The Effect of Dentine Conditioning Agents on Solubilisation of Bioactive Dentine Matrix Components and Dentine Regeneration

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<u>Summary</u>

The dentine-pulp complex is a unique, dynamic organ capable of responding to mild and aggressive injurious stimuli. Deep caries or tooth fractures that extend close to the pulp can lead to inflammation and ultimate pulpal necrosis if not managed appropriately. Current therapeutic regimes for such scenarios include the use of vital pulp (capping) treatments, which attempt to stimulate the pulp's auto-repair capacity which is still poorly understood and consequently not exploited to its full potential.

Amongst the possible mechanisms involved in inducing a dentinogenic response in vital pulp therapy, the ability of dental materials in solubilising cell signalling molecules from dentine has been proposed recently. As such, the role of growth factors belonging to TGF-β superfamily in particular has been highlighted during dentine repair and regeneration (Graham et al. 2006).

In recent times clinical practice has moved towards the use of dentine bonding agents and composite restorations even in deep dentinal cavities that extend close to the pulp in the hope of reducing the effects of microleakage, amongst others (Lynch 2008). However, although such treatments appear moderately successful in practice, the justifications for introducing new protocols to improve dental pulp protection and dentine bridge formation are not currently driven by biological knowledge.

This in vitro study investigated the effects of dentine conditioning by several clinically used etching agents/ dentine conditioners (components of adhesive restorative systems) on the solubilisation of bioactive dentine matrix components and dentine regeneration (by assessing the impact on DPSC phenotype and behaviour).

It was found that growth factors (TGF-β1, BMP2 and VEGF) were exposed and released following dentine treatment by EDTA, phosphoric and citric acid. Polyarcrylic acid on the other hand was ineffective in solubilising dentine matrix proteins. Dentine surface conditioning by EDTA, phosphoric and citric acid resulted in an up regulation in the expression of genes associated with osteoblast/odontoblast-like cell differentiation (OPN and ALP) in DPSCs, however surfaces treated with phosphoric acid appeared significantly less supportive of cell growth following 8 days in culture.

These findings provide direction for future research and take crucial steps for informing clinical practice as to the choice of materials and techniques in vital pulp treatments.

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Abbreviations

ADJ	Amelodentinal junction
ALP	Alkaline phosphatase
ASARM	Acidic serine and aspirate-rich motif
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
COL1	Collagen type I
EDTA	Ethylenediaminetetraacetic acid
DMEs	Dentine Matrix Extracts
DMP1	Dentine matrix protein 1
DPP	Dentine phosphoprotein
DPSCs	Dental pulp stem cells
DSP	Dentine sialoprotein
DSPP	Dentine sialophosphoprotein
ECM	Extracellular matrices
EDTA	Ethylenediaminetetraacetic acid
FGF	Fibroblast growth factor
IGF	Insulin-like growth factor
LAP	Latency-associated protein
LTBP	Latent TGF-β binding protein
MAPK	Mitogen-activated protein kinases
MEPE	Matrix extracellular phosphoglycoprotein
MMP	Matrix metalloproteinase

MTA	Mineral trioxide aggregate
MV	Matrix vesicles
NCPs	Noncollagenous proteins
OCN	Osteoocalcin
OPN	Osteopontin
PDGF	Platelet-derived growth factor
SHEDs	Stem cells from human exfoliated deciduous teeth
SIBLING	Small integrin binding ligand N-linked glycoprotein
SLC	Small latent complex
SLRPs	Leucine-rich repeat proteoglycans
TGF-β	Transforming growth factor-β
TLR	Toll-like receptors
TNAP	Tissue non-specific alkaline phosphatase
VEGF	Vascular endothelial growth factor

Chapter 1: General Introduction

1.1 Introduction

Dental caries continues to be one of the most prevalent infectious diseases in the world (Bagramian et al. 2009). The disease is initiated and progresses by activity of cariogenic bacteria, in the presence of substrates, on the tooth surface. The process involves demineralisation and then destruction of the dental hard tissues, following which pulpal tissue damage can occur.

Data from the most recent Adult Dental Health Survey reveals that 84% of the adults in England and Wales have at least one filling, with each adult having, on average, 7.2 filled teeth (Steele and O'Sullivan 2009). Let alone this, dental caries continues to be the most common disease of childhood: the Child Dental Health Survey 2013, England, Wales and Northern Ireland found that nearly a third (31%) of 5 year olds and nearly half (46%) of 8 year olds had obvious decay experience (Health and Social Care Information Centre 2015) . As well as this, recent data suggest caries prevalence is still high in some developing countries (Southeast Asia had significantly higher caries prevalence in the deciduous and Latin America in permanent teeth than the global mean in 2010) and parts of Europe (Central Europe, higher caries prevalence in both deciduous and permanent teeth compared with Eastern and Western Europe in 2010) (Kassebaum et al. 2015).

Dental caries accounted for almost half of all tooth extractions in the UK at the start of 21st century (McCaul et al. 2001; Richards et al. 2005). The problems posed by caries – in terms of pain and financial costs for patients and healthcare providers – is noted in

studies such as those reporting reasons for patient attendance at dental emergency clinics (Lynch et al. 2010).

While modern strategies in the management of dental caries are multifaceted, the management of cavitated caries lesion within dental practices still involves excision of the lesion and restoring the resultant cavity with an inert restorative material (Fontana et al. 2013; Gomez et al. 2014; Hurlbutt and Young 2014; Brennan et al. 2015). Despite many advances in the materials properties, failure rates remain relatively high. Within the UK, 11% of fillings placed in general practice require replacement within one year, while 20% require replacement after 3 years, and 50% require replacement after 10 years (Burke and Lucarotti 2009). As well as this, there is evidence to suggest that 56% of restorations placed by dentists are replacement restorations, necessitated by failure of existing restorations, rather than treatment of new lesions of caries (Deligeorgi et al. 2001).

Operative management of a lesion of caries places the tooth into a life-long "restorative cycle" (Lynch 2008). This involves additional loss of healthy hard dental tissues through cavity preparation and cycles of subsequent failure and replacement all of which involve iatrogenic loss of adjacent tooth tissue. Throughout this cycle the pulp may be subjected to insult including new lesions of caries, bacterial microleakage and trauma due to operative procedures. The insult can result in inflammation/ infection of the dental pulp and eventual loss of vitality and development of apical periodontitis. There will be a consequent need for root canal treatment to conserve the tooth which would otherwise have to be extracted.

Root canal treatment, which is the standard procedure for treating non-vital and severely inflamed vital pulps, is technically challenging and may require several treatment visits with obvious cost implications (ESE 2006). Furthermore, the procedure involves substantial loss of dentine through gaining access to the pulp chamber and cleaning and shaping of the root canals which weakens the tooth considerably and reduces its prognosis (Caplan et al. 2005b; Zadik et al. 2008). However, if forced to carry out this treatment, it means that the prognosis of the tooth is already compromised and the long-term success cannot be guaranteed. While approximately 80% success rate at the annual post-operative review for root canal treatment has been reported (Ng et al. 2011), there is evidence to show that root filled teeth are lost for reasons other than endodontic failure, such as fracture or periodontal or prosthodontic considerations (Vire 1991; Zadik et al. 2008). Epidemiological data suggests, for example that root filled molars are over 7 times more likely to be lost than their non-root filled counterparts (Caplan et al. 2005a). It should also be noted that the loss of teeth presents psychological and nutritional challenges for affected patients (Daly et al. 2003).

In addition to morbidity associated with loss of teeth, there are cost implications associated with cycles of restoration placement and replacement as well as subsequent tooth maintenance via root canal treatment and crowns. The burden on UK patients and the NHS in terms of cost of managing dental caries is estimated to have been approximately £270M in 2010/2011, providing 12 million restorations to adults in dental practices in the England & Wales*.

* Estimated from the Adult Dental Health Survey (Steele and O'Sullivan 2009) and http://www.ic.nhs.uk/webfiles/publications/007_Primary_Care/Dentistry/Dental_Stats _Eng_2010_11/NHS_Dental_Statistics_for_England_2010_11_Report_v2.pdf

Developing a strategy for promoting dentine regeneration/ auto-repair in response to a placed restorative material would be of benefit to patients as it may help improve the predictability of treatment and, in turn, improve the longevity of the tooth. This would represent a shift from a mechanistic approach to a biological approach for the treatment of lesions of dental caries and represent a significant improvement in treatment available to patients with compromised pulpal viability.

1.2 Enamel and dentine formation

The reparative processes that take place following caries and traumatic dental injuries appear to share common genetic and molecular mechanisms with those taking place during early dental development (Mitsiadis and Rahiotis 2004). It is therefore essential to consider biological interactions occurring during tooth formation as the auto-repair/regenerative potential of dentine-pulp complex is best understood in light of these factors. The next few sections of this chapter will provide a summary of key steps in tooth development.

Tooth formation is a result of a series of epithelial-mesenchymal interactions early in embryogenesis. The initiation of tooth formation involves synthesis and secretion of growth factors by the oral epithelium derived dental lamina which induces the expression of transcription factors in the underlying neural crest derived mesenchyme (Tummers and Thesleff 2009). A series of subsequent reciprocal interactions between the two tissues, via signalling pathways involving ligand-receptors, orchestrates the development of tooth organs (Thesleff 2003). While the exact molecular nature of these signals have not been fully characterized, members of the bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) families are suggested to play critical roles

in early tooth development (Jernvall and Thesleff 2000). During the bell stage of tooth development, cyto-differentiation occurs with odontoblasts differentiating from ectomesenchymal cells in the dental papilla and ameloblasts from the inner enamel epithelium (Tucker and Sharpe 2004). During the late bell stage of tooth development, the inner dental epithelium and its associated basement membrane signal the peripheral cells of the dental papilla to differentiate into odontoblasts. The onset of dentinogenesis coincides with the completion of crown form and enamel is secreted with a delay of around 30 hours as inductive signalling between the two tissues continues (Gaunt 1967).

1.2.1 Odontoblasts and their roles

Dentine matrix is laid down as an extracellular matrix by odontoblasts during odontogenesis (primary dentine) and throughout life (secondary and tertiary dentine). Odontoblasts are post-mitotic neural crest derived cells which differentiate from the ecto-mesenchymal cells of the dental papilla. Odontoblasts terminal differentiation is characterized by withdrawal from the cell cycle, elongation and cytological polarization which involves the nucleus occupying the proximal end of the cell. The fully differentiated odontoblasts are tall columnar cells which specialise in unidirectional secretory function and secrete all dentine matrix constituents (Bleicher 2014). Once the tooth erupts and formation of the root is completed, a significant change in odontoblast transcriptome is noted and they dramatically reduce the rate of dentine production, although their capacity to produce dentine is retained throughout life (Simon et al. 2009; Couve et al. 2013). The p38 MAPK pathway is proposed to be key in regulating the secretory behaviour of odontoblasts (Simon et al. 2010). In the absence of disease or trauma to the tooth, primary odontoblasts may persist for the lifetime of an individual with a life span of many decades. Although some decline in the numbers is seen with

age (Murray et al. 2002c; Murray et al. 2002d), similar to other static cell populations, they are not normally replaced during the life time of the organism (Rezzani et al. 2012).

Further to their secretory function, odontoblasts are able to recognise the invading oral microorganisms through their Toll-like receptors (TLR) (Veerayutthwilai et al. 2007; Keller et al. 2011). Engagement of odontoblasts TLRs with the bacterial compounds *in vitro* is shown to lead to NF-κB nuclear translocation, chemokine production and recruitment of dendritic cells which initiate an innate immune response against caries pathogens (Farges et al. 2009; Keller et al. 2010). However, there is also evidence to show odontoblasts upregulate the production of lipopolysaccharide-binding protein (LBP), an acute-phase protein known to attenuate proinflammotry cytokine production, when challenged by inter-dentinal bacteria *in vivo* (Carrouel et al. 2013). With these apparent contradictory functions, odontoblasts have been proposed to play a role in balancing and controlling inflammation when challenged by cariogenic bacteria (Farges et al. 2013). Furthermore, odontoblast layer barrier beneath the carious lesion is shown to reduce in thickness and integrity, allowing dendritic cell migration into predentine-dentine interface and sprouting of nerve fibres into the reactionary dentine (Couve et al. 2014). This evidence suggest a multifaceted role for odontoblasts involving a secretory function and contribution to neuroimmune response which would facilitate detection and containment of caries pathogens and ultimately promotion of dentine-pulp healing.

1.2.2 Dentine formation and mineralisation

Dentine is first secreted as an unmineralised matrix, called predentine which is 10-40μm thick and separates odontoblasts from the mineralised dentine. This is similar to osteoid that separates the osteoblasts from the bone mineralized matrix. Both predentine and

osteoid are type I collagen-rich unmineralized extracellular matrices which are subsequently mineralized when apatite crystals are deposited. Formation of predentine and its transformation to dentine by undergoing mineralization are highly controlled, orderly processes. It has been observed that collagen fibres in the distal zone of predentine are thicker than those at the proximal region (Beniash et al. 2000). This observation confirms transformation of predentine to dentine is a dynamic event in which predentine collagen fibres gradually mature and get prepared for initiation and growth of dentine mineral crystals (Waddington et al. 2003; Milan et al. 2004, 2006).

The first deposited coronal dentinal layer, mantle dentine, is produced by differentiating odontoblasts and contains large and long collagen type III fibrils arranged at right angles to dentine-enamel junction. After deposition of collagen fibrils and other extracellular matrix components in mantle dentine, numerous matrix vesicles (MV) bud off through the distal membrane of the differentiating odontoblasts. MVs are small (20-200 nm) spherical bodies which are also observed in pre-mineralised bone and cartilage. They are derived from the plasma membrane of mineralised tissue forming cells. However they are enriched in tissue non-specific alkaline phosphatase (TNAP), nucleotide pyrophosphatase phosphodiesterase, annexins and phosphatidyl serine relative to their originating membranes (Golub 2009). The MVs are considered as the primary nucleation sites for the initiation of mineralisation process in dentine, similar to immature bone and calcifying cartilage (Bonucci 2002). Although it is generally accepted that MVs play an important role in the calcification of dentine, bone and cartilage, the exact mechanisms involved in the process remain controversial (Golub 2009). It has recently been suggested that interactions between MV phospholipids, proteins and calcium and phosphate ions form a molecular architecture which favours

nucleation of apatite crystallization (Genge et al. 2007b, a). Mantle dentine is mainly atubular and its lower mineral content confer some elastic properties and provide resilience which may be important in dissipation of the applied occlusal forces (Wang and Weiner 1998).

When the odontoblasts fully differentiate, they no longer express MVs and secretion of noncollagenous proteins (NCPs) starts at this stage (Arana-Chavez and Katchburian 1998). The dentine secreted by the differentiated odontoblasts is called circumpulpal dentine and the NCPs are believed to play a key role in the mineralisation process of it. Among the NCPs, the Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGs) proteins and the small leucine-rich repeat proteoglycans (SLRP) family are shown to be of particular importance for the mineralization of the type I collagen network in bone where they actively promote and control the mineralization process and crystal growth in the ECM (Staines et al. 2012). The Ca^{2+} ions and inorganic phosphate necessary for mineralisation are actively transported to the mineralizing front by the odontoblasts (Lundquist et al. 2002; Tsumura et al. 2010).

With increasing thickness of dentine, odontoblasts recede towards the centre of the dental papilla/pulp and leave behind their cellular processes in the forming dentine matrix. Once dentine matrix mineralises around the odontoblast process, a dentinal tubule will form. The dentine formed initially between the odontoblast processes is called intertubular dentine which contains less mineral content compared to the peritubular dentine in immediate proximity of the odontoblast process. Odontoblasts continue secreting peritubular dentine throughout life, owing to which a gradual obliteration of dentinal tubules occurs with aging (Arana-Chavez and Massa 2004).

Understanding the processes involved in physiological dentine formation is fundamental in providing a blueprint for regenerative strategies in trauma/injury situations. Such knowledge may enable clinicians harness the intrinsic signalling molecules that mimic those involved in tooth development and exploit the recruitment of dental pulp cells during natural wound healing to trigger tertiary dentine formation and limit injury to the viable dental pulp as is the aim in vital pulp therapy techniques.

1.2.3 Types of dentine

Primary dentine is defined as dentine formed up to the completion of root development. Secondary dentine is secreted at a much reduced rate following completion of root formation and throughout life as a physiological phenomenon which leads to a reduction of pulp volume with increasing age. Secondary dentine has a tubular structure similar to primary dentine. Tertiary dentine however, is produced in response to an external stimulus or irritant/ insult and is categorised as 'reactionary' or 'reparative'. In response to mild environmental stimuli (such as slowly progressing caries) reactionary dentine is produced beneath the infected dentinal tubules by the stimulated odontoblasts (Charadram et al. 2012; Couve et al. 2014). Since the original primary odontoblasts are responsible for matrix secretion in reactionary dentine, tubules are continuous with those in secondary dentine, although they are narrower and less abundant compared to primary dentine (Charadram et al. 2013).

In contrast to reactionary dentine formation, reparative dentinogenesis occurs in response to more intense stimuli (rapidly progressing caries) triggering an intense immune and inflammatory response resulting in death of primary odontoblasts and pulpal tissue. In this situation if disease progression can be stopped and a suitable dental

material applied, reparative dentine may be formed by a population of odontoblast-like cells which differentiate from a precursor population within the pulp (Smith et al. 1990; Tziafas et al. 2000). Reparative dentine has a different structure compared to reactionary dentine in the majority of cases. Matrix secreted by the new generation of cells has discontinuity in the tubular structure and/or may be atubular. Cellular inclusions within the matrix give the reparative dentine an osteotypic appearance (Goldberg et al. 2011). Discontinuity and absence of a tubular structure (in reparative dentine) or reduction in tubular diameter and numbers (in secondary and reactionary dentine) may result in reduction of dentine permeability. This property of dentine is important in response to caries, reducing the risk of subsequent pulp tissue injury from tubular invasion of bacteria and diffusion of their products (Farahani et al. 2010; Couve et al. 2014).

1.3 Comparison of odontoblasts with osteoblasts

In light of the similarities of reparative dentine with both bone and dentine, it is useful to compare the characteristics of the cells producing the two tissues. Odontoblast-like cells differentiate from ectomesenchymal cells present in the pulp (Huang et al. 2006a). Reparative dentine produced by these cells has similarities to bone: it contains cellular inclusions as in bone, also some noncollagenous proteins (osteopontin, for example) that are more typical for bone than of dentine (Smith et al. 1995; Arana-Chavez and Massa 2004). Although osteoblasts of dermal and endochondral bone are derived from the neural crest, osteoblasts originate from the mesoderm also. Odontoblasts however, derive exclusively from the neural crest (Hall and Gillis 2013). In terms of morphology, odontoblasts differ from the osteoblasts by possessing single cell processes that arises from their tall columnar cell bodies and penetrates into the dentinal tubule, through the thickness of dentine. Such a structure may play a role in communications between the

cells and ECM as well as early detection of the invading oral pathogens, for example in caries (Love and Jenkinson 2002).

A 10-40μm thick layer of unmineralised matrix, predentine, separates the odontoblast layer from the mineralised dentine which is similar to the osteoblasts being separated from the mineralised bone by the osteoid. Consideration of dentine and pulp together as a functional organ implies great similarities to bone, while the tubular structure of dentine shows many morphological similarities to the osteocyte lacuno-canalicular network (Lu et al. 2007a). The significant remodelling of bone throughout life, in contrast with dentine, reflects the importance of bone as a homeostatic reservoir for calcium and phosphate ions. In terms of matrix formation activity, odontoblasts are very similar to osteoblasts and express almost all kinds of osteoblast marker genes such as alkaline phosphotase (ALP), bone sialoprotein (BSP), osteopontin (OPN) and osteocalcin (OCN) (Park et al. 2009). The diverse range of NCPs present in dentine (see the next section) is also present in bone, however their relative proportions differ. Dentine sialophosphoprotein (DSPP) transcript was originally thought to be specific to odontoblasts but later studies demonstrated the expression of DSPP in bone (Qin et al. 2002; Verdelis et al. 2008), cementum (Baba et al. 2004), and some non-mineralized tissues (Alvares et al. 2006; Ogbureke and Fisher 2007). However, the relative expression level of DSPP is considerably higher in dentine (Suzuki et al. 2012).

1.4 Dentine extra cellular matrix components and their biological activities

Dentine is composed of 70% inorganic hydroxyapatite crystals, 20% organic matter and 10% water (by weight). The organic matrix of dentine is known to contain collagen,

noncollagenous proteins (NCPs), phospholipids and growth factors. Type I collagen is the most abundant protein with types III, V and VI collagen also present in dentine matrix. Type I collagen fibrils are considered to be a template for calcium and phosphate binding and the nucleation and growth of apatite crystals in mineralised tissues (Silver and Landis 2011). The importance of normal collagen structure for mineralisation is clearly seen in osteogenesis imperfecta in which both bone and dentine structure are affected as a result of mutations in either of the two genes encoding the two chains of type I collagen (COL1 A1 and COL1 A2) (Martin and Shapiro 2007).

The NCPs form less than 10% of dentine organic matrix and are believed to actively control the mineralization process and apatite crystal growth within predentine resulting in conversion of it into dentine (He et al. 2003; George and Veis 2008). Mutations in NCP genes are shown to result in dramatic phenotypic abnormalities in dentine, confirming their crucial role in matrix organisation and mineralisation (Sreenath et al. 2003; McKnight et al. 2008).

1.4.1 Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGs)

SIBLINGs are a family of well-characterised, multifunctional bioactive molecules. They have important regulatory function in balancing the promotion and inhibition of the mineralisation process in bone and dentine. SIBLINGs are capable of interacting with other macromolecules, cell membranes and mineral crystals. They are highly acidic proteins and can act as nucleating factors in matrix mineralisation process by precipitating calcium ions (Ca^{2+}) and they regulate hydroxyapatite crystal formation (Fisher et al. 2001; He et al. 2003; He et al. 2005a).

A number of shared characteristics in SIBLINGs gene and protein structure have been reported in review studies (Staines et al. 2012; Suzuki et al. 2012). The genomic structures of all SIBLINGs are located on the human chromosome 4q21 and they share common gene structure features. The protein contains an Arg-Gly-Asp (RGD) integrin binding site which is important in cellular responses such as adhesion and migration (Suzuki et al. 2014). The effect of SIBLING proteins on hydroxyapatite mineral crystallite formation and growth *in vitro* appears to be dependent on the extent of phosphorylation of the protein (Boskey et al. 2012). Furthermore, it has been proposed that different regions of the protein may have distinct effects on the mineralization process. For example a domain within their structure, the acidic serine and aspirate-rich motif (ASARM), is shown to be critical in inhibiting mineralisation thus providing a mechanism for controlling the bone turnover (Addison et al. 2008; David et al. 2011). Major SIBLING proteins in dentine include dentine sialoprotein (DSP), dentine phosphoprotein (DPP), dentine matrix protein-1 (DMP-1) bone sialoprotein (BSP), osteopontin (OPN), and matrix extracellular phosphoglycoprotein (MEPE).

1.4.1.1 Dentine sialophosphoprotein (DSPP)

Odontoblasts secrete dentine sialophosphoprotein (DSPP), a large parental protein which is subsequently cleaved into DSP and DPP, also known as phosphophoryn (Guo et al. 2014). DPP is the major noncollagenous component of dentine, accounting for as much as 50% of the NCPs (Suzuki et al. 2009). DPP has a specific affinity for collagen molecules and has been shown to localise to the boundaries between the gap and overlap zones along the collagen fibrils at the mineralisation front of dentine (Dahl et al. 1998; Beniash et al. 2000). In vitro studies have demonstrated strong affinity of DPP for Ca^{2+} which promotes growth of hydroxyapatite crystals when bound to collagen fibrils

(Zanetti et al. 1981; Milan et al. 2006). The effects of DPP on mineralisation *in vitro* have been reported to be significantly variable depending on its concentration, degree of phosphorylation, and conformational state (whether surface-bound or in solution) (Boskey et al. 1990; He et al. 2005b). In contrast to DPP, DSP has been reported to have limited or no effect on initiation of mineralisation and subsequent growth of hydroxyapatite crystals *in vitro* (Boskey et al. 2000). However, the results of a recent study in mice indicated that DSP plays a role in regulating initiation of dentine mineralization while DPP is involved in the maturation of mineralized dentine (Suzuki et al. 2009).

Mutations of DSPP gene in human are associated with two most common forms of dentin genetic disorders, dentinogenesis imperfecta and dentine dysplasia (Kim and Simmer 2007; McKnight et al. 2008; Zhang et al. 2011a). Patients affected by these hereditary conditions have amber-brown, opalescent teeth and the enamel is often broken off soon after eruption, exposing the underlying dentine. This leads to accelerated tooth wear and eventually pulp exposure. The histological appearance of dentine in affected patients shows sparse and irregular tubules with large areas of uncalcified matrix (Aldred 1992). In vivo studies in DSPP knockout mice have reported tooth defects similar to those seen in humans suffering from DGI such as hypomineralized teeth, thin dentine and large pulp chambers (Sreenath et al. 2003). Additional developmental abnormalities including less organised mineral structure, ectopic dentine formation within the pulp as well as altered odontoblast differentiation have been reported in DSPP knockout mice (Fang et al. 2014; Guo et al. 2014).

