cells (Huang *et al.*, 2006). The up-regulation of *ALP*, *RUNX-2* and *OCN* may provide strong evidence of the osteogenic differentiation potential of the studied HDPCs. Similar findings were reported by Wang *et al.* who demonstrated an important role of active cellular NF- $\kappa$ B pathway in the up-regulation of *ALP*, *RUNX-2* and *OCN* and the formation of bone-like hard tissue bridge in injured dental pulps of rats (Wang *et al.*, 2013). Additionally, HDPCs were found to have strong osteogenic differentiation potential when isolated from healthy pulps of exfoliating primary teeth. Furthermore, the current findings may explain the undetectable expression on odontogenic markers (*DSPP* and *DMP-1*) as it has been identified by Li *et al.* *RUNX-2* inhibited odontoblast terminal differentiation and induced transdifferentiation of odontoblasts to osteoblasts at the late cell differentiation stage (Li *et al.*, 2011).

Our study demonstrated concurrent down-regulation of *RUNX-2* and up-regulation of *ALP* and *OCN* in later passages as shown in Figure 56. Similar findings were reported in a study by Paranjpe *et al.* who described a similar sequence for expression of osteogenesis transcription factors in HDPCs when incubated for various periods

(Paranjpe *et al.*, 2010). Our results are also in agreement with another study, which evaluated the effect of MTA on gingival and periodontal ligament fibroblasts. The study by Bonson *et al.* demonstrated up-regulation of osteogenic markers such as alkaline phosphatase, osteonectin, osteopontin, and osteonidgen, which may contribute to hard tissue matrix formation and/or mineralization (Bonson *et al.*, 2004).

Replicative ageing phenomenon is a particular type of cell ageing that can be encountered *in vitro* with repetitive cell replication or serially passaged cells. Telomere length shortening is largely implicated in this process (Jiang *et al.*, 2007, Westin *et al.*, 2007). Telomerase is a ribonucleoprotein complex that is responsible for enzymatically maintaining the telomere length and thereby prevents cell ageing. It has been shown that the telomerase enzyme has an effect on cellular senescence and ageing because it can be stimulated to maintain the length of the telomere. Telomerase is expressed mainly in embryonic and tumour cells, which explains their unlimited proliferation potential. The protein subunit of telomerase is coded for by the *TERT* gene that is normally expressed at a lower level than the RNA subunit which is coded for by the *TERC* gene (Lin and Yan, 2005, Shawi and Autexier, 2008). For telomerase gene to be activated, both subunits must be up-regulated to maintain the length of telomere (Wang *et al.*, 2008)

Ageing cells may exhibit loss of differentiation and proliferation potential (Huang *et al.*, 2006, Chambers and Goodell, 2007, Mehrazarin *et al.*, 2011). Similar findings were demonstrated in the current study, where HDPCs from later passages exhibited lower proliferation potential and viability. Additionally, qRT-PCR conducted in the present study revealed that serially passaged HDPCs had lower osteogenic differentiation capacity toward later passages. qRT-PCR data also revealed undetectable expression of *TERT* gene which is essential for the function of telomerase and thereby telomere length preservation as shown in Figure 47. Thus, the degradation of biological function

supported by undetectable telomerase function may point toward telomere length shortening or cell ageing in the serially passaged HDPCs. Furthermore, MTA induced proliferation of HDPCs which means that the cells may have gone through a higher number of cell divisions and possibly sustained a higher degree of cell ageing. The antioxidant effect of the novel, biological agent, propolis has been widely cited and may have a protective role against telomere shortening. Thus, the phenomenon of replicative senescence was investigated in serially passaged HDPCs that were cultured with MTA or propolis and compared to control cells in the current study. qRT-PCR studies on *TERT* and *TERC* genes indicated that the studied HDPCs will be susceptible to cell ageing but to an unknown level as shown in Figure 47. The level of cell ageing was determined by the extent of telomere shortening. The latter was quantified in the present study using a telomere specific probe in southern blot DNA hybridisation technique.