1.4.1.2 Dentine matrix protein1 (DMP1)

DMP1 is primarily expressed in bone. However, it was identified in dentine (George et al. 1993) prior to being detected in bone (Hirst et al. 1997). DMP1 is proposed to play a role in inducing cytodifferentiation of dental pulp stem cells into odontoblasts *in vitro* (Almushayt et al. 2006) and regulation of biomineralization events in bone and dentine as well as phosphate homeostasis (Feng et al. 2003; Ling et al. 2005; Feng et al. 2006; Lu et al. 2007b). DMP1 has also been shown to bind collagen and to affect collagen fibrilogenesis (He and George 2004). Depending on its level of phosphorylation, DMP1 alternatively regulates calcium phosphate nucleation or inhibits mineralization (Deshpande et al. 2011). DMP1 also affects crystal size depending on its concentration *in vitro* (Tartaix et al. 2004). Furthermore, it is shown in vitro that DMP1 can induce crystal nucleation and regulate the phase transition from amorphous calcium phosphate to carbonated apatite (He et al. 2003; He et al. 2005a).

The crucial role of DMP1 in bone and dentine physiology is confirmed through mutational or gene knockout analysis studies. Autosomal recessive hypophosphatemic rickets in humans involves a mutation affecting the DMP1 encoding gene (Feng et al. 2006). Defective osteocyte maturation and pathological changes in bone mineralization (Ling et al. 2005) as well as failure of maturation of predentine and dentine hypomineralization are associated with this condition (Ye et al. 2004). It has been reported that DSPP levels also were reduced in DMP1 knockout mice (Ye et al. 2004). This finding suggests that DMP1 may somehow have a regulatory effect on DSPP levels during dentinogenesis.

1.4.1.3 Bone sialoprotein (BSP)

BSP is a mineralized-tissue specific protein and is expressed in bone, dentine, mineralizing cartilage, and cementum (Qin et al. 2004). The biological functions of BSP in mineralized tissues are largely unknown, however the temporospatial deposition of it in the extracellular matrix and its ability to nucleate hydroxyapatite crystal formation, indicates a potential role for in the initial mineralization of mineralised tissue matrix. BSP has been shown to act as a nucleator for the formation of hydroxyapatite *in vitro* (Hunter and Goldberg 1993) and it is reported to act as inhibitor of the growth of crystals at later stages (Stubbs et al. 1997). Studies in BSP deficient mice have supported a role for BSP in bone development and mineralisation as well as regulating bone remodelling (Malaval et al. 2008; Boudiffa et al. 2010; Holm et al. 2015).

1.4.1.4 Osteopontin (OPN)

OPN is mainly expressed in bone but is also present in dentine. It is believed to play a role in osteoclast recruitment and function through which it acts as an inhibitor of hydroxyapatite crystal formation and growth in bone; animal studies in OPN knockout mice have revealed that the amount of bone mineral as well as mineral maturity, determined by crystal size and perfection, is significantly increased compared to the wild-type controls. Furthermore, osteoclasts in OPN knockout mice are reported to be hypomotile, displaying poor bone resorption activity compared to those of the wild type (Boskey et al. 2002; Chellaiah et al. 2003).

With regard to dentine, it has been shown that the expression of SIBLING proteins is altered in reparative compared with primary dentine in rats (Xie et al. 2014).While BSP and OPN were detected in reparative dentine only, the levels of DMP1 and DSP are

reduced in reparative dentine when compared to primary dentine. According to these findings, it may be suggested that the osteoid-like resemblance of reparative dentine produced by the odontoblast-like cells, may be associated with altered organic contents of the ECM in reparative dentine.

1.4.2 Non-phosporylated dentine matrix proteins

Dentine contains some non-phosphorylated proteins which are believed to be important in regulating mineralisation. Osteocalcin (OCN), decorin and biglycan are examples of this category (Chaussain-Miller et al. 2006).

OCN is most abundant in bone extracellular matrix, but has also been detected in the odontoblast cell body and process (Papagerakis et al. 2002). It has been reported that osteocalcin is up-regulated during the formation of reparative dentine and in the bone-like tissue formed in the pulp cavity after tooth replantation (Braut et al. 2003; Aguiar and Arana-Chavez 2007; Zhao et al. 2007). Furthermore, a role for osteocalcin in mediating nucleation, growth and development of apatite crystals in association with type I collagen has been demonstrated (Chen et al. 2015).

Decorin and biglycan are members of the small leucine-rich repeat proteoglycans (SLRPs) family which are widely distributed in mammalian tissues, including mineralized tissues such as bone and teeth (Iozzo and Murdoch 1996; Hocking et al. 1998; Reed and Iozzo 2002). Seventeen distinct genes are shown to be involved in encoding these proteins which are involved in a variety of cellular functions and signalling (Schaefer and Iozzo 2008). The SLRPs, are collagen-binding molecules and mediate the intermolecular cross-linking of collagen during collagen fibrillogenesis in the ECM (Kalamajski and Oldberg 2010). Matrix bound SLRPs have the ability to

sequestrate growth factors (TGF-β) in the ECM, and are proposed as regulators of matrix formation and organisation (Markmann et al. 2000; Ferdous et al. 2007; Chen and Birk 2013; Wang et al. 2014a). Fibromodulin and biglycan knockout mice exhibited disorganized collagen fibrils in periodontal ligament as well as disturbed homeostasis of hard tissues (Wang et al. 2014a). Furthermore, decorin and biglycan present in predentine are understood to play an important role in mineralisation of dentine via their ability to bind calcium (Goldberg et al. 2003). Absence of these proteins results in impaired dentine mineralisation in decorin and biglygan knockout mice (Haruyama et al. 2009).

1.4.3 Growth factors

Dentine matrix contains a cocktail of growth factors with potent biological effects (Smith 2003). The growth factors detected in the ECM of dentine include the transforming growth factor-β (TGF-β) superfamily, insulin-like growth factor (IGF), fibroblast growth factor (FGF), adrenomedullin and several angiogenic growth factors including platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (Finkelman et al. 1990; Cassidy et al. 1997; Roberts-Clark and Smith 2000; Zhao et al. 2000; Musson et al. 2010). These bioactive molecules have been secreted, probably largely by odontoblasts, during odontogenesis (Sloan et al. 2000). After secretion, the growth factors interact with other extracellular matrix components and thereby become fossilised within dentine matrix, a process which may help retain their biological activity (Smith et al. 1998; Sloan et al. 2002; Baker et al. 2009).

Growth factors have been implicated in signalling tooth morphogenesis and odontoblasts differentiation during tooth development. Similarly in mature teeth they may be important in signalling odontoblast-like cell differentiation and repair during

tissue injury (Smith 2003). The sequestrated growth factors within dentine matrix provide one possible source of cell-signalling molecules in injury situations, however the role of other possible sources should not be overlooked. Both the physiological cell populations of the pulp and the inflammatory cells, which locally infiltrate the tissue at sites of injury express a number of cell signalling molecules which may contribute to the overall tissue response (Sloan et al. 2000).

1.4.3.1 Transforming growth factor-β (TGF-β) superfamily

The TGF-β superfamily includes various TGF-β isoforms, bone morphogenetic proteins (BMPs), activin and related proteins. They regulate a diverse range of developmental and homeostatic processes including maintenance of stem cell pluripotency, cell fate determination during development, chemotaxis and apoptosis as well as carcinogenesis and metastasis (Shen 2007; Watabe and Miyazono 2009; Beyer et al. 2013). Within the TGF-β superfamily, TGF-β1, TGF-β2, TGF-β3, BMP2, BMP4, BMP7 are expressed in the inner enamel epithelium and dental papilla during odontogenesis and are believed to mediate epithelial–mesenchymal interactions critical in tooth development and morphogenesis (Li et al. 2011a). Analysis of the expression patterns of growth factors during odontogenesis confirms that members of the transforming growth factor β (TGF-β) superfamily amongst other cell signalling molecules such as IGFs, WNTs and FGFs contribute to odontoblast terminal differentiation (Begue-Kirn et al. 1992; Cam et al. 1992; Thesleff and Vaahtokari 1992; Begue-Kirn et al. 1994; Lohi et al. 2010).

BMPs are expressed throughout embryonic tooth development. Bmp2, Bmp4 and Bmp7 are expressed in the enamel knot during odontogenesis and subsequent reciprocal interactions between the mesenchyme and epithelium will follow; members of BMP

family including Bmp2, Bmp4, Bmp6 and Bmp7 are also expressed during odontoblast differentiation (Heikinheimo 1994; Aberg et al. 1997). BMP signalling has been shown to be crucial in regulating tooth morphogenesis and particularly patterning the crown (Thesleff and Jernvall 1997; Thesleff and Mikkola 2002; Unterbrink et al. 2002; Thesleff 2003; He et al. 2004). Inhibition of TGF-β and BMP signalling in mice has been shown to result in abnormal dentine formation (Plikus et al. 2005; Oka et al. 2007; Gao et al. 2009). Further to the crucial role of TGF-β signalling in tooth development, in vivo studies in animal models have shown that application of exogenous TGF-β1 and BMPs to exposed pulp induced tertiary dentine formation implicating a role for these cell signalling molecules in response to injury in mature teeth (Rutherford 2001; Six et al. 2002; Nakashima 2005; Kalyva et al. 2010).

Although in human teeth odontoblasts express all three isoforms of TGF-β (TGF β1, 2, and 3), only TGF-β1 becomes sequestrated within the matrix (Cassidy et al. 1997). It has been suggested that this may be due to the specific affinity of TGF-β1 with proteoglycans of dentine, decorine and biglycan (Baker et al. 2009). TGF-βs are synthesized as latent dimeric complexes consisting of the mature dimeric growth factor bound noncovalently to a latency associated peptide (LAP). This small latent complex (SLC) may be covalently cross-linked to the latent TGF-β binding protein (LTBP) through disulphide bonds, forming a large latent complex (Clark and Coker 1998). The growth factor is immobilised in dentine ECM via the binding of LTBP to other matrix proteins such as proteoglycans, glycoproteins and collagen. This is important for protecting the biological activity of growth factors which would otherwise have a very short half-life (Wakefield et al. 1990). The critical role of the TGF-β binding proteins in relation to odontogenesis has been demonstrated in mutation and genetic analysis

studies. It has been reported that mutation in the LTBP3 encoding gene results in oligodontia in humans (Noor et al. 2009). Furthermore, a phenotype involving amelogenesis imperfecta together with significant short stature in individuals affected by LTBP3 gene mutation has been reported (Huckert et al. 2015).

1.4.3.2 TGF-β/BMP signalling pathway

The TGF-β/BMP signalling pathway involves the ligand binding to type I and type II transmembrane serine/threonine kinase receptors. Different type I and type II receptors are involved for TGF-βs and BMPs (Janssens et al. 2005; Miyazono et al. 2005). There are a total of seven type I and four type II receptors for the TGF-β family of ligands, of which three of each type bind BMPs (Wang et al. 2014b).

Ligand binding induces formation of hetero-tetramers composed of two type II and two type I receptors, which allows the constitutively active type II receptor to induce phosphorylation of type I receptor. This in turn initiates intracellular signalling cascade through the SMAD pathway. The activated receptor complex phosphorylates the receptor-activated Smad proteins (R-SMADs). The R-SMADs dissociate from the type I receptor and binds Smad4 to form a complex. The activated Smad complexes then translocate into the nucleus where they regulate the transcription of target genes (Shi and Massague 2003). Although the majority of transcriptional responses induced by the TGF-β family are mediated through the Smad pathways, there are numerous other intracellular kinase cascades (non-SMAD) which are also regulated by TGF-β signalling. These include ERK, JNK, and p38 MAPK pathways which may link with the gene-expression programs controlled by Smads to generate an integrated response to TGF-β signals (Mu et al. 2012). Furthermore, there is evidence to suggest that the

TGF-β signalling interacts with the WNT (via Lef/Tcf transcription factors and β-catenin), Hedgehog (via Gli transcriptional regulators) and Hippo (via TAZ and YAP) pathway signalling and the two pathways may regulate one another synergistically or antagonistically (Ohazama et al. 2008; Ahn et al. 2010; Attisano and Wrana 2013). These complex interactions provide an interconnected signalling network within the cells which allow for contextual interpretation of the signals in diverse biological settings and provide the means for controlling different activities in response to the same morphogen (Wrana 2013; Kim et al. 2014b).

1.4.3.3 Vascular epithelial growth factor (VEGF)

The VEGF gene family consists of a few members including VEGF-A, -B, -C, -D and PlGF of which VEGF-A is the well-characterized member. VEGF-A is a homodimeric glycoprotein comprising two identical 23 kDa subunits. (Ferrara and Henzel 1989). VEGF-A exists as a number of alternatively spliced isoforms in humans of which $VEGF_{165}$ is the most abundant and mitogenic isoform (Ferrara et al. 2003). VEGF is expressed in most tissues and has a critical role in angiogenesis, vasculogenesis and lynphagenesis during embryonic and postnatal development in both physiological and pathological processes (Ho and Kuo 2007; Kajdaniuk et al. 2011). Several mechanisms are known to be involved in modulating the expression, availability, and activity of VEGF-A including hypoxia, oncogenes, transcription factors, inflammatory mediators, and mechanical forces applied on the cells (Ho and Kuo 2007). Only a fraction of the secreted VEGF protein becomes sequestered within the ECM, which may provide a reserve of available growth factor. Growth factors may also directly bind other matrix proteins. For example, the VEGF ability to bind matrix glycoproteins has been demonstrated (Wijelath, 2006). There is also evidence indicating that larger ECM

proteins, such as collagens may potentially be involved in immobilising and modulating the signalling factors activity in the mineralised tissues (Paralkar, 1990, 1991). Moreover, it has been shown that pro collagens II and IV contain binding sites for BMP and TGF-β consisting of chordin-like motifs (Paralkar 1991, Zhu 1999, Larrain 2000). The soluble bioactive peptide can be released through proteolysis by either plasmin or matrix metalloproteinases (Houck et al. 1992; Lee et al. 2005).

VEGF binds to tyrosine kinase receptors VEGFR-1 and VEGFR-2 with greater affinity for the later through which VEGF signals key angiogenic related activities in endothelical cells including permeability, proliferation, migration and survival. VEGFR-2 is expressed in other cell types, for example in osteoblasts, neurons, pancreatic duct cells and retinal progenitor cells, although at lower levels compared to vascular endothelial cells (Matsumoto and Claesson-Welsh 2001). Binding of VEGF to VEGFR-2 results in dimerization and autophosphorylation of the receptor, which in turn, activates various signalling cascades, for example the Ras-Raf-MEK-ERK and MAPK pathways for cell proliferation, PI3-Kinase and Akt/PKB activation for cell survival and either FAK and Paxillin, PI3Kinase/Akt, or MAPK activation for cell migration (Byrne et al. 2005).

In addition to its role during embryonic development, VEGF stimulates differentiation of mesenchymal stem cells (MSCs) to osteoblasts and represses adipocytes differentiation through which it plays a critical role in maintaining bone homeostasis after birth (Liu and Olsen 2014). The role of VEGF in odontogenesis has been investigated through animal studies. It has been demonstrated that inhibiting VEGF signalling can result in delays in developing replacement teeth in zebrafish (Crucke and

Huysseune 2015) and degeneration of odontoblasts and ameloblasts with decreased mineralization of dentine (Patyna et al. 2008; Fletcher et al. 2010). These problems were manifested clinically as discoloration and fracture of incisors in rats. Furthermore, the application of exogenous VEGF is shown to promote neovascularization in a tooth slice model transplanted into immunocompromised mice (Mullane et al. 2008). Based on this evidence, it may be suggested that the release of angiogenic growth factors from dentine matrix may play a role in promoting angiogenesis which would be crucial for the healing process in injury situations.

1.4.4 Serum/plasma proteins

Presence of blood derived proteins such as serum albumin, transferrin and the immunoglobulins (IgG, IgA and IgM) have been reported in dentine (Thomas and Leaver 1975; Okamura et al. 1979; Park et al. 2009). Considering the intimate proximity of odontoblasts with the dental pulp vasculature it is hardly surprising that such molecules have been identified in dentine extracellular matrix (ECM). There is little evidence on the possible role of these molecules on dental pulp tissue following their release. However, it seems possible that immunoglobulins present in dentine may play a role in modulating host response against cariogenic bacteria. Following mobilisation from the ECM and binding to the bacteria, immunoglobulins may be important in facilitating the clearance of bacteria by immune system cells (Smith et al. 2012a). In addition, other serum derived molecules, such as alpha2-macroglobulin, may be important in binding and stabilising growth factors such as TGF-β1 within dentine ECM (Arandjelovic et al. 2003).

1.5 Release of bioactive molecules from dentine matrix

In recent years investigators have demonstrated that certain dental materials are capable of releasing growth factors from dentine (Graham et al. 2006; Tomson et al. 2007; Ferracane et al. 2013). These bioactive molecules have the potential to signal the recruitment of undifferentiated dental pulp stem cells to differentiate to odontoblast-like cells and induce dentine repair and regeneration (Huang et al. 2006a; Galler et al. 2011; Pang et al. 2014).

Current evidence suggests that both acidic and basic as well as neutral chelating agents are capable of extracting ECM molecules from human dentine under routine conditions (Goldberg and Smith 2004). Acids have a demineralising effect on dentine such as occurs in dental caries. Cavity etchants used in adhesive restorative systems, result in localized demineralization and exposure of collagen fibrils. The effect of the etchants also locally releases soluble matrix-bound bioactive molecules, exposing them to the cells (Ferracane et al. 2013; Pang et al. 2014). In relation to an alkaline material, such as calcium hydroxide, it has been suggested that its interactions with mineralised tissue may involve protein solubilisation and degradation (Partridge and Elsden 1961), resulting in the release of proteins, close to the surface (Smith et al. 2011). With regard to a chelating agents such as EDTA, solubilisation of dentine is related to chelation of calcium ions, it is not clear however, if this results in simply releasing the matrix bound bioactive components or whether more complex mechanism are involved in the process (De-Deus et al. 2008).

As well as the possibility of growth factor release, other matrix components such as MMPs may also be released from dentine. Low pH conditions, for example, in the acidic

environment produced during caries process, can cause the release and activation of MMPs (Mazzoni et al. 2006; Mazzoni et al. 2013; Mazzoni et al. 2015).

1.5.1 Role of MMPs in dentine

MMPs are endogenous zinc and calcium ion dependent enzymes and are capable of degrading almost all ECM components (Visse and Nagase 2003). In humans, 23 members have been identified in the MMP family, which based on substrate specificity and homology are classified into 6 groups (Visse and Nagase 2003). Several MMPs (MMP-2, -8, -9, -3, and -20) have been reported to be present in dentine ECM and are believed to have collagenase, gelatinase and stromelysin activity in dentine (Sulkala et al. 2007; Boukpessi et al. 2008; Mazzoni et al. 2009; Santos et al. 2009; Mazzoni et al. 2011). MMPs are usually expressed as inactive proenzyme, and low pH conditions can cause a conformational change within their pro-domain resulting in its dissociation from the catalytic domain resulting in the activation of the enzyme (Hannas et al. 2007). The capacity of acidic pH ranging from 4.5 to 6 in activating saliva derived MMPs has been investigated *in vitro* (Sulkala et al. 2001) and highest level of activation was observed at the lowest pH investigated (4.5).

Current evidence suggests that in dental caries, following the dissolution of dentine minerals by bacterial acids and initial degradation of the ECM by bacterial proteases, the activity of host-derived MMPs can play a major role in further degradation of the matrix (Chaussain et al. 2013). Furthermore, there is evidence to show MMPs are released from dentine upon application of etching and acidic bonding agents and may play a detrimental role on the bond effectiveness via the breakdown of collagen in the hybrid layer (Tay et al. 2006; Mazzoni et al. 2013; Mazzoni et al. 2015). It has been

reported that acidic agents are more efficient in liberating the MMPs in comparison to the neutral EDTA (Mazzoni et al. 2007).

The activity of MMPs may be important in inducing a biological response in dentine-pulp complex facilitating a healing response (Caley et al. 2015) as enzymatic cleavage of large insoluble ECM components may result in the liberation of bioactive peptides from dentine matrix (Mott and Werb 2004; Chaussain-Miller et al. 2006). The liberated peptides are shown to retain their biological activity (Dean and Overall 2007) and may display diverse properties in terms of inducing cell proliferation, angiogenesis and differentiation which may ultimately favor the healing process in the pulp for example in caries (Chaussain et al. 2013) or subsequent to the application of acidic dental materials to dentine.

1.6 Dental caries pathology

Dental caries is a localised destruction of the tooth and is often described as a chronic disease caused by the metabolic activity of microbial communities within the biofilm, also known as plaque. Some studies have indicated that dental caries is a specific infection caused by mutans streptococci (Loesche 1986; Tanzer 1995) whereas others concluded dental caries does not meet the classic criteria of a specific infectious disease (Fejerskov 2004). The essential role of fermentable sugars in the diet as part of the disease process had been demonstrated by (Fitzgerald and Keyes 1960). The expansion of genes related to carbohydrate uptake and metabolism in the evolutionary advancement of S. mutans, has been essential in the success of the microorganism as a caries pathogen (Adler et al. 2013; Cornejo et al. 2013). Acid produced by bacteria following metabolism of sugars causes the pH of the biofilm to drop rapidly. The acidic

biofilm environment provides a competitive ecological advantage to the acidogenic and acid stress-tolerant S. mutans (Lemos and Burne 2008). Repeated acid attacks result in demineralisation of the enamel initially, and dentine subsequently. Caries is a dynamic process and progression of the lesion depends on degree of metabolic activity of plaque bacteria and host factors (Moye et al. 2014). Accordingly, lesions may be progressing rapidly, slowly or even become arrested (Pretty and Ellwood 2013). During disease activity while the enamel remains intact although demineralised, no serious microbial invasion takes place in the dentine and the bacterial invasion is associated with gradual break down of enamel (Ricketts et al. 2002). Once the structure of enamel breaks down and effective plaque control cannot be achieved, pH is kept low over much longer periods in the cavity than in microbial biofilms on the enamel surface (Fejerskov et al. 1992). It may not be possible to arrest the lesion at this stage and operative interference becomes necessary.

Host-derived MMPs originate both from saliva and dentine and may be activated by the acidic pH following lactate release from cariogenic bacteria (Tjaderhane et al. 1998). Once activated, MMPs remain functional and digest demineralized dentine matrix even after pH neutralization by salivary buffers (Chaussain-Miller et al. 2006).

1.6.1 The response of the dentine-pulp complex to caries

Dentine and pulp are inter-related via extension of odontoblast processes into dentine. Dentine therefore is considered a vital tissue. Exposure of dentine to the oral environment as a result of tooth wear or caries can elicit a response in the pulpo-dentinal organ (Bleicher 2014; Ganss et al. 2014). The first histological change in dentine related to slowly progressing caries is hypermineralisation in dentinal tubules. This cellular

driven defence can result in plugging of tubules by minerals, making the dentine less permeable (Charadram et al. 2013). The permeability of dentine adjacent to caries lesion has been shown to be only 14% of the unaffected dentine in the same age group (Tagami et al. 1992).

Inflammatory reactions in the pulp also have been observed in the early stages of caries process when the lesion is still in enamel (Brannstrom and Lind 1965). The rate of progression of caries can affect the histological appearance. In active caries when the demineralisation front reaches the amelodentinal junction (ADJ), the odontoblasts corresponding to the area change appearance and show a significantly lower cytoplasm: nucleus ratio (Bjorndal et al. 1998). Since bacteria are not able to penetrate through intact enamel, microbial invasion is related to breakdown of the enamel (Ricketts et al. 2002). At this stage cariogenic bacteria gain improved growth conditions and if the lesion is left untreated further tissue breakdown takes place. Pulp reaction to deep caries lesion may exhibit acute infiltrate comprising predominantly neutrophils admixed with lymphocytes and plasma cells or chronic inflammatory infiltrate composed of lymphocytes, plasma cells and macrophages together with proliferation of blood vessels and some degree of fibrosis (Gopinath and Anwar 2014). Mediators of the inflammatory response have been shown to be produced by odontoblasts at early stages of infection and fibroblasts, endothelial cells, pulp stem cells, and tissue resident immune cells at later stages (Barkhordar et al. 2002; Yang et al. 2003; Coil et al. 2004; Veerayutthwilai et al. 2007; Horst et al. 2011; Staquet et al. 2011).

The reaction of dentine pulp complex to caries is somewhat variable. Evidence suggests that regeneration may be triggered in relatively slowly progressing caries, with

reactionary dentine formation by primary odontoblasts (Bjorndal 2008). Whereas in rapidly progressing caries, primary odontoblasts may die and reparative dentine may be formed by odontoblast-like cells or chronic inflammation may ensue resulting in abrogation of regenerative processes altogether (Bjorndal 2008; Cooper et al. 2010). Progression of the caries lesion and pulp infection can eventually bring about irreversible inflammatory reactions and ultimately pulp necrosis (Zero et al. 2011). Similar to dentine, it has been suggested that low-level inflammation may be beneficial in stimulating bone metabolic activity and regeneration whereas chronic and/or severe inflammation may inhibit reparative processes completely (Thomas and Puleo 2011).

1.7 Dental pulp progenitor cells

Stem cells are broadly classified into embryonic and adult stem cells. Embryonic stem cells are pluripotent stem cells and have the capacity to differentiate into cells types from all three germ layers, ectoderm, mesoderm and endoderm (Rao and Zandstra 2005) while adult stem cells, derived from adult somatic tissues, may have the potential to differentiate into (several) specific cell types (Pittenger et al. 1999). Stem cells in an adult organism provide a reservoir for replenishing specialized cells in various tissues during growth and repair (Keating 2012).

In relation to dental pulps, mesenchymal stem cells have been isolated from human exfoliated deciduous teeth (SHEDs) (Miura et al. 2003) and permanent teeth (dental pulp stem cells/DPSCs) (Gronthos et al. 2000). Both categories share a common embryonic origin and are derived from the neural crest. They demonstrate generic properties of mesenchymal stem cell, including expression of marker genes and the ability to differentiate into various mesenchymal cell lineages (Volponi et al. 2010).

1.7.1 Stem cells from human exfoliated deciduous teeth (SHEDs)

It was reported by Miura et al that dental pulp of human deciduous teeth could potentially be a source of stem cells. Similar to DPSCs, SHEDs are multipotent cells derived from dental pulp explants and have been reported to have immunosuppressive properties (Miura et al. 2003).

The morphology of SHEDs, is similar to that of DPSCs. However, compared with DPSCs, SHED show higher proliferation rates and increased population doublings and can form spherical aggregations in culture and therefore are considered distinct from DPSCs. SHEDs express Oct4, CD13, CD29, CD44, CD73, CD90, CD105, CD146 and CD166, but not CD14, CD34, or CD45 (Huang et al. 2009; Pivoriuunas et al. 2010). SHEDs have been reported to be capable of differentiating into a variety of cells including neural cells, osteoblasts, chondrocytes, adipocytes and myocytes (Miura et al. 2003; Kerkis et al. 2006; Wang et al. 2010).

1.7.2 Dental pulp stem cells (DPSC)

DPSCs are multipotent cells that proliferate extensively in culture and can be cryopreserved retaining their multipotential differentiation ability (Papaccio et al. 2006). They express CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD146 and STRO-1, but the lack expression of CD14, CD24, CD34, CD45 and CD19 (Huang et al. 2006b; Lindroos et al. 2008; Karaoz et al. 2010). The ability of DPSCs to differentiate into odontoblast-like cells, osteoblasts, adipocytes, neural cells, chondrocytes and myocytes has been reported by various in vitro studies (Zhang et al. 2006; d'Aquino et al. 2007; Carinci et al. 2008; Arminan et al. 2009). The DPSCs represent less than 1% of the total cell population of dental pulp (Sloan and Waddington

2009) and are believed to reside within specialised microenvironments called "stem cell niche" which are formed at specific anatomic locations in the pulp. The stem cell niches are usually quiescent and become activated only after injury (Waddington et al. 2009). (Zhang et al. 2008).

So far, at least two different stem cell populations have been identified in adult dental pulps, one originating from the neural crest and the other of mesenchymal origin (Waddington et al. 2009). Both populations express STRO-1 and classical DPSC markers, as well as of Msx-1 and CD31. The cells with mesenchymal origin are reported to be strongly positive for β1-integrin which has strong affinity for fibronectin of the extracellular matrix in dental pulp whereas the neural crest derived cells show high expression of neural crest–associated low affinity nerve growth factor receptor (LANGFR^{+} cells). Both populations were reported to have the ability to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro* (Waddington et al. 2009). Using antibodies against STRO-1 and CD146, DPSC niches localised to the perivascular and perinural sheath regions within the pulp have been identified (Shi and Gronthos 2003). Furthermore, the progenitor/stem cell niches residing in the perivascular regions of the pulp, have been shown to migrate to the site of injury in studies monitoring *ex vivo* BrdU uptake by proliferative progenitor cells (Tecles et al. 2005). Using raised Notch expression levels following pulpal injury *in vivo* as a marker for identification of stem/progenitor cells, has identified niches in the pulpal stroma and subodontoblast layers, in addition to the previously identified perivascular region (Lovschall et al. 2005). However, a recent lineage tracing study in adult mice and embryos demonstrated that a significant population of mesenchymal stem cells during development, self-renewal and repair of mouse incisors originate from peripheral nerve-associated glia

(Kaukua et al. 2014). This differs from the previous speculations regarding the distinct locations of progenitors cells within the dental pulp.

DPSCs have been reported to differentiate into odontoblast-like cells and generate mineralized deposits after treatment with dentin matrix extracts and in the presence of mineralisation supplements *in vitro* (Liu et al. 2005). Furthermore, in vivo transplantation of DPSCs, within a tooth slice, in animals demonstrated the ability of these cells to generate dental tissue in the form of dentine/pulp-like complexes (Gronthos et al. 2000; Batouli et al. 2003; Iohara et al. 2004). Although, it is generally agreed that true dentinogenic events are not seen outside the dentino-pulpal environment, the search for factors determining the specificity of this environment will be important for our understanding of reparative dentinogenesis *in vivo* (Tziafas et al. 2000).

1.8 Vital pulp therapy techniques

Vital pulp therapy techniques exploit the dentine-pulp complex potential in regeneration to maintain pulp vitality and function, and avoid the more complex and costly root canal treatment or extraction (Tziafas 2004; Trope 2008). The current therapeutic approaches include indirect pulp treatment in deep dentinal cavities and direct pulp capping or pulpotomy in cases of pulp exposure. Successful outcomes in all vital pulp therapy approaches rely on an appropriate treatment strategy including: removal of the injurious challenge via operative procedure and control of infection, application of a capping biomaterial which should ideally stimulate a biological reaction leading to a specific dentinogenic response and finally, cavity restoration by a material which is capable of sealing the treated area and protecting it from bacterial microleakage (Tziafas et al.

2000). Although vital pulp treatment has been attempted and produced some encouraging results in symptomatic permanent teeth (with a diagnosis of irreversible pulpitis) in the short term (Asgary et al. 2014a), the treatment is normally recommended where the diagnosis of reversible pulpitis is established (Al-Hiyasat et al. 2006; Aguilar and Linsuwanont 2011).

The true status of the pulp in terms of severity of inflammation is most reliably determined by histological analysis, however the clinicians have to rely on clinical assessment methods to establish a diagnosis (Mejare et al. 2012). These methods include description of symptoms by the patient (when present), clinical examination including pulp sensibility testing and radiographic appearance. Evidence suggests that clinical and histological diagnoses of pulp conditions may be weakly related (Mejare et al. 2012). This can make it difficult for clinicians to distinguish between a treatable and non-treatable pulp inflammation, with obvious implications for teeth affected by deep caries or traumatic injuries. There is therefore a need for improved means to enable reliable diagnosis of pulp condition in a clinical setting (Ricucci et al. 2014).

1.8.1 Indirect pulp treatment

When removing caries in deep lesions the procedure where caries removal is stopped close to a vital pulp to avoid the risk of exposure, and the cavity covered with a biocompatible material, is termed an indirect pulp cap (AAoPD 2014). The importance of avoiding pulpal exposure in indirect pulpal treatment is related to the fact that restoring an exposed cavity is associated with a more traumatic pulp injury. Also success of the treatment associated with indirect pulp treatment does not rely as much on pulpal tissue repair response as with cases of direct pulp capping (Murray et al.

2002b). Various approaches have been described in the literature in relation to indirect pulp treatment. These approaches can be broadly divided into two categories: a) where the residual caries is sealed into the cavity and no further attempt is made to remove the caries at a later date and b) where caries is removed over two stages several months apart to allow pulp lay down reparative dentine (stepwise excavation technique).

A recent Cochrane review on the subject of caries management in primary and permanent teeth (Ricketts et al. 2013) has revealed that stepwise and partial excavation reduce the incidence of pulp exposure in symptomless, vital, carious teeth. The authors concluded that although there is no evidence of a difference in signs or symptoms of pulpal disease between stepwise excavation, and complete caries removal, and insufficient evidence of such difference between partial and complete caries removal, these techniques show clinical advantage over complete caries removal in the management of dentinal caries. With regard to the choice of a lining material in indirect pulp capping, it has been suggested that this may be less important for success than placement of a well-sealed restoration in the cavity (Hilton 2009).

1.8.2 Direct pulp capping and partial pulpotomy

Direct pulp capping procedure involves the application of a biocompatible material over the (mechanically or traumatically) exposed pulp before placing the restoration, to allow maintenance of pulp vitality and normal function (AAE 2013). Vital pulp therapy carries a higher rate of success in traumatically compared with cariously exposed teeth; in a study by (Al-Hiyasat et al. 2006) it was reported that after a follow up period of at least 3 years, success rate of direct pulp capping of traumatically exposed pulps was 92% compared with only 33% of carious exposures. It can be speculated that the

difference in the success rates is related to the status of the pulp at the time the interference was carried out. Caries penetration to the pulp will result in bacterial invasion resulting in severe pulpal inflammation (Bergenholtz and Lindhe 1975). This may leave the pulp less able to develop a healing response to treatment, compared to a mechanical exposure in which pre-existing inflammation is either non-existent or low-grade, which may be in favour of a reparative response (Cooper et al. 2010).

The lack of reliable clinical methods for assessing the extent of pulpal inflammation has resulted in some controversy in relation to the treatment of teeth affected by carious exposure (Aguilar and Linsuwanont 2011). Amongst the methods for preservation of vitality in a cariously exposed pulp, partial pulpotomy has yielded a reasonably high success rate in permanent teeth (Mejare and Cvek 1993; Qudeimat et al. 2007; Chailertvanitkul et al. 2014). The procedure involves the removal of a small portion of the coronal pulp (in a superficially inflamed pulp) and preserve the residual presumed "healthy" coronal and radicular pulp (Ghoddusi et al. 2014). Subsequent rinsing the surgical wound with 1.25-6% sodium hypochlorite solution is commonly recommended (Witherspoon et al. 2006; Bogen et al. 2008b).

Evidence provided from historical animal studies suggests that success in vital pulp exposure treatments relies significantly on the sealing ability of the restorative material against bacterial microleakage. It has been reported that the exposed pulps in germ free rats maintain vitality and exhibit similar dentine bridge formation against a wide variety of materials (Kakehashi et al. 1965). Other studies have supported the importance of maintaining a "bacteriometic" seal and prevention of bacterial microleakage for the success of pulp capping procedure in rats (Kozlov and Massler 1960; Rowe 1967). A

further study by Cox et al. (1996), investigated the outcome of direct pulp capping with zinc phosphate, silicate, amalgam and auto-cured composite in mechanically exposed dental pulps in monkeys. Some cavities were filled solely by the above mentioned materials and others only to the half way in the cavity and sealed over with zinc oxide eugenol to the cavosurface margin of the cavity to prevent microleakage. It was demonstrated that dentine bridge formation occurred regardless of the type of the material as long as the cavity was sealed on top. In interpreting the findings of these studies, it must be considered that the above animal studies have been carried out in caries free teeth, in highly controlled conditions, and may not reflect the clinical situation where teeth are caries affected or traumatised. In such clinical situations there is expected to be pre-existing bacterial contamination and ongoing inflammation, which may well alter the pulp response to the treatment. Exploitation of the biomaterials full potential in such circumstances, for inducing reparative processes in the dentine pulp complex, is expected to maximise the chance of clinical success in vital pulp treatments. Nevertheless, the findings from the above mentioned animal studies are valuable in highlighting an additional aspect of materials properties which may be important for the overall success of pulp capping treatment.

1.8.3 Pulp capping materials

Despite the ability of the pulp tissue to tolerate proximity or even contact with a variety of dental materials, it must be kept in mind that chemical stimuli may provoke a response in the pulp. In the past, investigation of materials interaction with the pulp mainly focused on biocompatibility of materials (Hanks et al. 1996). A number of materials-related adverse effects associated with direct pulpal contact with certain materials, including cytotoxicity and immune suppression have been reported. While

the former results in destruction of pulp cells, the latter can reduce the ability of the pulp to respond to bacterial invasion (Hilton 2009). In addition to the direct effect of a material, the methods used in conjunction with their application may have an effect on the pulp, for example light curing required for polymerization of resin based materials, may result in raising the intra-pulpal temperature to levels incompatible with pulp cell survival (Cox et al. 1987; Jontell et al. 1995; Pameijer and Stanley 1998; Hebling et al. 1999a).

Notwithstanding the importance of biocompatibility of materials for their successful application, more recently investigators have switched their focus on the specific cellular responses to the materials in an attempt to understanding how materials may contribute to regenerative processes in the tooth (Smith et al. 2008). Several clinically used materials, such as calcium hydroxide and mineral trioxide aggregate, in addition to acidic materials, such as cavity etchants and dentin bonding agents, are capable of releasing bioactive proteins, such as TGF-β1 (Zhao et al. 2000) and BMP2 (Casagrande et al. 2010) from dentine matrix. The released bioactive molecules have the ability to modulate gene expression in DPSCs resulting in odontoblast-like cell differentiation, which highlights the potential of materials in inducing reparative processes in dentine-pulp complex (Graham et al. 2006). The released molecules may directly stimulate the pre-existing primary odontoblasts to deposit reactionary dentine, or recruit the progenitor cells with their subsequent differentiation into odontoblast-like cells to produce new mineral through reparative dentinogenesis (Ferracane et al. 2010).

1.8.3.1 Calcium hydroxide

Since the introduction of calcium hydroxide by Hermann in 1930s for vital pulp therapy, it has been recommended by many authors as a multipurpose agent in various clinical applications (Modena et al. 2009). Some of its applications include direct and indirect pulp capping, apexogenesis, apexification and treatment of root resorption, iatrogenic root perforations, root fractures and intra-canal dressing between appointments (Farhad and Mohammadi 2005). Many studies still consider $Ca(OH)_2$ as a gold standard for comparison and evaluation of the newer biomaterials, recommended for similar applications (Sangwan et al. 2013).

Several mechanisms for the action of calcium hydroxide in inducing repair when used as a pulp capping agent has been proposed. With an alkaline pH of 12.5, $Ca(OH)_2$ possesses strong antimicrobial properties at which very few bacteria can thrive (Bystrom et al. 1985). Also, when in direct contact with dental pulp, calcium hydroxide has cytotoxic properties due to its alkaline pH and it also causes formation of a zone of coagulation necrosis which has been proposed to be important in initiating mineralisation process (Yoshiba et al. 1996). It has been observed histologically, in human teeth planned for orthodontic extractions, that the pulp sub-adjacent to the necrotic layer, on day 7 after direct pulp capping with $Ca(OH)_2$, was associated with severe inflammatory reaction and disorganised pulp tissue with dilated blood vessels. Dentine matrix appeared around 30 days later, concomitant with fading of the cellular and vascular inflammatory events and arrival of new cells with secretory potential at the site (Lu et al. 2008). It has been proposed that the Ca^{2+} gradient in the pulp tissue following its release from the applied $Ca(OH)_2$, may be capable of activating cells, for example stem cells (Adams et al. 2006) and fibroblasts (Lansdown 2002), therefore a

potential regulator in the healing process. Furthermore, raised concentration of Ca^{2+} induces fibronectin synthesis in the pulp cells (Mizuno and Banzai 2008), which is a glycoprotein involved in a wide array of cellular events such as adhesion, chemotaxis and cyto-differentiation (Yoshiba et al. 1994). Further to its potential direct role in regeneration, fibronectin is believed to possess the ability to bind TGF-β (Mooradian et al. 1989), which in turn may stimulate cytodifferentiation of odontoblasts. It has been proposed that further to the possibility of $Ca(OH)_2$ itself acting as a suitable substrate for mineralisation, it may react with the surrounding environment to facilitate formation of inorganic crystal structures at sites of direct application to the pulp (Faraco and Holland 2001). Furthermore, odontoblast-like cell differentiation of human pulp cells has also been demonstrated *in vitro* in the presence of crystals produced via the reaction of $Ca(OH)_2$ with the containing culture medium (Seux et al. 1991).

More recently the ability of calcium hydroxide in solubilizing the sequestrated regulating molecules from dentine matrix has been demonstrated *in vitro*, and it has been suggested that release of the cell signalling molecules from dentine by $Ca(OH)_2$ may be an important mechanisms through which it exerts its effect on dentine regeneration (Graham et al. 2006). In summary, it may be stated that a combination of non-specific actions providing an optimal environment for the pulp to best utilize its inherent healing capacity combined with specific pathways may be involved in the action of $Ca(OH)_2$ inducing tertiary dentinogenesis. There is a possibility that these may not be exclusive, but rather interrelated (Sangwan et al. 2013).

Despite its desirable ability in inducing dentine bridge formation, pulp capping with calcium hydroxide has the disadvantage of being an unpredictable method of treatment in permanent teeth. The predictability of the calcified hard tissue barrier formation after capping by calcium hydroxide has been challenged by evidence showing multiple tunnel defects and cell inclusions in the bridges (Kitasako et al. 2008). Another disadvantage is the inability of the material in providing a bacterial tight seal over the exposed pulp tissue which can be particularly troublesome when there is microleakage associated with the overlying restorative material. Furthermore, the lack of mechanical and chemical adhesiveness combined with potential accelerated degradation of the material after being acid etched during adhesive bonding process has reduced the popularity of calcium hydroxide as a pulp capping agent in modern dentistry (Cox et al. 1999; Silva et al. 2006).

1.8.3.2 Mineral trioxide aggregate

MTA was first introduced as a root-end filling material in 1993 and was subsequently recommended for other endodontic applications including pulp capping and pulpotomies (Parirokh and Torabinejad 2010a). The main soluble component from MTA has been shown to be calcium hydroxide but it also contains tricalcium silicate, tricalcium aluminate, calcium silicate, and tetracalcium aluminoferrite. Addition of bismuth oxide to MTA makes the material radio-opaque (Fridland and Rosado 2005; Islam et al. 2006).

The sealing ability of MTA as a coronal as well as apical barrier material has been tested *in vitro* by various methods including dye penetration, fluid filtration, protein leakage and bacterial leakage methods (Tselnik et al. 2004; Valois and Costa 2004; Jenkins et

al. 2006; Vogt et al. 2006; Zakizadeh et al. 2008). Evidence supports biocompatibility as well as excellent sealing ability of MTA, which makes it a suitable material for diverse clinical applications (Torabinejad and Parirokh 2010).

Clinical studies on MTA

Successful outcomes after clinical application of MTA as a pulp capping agent in mechanically and cariously exposed human pulps have been reported in many studies (Aeinehchi et al. 2003; Farsi et al. 2006; Iwamoto et al. 2006; Bogen et al. 2008b; Min et al. 2008; Nair et al. 2008; Sawicki et al. 2008) and MTA has been reported to be a suitable capping material in partial pulpotomy (Karabucak et al. 2005; Barrieshi-Nusair and Qudeimat 2006).

Application of MTA as capping agent in pulpotomy, in premolars planned for extraction for orthodontic reasons, revealed that the pulps capped with MTA showed significantly less inflammation and better dentine bridge formation compared with those capped with $Ca(OH)_2$ (Chacko and Kurikose 2006). A case series study of permanent molars with a clinical diagnosis of irreversible pulpitis and planned for extraction, revealed that application of MTA on pulpotomy sites subsequent to carious exposure resulted in complete dentine bridge formation 2 months after treatment in all of the treated teeth (Eghbal et al. 2009). A systematic review (Parirokh and Torabinejad 2010b) on clinical applications of MTA proposed MTA as the material of choice for pulp capping in permanent teeth. Subsequent to this review, results of randomised clinical trials and long term cohort studies in humans, further supported superior outcomes for MTA compared to $Ca(OH)_2$ when applied as a pulp capping agent in vital pulp treatment of permanent teeth (Eskandarizadeh et al. 2011; Hilton et al. 2013; Mente et al. 2014).

With regard to application in primary dentition, histologically, MTA is shown to induce a thick dentine bridge at pulp amputation and MTA application site which reflects its bio-regenerative properties (Maroto et al. 2006; Zarrabi et al. 2011). Furthermore, in a recent systematic review and meta-analysis, MTA demonstrated superior long-term treatment outcomes compared with ferric sulphate following pulpotomy in cariously exposed vital primary molars (Asgary et al. 2014b). Also, a superior outcome for MTA compared with formocresol also has been reported in vital pulpotomy of primary molars after 1 year follow up (Olatosi et al. 2015). The authors concluded that MTA has the potential to become a replacement for the traditional materials in primary molars pulpotomy.

Despite its desirable properties in providing an effective seal and inducing repair, MTA suffers a few draw backs including a long setting time, high cost, and potential of discoloration of teeth (Parirokh and Torabinejad 2010a). Furthermore, similar to $Ca(OH)_2$, the material does not bond to dentine and its application underneath adhesive restorations carries the disadvantage of reducing the bond area to dentine in relation to adhesive restorations.

1.8.3.3 Glass ionomer and resin-modifed glass ionomer (RM GIC)

Glass ionomer and resin modified glass ionomer are cytotoxic in direct contact with cells. However due to the materials ability to chemically bond to tooth structure, it is considered as a suitable bacterial tight lining in deep cavities where the remaining dentine layer can prevent the material contacting the pulp (Heys and Fitzgerald 1991; Murray et al. 2002a; Costa et al. 2003).

A study of mechanically exposed healthy human premolars, scheduled for orthodontic extractions, reported poor outcome following direct pulp capping with RMGIC (do Nascimento et al. 2000). Chronic inflammation and lack of dentine bridge formation up to 300 days post-pulp capping was reported compared with complete pulpal healing and dentine bridge formatioon in calcium hydroxide control group. When using RMGICs to line deep cavities however, a mild initial inflammation and pulp tissue disorganising, which decreased with time was observed, indicating acceptable biocompatibility in such circumstance (Costa et al. 2011).

1.8.3.4 Adhesive restorative materials

Concern over the toxicity of dental amalgam in recent years combined with increased patients demand for more aesthetic restorations has led to the increased popularity of resin composite restorations. Resin composites are also very popular amongst dental professionals due to their less invasive cavity preparation requirements compared to dental amalgam. Since resin composites bond to the adjacent tooth substance, the need for creating mechanical undercuts by removal of sound tooth substance is significantly reduced. Also while dental amalgam requires a minimum thickness of 2mm irrespective of the extent of caries to achieve sufficient compressive strength, the same limitation does not always apply to resin composite (Hamburger et al. 2014; Johnson et al. 2014).

The use of adhesive restorative systems as potential direct pulp capping agents has been suggested in recent years. Some authors have recommended the application of adhesive systems as pulp capping materials in spite of the manufacturer's instruction to the contrary (Costa et al. 2000). Adhesive resins are shown to be cytotoxic to odontoblast-

like cells in vitro. Toxicity was seen in both multi- and single-component adhesive systems, and the unpolymerized components were shown to be more toxic than when the fully polymerized material (Costa et al. 1999). It is important to consider that complete polymerization of adhesive resins may not be achievable in direct pulp capping of oedematous pulps also due to oxygen inhibition of the polymerization process (Modena et al. 2009).

In vivo application of an adhesive resin directly onto a site of pulp exposure, or to a thin layer of dentine (less than 0.5 mm), has been shown to cause vascular reactions as well as chronic inflammatory pulpal response in premolars scheduled for orthodontic extractions (Hebling et al. 1999b, a). Although there are reports of encouraging results in the mechanical, non-contaminated, pulp exposures capped with adhesive systems in experimental animal studies (Cox et al. 1998; Kitasako et al. 1998; Fujitani et al. 2002), the outcome is different in studies of bacteria contaminated mechanical pulp exposures in primates. These experimental regimens which more closely resemble a clinical situation of a contaminated pulp exposure, have shown a significantly poorer pulp healing when using adhesive agents for pulp capping compared with calcium hydroxide (Pitt Ford and Roberts 1991; Pameijer and Stanley 1998).

Clinical application of resin-based materials as a liner in deep cavities in close proximity, or in contact, with the pulp is still controversial and not well documented. In the absence of sufficient evidence, the introduction of a new clinical procedure should be preceded by a careful assessment of the potential risks and benefits. Currently, there are several potential problems associated the above mentioned procedure. Resin composite may release substances that are potentially harmful to the pulp and may

interfere with the pulp potential to resist a bacterial challenge (Bergenholtz 2000). Furthermore, the risk for bacterial leakage in gaps along restoration margins is still considered a major threat. Such leakage is likely to induce or aggravate the pre-existing existing inflammatory response in the pulp (Modena et al. 2009). Due to a lack of sufficient clinical data at present, caution should be exercised when considering resin composites for direct restoration of deep cavities.

1.8.4 Current mechanisms of bonding

The fundamental mechanism of bonding to enamel and dentine involves an exchange process between the minerals removed from the tooth tissue and the components of the adhesive system (De Munck et al. 2005; Cardoso et al. 2011). Bonding to enamel involves penetration of the photo-polymerisable bonding agent to the pits that have been created by the acid etching process. The polymerised resin tags provide micromechanical retention between the enamel and composite restoration overlying the adhesive bonding agent. Bonding to enamel is reliable and less technique sensitive compared to dentine (Van Meerbeek et al. 2003).

When bonding to dentine, acid etching removes the mineral contents and uncovers the dentine collagen fibrils as a network for penetration of the adhesive bonding agent (Van Meerbeek et al. 2001; Erickson et al. 2009; Manuja et al. 2012).

Depending on the material used and the number of clinical application steps, three mechanisms are currently available for bonding adhesive restorative materials onto enamel and dentine (Van Meerbeek et al. 2005). The first two mechanisms are used in conjunction with composite resins:

1) Etch and rinse approach, where the tooth is first conditioned (etched) with 30-40% phosphoric acid and rinsed off followed by application of the primer and adhesive resin. Conventionally, this approach was applied as a three step process. To simplify and shorten the process, a two-step etch and rinse approach was later introduced combining the primer and the adhesive resin into one application.