In the present study we have demonstrated progressive attrition of telomere length as a function of passage number in control and MTA groups. The MTA group, however, showed higher mean telomere length shortening ( $3.16 \pm 0.09$  kb) between passage 2 and 12 in comparison to control ( $3.11 \pm 1.18$  kb) as shown in Figure 58. The difference could be attributed to the higher number of cell divisions HDPCs underwent in the MTA group as a result of the positive proliferative effect of the material. However, the difference was not statistically significant. Our study also demonstrated excessive telomere length reduction in HDPCs between passage 2 and 12 ( $10.71 \pm 0.50$  kb) as shown in Figure 57. Despite the increase in proliferation and cell division, the reduction of TRF was not statically significant in MTA treated groups as shown in Figure 58.

Indirect methods that measure T/S ratio that is indicative of the telomerase function are available to investigate cell ageing (Cawthon, 2009). The use of specific probes in southern blot hybridisation technique, as in the present study, may be regarded as a

superior technique to identify and quantify cell ageing. It is a direct technique where the exact amount of telomere shortening can be determined. In previous studies, progressive telomere length attrition was identified in serially passaged HDPCs as indicated by the decreasing T/S ratio as a function of passage number (Mokry *et al.*, 2010, Mehrazarin *et al.*, 2011). The effect of continuous replication of cells *in vitro* on the telomere length shortening is not an exclusive issue with HDPCs. Rather it has been reported with many other cell types including, epidermis keratinocytes and bone marrow mesenchymal cells (Miyata *et al.*, 2004, Bonab *et al.*, 2006).

The effect of MTA on telomere length shortening of HDPCs has not been reported yet in the literature to the knowledge of the author of this thesis. However, there is a consensus that cells from later passages may lose proliferation and differentiation capacity (Huang *et al.*, 2006). With regard to propolis, the great reduction of telomere length can be attributed to the anti-telomerase and pro-apoptotic effects of the material. It has been cited that propolis may reduce cell counts, decrease cell proliferation, induce apoptotic or cytotoxic effect and suppress telomerase activity in tumour cells (Gunduz *et al.*, 2005). The same effects may be implicated in the lower proliferation potential, viability, differentiation capacity and excessive telomere shortening identified in the present study among propolis treated HDPCs. However, these adverse effects are not compatible with the comparable pulp/dentine regeneration that is noticed when using MTA and propolis (Parolia *et al.*, 2010). One explanation can be that these findings were after a short observation period when the ageing/degenerative effect of the material was fully expressed. Additionally, the strong anti-inflammatory and anti-bacterial effects beside the high pulp sealing ability of propolis may collectively promote healing and subsequent regeneration.



The low frequency of subpopulation of stem cells in dental pulp necessitates *in vitro* expansion prior to clinical use. According to the result of the current study, serial passage of HDPCs reduced the viability, caused phenotypic changes and telomere shortening which is a causative factor for cellular ageing as shown in Figures and 28, 29 and 57. Consequently propagating HDPCs *in vitro* for the purpose of growing them in scaffolds to be utilized in tissue engineering should be re-evaluated. Therefore in order to use HDPCs for clinical application and tissue engineering, the ageing process needs to be overcome. In the current study, in an attempt to overcome this problem, new strategy was investigated to minimise the ageing of HDPCs. In the present study, exogenous telomere specific oligonucleotide primers were added to HDPCs in culture to investigate if such primers can suppress telomere length shortening. These primers were custom designed to prime to the lagging strand of the parental DNA which can theoretically ameliorate the DNA end replication problem and thereby, reduce telomere length shortening. Such primers prime to telomere sequence and provide a subunit for polymerase I to synthesise complementary daughter DNA strand to the lagging parental DNA strand at both ends of the chromosome as shown in Figure 59.

In our study, the effect of adding exogenous “Telome 3” and “Telome 5” complimentary oligonucleotides was investigated. The effect of the oligonucleotide primers (“Telome 3” and “Telome 5”) on the viability and proliferation of HDPCs was studied. As expected, viability of control cells gradually decreased in later passages. Addition of “Telome 3” and “Telome 5” oligonucleotide primers and to a lesser extent “Telome 5” has further decreased the viability of HDPCs in comparison to control samples. However, addition of “Telome 3” oligonucleotide primers to the HDPC culture maintained the viability level even in later passages. After passage 6, the viability of HDPCs treated with “Telome 3” oligonucleotide primers was significantly higher than

all the other groups as shown in Figures (60-63). These findings were reproducible upon repeating the experiment. The current findings may suggest that the uptake of “Telome 3” oligonucleotide primers by HDPCs may perhaps influence the telomere shortening process and thereby, suppress ageing, reduced apoptotic activity and maintain biological functions of the cells in later passages as shown in Figures 60 and 62.

Addition of oligonucleotide primers has been previously used to suppress ageing of cell types other than HDPCs. A study reported that treatment of human endothelial cell populations with reverse primer to the human interleukin 1 (IL-1 alpha) transcript prevented cell senescence and extended the proliferative life-span of the cells *in vitro*. Removal of the IL-1 alpha primer resulted in the generation of the senescent phenotype and loss of proliferative potential (Maier *et al.*, 1990). Another study suggested exogenously added 3' overhang-oligo induced senescent phenotype of neonatal fibroblasts from early passages (Li *et al.*, 2003).

The current study further verified the effect of “Telome 3” oligonucleotide primers on the extent of telomere shortening in serially passaged HDPCs. Our results may indicate that treatment of HPCs with “Telome 3” oligonucleotide primers may perhaps lead to maintain telomere length in serially passaged HDPCs (P2-12). TRF length was estimated to be 13.2 Kb in HDPCs from passage 2. Upon serially passaging HDPCs treated with “Telome 3” oligonucleotide primer, telomere length was maintained at passage 12 (13.4 Kb). Our results indicated that the treatment of HDPCs with “Telome 3” oligonucleotide primer may prevent telomere length shortening and perhaps delay HDPCs ageing. Furthermore, the current findings may account for the improved biological characteristics in terms of proliferation and cell viability of HDPCs. Additionally, the development of such novel primer has a significant impact in providing a strategy to delay ageing, especially in HDPCs that are grown and amplified

for tissue engineering applications. However, the effect of “Telome 3” oligonucleotide primer on telomere shortening should be assessed more thoroughly using multiple experiments on different batches of HDPCs. In addition, telomere length degradation/maintenance at different passages and using different methods should be analysed to confirm such effect as shown in Figure 64.

## 4.2 Conclusions

We have investigated the biological characteristics and cell ageing of HDPCs. The study focused on the effects of various pulp capping materials on the proliferation, differentiation and replicative senescence in the studied HDPCs. Furthermore, a novel exogenous oligonucleotide primer was designed and investigated for its effect on replicative senescence, proliferation and viability of serially passaged HDPCs. The findings of the current study can be summarised as follows:

- HDPCs were successfully isolated from human third molar teeth. Isolating HDPCs by the collagenase method was superior to the explant outgrowth technique as indicated by the higher proliferation rate, lower doubling time and higher viability percentage of the former. However, HDPCs isolated using both techniques exhibited similar morphology. The collagenase isolated HDPCs did not express specific odontogenic markers which pointed toward them being a mixed population of cells that may exhibit alternative differentiation pathways.
- Dycal exhibited very high cytotoxicity toward HDPCs from different passages and after different incubation periods. This effect was not related to the treatment method, i.e. direct or indirect contact with the cells. MTA possessed pro-proliferative qualities and had a highly biocompatible effect on HDPCs from different passages when delivered in concentrations according to the manufacturer's recommendations. At high concentration, propolis negatively affected the proliferation potential and viability of the studied HDPCs. Reduction of the delivered concentration significantly improved proliferation and viability of HDPCs. Furthermore, both materials enhanced osteogenic differentiation potential as indicated by the up-regulation of *ALP*, *RUNX-2* and *OCN* markers in comparison to control.