2) Self-etch adhesives use a non-rinse acidic monomer which conditions and primes the dentine in one application step. They are available in a two and one step (all in one) applications. The two step application consists of a self-etching primer and an adhesive resin in separate bottles. Over the years there has been a tendency towards the more user friendly systems with shorter and simplified application. However, some simplified systems seem to have resulted in loss of bond effectiveness as evident by inferior clinical performance of the restoration (Peumans et al. 2005). It is widely acknowledged that three-step etch and rinse and two-step self-etch systems produce more superior outcomes (Tjaderhane 2015).

3) Glass-ionomers which are considered as the only self-adhering material to the tooth substance. Use of a weak polyalkenoic acid primer is recommended with this approach to clean the surface and remove the smear layer and expose the collagen fibrils up to 0.5-1 μm depth (Inoue et al. 2001). The bond with the tooth structure is then established via diffusion of glass-ionomer components and formation of micromechanical bonds. Chemical bonding is also established by ionic interaction of the carboxyl groups of the polyalkenoic acid and calcium of hydroxyapatite remaining attached to the collagen fibrils (Yoshida et al. 2000).

The major problem associated with early composites, the volumetric shrinkage and associated microleakage, has been significantly overcome by developments in filler

technology and bonding systems. Despite these advances, the occurrence of microleakage at dentine- restoration interface is still considered a significant risk associated with composite restorations (Samimi et al. 2012; Sooraparaju et al. 2014). This is due to the fact that bonding to dentine while predictable, is technique sensitive which may lead to problems with sensitivity/ new lesion of caries. Concerns have been expressed regarding the role of MMPs within dentine matrix and their activation by the acidic bonding agents which may contribute to degradation of the hybrid layer (Mazzoni et al. 2015). In an attempt to improve durability of dentine bonding, approaches such as application of a hydrophobic adhesive resin after the hydrophilic primer and more recently application of chlorhexidine to inhibit dentine collagenolytic enzymes have been advocated (Breschi et al. 2008; Tjaderhane et al. 2013).

1.9 Managing operatively exposed dentine

Dental practitioners face dilemmas when managing operatively exposed dentine. These include whether to 'bond' a composite restoration or to 'base' it with glass-ionomer cement. A systematic review (Peumans et al. 2005) on the clinical effectiveness of contemporary adhesives revealed that amongst the contemporary three mechanisms of adhesion glass-ionomers most effectively and durably bond to tooth tissue.

Using a glass-ionomer base under resin composite restorations in deep cavities may offer the advantage of preventing bacterial leakage along the tooth-base interface (Giorgi et al. 2014; Karaman and Ozgunaltay 2014). However, the use of dentine bonding agents instead of a base of glass ionomer simplifies placement of the restoration and may enhances the biomechanics of the restoration due to the increased thickness of the restorative material (Lynch 2008). Information from surveys of dental school

programmes and clinical practice suggest that in relation to indications for resin composite restorations as well as bonding and basing techniques conflicting approaches are often employed, and some without any obvious evidence base (Lynch et al. 2007; Gilmour et al. 2009).

Recent attention has begun to focus on the development of materials which may facilitate or even promote natural dental tissue regenerative response (Ferracane et al. 2010). Clearly if certain materials/techniques offer an improved regenerative response compared to others, then their use should be preferred as they would be of benefit in generating new natural tissue, to maintain pulpal vitality/ viability and avoid future damage. However, more research is needed to provide sound biological evidence to justify introducing new protocols.

The present study hypothesised that growth factors, and other bioactive molecules within dentine, may be released by the action of currently used dentine bonding systems/ materials and signal regenerative processes within the dentine-pulp complex. The aim of the study was to characterize the effects of selected currently used dentine conditioning/ etching agents on the cells of the dentine-pulp complex employing histological, immunohistochemical and molecular biology techniques and human pulpal primary cell lines. A better understanding of the reparative and regenerative control in these cells will enable optimisation of the biological efficacy of such materials and development of novel tissue engineering approaches for dental disease treatment. Such translational research is at the forefront of basic scientific/ clinical interface and may greatly improve current treatment strategies for restorative dentistry, reducing the population morbidity and rates of disease incidence.

Chapter 2: Solubilisation of Dentine Matrix Proteins by Dentine Conditioning Agents

2.1 Introduction

Dentine is a complex mineralised tissue, arranged in an elaborate three dimensional structure, with dentinal tubules extending from the pulp to amelo-dentinal junction in the crown. The dentine tubular structure and the close proximity of odontoblasts cellular processes with dentine matrix, provides opportunities for communication between the cells and the ECM (Larmas 2001; Smith et al. 2001a). With the absence of turnover in dentine, dentine matrix acts as a reservoir of bio-active molecules which have been sequestrated during dentinogenesis, and play a subsequent role in regulating dental tissue repair processes much later in life (Smith et al. 2012a). Bioactive proteins and growth factors have a short half-life and to maintain their biological activity and prolong their life span as well as for their controlled release, they are bound to other extracellular matrix components in dentine. Examples of possible growth factor binding components are proteoglycans, mainly heparin sulphate (Dreyfuss et al. 2009), glycoproteins (Rahman et al. 2005), collagen (Somasundaram et al. 2000) and specific binding proteins (Arai et al. 1996; Hyytiainen et al. 2004).

Current evidence suggests that a wide range of clinically used materials including $Ca(OH)_2$, mineral trioxide aggregate (MTA), EDTA and self-etching adhesive primers have the ability to release sequestrated bioactive molecules from powdered dentine (Graham et al. 2006; Tomson et al. 2007; Ferracane et al. 2013). Furthermore, EDTA solubilised DMEs are shown to stimulate odontoblast-like cell differentiation and reactionary dentinogenesis *in vivo* in animals (Tziafas et al. 1995; Smith et al. 2001b)

and DPSC differentiation and dentine formation *ex vivo* (Chun et al. 2011), which would be regarded as desirable outcomes in clinical vital pulp therapy treatments in humans.

In recent times, aesthetic adhesive restorations and bonded ceramic restorations have gained popularity. The conditioning/etching agents used in conjunction with adhesive restorative materials, remove the smear layer (in etch and rinse composite systems and glass ionomer) and demineralise dentine at sites of application. This effect is necessary for effective bond formation between glass ionomer and dentine. It is also important for the penetration of adhesive bonding agents (resins) used in conjunction with composite restorations which form a hybrid layer between the resin and dentine (Pashley 2003; Raggio et al. 2010).

Solubilisation and release of dentine matrix components by conditioning/ etching agents may extend their property to eliciting a physiological response in dentine-pulp complex and beyond a mere physicochemical impact for which they had been originally developed (Galler et al. 2015). Amongst the growth factors present in the extracellular matrix of dentine, TGF-β1 and BMPs are believed to play important roles in tooth morphogenesis and odontoblast differentiation, and VEGF regulates blood vessels growth during embryogenesis (Begue-Kirn et al. 1994; Aberg et al. 1997; Zhang et al. 2011b; Yang et al. 2012a; Arany et al. 2014). These growth factors are believed to play crucial roles in wound healing and repair processes also (Zhang et al. 2011b; Pakyari et al. 2013; Alaee et al. 2014).

The aim of this chapter was to investigate the ability of several clinically used dentine conditioning and etching agents in solubilising three important growth factors, TGF-β1, BMP2 and VEGF from human powdered dentine. The agents used were as follows:

- EDTA (ethylenediaminetetraacetic acid) - a dentine conditioning agent used for the removal of the smear layer in endodontics (served as control here),

- phosphoric acid- an etching agent used with "etch and rinse" resin composites,

- citric acid

- polyacrylic acid

  with the latter two being examples of dentine conditioning agents used for smear layer removal in conjunction with composite resin and glass ionomer adhesive systems respectively.

2.2 Materials and Methods

2.2.1 Isolation of dentine matrix extracts (DMEs)

Thirty caries free human molar teeth were collected immediately post extraction from the Oral Surgery clinics in Cardiff Dental Hospital, in accordance with ethical approval from the South East Wales Research Ethics Committee of the National Research Ethics Service (NRES), and patients' consent (07/WSE04/84, Wales REC1, Appendix 1). Teeth were cut into 5 mm thick slices using a segmented diamond edged rotary saw and enamel and cementum removed using a dental turbine with water coolant. Following the removal of pulpal tissue, the remaining dentine was crushed to a fine powder using a 6750 Freezer/Mill$^{®}$ (SPEX SamplePrep LLC, USA) cooled with liquid nitrogen. Dentine powder was weighed and equal amounts of 4g were placed in universal tubes (Starstedt, Leicester, UK). Dentine matrix proteins were extracted from powdered dentine using the etching and conditioning agents in commercially applicable concentrations as follow: 10% w/v EDTA (Fisher scientific, Loughborough, UK) pH adjusted to 7.2, 37% (v/v) phosphoric acid (VWR International, Lutterworth, UK) pH <1, 10% (w/v) citric acid (Fisher Scientific, Loughborough, UK) pH 1.5 and 25% (v/v) polyacrylic acid (Fischer Scientific) pH 3.9. One complete protease inhibitor cocktail tablet (Roche, Hertfordshire, UK) was added per 50ml of each solution as per manufacturers' instruction. Extractions were performed for 14 days at 4°C, with constant agitation, with the extraction solution changed daily. The recovered extracts were pooled for each solution and stored at 4°C, over the 14-day period. The pooled extracts were dialysed exhaustively for 10 days, in dialysis tubes (Sigma-Aldrich, Dorset, UK) placed in buckets filled with distilled water. The buckets were emptied on alternate days and distilled water was replaced.

Contents of the dialysis tubs were stored at -20ºC. Once completely frozen, lyophilisation using a Modulyo freeze dryer (Edwards, Crawley, UK) followed. Freeze dried dentine matrix extracts were stored at -20°C prior to use.

2.2.2 Quantification of protein in samples

Concentration of protein in each sample was quantified using a Pierce™ BCA (bicinchoninic acid) assay kit (Life Technologies, Paisley, UK). The kit provides BSA (bovine serum albumin) standards in a known concentration ranging from 25µg/ml to 2000µg/ml. Distilled water (assay diluent) placed in triplicate wells served as blank. 25µl of the standards were placed in triplicate wells of a 96 well plate. 25µl of each sample made up by solubilising DMEs in distilled water to a concentration of 1mg/ml was also placed in triplicate wells of the same plate. Working BCA reagents were prepared by mixing 50 parts of a sodium bicinchoninate solution (Reagent A) with 1 part of a cupric sulphate solution (Reagent B). 200 µl of the resulting bicinchoninate/cupric solution was added to each well containing either the standard or the sample and mixed thoroughly on a plate shaker for 30 seconds prior to being incubated at 37°C. Absorbance was read by FLUOstar OPTIMA (BMG Labtech, USA) at 570nm.

A standard curve was generated by plotting the concentration against absorbency for the standards on Excel, based on a best fit line, which allowed the protein concentration for the samples to be calculated. Regression analysis was used to assess the linearity of the best fit line which was found to have R^2 (coefficient of determination) >0.9, indicating linearity.

2.2.3 Protein separation using polyacryamide gel electrophoresis

Thirty micrograms (for gels to be silver stained) and one hundred and fifty micrograms (for gels to be electroblotted) of DMEs were solubilised in 22.5μl of the sample loading buffer, which contained 62.5 mM Tris HCl (pH 6.8), 2% SDS (w/v) (Fisher Scientific), 10% glycerol (v/v), 0.5% (w/v) bromophenol blue and 2.5% (v/v) 2-β-mercaptoethanol (all Sigma-Aldrich) in double distilled water. Samples were heated at 95°C for 5 min prior to loading 17μl onto pre-cast polyacrylamide 4-15% Mini-PROTEAN® TGX ^{TM} gels (BIO-RAD, Hemel Hempstead, UK). Gels were run in the SDS-PAGE running buffer containing 3.03% (w/v) Tris HCl, 14.4% (w/v) glycine and 1% (w/v) SDS in double distilled water, pH adjusted to 8.3 in an electrophoresis tank (BIO-RAD) at 200V, 400 mA for 35 min.

2.2.4 Visualisation of the separated proteins

The Color Silver Stain Kit (Pierce, Life technologies) was used to help visualise and compare the profiles of separated proteins in DMEs solubilized by various agents. After electrophoresis, the gel was fixed in 20ml fixative buffer containing 50% ethanol and 5% acetic acid in distilled water for 2 hr with frequent changes of the buffer. Gels were then washed four times with ultrapure (deionised and distilled) water for 40 min each time. The Silver Working Solution, Reducer Aldehyde Working Solution, Reducer Base Working Solution and Stabilizer Working Solution were prepared by diluting the ready-made reagents in water as per manufacturer's instructions. The gel was incubated in the Silver Working Solution for 30 min followed by water rinse for 20 sec. Equal volumes of Reducer Aldehyde Working Solution and Reducer Base Working Solution were combined immediately before use to make up the Reducer Working Solution in which the gel was incubated for 5 min. The gel was rinsed with

water for 5 sec and incubation in Stabilizer Working Solution for 40 min followed. The gel was visualised using Gel Doc (Bio-Rad Laboratories, UK) and digital images recorded.

2.2.5 Protein electroblot

Proteins were transferred to an Amersham Hybond^{TM}-P membrane 0.45μm minimal pore size (GE Healthcare Life Sciences, Buckinghamshire, UK). The membrane was soaked in 10ml methanol for 5 min, washed in distilled water for 5 min and stored in the protein transfer buffer, containing 0.145% Tris HCl (w/v), 0.72% glycine (w/v) and 20% methanol (v/v), pH adjusted to 8.3, until running the gels were ready for transfer. A Trans-Blot$^®$ SD semi-dry transfer cell (BIO-RAD) was used for the electroblot. Two pieces of extra thick blot paper (BIO-RAD) were soaked in the transfer buffer. The prepared Hybond membrane was placed on the soaked blot paper with the gel atop followed by the other piece of blot paper. Transfer was carried out in the transfer cell at 15 V for 30 min. At completion, the membrane was recovered for protein detection.

2.2.6 Protein detection

The blotting membrane was placed in a plastic plate and blocked for 1 hr at room temperature, with constant agitation, in blocking solution containing 5% (w/v) skimmed milk (ASDA's) and 0.05% (v/v) Tween 20 in TBS, pH adjusted to 7.6. Primary antibodies (Table 2.1) were diluted in the blocking solution and incubated with the membrane at room temperature for 1 hr with agitation and subsequently washed 3 times for a total duration of 40 min with wash buffer containing 0.05% Tween 20 in TBS. Horse radish peroxidase (HRP) conjugated secondary antibodies

(Table 2.1) were diluted in the blocking solution and incubated with the membrane for 1 hr at room temperature with agitation and the membrane was subsequently washed 3 time with wash buffer for 40 min.

Finally, a Western blotting detection reagent (Amersham^{TM} ECL^{TM} Prime, GE Healthcare Healthcare Life Sciences) was made up by mixing solutions A and B in equal volumes of 3ml. The membrane was incubated in the reagent for 3 min and chemoluminescence was subsequently detected by exposing the membrane to Hyperfilm ECL (GE Healthcare Healthcare Life Sciences). The film was developed in a Curix 60 auto-developer (AGFA, Mortsel, Belgium) in which it passed automatically through a developer (AGFA) and fixative (AGFA). The developed films were then scanned and digital images obtained.

Blocking peptide for the primary antibodies to TGF-β1, VEGF and BMP2 were used to produce negative controls (Table 2.1). Primary antibodies were incubated in blocking solution with 10× excess of blocking peptide for 30 min at room temperature with agitation and used as per detection protocol explained above.

Primary antibody	Antibody source	1° isotype and dilution used	HRP conjugated 2° and dilution used
Anti- human TGF-β1 (V)	Santa Cruz Biotechnology, Inc., CA, USA	IgG, Rabbit polyclonal; 1:100 SC-146	Goat anti-rabbit IgG-HRP; 1:3000 SC-2004
Anti- human VEGF (A-20)	Santa Cruz Biotechnology, Inc., CA, USA	IgG, Rabbit polyclonal; 1:100 SC-152	Goat anti-rabbit IgG-HRP; 1:3000 SC-2004
Anti- human BMP2 (N-14)	Santa Cruz Biotechnology, Inc., CA, USA	IgG, Goat Polyclonal; 1:300 SC-6895	Rabbit anti- goat IgG-HRP; 1:10000 SC-2768
TGF-β1 Blocking peptide	Santa Cruz Biotechnology, Inc., CA, USA	n/a	Goat anti-rabbit IgG-HRP; 1:3000 SC-2004
VEGF Blocking peptide	Santa Cruz Biotechnology, Inc., CA, USA	n/a	Goat anti-rabbit IgG-HRP; 1:3000 SC-2004
BMP2 Blocking peptide	Santa Cruz Biotechnology, Inc., CA, USA	n/a	Rabbit anti- goat IgG-HRP; 1:10000 SC-2768

Table 2.1: Antibodies and blocking peptides used in Western blots and their dilutions.

2.2.7 Identification of the extracted proteins using 2-D gel electrophoresis and mass spectrometry

2.2.7.1 Sample clean-up procedure

In order to purify the protein sample, remove any interfering contaminants and improve the 2-D gel results, prior to loading the DME sample onto the gels, it was pre-treated using the 2-D Clean-Up Kit (GE Healthcare Healthcare Life Sciences). 100µg EDTA extracted DMEs were solubilised in 100µl distilled water in an eppendorf (Fisher Scientific) and the clean-up procedure was followed. 300µl precipitant was added to the sample and vortex mixed prior to being incubated on ice for 15 min. 300µl of co-precipitant was added to the solution and vortex mixed briefly. The mix was then centrifuged at 12000×g for 5 min and the supernatants were discarded. A further 40µl of co-precipitant was added on top of the sample pellet prior to incubation on ice for 5 min followed by centrifugation at 12000×g for 5 min. The liquid was discarded and 25µl distilled water was added and the pellet dispersed in the liquid by vortex mixing. 1 ml chilled wash buffer and 5µl of wash additive was added to the tube and incubated in -20ºC subsequently with removal from the freezer every 10 min for vortex mixing over a 30 min period. The solution was centrifuged for 5 min again and the liquid discarded. The pellet was resuspended in 130µl lysis/rehydration buffer which contained 7M urea (Simga-Aldrich), 2M thiourea (Sigma-Aldrich) and 2% (w/v) cholamidopropyl dimethylamino-1-propane sulfonate (CHAPS) (Sigma-Aldrich) in distilled water. The cleaned-up sample was centrifuged one last time at 12000×g for 5 min to separate off any insoluble material. The supernatants (protein sample) were transferred to another tube to be used in the next step.

2.2.7.2 Isoelectric focusing

116µl of the above protein sample was mixed with 1 µl of 1% (w/v) bromophenol blue, 6.25 µl of 50 mM dithiothreitol (DTT) (both Sigma-Aldrich) and 2µl IPG buffer (GE Healthcare Life Sciences). The resulting 125µl sample-rehydration solution was then evenly distributed into a special strip holder (Amersham plc, UK) over its 7cm length. After gently pulling off the cover coil of a 7cm Immobiline Drystrip pH 3-10 NL gradient gel (GE Healthcare Life Sciences), the gel was placed in the holder on top of the sample-rehydration solution, with the gel side down and avoiding any bubble formation. Care was taken to avoid getting any solution on top side of the strip. The sample and the strip were then covered with DryStrip Cover fluid (GE Healthcare Life Sciences) subsequently and the lid of the strip holder was shut and isoelectric focusing was carried out at 20ºC with an Ettan IPGphor (Amersham plc, UK). After rehydration which took 12 hr in the machine, proteins were focused in five steps: 500 V for 1 hr, gradient 1000 V for 2 hr, 1000V 1 hr, gradient 8000 V for 2 hr and 8000V for 8 hr.

2.2.7.3 SDS-PAGE

Following completion of isoelectric focusing, the strip was incubated for 15 min in 5ml reducing solution containing 4.5ml 1× NuPAGE LDS sample buffer (Life Technologies) and 0.5ml 10× NuPAGE LDS sample reducing agent (Life Technologies). The strip was then taken out of the reducing solution and gently placed sideways on absorbent tissue to blot off excess liquid and subsequently immersed in alkylating solution containing 116mg of iodoacetamide in 5ml 1× NuPAGE LDS sample buffer (Life Technologies) for a further 15 min. The strip was then carefully removed from the solution with tweezers and gently placed sideways on absorbent

paper to remove any excess liquid and was subsequently transferred to the well of NuPAGE 4-12 % Bis-Tris Zoom Gel (1mm) (Life Technologies) for electrophoresis. The Mark12 Unstained Standard (Invitrogen, Life Technologies) was used for band identification. The running buffer was made up by adding 40ml ×20 MOPS to 760ml deionised water. The gel was run in an electrophoresis tank (Life Technologies) at 200V and 120mA for 50 min.

The gel was silver stained subsequently using the PlusOne Silver Staining Kit (GE Healthcare Healthcare Life Sciences) and following their recommended methods as shown in table 2.2. At completion of staining, the gel was stored in the cold room ready for the next step. The gel was visualised using Gel Doc (Bio-Rad) and digital images recorded.

Step		Solution	Volume	Time
1	Fixing	10 % acetic acid; 40 % ethanol; Water to 250ml	100 ml	30 min
2	Sensitizing	75 ml ethanol; 17 g Na-acetate; 10 ml Na-thiosulphate (5 % (w/v) stock). No gluteraldehyde	100 ml	30 min
3	Washing	Distilled water	100 ml	5 min
4	Washing	Distilled water	100 ml	5 min
5	Washing	Distilled water	100 ml	5 min
6	Silver solution	25 ml silver nitrate (2.5 % w/v stock) Water to 250 ml	100 ml	20 min
7	Washing	Distilled water	100 ml	1 min
8	Washing	Distilled water	100 ml	1 min
9	Developing	6.25 g Sodium carbonate; 0.1 ml formaldehyde (37% w/v stock)	100 ml	Up to 4 min
10	Stopping	3.65 g EDTA Water to 250 ml	100 ml	10 min
11	Washing	Distilled water	100 ml	5 min
12	Washing	Distilled water	100 ml	5 min
13	Washing	Distilled water	100 ml	5 min
14	Preserve	5 ml acetic acid Water to 500 ml	100 ml	-

Table 2.2: Details of methodology and reagents used for silver staining 2-D gel.

2.2.7.4 In-gel digestion and mass spectrometry

In order to prepare samples for proteomic analysis, protein plugs of 1.5 mm diameter were manually excised from the silver stained 2-D gels using a Spot Picker OneTouch Plus (Web Scientific Ltd, Cheshire, UK) and placed in microtiter 96 well plates (Greiner Bio-One, Stonehouse, Gloucestershire, UK). Sample preparation protocol was as follow; each plug was covered with 50μl acetonitrile MS grade (Sigma-Aldrich) for 5 min to dehydrate the gel and the liquid removed before adding the de-staining solution. The de-staining solution contained 1vol ratio of freshly made 30 mM potassium hexacyanoferrate (Sigma-Aldrich) and 100 mM sodium thiosulphate (Fisher Scientific, Loughborough, UK). 50μl of the above solution was added to each well containing a gel plug and the plate was covered and placed on a shaker for 1 hr. The liquid was removed before washing the de-stained gel, in 50μl of 25mM ammonium bicarbonate (Sigma-Aldrich), 3 times. For dehydration, the plug was then covered with 50μl of acetonitrile (Sigma-Aldrich) for 15 min for dehydration. The supernatants were removed before covering the gel with 50μl of 25mM ammonium bicarbonate for 10 min to rehydrate the gel. After removal of the supernatants a final dehydration step was carried out by incubating the gel plug in 50μl of acetonitrile. Dehydration was completed by incubating the plates in an oven at 60-65°C for 20 min without lid or PCR adhesive cover. The gels were removed from the oven and allowed to cool down for 10 mins at room temperature. For in-gel reduction of proteins, a solution containing 25μl 10mM dithiothreitol (Sigma-Aldrich) in 25mM ammonium bicarbonate was added to each well and the plate incubated in the oven at 56 °C for 1 hr. The plated were removed from the oven and allowed to cool for 15 min at room temperature. Any residual liquid was removed from the wells and discarded.

For in-gel alkylation of proteins, 25µl 55mM iodoacetamide (GE Healthcare Life Sciences) in 25mM ammonium bicarbonate was added to each well, plates covered with plate lid and incubated for 45 min at room temperature, in the dark. The liquid was discarded and the gel washed with 25mM ammonium bicarbonate and the liquid discarded subsequently. Dehydration step followed by rehydration and another dehydration step as explained in previous paragraph. Again, to completed dehydration, the plates were incubated in the oven at 60-65ºC for 20 min without lid or PCR adhesive cover.

The next step involved gel digestion using trypsin. Sequencing grade modified trypsin (Promega UK Ltd, Southampton, UK) was prepared as described by the manufacturer and made to a final concentration of 12.5ng/µl in 25 mM ammonium bicarbonate. 5µl trypsin was added to each well plate to fully rehydrate gel, the plate sealed and placed into 37ºC incubator for 20mins. Once fully rehydrated, the gel plug was covered with 10µl of 25mM ammonium bicarbonate to keep gel wet during digestion and incubate at 37ºC for 4 hr with adhesive film covering the plate. The plate was then removed from the incubator and sonicated in ultrasonic water bath for 10 min to encourage peptides to leave the gel plug. The digestion buffer surrounding the gel plug was removed and placed in a clean plate. The new plate containing the digestion buffer (peptide plate) was incubated at 60-65 ºC for 45 min without lid or adhesive cover.

In order to further extract peptide digestion products from the gel, 20µl acetonitrile was added to the gel plug (in the old plate), covered with adhesive film and left on bench until peptide plate had dried in the oven. Acetonitrile covering the plug was removed and added to appropriate well of the peptide plate and the acetonitrile

peptide-containing samples were incubated to completion in an oven at 60-65ºC for approximately 30 min. The plate was allowed to cool to room temperature and dried extracts were stored in the -20 °C freezer with an adhesive cover on the plate.

Mass spectrometry was performed at the Central Biotechnology Services, School of Medicine, Cardiff, using a MALDI TOF/TOF mass spectrometer (Applied Biosystems 4800 MALDI TOF/TOF Analyzer; Foster City, CA, USA) with a 200 Hz solid state laser operating at a wavelength of 355nm.

2.2.8 Sandwich Enzyme-linked Immunosorbent Assay (ELISA)

2.2.8.1 Preparation of the ELISA plates

ELISA was performed to quantify the concentrations of TGF-β1, BMP2 and VEGF in DMEs. Commercial ELISA development kits were used (Human/Mouse Ready-Set-Go, eBiosciences for TGF-β1 and Peprotech for BMP2 and VEGF). The TGF-β1 kit contained ready- made buffers. For BMP2 and VEGF, buffers were prepared according to the manufacturer's instructions. All kits contained specific capture antibodies for each protein, biotinylated detection antibodies and avedin-HRP. The standards were also provided which contained a known concentration of the proteins of interest.

For TGF-β1, the capture antibody was diluted 1 in 250 with the coating buffer provided in the kit. For BMP2 and VEGF the capture antibodies were diluted to 1 and 0.5μg/ml respectively in PBS. Pierce™ 96-Well Microplates (Fisher Scientific) were coated with 100μl of each capture antibody and incubated over night at 4ºC for TGF-β1 and at room temperature for BMP2 and VEGF according to the manufacturers'

instructions. The liquid was then aspirated and the wells washed 4 times with the wash buffer containing 0.05% (v/v) Tween-20 in PBS. Wells were then blocked, for 1 hr at room temperature, with 200µl 1× assay diluent for TGF-β1 and 300µl block buffer containing 1% w/v BSA in PBS for BMP2 and VEGF, as per manufacturers' instructions. Plates were washed 4× in wash buffer.

2.2.8.2 Preparation of ELISA standards and samples

The supplied standards were diluted in diluent buffer, which was prepared by mixing the 5× ready-made assay diluent, 1 in 5, with distilled water for TGF-β1 ELISA and was made up of 0,05% (v/v) Tween-20, 0.1% (w/v) BSA in PBS for VEGF and BMP2 ELISAs. Standard dilutions ranged from 8, 3 and 1ng/ml to zero for TGF-β1, BMP2 and VEGF ELISAs respectively using 2 fold dilutions. 100µl of each standard concentration was added, in triplicates, to each coated ELISA plate. Triplicate wells loaded with diluent buffers alone were used to represent zero concentration.

DMEs sample preparation involved solubilising the DMEs in the diluent buffer to a maximum concentration of 1mg/ml which were further diluted using 2 fold dilutions. 100µl of each sample was added, in triplicates, to each coated ELISA plate next to the standards. Plates were incubated at room temperature for 2 hr. The solutions were then aspirated and the wells washed 4 times using the wash buffer. Plates were incubated for a further 1 hour following addition of 100µl of the kit biotin conjugated detection antibody, diluted 1 in 250 with the 1× assay diluent for TGF-β1 to each well. Biotinylated detection antibodies were diluted in diluent buffer to 0.5 µg/ml and 0.25 µg/ml for BMP2 and VEGF respectively and 100µl were added to each well followed by 1 hr incubation for TGF-β1 and 2 hr incubation for the remaining two ELISAs, at

room temperature. The liquid was aspirated and the wells washed 4 times before adding 100µl of avidin-HRP, diluted in diluent buffer to 1:250 for TGF-β1 and 1:2000 for the remaining two ELISAs, was added to each well. Incubation at room temperature for 30 minutes followed. The liquid was aspirated and the well washed 4 times prior to adding 100µl of the provided substrate solutions to each well. For TGF-β1, plates were incubated at room temperature for approximately 15 min before stopping the reactions by adding 50µl of 1M phosphoric acid to each well, as per manufacturer's instructions.

For BMP2 and VEGF, after adding the substrate solution and incubation at room temperature for colour development, the absorbency was quantified straight away, with no addition of a stop solution, as the manufacturer did not recommend this step.

2.2.8.3 ELISA signal quantification

The plate absorbance was read in a Microplate^{TM} reader (BioTek instruments Limited, Winooski, USA) at 450nm for TGF-β1 and 405nm for VEGF and BMP2 ELISAs. Standard curves was generated by plotting the concentration against absorbency for the standards on Excel programme, based on a best fit line, which allowed concentrations of TGF-β1, BMP2 and VEGF in samples to be calculated. Regression analysis was used to assess the linearity of the best fit line which was found to have $R^2 > 0.9$, indicating linearity. The ELISA experiment was repeated and the results were presented as means and standard errors from n=6.

2.3 Results

2.3.1 Isolation of dentine matrix proteins

Comparison of percentage recovery of extracted proteins from dentine powder revealed highest total yield for phosphoric acid at 3.3% with EDTA yielding 1.38% and citric acid 0.8% of the starting weight of dentine (Table 2.3). Weight of the freeze dried supernatants following exposure of dentine powder to polyacrylic acid was higher than that of the dentine powder used at the start.

Conditioner/ etchant	EDTA	Phosphoric acid	Citric acid	Polyacrylic acid
Weight of dentine powder (g)	4	4	4	4
Total weight of extracts (g)	0.055	0.132	0.032	6.5 (acid-protein precipitate)
Percentage yield	1.38%	3.3%	0.8%	NA

Table 2.3: Percentage recovery of dentine matrix protein from powdered human dentine following extraction for 14 days in various solutions showing highest yield with phosphoric acid treatment and acid-protein precipitation with polyacrylic acid treatment.

2.3.2 Quantification of protein in samples

Protein concentrations in DMEs were measured using the BCA assay. Phosphoric acid extracted DMEs contained the highest protein level (181.87µg/mg of DMEs) followed by citric acid (111.87 µg/mg of DMEs) and EDTA (104 µg/mg of DMEs) (Table 2.4). The standard curve for BCA assay is are provided in figure 2.1

Extraction solution:	EDTA	Phosphoric acid	Citric acid
Concentrations, µg/mg of DMEs	104	181.87	111.87

Table 2.4: Concentrations of protein in DMEs of different etching agent/ conditioners.
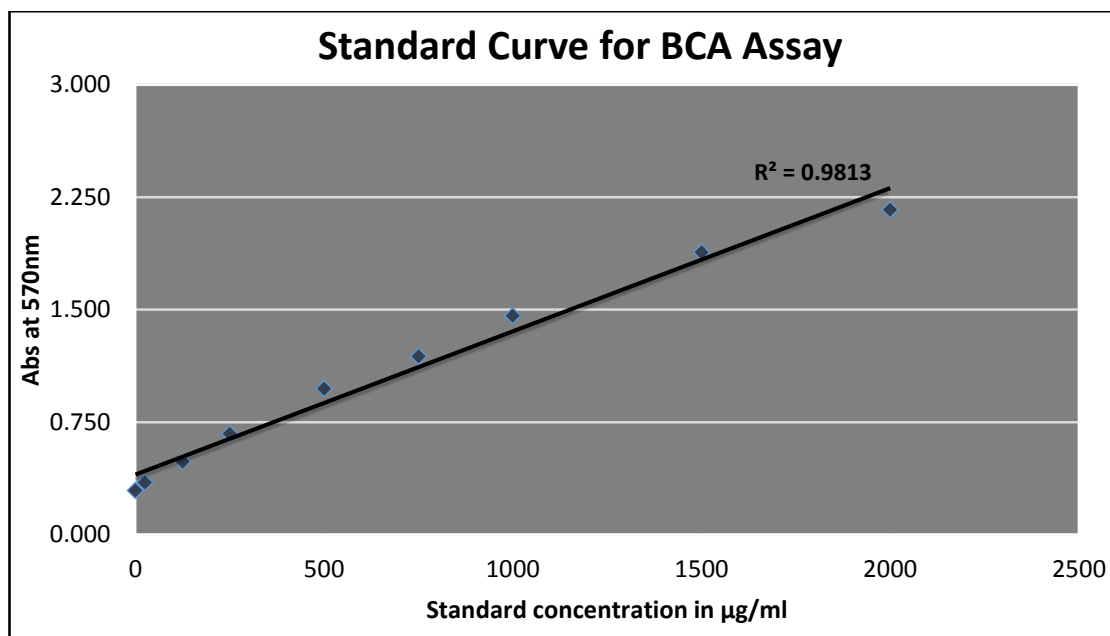
Figure 2.1: Standard curve generated for the BCA assay. The line equation was used to calculate protein concentration in samples.

2.3.3 Comparison of dentine matrix protein profiles

Silver staining of the one dimensional polyacrylamide gel electrophoresis (1D SDS-PAGE) revealed banding patterns for DMEs extracted by different agents (Figure 2.2). Distinct protein profiles were observed for DMEs extracted by all solutions other than polyacrylic acid (Table 2.5). No clear bands could be detected in the gel with visual inspection related to polyacrylic acid extracts which suggests little or no detectable protein in these extracts.
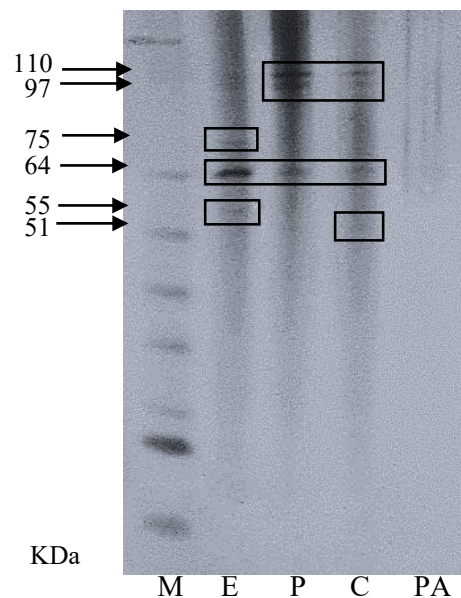
Figure 2.2: Silver stained gel showing the protein profiles of DMEs extracted by EDTA (E), phosphoric acid (P), citric acid (C) and polyacrylic acid (PA). No obvious band formation is identified in PA lane. Lane marked as M is the molecular weight marker.

MW (KDa) of protein bands	EDTA DMEs	Phosphoric acid DMEs	Citric acid DMEs	Polyacrylic acid DMEs
110	–	++	+	–
97	–	+	faint	–
75	+	–	–	–
64	++	+	+	–
55	+	–	–	–
51	–	–	faint	–

Table 2.5: Subjective assessment of protein band intensity revealed an obvious band at 64 KDa level for EDTA, phosphoric and citric acid extracts. The bands detected at 97 and 110 KDa in phosphoric and citric acid extracts were not evident in EDTA extracts. Faint bands at 55 and 51 KDa levels were detected in EDTA and citric acid extracts respectively. No bands could be detected in polyacrylic acid extracts.

2.3.4 Identification of proteins in DMEs

2.3.4.1 Western blots

TGF-β1and VEGF were identified in DMEs extracted by EDTA, phosphoric and citric acid. For both growth factors the signal was detected at three different molecular weight levels corresponding to 83 and approximately 64 and 50 KDa (Figure 2.3). Negative controls in which the primary antibodies were incubated with corresponding blocking peptides revealed no immunoreactivity (Figure 2.4).
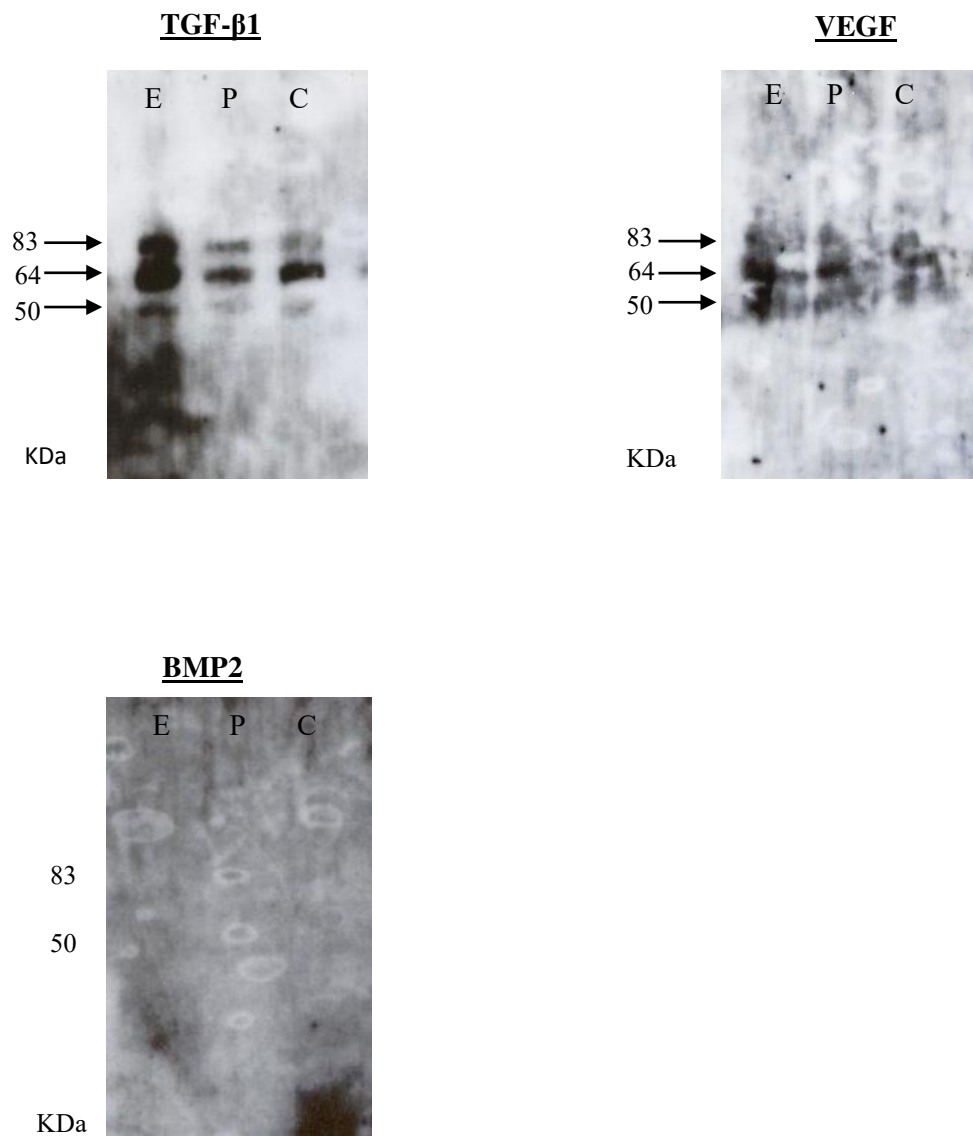
TGF-β1

VEGF

BMP2

Figure 2.3: Western blots showing the presence of TGF-β1 and VEGF in DMEs extracted by EDTA (E), phosphoric (P) and (C) citric acid. BMP2 could not be detected with this method.

TGF-β1   **VEGF**
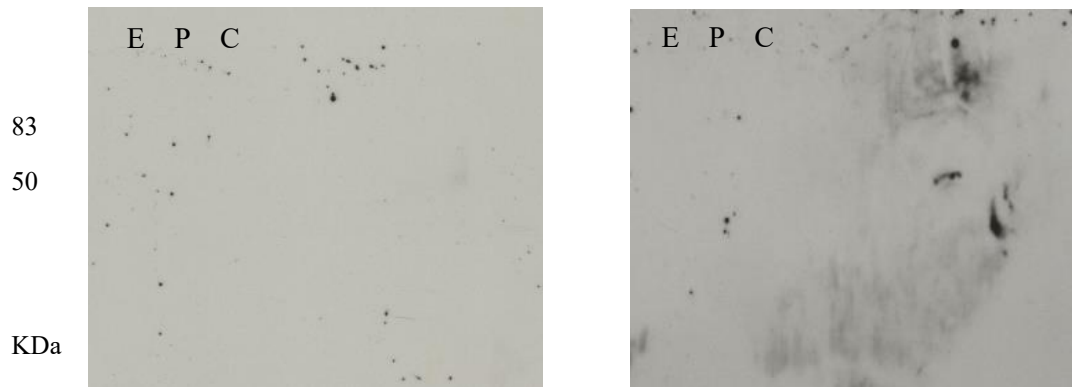
Figure 2.4: Negative controls for Western blots, in which the primary antibody was incubated with a blocking peptide for TGF-β1 and VEGF, prior to use in the blot. Lanes loaded with DMEs extracted by EDTA (E), phosphoric (P) and (C) citric acid.

2.3.4.2 2-D gel electrophoresis and mass spectrometry

2D gel electrophoresis of EDTA extracted DMEs was performed. A distinct band appeared at approximately 66 KDa and spots at 55, 21-31 and 14-21 KDa molecular weight levels. Mass spectrometry identified these areas as albumin, collagen1A2 and haemoglobin (Figure 2.5). Albumin was identified in two spots, indicating the presence of an isoform or a protein fragment.

Figure 2.5: The silver stained two-dimensional gel of EDTA solubilised DMEs. The marked spots (1-4) were subsequently identified by mass spectrometry as 1 and 2) albumin, 3) collagen and 4) haemoglobin.

2.3.4.3 Sandwich Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was performed to quantify TGF-β1, BMP2 and VEGF in proteins extracted by various solutions. TGF-β1 was identified at highest concentration in EDTA extracted DMEs followed by phosphoric acid and citric acid extracted DMEs. BMP2 and VEGF were detected in EDTA extracted DMEs only, and in considerably lower concentrations compared to TGF-β1 levels (Figure 2.6). The standard curve for ELISA experiments are provided in figure 2.7.
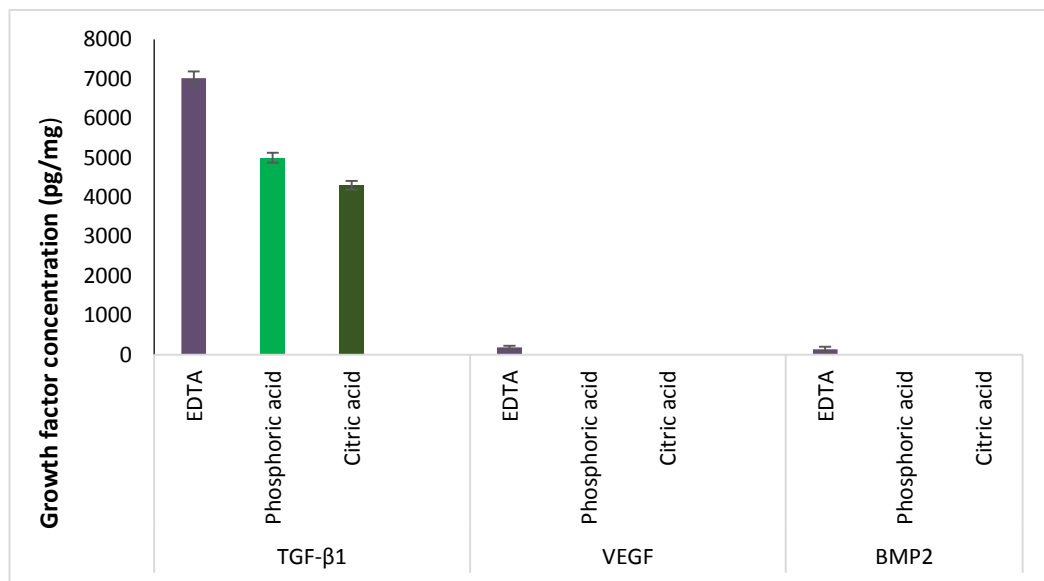
Figure 2.6: Concentrations of growth factors (pg/mg) in DMEs extracted by different agents as quantified by ELISA.
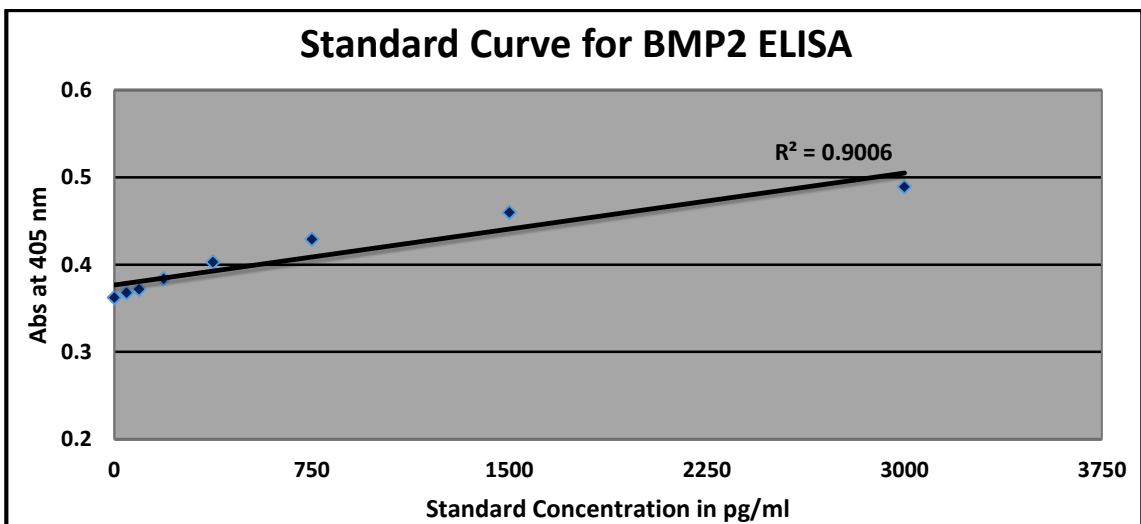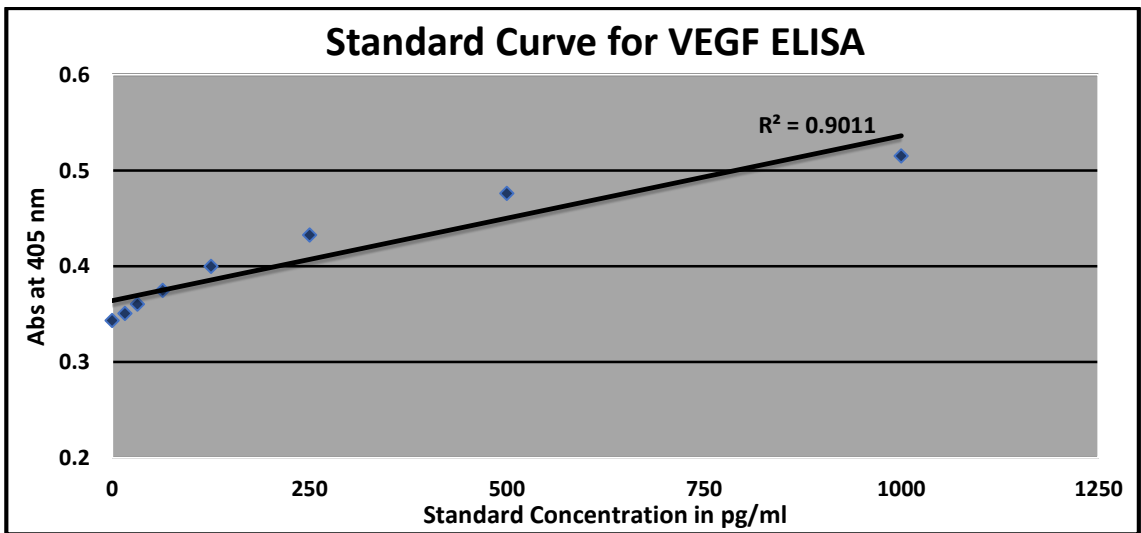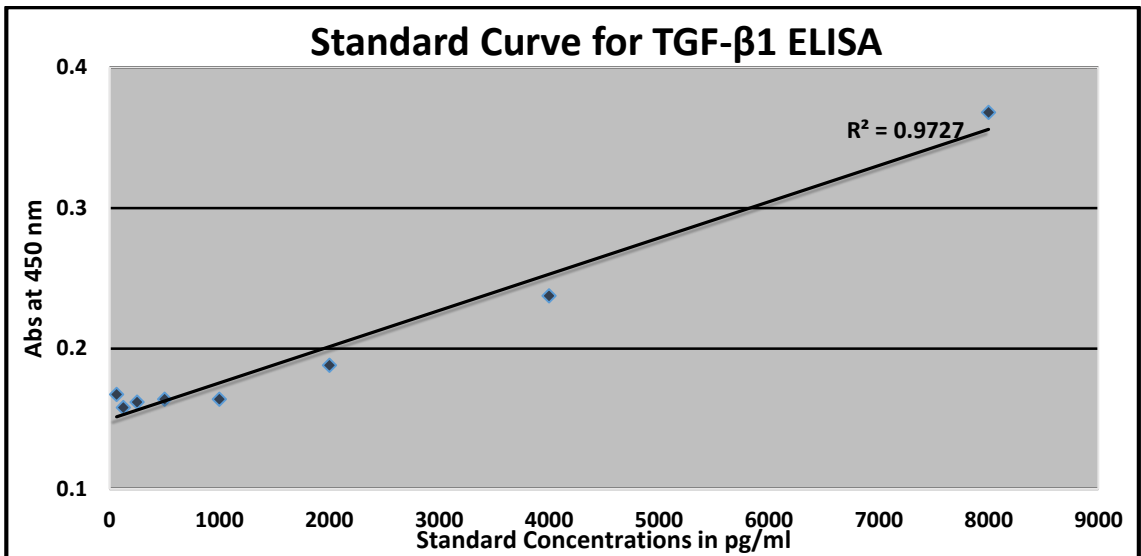
Figure 2.7: Standard curves generated for TGF-β1, VEGF and BMP2 ELISAs. The linear relationship between absorbance and concentration values demonstrates reliability of predictions by the line equation.