- Serially passaging HDPCs with or without MTA resulted in gradual loss of their proliferation potential and viability. However, the reduction in cell viability was significantly higher in later passages of HDPCs treated with propolis. Furthermore, all studied groups exhibited significant loss of differentiation capacity toward later passages. Treatment with MTA and propolis did not have a notable effect on expression of osteogenic markers (apart from slight up-regulation of *ALP*) in late passages.
- In tandem with degradation of biological functions, serially passaged HDPCs sustained a high degree of replicative senescence as indicated by undetectable expression of telomerase enzyme subunits and telomere length shortening in control, MTA and propolis groups. The highest degree of telomere length shortening was noticed upon treating HDPCs with propolis. It was concluded that propolis caused significant cell ageing and apoptotic activity in serially passaged HDPCs.
- The use of oligonucleotide primer “Telome 3” complimentary to 3’ hydroxyl end of DNA, significantly improved viability and proliferation of HDPCs in later passages. Additionally, this effect was possibly due to the ability of such primer to prevent telomere length attrition throughout serial passaging.

In conclusion, the current study provided better understanding of the interaction between pulp capping materials and HDPCs which is essential to the performance of pulp capping materials *in vivo*. MTA seemed to have superior effect on cell proliferation, viability and replicative senescence which may explain the optimum outcome of this material when used in clinical situations. Human dental pulps of third molar teeth may be an abundant source for multipotent stem cells which have high osteogenic potential and can be used for tissue engineering. However, amplification of HDPCs *in vitro* may

negate their use as a result of cell ageing and subsequent degradation of biological functions. The current study represents a major advance in our understanding of cellular ageing and associated biological changes in HDPCs in serial passage. The use of novel tissue engineering solutions, such as treatment of HDPCs with oligonucleotide primer “Telome 3” to preserve telomere length during *in vitro* replication may overcome such problem and allow propagation of a high number of cells to be used in tissue engineering therapies.

### 4.3 Future work

The complex nature of odontogenesis and pulp regeneration provides a large scope for future work. Replicative cell senescence can be further studied using several, complementary techniques such as TRF measurements and Q-FISH technique to determine telomere length in each chromosome. Such techniques can be used to further analyse and confirm the effectiveness of the exogenous oligonucleotide primers “Telome 3” in suppression of telomere attrition and thereby cell ageing when added to HDPCs culture from other donors and in other cell types. The effect of “Telome 3” can be further studied in conjunction with various pulp capping materials and propolis in particular. It could also be interesting to investigate whether “Telome 3” may allow amplifying cells *in vitro* for a very high passage numbers (above 15) without problems related to cell ageing and telomere shortening which may allow seeding these cells on scaffolds for tissue engineering applications. The biological characteristics can also be reappraised in serially passaged cells that were incubated with “Telome 3” using viability and qRT-PCR assays.

Characterisation of the isolated cells from the human dental pulp can be performed using cell sorting techniques. Studying the effects of different pulp capping materials on the biological characteristics, differentiation potential and cell ageing within each subpopulation may deliver important information regarding the cell response to pulp capping materials especially when incubated with the “Telome 3” oligonucleotide primer. MTA and propolis can also be compared to the relatively new pulp capping agent, Biodentine<sup>®</sup> which exhibited optimum performance as indicated by the clinical studies.

The use of various imaging techniques, with or without staining techniques such as SEM or confocal laser microscopy may be an adjunct to analyse morphological changes (attachment and migration) among the cells when incubated on different pulp capping materials for different periods. More sophisticated techniques can be used to investigate proliferation and viability such as MTT or flow cytometry techniques. The latter technique can elaborate on the causes of cell death and categorise cells into viable, pre-apoptotic, apoptotic and dead as a result of necrosis.

Although *in vitro* tests provide an insight into the biological changes, including cell ageing in HDPCs, they fail to recreate the complex environment *in vivo*. Pulp regeneration, repair and hard tissue bridge formation and cell ageing could be further investigated in depth in animal and human dental pulps.



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