2.4 Discussion

Interactions between dental materials and underlying tooth tissues may be influenced by a number of factors including the chemistry and concentration of the materials used as well as the ability of the dental tissue to respond to the agent. The response of the tissues will vary in health and disease, for example in caries and/or pulp inflammation (Bjorndal et al. 2014). In addition to their possible physicochemical effect, when placing the materials in contact with a living dynamic tissue such as dentine-pulp complex, a biological response may be triggered (Ferracane et al. 2010). Such a response may be mediated by the signaling molecules released from dentine following treatment by the chemicals used during the process of dentine conditioning in dental restorative procedures (Pang et al. 2014).

In this chapter, the ability of three clinically used etching/conditioning agents in solubilising TGF-β1, VEGF and BMP2 from human dentine were investigated. The chemical agents used included an etchant (phosphoric acid) and two dentine conditioning agents, (citric and polyacrylic acids). EDTA was selected as a control because its ability in solubilising bioactive molecules from dentine is well documented (Roberts-Clark and Smith 2000; Graham et al. 2006; Tomson et al. 2007). Furthermore it has been shown that EDTA treatment of the dentine surface can promote stem cell attachment and odontoblast/ osteoblast differentiation of the attached cells (Galler et al. 2011; Trevino et al. 2011).

To preserve dentine matrix protein and avoid degradation and possible loss of some proteins, protease inhibitors were added during the process of DME extraction. Protein

detection and identification methods included Western blot and mass spectrometry following 2-D gel electrophoresis. Furthermore, ELISA was used to quantify growth factor concentrations in DMEs. The silver stained 2-D gel, for EDTA solubilised DMEs, revealed a prominent band at the 65 kDa level extended horizontally along the pH gradient. There were also distinct spots at 50, 26 and 14 KDa levels. The silver stained 1D gel had also revealed band formation at the 64 and 55 KDa level. For identification of the prominent spots on the 2D gel, mass spectrometry was carried out and three proteins known to be abundant in dentine, collagen, albumin and haemoglobin were identified. The limitations of 2D gel electrophoresis in identifying proteins with low concentrations (such as growth factors) has been acknowledged in a recent comprehensive proteomic analysis study on human dentine (Jagr et al. 2012). Also, proteins at extremes of pH could not be readily separated with this method since the pH range of the IPG strip used was between pH 3-10. Furthermore, at this stage of the experiment it was not known at what molecular weight level the growth factors of interest might have appeared in the gel. Their detection by mass spectrometry would have involved excising an excessively large number of spots from the gel for analysis, which would have proven unpractical for time and cost implications. Therefore, western blots were used instead to identify the growth factors in DMEs.

TGF-β1 was detectable in EDTA, phosphoric and citric acid DMEs by both Western blot and ELISA. The growth factor appeared on three bands, between 60 and 83 KDa on Western blot. This is a higher molecular weight than the TGF-β1 dimer (25 KDa) suggesting the identified growth factor is bound to other ECM proteins/ protein fragments. This finding is not surprising. Although most in vitro studies describing the biological effects of TGF-β have used the active, soluble TGF-β, the growth factor is

secreted into the ECM as a latent, inactive protein, bound to its latency associated proteins *in vivo*. The endogenous TGF-β1 has been previously shown to appear at a molecular weight of just over 60 KDa by Western blotting (Boivin et al. 2012) which is consistent with findings in this chapter. The mechanisms by which the latent TGF-β is activated *in vivo* are not fully understood. The latent TGF-β binding protein (LTBPs) are proposed to be important for targeting and localization of TGF-β on cells; it has been suggested that TGF-β activation involves cellular recognition of the extracellular associated LTBP initially, followed by subsequent recognition of the bound TGF-β (Hyytiainen et al. 2004). Recognition by specific cell-associated molecules like integrins and plasmin are believed to play important roles in the regulation of TGF-β activation (Munger et al. 1997; Hyytiainen et al. 2004). It has been proposed that simultaneous binding of the latent growth factor and ECM proteinases to integrins, improves enzymatic cleavage of the latent complex, facilitating the release of active growth factor (Wipff and Hinz 2008). Furthermore, binding with integrin results in conformational changes in the latent TGF-β1 resulting in the release the active growth factor (Wipff and Hinz 2008). The present study did not identify TGF-β in its soluble dimeric form in DMEs, nevertheless it may be suggested that the bound growth factor can initiate a cellular response via the mechanisms proposed above.

In addition to its latency associated proteins, acitve TGF-β1 may be bound to the ECM proteoglycans (Hildebrand et al. 1994). Decorin and biglycan, in particular, have strong affinity for TGF-β1 (Baker et al. 2009). Interestingly, binding of TGF-β1 to decorin has been reported to enhance its bioactivity in bone (Takeuchi et al. 1994). Considering that the molecular size of decorin and biglycan are around 40 KDa, a

further explanation for finding TGF-β1 at 64 KDa would be the binding of the dimer to these ECM proteoglycans.

Similarly VEGF was detected with Western blot, on three bands between 60 to 83 KDa, a higher molecular weight level than the soluble growth factor with molecular size of approximately 21 KDa, which suggest the detected growth factor is bound to other molecules. Within the ECM, VEGF may be bound to a number of components. A number of matrix binding sites have been identified for VEGF including GAGs (Park et al. 1993), collagen (Chen et al. 2010), fibrin (Sahni and Francis 2000) and fibronectin (Wijelath et al. 2004). Interestingly, the matrix-bound VEGF is shown to be bioactive and capable of activating the VEGF receptors, albeit via a different signaling pattern compared to soluble VEGF (Chen et al. 2010; Anderson et al. 2011). Furthermore, it has been demonstrated that coordinated binding of the VEGF/ fibronectin complex with their corresponding receptors on the cell surface enhanced the specific cellular response to the growth factor, promoting VEGF- induced endothelial cell proliferation and migration *in vitro* (Wijelath et al. 2006). This evidence suggests that for VEGF to exert its biological activity, it is not always necessary for the growth factor to be broken free from the ECM components in its soluble form.

In this chapter both detection methods, Western blot and ELISA, failed to detect BMP2 in phosphoric acid and citric acid extracted DMEs. Studies involving proteomic analysis of human dentine, have managed to identify in excess of 200 proteins in dentine (Park et al. 2009; Jagr et al. 2012). The mentioned studies have used EDTA for solubilisation of dentine matrix components prior to identification of proteins by

mass spectrometry. Both studies identified TGF-β1 in EDTA solubilised extracts but failed to detect VEGF or BMPs. Their explanation for the failure in detection of certain growth factors and cytokines was the low expression of these proteins onto dentine or the detachment of the protein during the demineralisation process rendering them undetectable. Similarly in this chapter, failure in detecting BMP2 in phosphoric acid and citric acid extracted DMEs, and the low concentration in EDTA extracted DMEs may be due to the loss of the growth factor through dentine demineralisation and protein isolation process. However, an alternative explanation would be the possible degradation of growth factor as a result of prolonged exposure to strong acids used here. Phosphoric acid at a 37% and citric acid at 10% concentration are potent acids with pH of <1 and 1.5 respectively and capable of denaturing proteins including BMP2. However EDTA, being a calcium chelating agent pH adjusted to 7.2, may act as an efficient agent in matrix dissolution without denaturing matrix proteins.

Furthermore, the results of this chapter showed that in relation to polyacrylic acid, the weight of the extracts were higher than the initial dentine powder used. This phenomenon may be due to acid-protein precipitation which can occur through binding −COOH groups of polyacrylic acid with cationic proteins through ionic exchange interactions, resulting in the formation of insoluble complexes (Bhattacharjee et al. 2012). Polyacrylic has been shown to have high affinity for proteins in the pH range of 3-6 and that shifting the pH outside this range results in the proteins being released form the complex (Sternberg and Hershberger 1974). Although immobilisation of proteins is not considered desirable in the context of this study, this property of polyacrylic acid allows controllable and selective separation of proteins form crude biological mixtures which is exploited in the industry, for example

in antibody purification (McDonald et al. 2009). The pH of 20% polyacrylic acid used in this study was 3.9. At this pH high density of polyacrylic acid –COOH groups are available (Bhattacharjee et al. 2012), facilitating the formation of covalent bonds with protein in the pooled extracts and resulting in the formation of large complexes which could not be filtered out of the dialysis tubes. This may explain the increase in weight of freeze dried dialysis tube contents. SDS-PAGE followed by silver staining of the gel, did not reveal any protein bands in the freeze dried polyacrylic acid DMEs, probably due to dilution of proteins in high volumes of the bound polyacrylic acid. The use of the polyacrylic extracted compounds was therefore abandoned in the subsequent chapters of this study.

With regard to the clinical view point in relation to this finding, applying polyacrylic acid to a prepared cavity may results in immobilising dentine proteins at sites of application. This property of polyacrylic acid as a dentine conditioner and a constituent of glass ionomer restorative materials may render it ineffective in triggering a biological response mediated by the ECM bioactive proteins. Comparatively, the other dentine conditioning agents (EDTA and citric acid) and phosphoric acid were shown to have the ability to release the growth factors from powdered dentine. This finding may have implications for clinical practice as to the choice of materials in vital pulp therapy. However, this information should be interpreted in the broader context since in relation to dental materials, a number of factors may contribute to a successful outcome in vital pulp therapy. As well as the ability to stimulate a biological response leading to reparative dentine formation, release of fluoride to prevent secondary caries, antibacterial properties and reliable bonding to dentine to obtain a bacterial tight seal are also important for maintaining pulp vitality and health (Qureshi et al. 2014).

Notwithstanding this, the present study has focused on investigating the ability of dentine etching/ conditioning agents to induce a biological response in dental pulp. We hypothesise that such a response may be mediated through the release bioactive ECMs. To test this hypothesis the effect of the released DMEs on phenotype and behaviour of DPSCs will be investigated in the next chapter.

Chapter 3: Effects of Dentine Matrix Extracts on Phenotype and Behaviour of Dental Pulp Progenitor Cells

3.1 Introduction

Adult tissue stem cells are undifferentiated cells with the capacity to self-renew and differentiate into different specific cell types that replenish lost cells throughout an organism's life time (Pittenger et al. 1999). Adult stem cells reside in special microenvironments termed "niches", which beside the intrinsic genetic programme within the cell, play a crucial role in regulating stem cell behaviour *in vivo* (Li and Xie 2005; Pentinmikko and Katajisto 2014). In vitro studies, have highlighted a number of parameters, including topographical, chemical and genetic cues, to be important factors in guiding stem cell behaviour (reviewed in Griffin et al (2015)).

In the mature dental pulp, multipotent dental pulp stem cells (DPSCs) are believed to reside within several niches (Gronthos et al. 2002; Shi and Gronthos 2003; Lovschall et al. 2005). Identification of parameters governing stem cell fate may provide opportunities for controlling their behaviour, including help to induce repair and regeneration. The natural ability of dentine-pulp complex in producing tertiary dentine in response to mild to moderate injury is well known (Couve et al. 2014). The prospect of inducing dentine regeneration clinically is attractive as it is likely to preserve vitality in viable dental pulps thereby avoiding the need for root canal treatment or extraction (Huang 2011). The clinical success of capping materials such as calcium hydroxide and MTA, in inducing tertiary dentine formation in vital pulp therapy is well documented (Olsson et al. 2006; Bogen et al. 2008a; Mente et al. 2014). However, trends in operative dentistry have shifted towards minimum intervention dentistry,

mediated by adhesive restorative materials and techniques (glass ionomers or resin based composite). As well as this, concern exists regarding the quality and outcome of dentine repair/ 'dentine bridge' formation achieved by 'traditional' pulp-capping with calcium hydroxide (Nair et al. 2008). In vital pulp treatments, the direct effect of materials on the pulp tissue is a crucial consideration for clinical success (explained in Chapter one, 6.3.1-6.3.4). However, their indirect effect, mediated through the released bioactive molecules from dentine, may provide an important mechanism for inducing a dentinogenic response in the pulp (Smith et al. 2001a).

It has been speculated that glass ionomer cements (GICs), are potentially capable of solubilising the bioactive dentine matrix components, attributed to their acidic pH, via which mechanism they may induce the process of dentine regeneration (Ferracane et al. 2010). There are currently no reported studies having investigated this hypothesis and the results of the previous chapter of this thesis provided evidence to the contrary. In relation to composite resin restorations, an etchant is first applied to demineralise dentine and enhance the formation of micromechanical bond between restoration and the tooth. Acids are shown to have the ability to solubilise the matrix components of powdered dentine (Klont and ten Cate 1990; Ferracane et al. 2013). However, there is little information on the biological impact of matrix components solubilised by the clinically used acids. Previous studies have mainly focused on the biological effect of EDTA soluble fractions of powdered dentine. EDTA extracted dentine matrix components have been shown to influence cell behaviour of the pulp progenitor cells, leading to cell differentiation and mineralisation *in vitro* (Liu et al. 2005; Chun et al. 2011). However, no similar studies in relation to acid solubilised dentine matrix components have been reported in the literature.

Against this background, the aim of this chapter was to investigate the effects of phosphoric acid and citric acid extracted DMEs compared with that of EDTA extracted DMEs on phenotype and behaviour of dental pulp progenitor cells.

3.2 Materials and Methods

3.2.1 Cell culture

A single-colony derived population of DPSCs (A31, at passage 11) was obtained from the Mineralised Tissue Group Cell Bank, School of Dentistry, Cardiff. The clone was previously isolated, via enzymatic digestion and fibronectin adhesion (Jones and Watt 1993) (methodology attached in Appendix 2), from human caries free third molars, obtained in accordance with ethical approval and patients' consent (Appendix 1).

To obtain sufficient number of cells, they were expanded in culture at the beginning. Culture was performed using tissue culture plates and flasks (Sarstedt), under sterile conditions in aseptic class II tissue culture hoods (Astec Microflow, Andover, UK). Cells were maintained in a humidified chamber (BINDER, Tuttlingen, Germany) with 5% CO_2 at 37°C. Culture media and accutase were first pre-warmed in a water bath to 37°C, prior to exposure to cells. The working medium was composed of α-modified Minimum Essential Medium (α-MEM) (containing ribonucleosides, deoxyribonucleosides, L-glutamine and phenol red), with 1% antibiotics (10000 units/ml penicillin G sodium, 10μg/ml streptomycin sulphate and 25μg/ml amphotericin; Invitrogen), 10% foetal bovine serum (FBS) (Invitrogen) and 100μM L-ascorbate 2-phosphates (Sigma-Aldrich) added.

When approximately 80% confluent in T-75 or T-125 flasks, the cells were washed with 0.1M phosphate buffered saline (PBS, pH 7.4) then incubated with 2ml of the pre-warmed accutase at 37°C. Incubation was continued until the cells became separated from the culture vessel (after approximately 3-5min). To inactivate accutase,

5-8ml serum-containing culture medium was added and the mix centrifuged at 1800rpm to separate the cells. The cell pellet was re-suspended in 1ml of culture medium for cell counting and cryopreservation. To count the cells, they were stained with trypan blue (Sigma, UK). To cryopreserve cells, after counting, they were re-suspended in 1ml freezing mix containing 10% Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich) and 90% FBS, placed in cryovials and put in freezing pot at -80°C. After 24 hr, the vials were transferred to liquid nitrogen for long term storage. To start each experiment, cells were taken out of liquid nitrogen and were rapidly thawed by transferring to a 37°C water bath. Once thawed, 5ml medium was added immediately and centrifuged as stated above. The pellet was then re-suspended in medium and seeded in appropriate vessels for each experiment.

Passage 13-14 cells were seeded in 96-well plates (Sarstedt) at 3000 cells/cm^2 for viability assay and at 2400 cells/cm^2 in 6-well plates (Sarstedt) for RNA extractions, immunocytochemistry, alizarin red and Oil-Red-O staining. Cells were cultured in medium containing α-MEM, 10% FBS, 1% antibiotics and 100µM L-ascorbate 2-phosphates for 24hr after seeding. They were then starved from serum for the next 24 hr, after which point 3.5% FBS was added to the medium for control and test groups. This point in time was considered as baseline at which point stimulation of the test groups with DMEs (10µg/ml) commenced (Table 3.1). Media for all groups was changed on alternate days.

Control	α-MEM 95%, FBS 3.5%, Antibiotics 1%, 100μM ascorbic acid
Test groups contained DMES extracted by: EDTA, phosphoric acid, citric acid	α-MEM 95%, FBS 3.5%, Antibiotics 1%, 100μM ascorbic acid, 10μg/ml DMPs

Table 3.1: Contents of the media for control and test groups from the baseline onwards.

3.2.2 Cell viability assay

Cell viability and cellular metabolic activity was assessed by MTT assay (CellTiter96® AQueous One Solution Cell Proliferation Assay, Promega, Southampton, UK). The test was carried out on day 1 (24 hr), day 3, day 5, day 7 and day 9. The CellTiter 96® AQueous One Solution reagent provided in the kit had to be stored in -20°C and protected from the light. It was removed from the freezer before use and placed in a water bath at 37°C, 10 min before use. 20μl of the reagent was added to each well of the 96-well plate containing 100μl culture medium. The plates were covered with foil to protect from the light and placed back in the incubator for 3 hours before recording the absorbance at 490nm with a Microplate Autoreader (BIO-TEK Instruments, Potton, UK). Data were presented as the mean and standard deviation for readings from 10 wells for each group at every time point. Statistical analyses were conducted using one-way ANOVA for matched samples followed by pairwise multiple comparisons using Bonferroni adjustment on statistical package SPSS. Significance levels were defined as *= $P<0.05$ (significant), ** = $P<0.01$ (very significant) and *** = $P<0.001$ (extremely significant).

3.2.3 Alizarin red staining

In order to detect mineralisation of the matrix produced by the cultured cells, alizarin red staining was used. The stain binds to areas containing calcium ions identifying areas of mineralisation as bright red. Media was removed from the culture wells, and cells in each well incubated in 0.5ml/well alizarin red (Sigma-Aldrich) (2% w/v in double distilled water) solution, pH adjusted to 4.2 for approximately 3 min. The solution was then emptied and the cells washed with double distilled water to remove excess stain. Wells were viewed under a Nikon Eclipse TS100 (Nikon UK Ltd., Surrey, UK) light microscope when dry. Images were captured using a Canon Powershot S5 IS digital camera. The process was undertaken on days 5, 8, 12, 15, 18 and 21 for control and test groups.

3.2.4 Oil Red O staining

To assess any possible phenotypic change of cultured cells to adipocytes following prolonged culture, Oil Red O staining was used. Oil Red O stock of 3.5g/l was made by dissolving powder (Sigma-Aldrich) in isopropanol. Working stain was prepared fresh by mixing a 4:6 ratio of double distilled water with the stain stock. The working solution was incubated at room temperature for 10 min on a shaker, and subsequently filtered through Whatman grade 42 filter paper (Fisher Scientific, UK). Media was removed from the culture wells and cells incubated in 0.5 ml/well Oil Red O working solution for 10 min at room temperature, with gentle agitation on a shaker. Wells were subsequently washed with double distilled water followed by a brief wash with 60% isopropanol, and finally washed again with double distilled water to remove the excess stain. Oil Red O staining was performed on day 21 for control and all test groups and

wells viewed under a Nikon Eclipse TS100 light microscope and images captured with a Canon Powershot S5 IS digital camera.

3.2.5 Immunocytochemistry

Immunohistochemistry was performed to assess the synthesis, by cells, of collagen1A1 and DSP. Glass cover slips were placed in sterilisation paper bags, sterilised in an autoclave and placed at the base of wells of 6-well plates. DPSCs were then seeded in the wells at 2400 cells/well in culture conditions as described before. The immunocytochemistry was carried out on days 2 and 14 post-stimulation. On the day of the experiment, media was emptied from the wells and the cells washed with PBS. The cells were then fixed in 4% paraformaldehyde for 20 min and washed twice with PBS. 1% BSA in PBS was used as the blocking buffer in which cells were incubated at room temperature for 1 hr. Anti DSP and collagen1A1 rabbit polyclonal primary antibodies (Santa Cruz, Insight Biotechnology Ltd, Middlesex UK) were diluted in 0.1% BSA in PBS solution to a concentration of 1 in 50. The cells were incubated overnight at 4°C. The cells were then washed 3 times in PBS, for 5 min each time. The cells were subsequently exposed to goat anti-rabbit IgG FITC secondary antibody diluted in 0.1% BSA/PBS solution to a concentration of 1 in 250 and incubated for 1 hour at room temperature. They were subsequently washed 3 times in PBS for 5 min each time. The glass cover slips were then removed from the bottom of the wells, a droplet of mounting medium for fluorescence microscopy viewing containing Dapi (Vector Laboratories, Inc., Burlingame, USA) was placed on the slip and the slip inverted on a glass microscope slide to help visualise the nuclei. The cells were then viewed under an Olympus AX70 fluorescent microscope and images were captured using a Nikon digital camera DXM 1200 and ACT-1 software. For negative

controls, primary antibodies were incubated in corresponding blocking solution with 10× excess of blocking peptide (Santa Cruz) for 30 min at room temperature with agitation and used as per detection protocol explained above. Nuclei were stained with Dapi.

3.2.6 mRNA extraction from cultured DPSCs

RNA was extracted from DPSCs in order to assess the expression of a number of mesenchymal, developmental, proliferation and differentiation cell markers through RT and q-PCRs subsequently. RNA extractions were performed using the RNeasy mini Kit (Quigen, Manchester, UK), following the manufacturer's protocol, on day 1 (24 hr), 7, 14 and 21 for RT-PCRs and day 3, 10 and 21 for Q-PCRs. The kit contains various buffers namely RLT lysis buffer and RW1 and RPE wash buffers. There are also spin columns which contain membranes for binding RNA.

Three hundred and fifty microliters of RLT buffer with addition of 1% β-mercaptoethanol (Sigma-Aldrich) was placed onto each well and for better lysis of the cells repeatedly aspirated and poured into the well. The resulting lysates from each group were placed into QiaShredder columns and centrifuged at 12,000×g for 2 min in a microcentrifuge (Spectrafuge 24D, Jencons, UK) in order to shear cellular components, including DNA, and separate debris. Subsequently, 350µl of 70% molecular grade ethanol (Sigma-Aldrich) was thoroughly mixed with the cell lysate by pipetting and the sample was transferred into an RNeasy Mini Kit spin column (Qiagen) and centrifuged at 10,000 ×g for 15 sec and the flow through discarded. The column was washed with 350µl of RW1 buffer and centrifuged again at 10,000 ×g for 15 sec. The flow-though was discarded, 10µl of DNase 1 stock was mixed with 70µl

of buffer RDD (both Qiagen) and added to each column and columns incubated for 15 min at room temperature in order to digest any genomic DNA contamination. A wash with 350µl buffer RW1 and 15 sec spin at 10,000×g followed. Two further washes with 500µl of buffer RPE and spins at 12,000×g for 20 sec and 2 min were followed and the flow-through discarded each time. The column inserts were transferred into a clean RNase free 1.5ml eppendorf (Fisher Scientific, Loughborough, UK) and centrifuged again for 1 min. The RNA was eluted by adding 30µl of RNase free water before the last spin at 12,000×g for 1 min.

The amount of RNA in each sample was quantified using 2µl of each sample by a Nanovue spectrophotometer (GE Healthcare Life Sciences). Prior to use, the NanoVue was standardised with 2µl of DNase free water as a blank. After quantification, RNA samples were stored in -80°C prior to being used for reversed transcription.

3.2.7 Reverse transcription (RT) for RT-PCRs

Appropriate volumes containing 1µg of RNA were treated with 1µl of Random Primers (Promega) and the volume made up to 15µl with the addition of nuclease free water before incubating the tubes in a G-storm GS1 thermal cycler (Genetic Research instrumentation Ltd, Braintree, UK) for 5 minutes at 70°C to unravel RNA. The tubes were then removed and immediately cooled by placing on ice. Five microliters of 5×M-MLV (Moloney murine leukaemia virus) buffer, 1.25µl of 10mM dNTPs, 0.6 µl of RNasin and 1µl of M-MLV reverse transcriptase (all Promega) and 2.15µl water was added to each tube. Each reaction volume was therefore 25µl in total which was mixed thoroughly. Sterile water instead of mRNA was used as negative control. An additional RT-negative control was set up by excluding the reverse transcriptase. The

tubes were then placed in the thermal cycler and incubated at 37°C for 1 hr. The generated cDNA was stored at -20°C until use in PCR reaction.

3.2.8 RT-Polymerase Chain Reaction (RT-PCR)

RT-PCR reactions were set up by mixing 1µl of cDNA/control with 5µl 5× Green GoTaq™ Flexi Buffer (Promega), 1µl of 25mM $MgCl_2$, 0.5µl 10mM nucleotide mix, 0.25µl 5U/µl GoTaq™ DNA polymerase, 1.25µl of 10 mM of each of the forward and reverse primers. Primer sequences were designed using the Primer-BLAST software on online internet National Centre for Biotechnology Information (NCBI), see table 3.2. All reactions were made up to 25µl by adding nuclease free water. Reactions were run on the G-Storm GS1 thermal cycler starting with an initialization step at 95°C for 5 min followed by 28 or 35 cycles of 1 min 95°C denaturation step, 1 min at appropriate annealing temperature (53-68ºC) and a 1 min 72°C extension step. In the end a final extension step of 5 min at 72°C was run. The PCR products were then run in agarose gels for visualisation using UV light.

Gene marker	Product size (bp)	Annealing temp. (°C)	Sequence 5' 3'	Source
ALP	613	68	TGTGCCTGGACGGACCCTCGCCAGTGCT CCAGCGTCCTTGGCCCAGCGCAGGATGG	XQ Wei Cardiff University
Bax	300	55	GGGGACGAACTGGACAGTAA CCCAAAGTAGGAGAGGAGGC	Chi Lee Cardiff University
β-actin (actb)	480	62	AGGGCAGTGATCTCCTTCTGCATCCT CCACACTGTGCCCATCTACGAGGGGT	XQ Wei Cardiff University
CD105 (Endoglin)	483	55	GAAGGGCTGCGTGGCTCAGG CCTTCCAAGTGGCAGCCCCG	Chi Lee Cardiff University
CD146 (MCAM)	321	55	CGACAACGGGGTCCTGGTGC CAGCGATAGCCGCCTCCTGC	Chi Lee Cardiff University
CD73	351	65	GTCGCGAACTTGCGCCTGGCCGCCAAG TGCAGCGGCTGGCGTTGACGCACTTGC	Amr Alraies Cardiff University
CD90	425	55	ATGAACCTGGCCATCAGCATC CACGAGGTGTTCTGAGCCAGC	Amr Alraies Cardiff University
Collagen1 A1	401	68	AGCAGGAGGCACGCGGAGTGTGAGGCCA TCGGTGGGTGACTCTGAGCCGTCGGGGC	L Sadaghiani Cardiff University
DSPP*	836	68	TGCATTTGGGCAGTAGCATGGGCCATTCCA CCATGGTCCTGGCCCTCCTGGCCCTTG	XQ Wei Cardiff University
MSX-1 (Msh homebox1)	410	55	GAAGCCCGAGAGGACCCCGT AGGCACCGTAGAGCGAGGCA	Chi Lee Cardiff University
PCNA	571	53	GGCGTGAACCTCACCAGTAT CTTCATCCTCGATCTTGGGA	L Sadaghiani Cardiff University
PPARG2	349	60	GCCATCAGGTTTGGGCGGATGCCACAG CCTGCACAGCCTCCACGGAGCGAAACT	Amr Alraies Cardiff University
RUNX2*	631	68	CCGTGGCCTTCAAGGTGGTAGCCCTCGGAG GGGCCCAGTTCTGAAGCACCTGAAATGCG	XQ Wei Cardiff University
TGF-β1*	511	68	CGCCAAGGAGGTCACCCGCGTGCTAATGGTGG GGAGCTTTGCAGATGCTGGGCCCTCTCCAGCG	L Sadaghiani Cardiff University

* 35 PCR cycles

Table 3.2: Human primer specification RT-PCR amplification. β-actin was used as the housekeeping gene.

3.2.9 Agarose gel preparation and visualisation of PCR products

To make 2% agarose gels as well as running buffer, 5× TBE buffer containing 0.5M tris-base (Sigma-Aldrich), 0.5M boric acid (Sigma-Aldrich) and 0.03M EDTA, pH 8.1 was made up. The buffer was diluted to a concentration of 0.5× prior to use. To make each gel, 1.4gr agarose was dissolved in 70 ml of 0.5× TBE and heated in a microwave for approximately 20 sec until the solution was clear. 4µl of 10mg/ml ethidium bromide (Promega) was added to the solution. The mix was then cast in trays with toothed combs positioned in the gel to create wells. The gels were then left at room temperature for 20 min to cool down and set. The comb was carefully removed and 7µl of a 100 base pair (bp) DNA ladder (Promega) was loaded alongside 10µl of each sample in the wells of the gel. The loaded gel was run in 0.5 TBE buffer at 90 mA for approximately 50 min. Gels were then removed from the tank and visualised in a Gel Doc 2000 scanner (Bio-Rad, Hemel Hampstead, UK), under UV light (312nm) and images were saved by the Quality One image software (Bio-Rad) on the computer attached to the scanner.

3.2.10 Quantitative real-time PCR (qPCR)

A SYBR green technique was used for the qPCR. The technique involves using fluorescence cyanine dye which binds to double-stranded DNA. The resulting DNA-dye-complex absorbs blue light (497 nm) and emits green light (520 nm). As the number of duplicates increases during the reaction so does the fluorescence signal strength, which is monitored by measuring the increase in fluorescence throughout the cycle.

A SYBR® Green JumpStart™ Taq ready mix™ (Sigma- Aldrich, UK) was used for the qPCR reactions. The pack contains a ready master mix and a reference dye. Each reaction was set up by mixing 12.5µl of the 2×mastermix, 0.25µl of reference dye, 2µl of 3µM forward primer, 2µl of 3µM reverse primer, 3.25µl sterile water and 5µl of 1 in 10 cDNA. Reactions were set up in Bright White 96-well plates (Primer Design Ltd), in triplicates. Quantitative real-time polymerase chain reactions (qPCR) were performed using an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Carlsbad, USA) and the data was processed and analysed by the software.

The cycles started with an initial denaturing cycle for 2 min at 94°C followed by 40 cycles of 15 sec at 94°C, 30 sec at annealing temperature (Table 3.3) and 30 sec at 72°C extension step. The output from the real-time PCR reactions was provided in the form of graphs showing the number of cycles against the increasing fluorescence (amplification plot). The gene expression levels were quantified by setting the Ct (cross threshold) value within the geometric phase for the housekeeping gene GAPDH, against which genes of interest were compared. The expression was considered specific and reliable for analysis only when a dissociation curve peak at a single melting temperature was observed. Using relative quantification, gene expression levels were presented as bar graphs as a percentage of reference gene, GAPDH Ct value. Data were presented as the mean and standard deviation values of percentages and used for the statistical analysis. Statistical analyses were conducted using one-way ANOVA for matched samples (as the cells used in the experiment were a single colony population). To reduce the chances of obtaining false-positive results (type I errors) when multiple pair-wise tests were performed on the data set,

Bonferroni adjustment on statistical package SPSS was used. Significance levels were defined as * = $P<0.05$ (significant), ** = $P<0.01$ (very significant).

Gene	Product size (bp)	Annealing temperature (°C)	Sequence 5' 3'	Source
ALP	137	55	GGACCATTCCCACGTCTTCAC CCTTGTAGCCAGGCCCATTG	XQ Wei Cardiff University
CD105	186	55	AACAGTCCATTGTGACCTTC TTTTGCTTGGATGCCTGG	L Sadaghiani Cardiff University
CD146	151	55	AAGTCATCTGGTACAAGAATGG AACTGGGCATCTTTGTCTTC	L Sadaghiani Cardiff University
GAPDH	253	55	GGTCGGAGTCAACGGATT ATCGCCCCACTTGATTTTG	XQ Wei Cardiff University
GAPDH	231	62	TCCCGCTTCGCTCTCTGCTCCTC GACCAGGCGCCCAATACGACCAAAT	XQ Wei Cardiff University
Osteopontin	430	55	ATCACCTGTGCCATACCA CATCTTCATCATCCATATCATCCA	L Sadaghiani Cardiff University
PCNA	184	55	CTCTTCAACGGTGACACTC TGGCATCTTAGAAGCAGTTC	L Sadaghiani Cardiff University
Runx2	105	62	CGCCTCAGAACCCACGGCCC ACGGCGGGGAAGACTGTGCC	XQ Wei Cardiff University

Table 3.3: Human primer sequences and PCR annealing temperatures for qPCRs.

3.3 Results

3.3.1 Cell viability assay

In all groups a gradual increase in mean absorbance was observed until day 7, indicating an increase in the levels of metabolic activity. The absorbance reached a plateau by day 7, only a slight increase for control and the group exposed to EDTA extracted DMEs was observed between days 7 and 9 (Figure 3.1). The difference between mean absorbance for control was significantly lower than phosphoric and citric acid DME stimulated groups on day 1 ($P<0.01$). The difference between control and all test groups were significantly different on days 5, 7 and 9 ($P<0.001$).
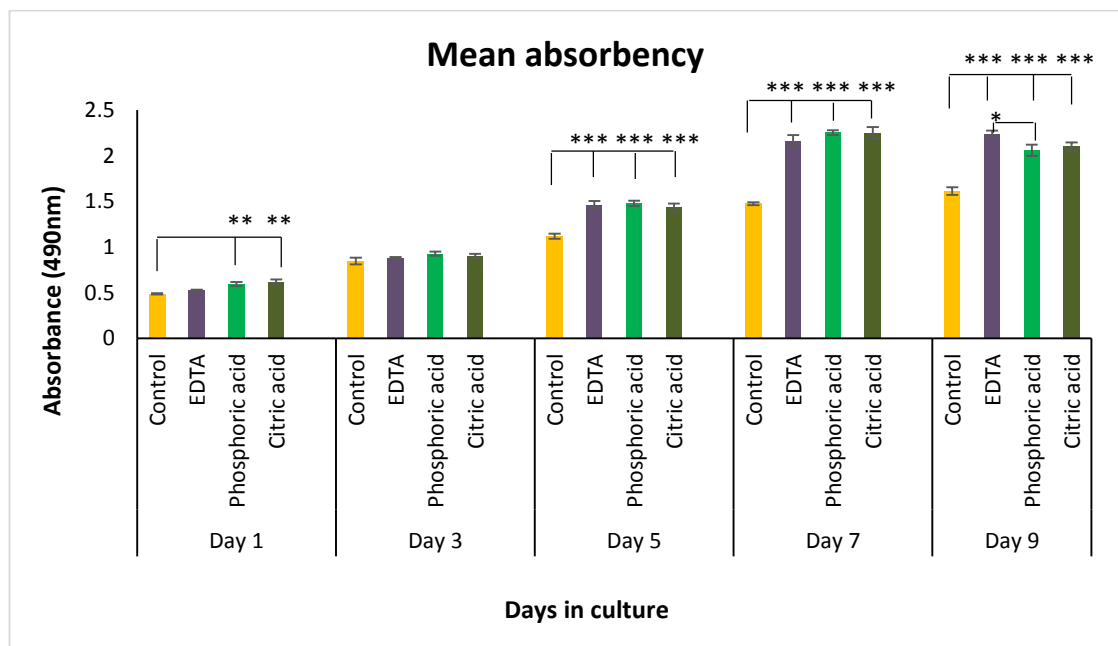
Figure 3.1: MTT assay showing mean absorbance of control and test groups on day1-9 post stimulation. * represents significant difference at $P<0.05$, ** at $P<0.01$ and *** at $P<0.001$.

3.3.2 Phenotype differentiation

3.3.2.1 Alizarin Red staining

Cells were observed to be tall, spindle shaped, and fibroblast like in morphology at the baseline (Figure 3.2). Alizarin red staining revealed the presence of calcium containing nodules as early as day 5 in DME stimulated cultures. The orientation of cells appeared different in areas of mineralisation with cells clustering around the nodules in the stimulated cultures. In control cultures, cells were relatively sparse with the outline of individual cells still identifiable at this early stage.

With increasing time, mineralised nodules increased in number and size in all DME stimulated groups. Controls however, did not exhibit mineralised nodule formation until day 15. Even then, the identified nodules were small and sparse compared to the test groups (Figure 3.3-3.5).
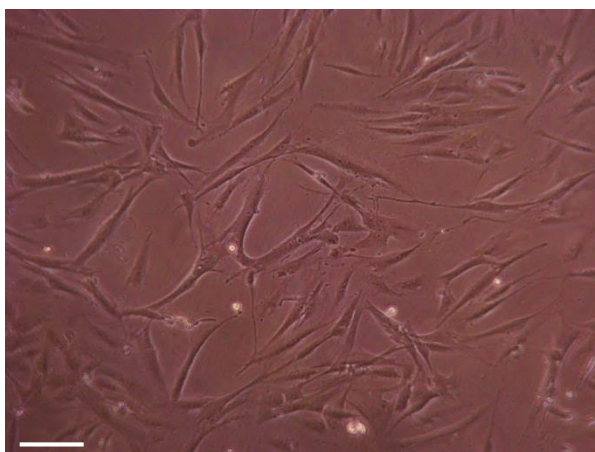
Figure 3.2: Tall spindle shaped morphology of DPSCs at base line (24 hr after start of culture and prior to stimulation). ×10 magnification, scale bar 100μm.
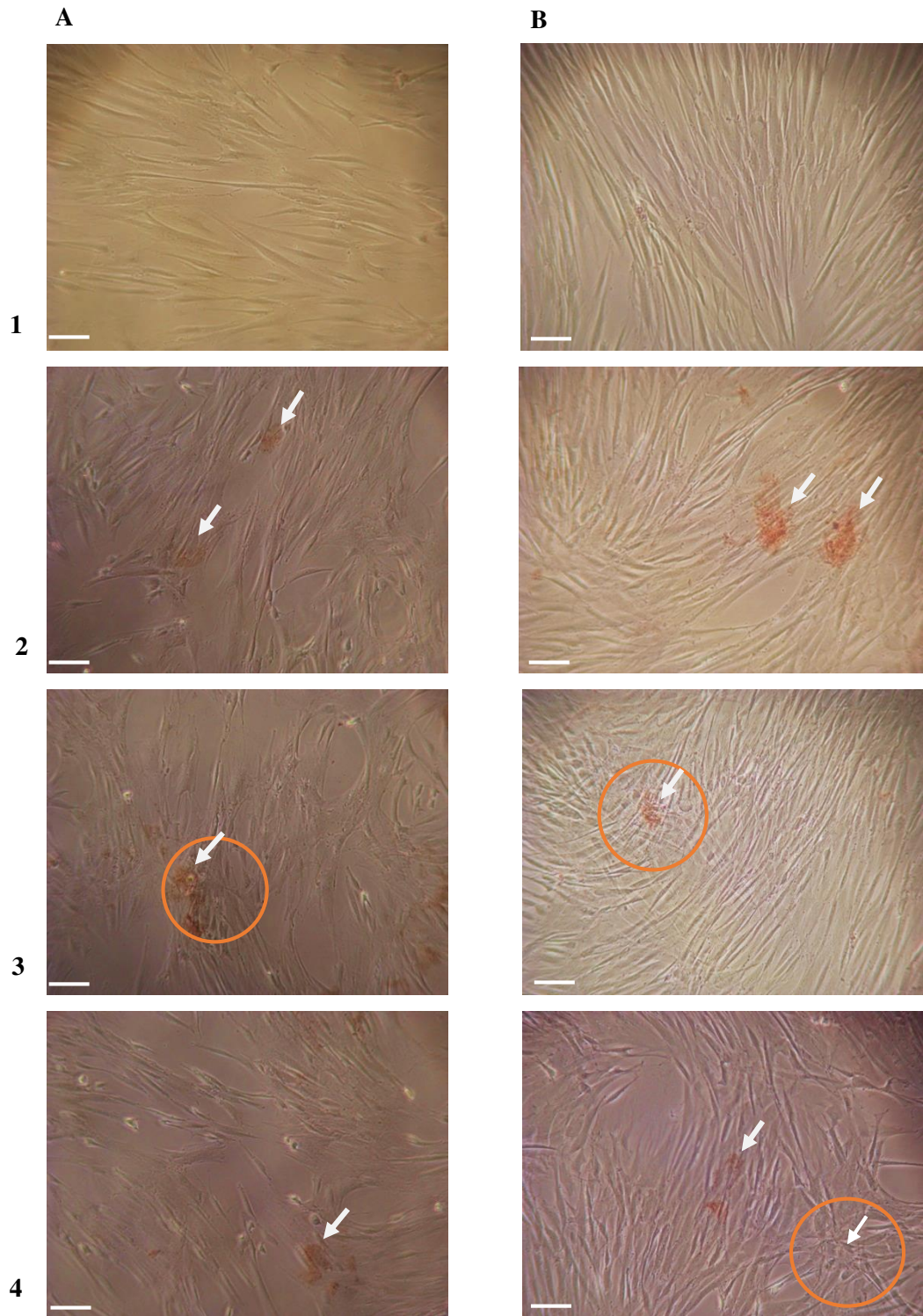
Figure 3.3: Representative light microscopy images of alizarin red stained cell cultures on day 5 (A) and day 8 (B). 1) Control, 2) stimulated with EDTA, 3) phosphoric acid and 4) citric acid extracted DMEs. Faintly stained mineralised nodules are present on day 5 and 8 in all test groups (arrows) with some cell clusters (circle). No staining is observed in the control group at this stage. ×10 magnification, scale bar 100μm.
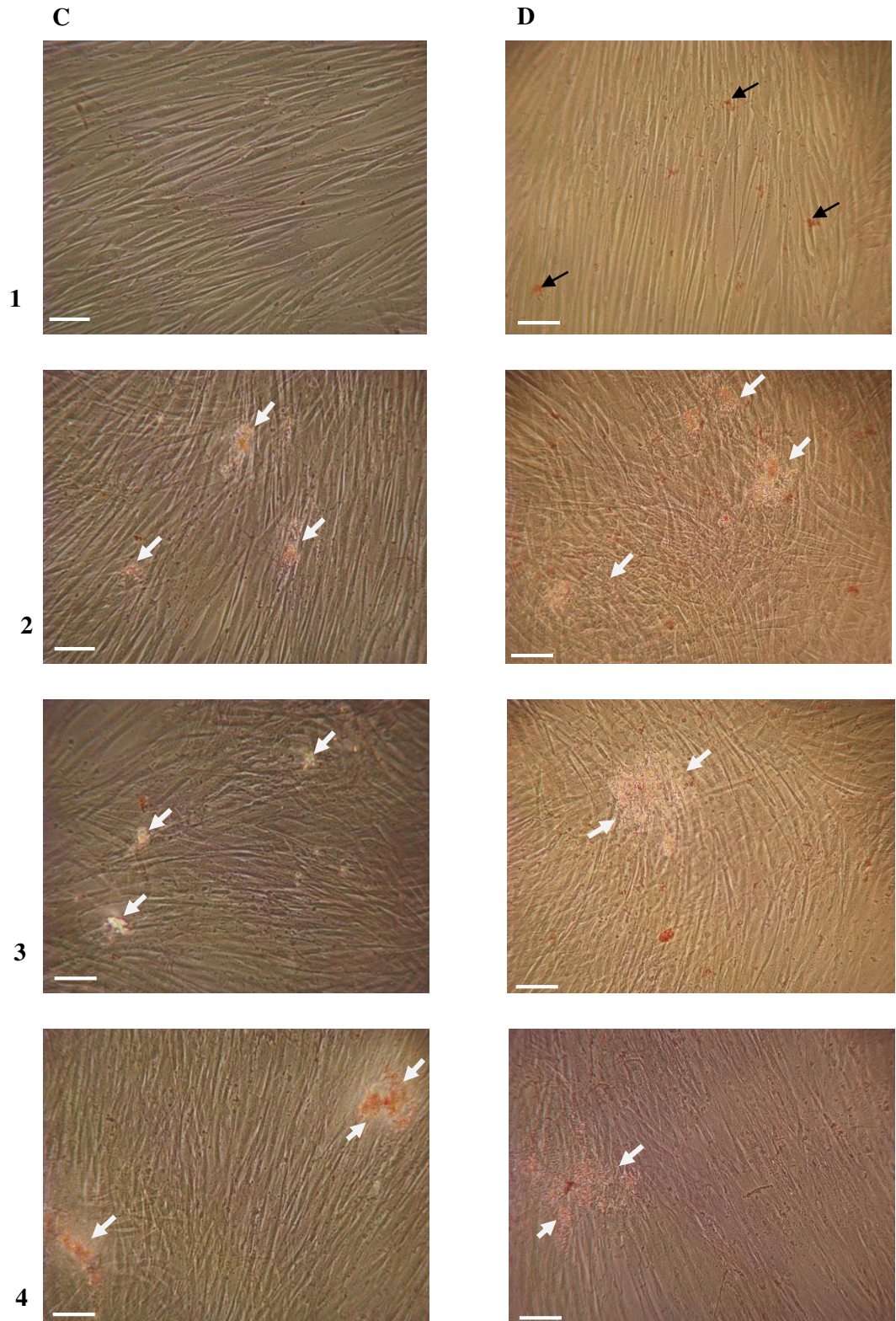
Figure 3.4: Representative light microscopy images of alizarin red stained cultures on day 12 (C) and day 15 (D). 1) Control, 2) stimulated with EDTA, 3) phosphoric acid and 4) citric acid extracted DMEs. Mineralised nodules have increased in numbers and size. No staining is observed in control on day 12, however, on day 15 some small nodules have appeared. ×10 magnification, scale bar 100μm.
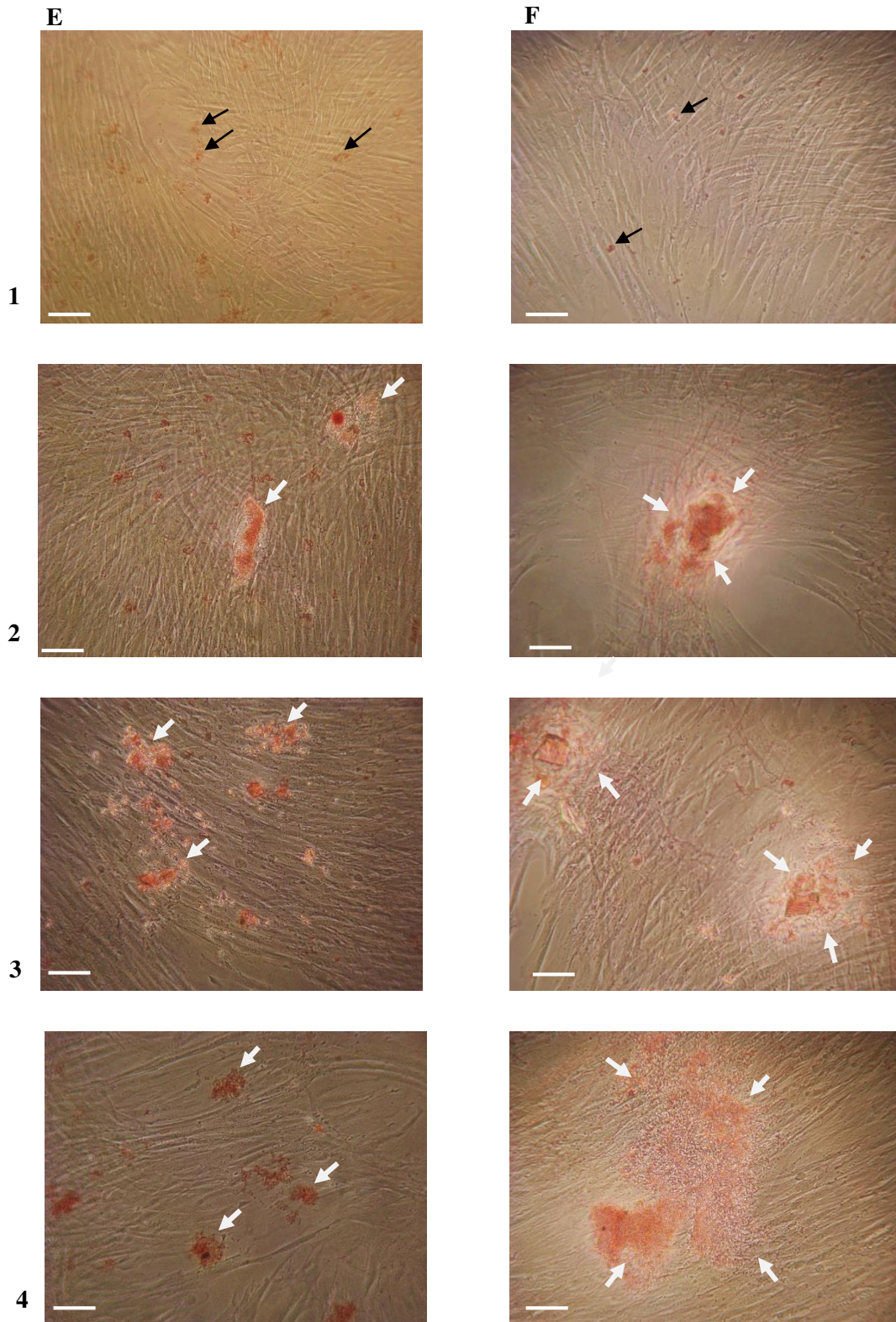
Figure 3.5: Representative light microscopy images of alizarin red stained cultures on day 18 (E) and day 21 (F). 1) Control, 2) stimulated with EDTA extracted DMEs, 3) stimulated with phosphoric acid extracted DMEs and 4) stimulated with citric acid extracted DMEs. Higher levels of staining is apparent also the nodules have increased in number and size. ×10 magnification, scale bar 100μm.

3.3.2.2 Oil Red O staining

Oil Red O staining on day 21 revealed presence of one or two lipid containing cells in each well in the cultures of control, EDTA and citric acid stimulated cultures. Although very few in numbers, rendering a quantitative comparison of a little value, the cells in control cultures represented the characteristic mature adipocyte shape and the accumulation of lipid droplets more clearly compared with those in the test. No staining could be detected in the group stimulated with phosphoric acid extracted DMEs (Figure 3.6).
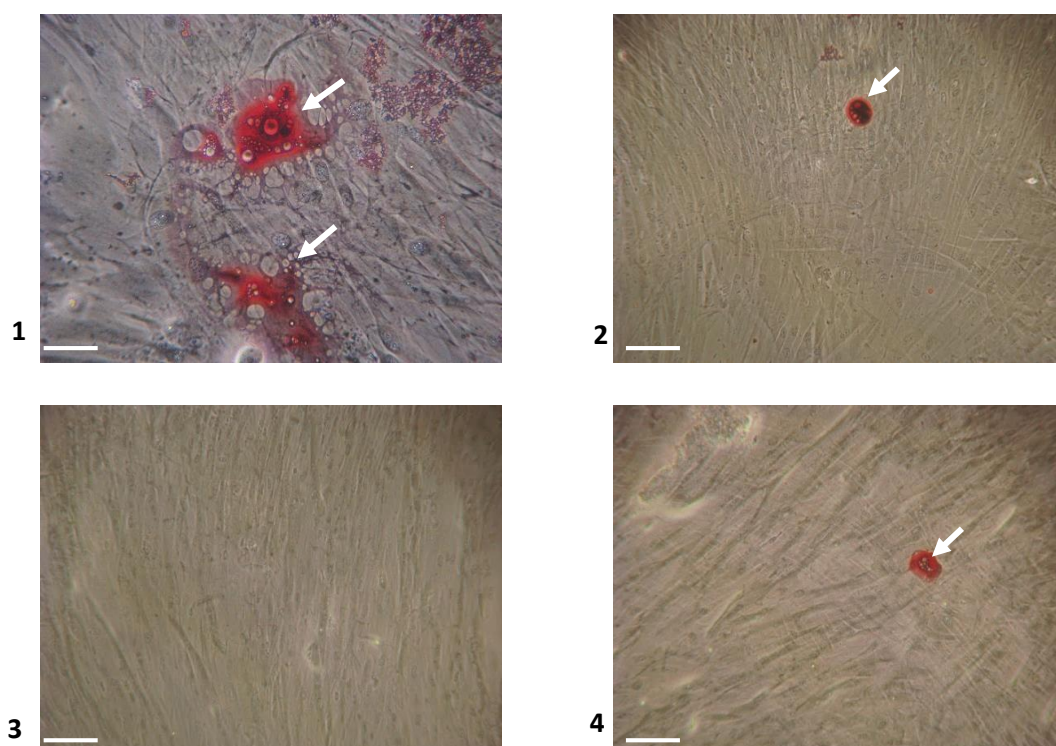
Figure 3.6: Representative light microscopy images of Oil Red O stained cultures on day 21 showing cells containing lipid droplets (arrow). 1) Control, 2) stimulated with EDTA extracted DMEs 3) stimulated with phosphoric acid extracted DMEs (no stained cells) and 4) stimulated with citric acid extracted DME. ×10 magnification, scale bar 100μm.

3.3.3 Immunocytochemistry

3.3.3.1 Collagen1A1 immunolocalisation

Collagen1A1 was detected in all the test cultures on day 2. No expression was detected in control cultures at this stage. By day 14 however, the protein was detected in control as well as test groups. Visual inspection of cultures by fluorescent microscopy revealed wide spread fluorescence in all groups at this stage (Figure 3.7). No non-specific staining was detected as confirmed by negative controls in which primary antibody were omitted (Figure 3.9).

3.3.3.2 DSP immunolocalisation

DSP was detected in all the test groups on day 2. No expression could be detected in control cultures at this stage. By day 14 however, the protein was detected in control and test groups. Visual inspection of cultures by fluorescent microscopy revealed more widespread fluorescence in all test groups compared with control at this stage (Figure 3.8). No non-specific staining was detected as confirmed by negative controls in which the primary antibody were omitted (Figure 3.9).
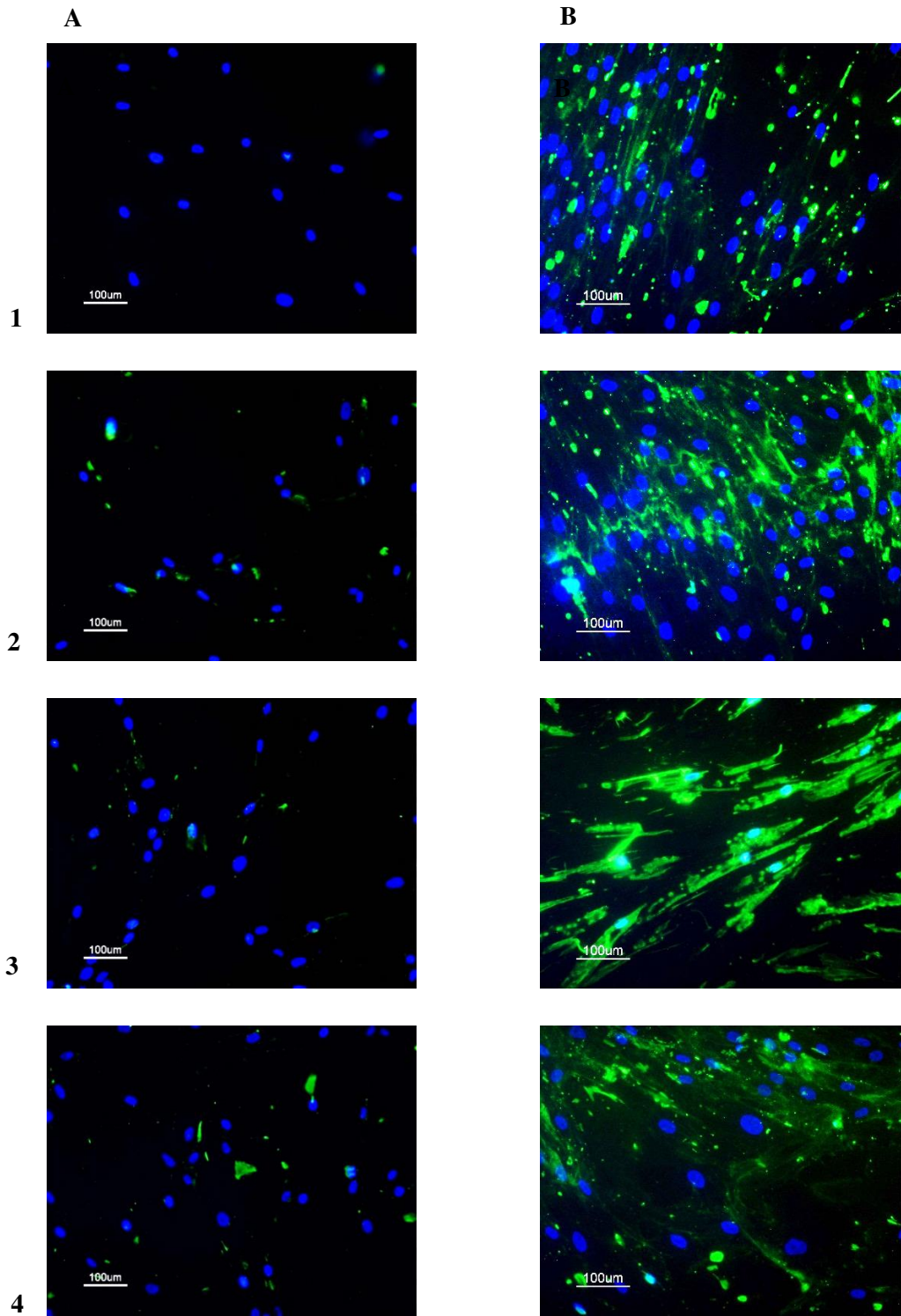
Figure 3.7: Representative fluorescent microscopy images for immunolocalisation of collagen1A1 on day 2 (A) and day 14 (B). 1) Control, 2) stimulated with EDTA extracted DMEs 3) stimulated with phosphoric acid extracted DMEs and 4) stimulated with citric acid extracted DME. The localisation of collagen is presented in green and nuclei are blue.
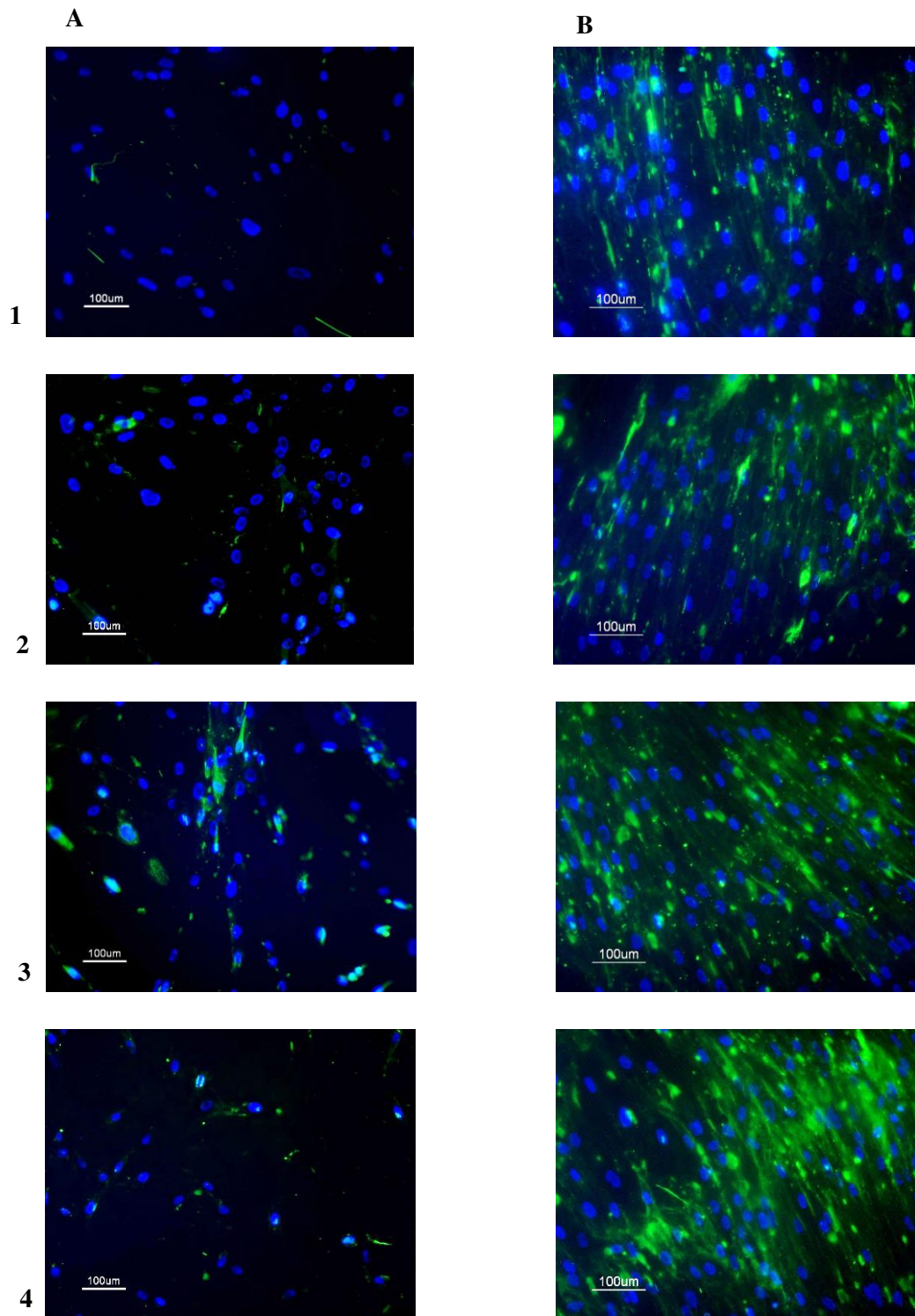
Figure 3.8: Representative fluorescent microscopy images for immunolocalisation of DSP on day 2 (A) and day 14 (B). 1) Control, 2) stimulated with EDTA extracted DMEs 3) stimulated with phosphoric acid extracted DMEs and 4) stimulated with citric acid extracted DMEs. The localisation of DSP is presented in green and nuclei are blue.
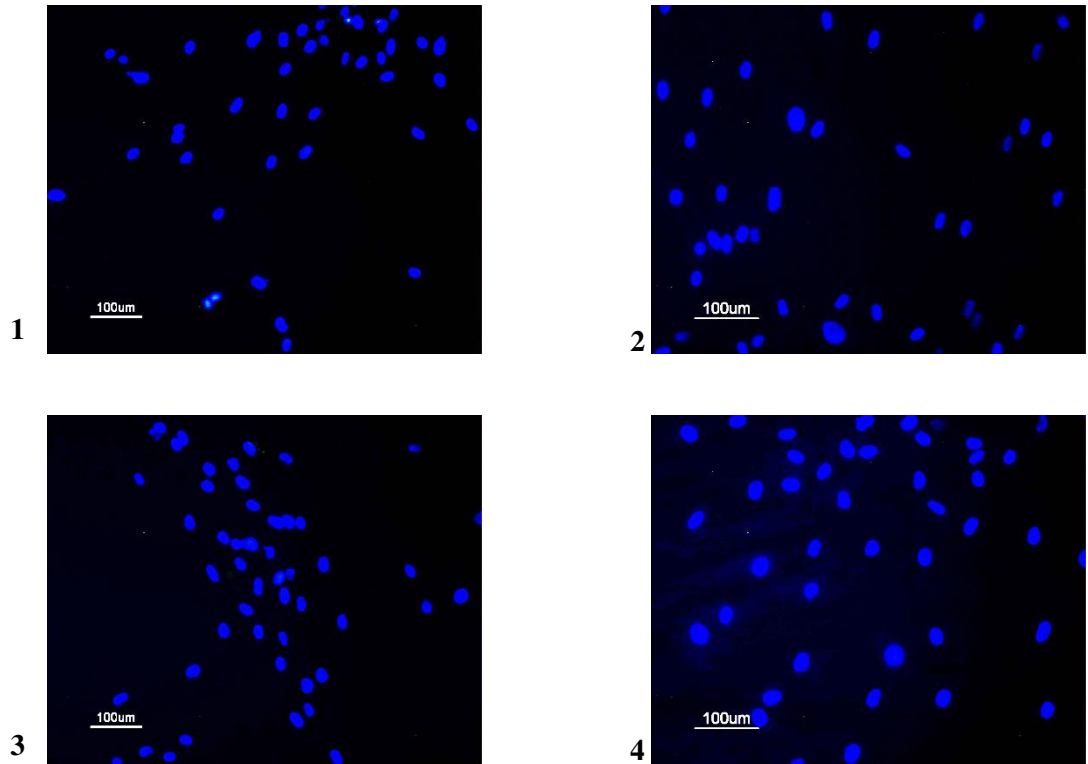
Figure 3.9: Immunocytochemistry negative controls: 1) Collagen1A1 day 2, 2) Collagen1A1 day 14, 3) DSP day 2 and 4) DSP day 14. Nuclei appear blue and there is absence of FITC fluorescence when primary antibodies are blocked.

3.3.4 Gene expression changes in cells exposed to DMEs

3.3.4.1 RT-PCR

β-actin was used as the house keeping gene for RT-PCRs. The expression levels of the other genes were visually compared with the house keeping gene which had a constant expression level throughout the experiment. Expression of mesenchymal stem cell markers CD 73, CD 90, CD146 and CD105 were detectable throughout the period of study (up to day 21) however, RT-PCR could not detect any change in the levels of expression over time or differences between groups. Similarly, the method could not detect any noticeable differences over time nor between groups for the apoptosis marker, Bax.

As observed by decreasing intensity of the product band, the expression levels of the proliferation marker PCNA appeared to decrease over time in all groups. The transcriptional repressor, MSX1 expression levels appeared to take an opposite trend to that of PCNA and a gradual increase in intensity of the product band was observed over time. Collagen1A1 and TGF-β1 product band were detected throughout the period of experiment and RT-PCR could not detect any noticeable difference in the intensity of the products band. Only a slight weakening of TGF-β1 product band for groups exposed to phosphoric and citric acid extracted DMES was suggested on day 21. The adipogenic differentiation marker gene, PPARγ2, expression could not be detected at day 1 in any group. On day 7, a faint signal was detected in control group and the intensity of product band increased over time. For test groups, faint product band appeared on day 14 and even on day 21, signal intensity was barely detectable in groups stimulated with phosphoric and citric acid DMEs. Expression of the

mineralising tissues differentiation marker alkaline phosphatase was detected from day 14 onwards in the group exposed to phosphoric acid extracted DMEs (as suggested by a faint product band) and on day 21 weak signals appeared for the remaining test groups. No signal could be detected for control at any time point (Figure 3.10).
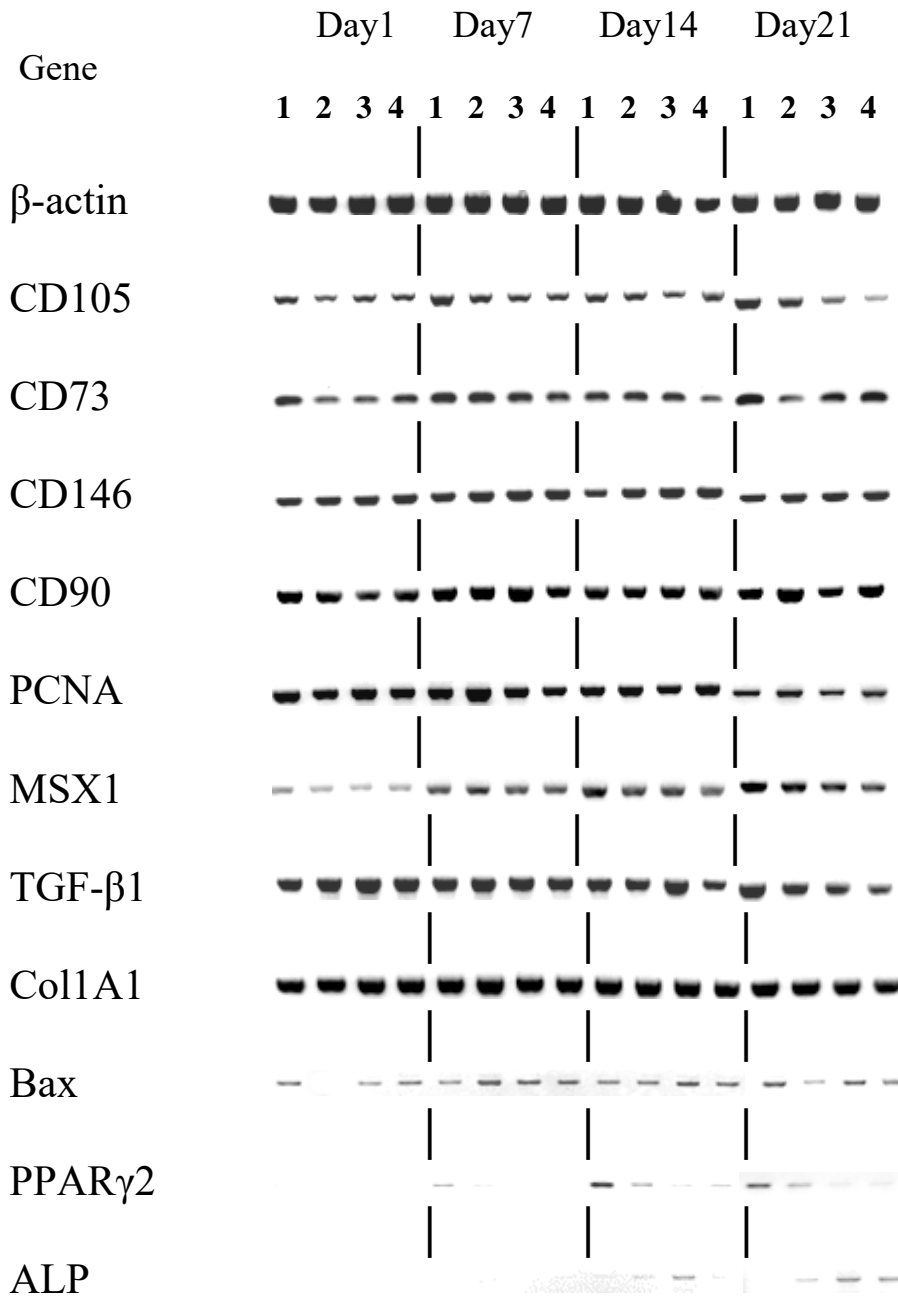
Figure 3.10: RT-PCR results showing the mRNA expression profile of DPSCs: 1) control, 2) stimulated with EDTA extracted DMEs 3) stimulated with phosphoric acid extracted DMEs and 4) stimulated with citric acid extracted DMEs on day1-21.

3.3.4.2 Quantitative Real-Time PCR

The expression of mesenchymal stem cell marker CD105, was found to be lower in the DME stimulated groups compared to the unstimulated control, at all the time points. The difference between tests and the control was statistically significant on day 21 (34-46% in test compared with 110% in control group, $P<0.05$) (Figure 3.11).

The gene expression of CD146 was down regulated over time for all groups (58-89% in the test groups and 12% in control on day 3 to 31-57% in the tests compared with 7% in control group on day 21). The expression was significantly higher for the DME stimulated groups compared with controls at all the time points ($P<0.01$) (Figure 3.12).

Figure 3.11: Gene expression of CD105 in 1) controls and, cells stimulated with 2) EDTA extracted DMEs 3) phosphoric acid extracted DMEs and 4) citric acid extracted DMEs on days 3, 10 and 21. Expression in control group is significantly higher than the DME stimulated groups throughout. * and ** represents significant difference with control at P<0.05 and 0.01 respectively. No differences were detected between the test groups (P>0.05).
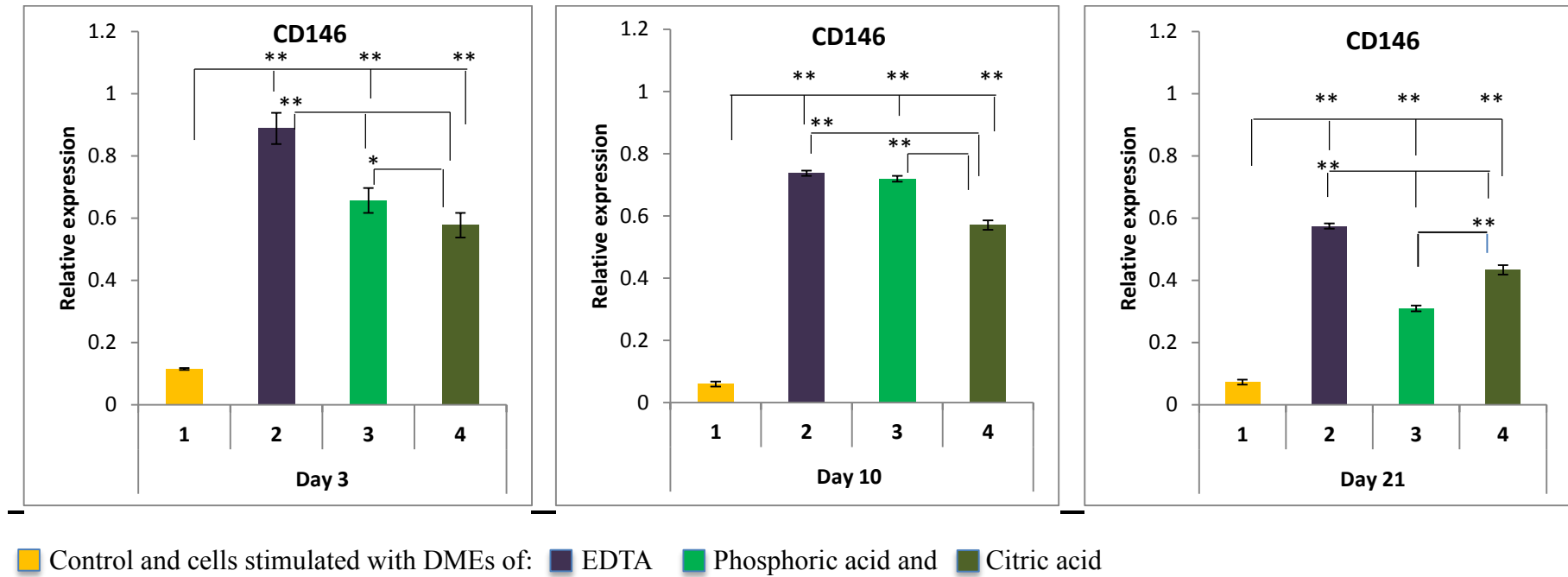
Figure 3.12: Gene expression of CD146 in 1) controls and, cells stimulated with 2) EDTA extracted DMEs 3) phosphoric acid extracted DMEs and 4) citric acid extracted DMEs on days 3, 10 and 21. Expression in control group is significantly lower than the DME stimulated groups throughout. * and ** represent significant differences between groups at P<0.05 and 0.01 respectively.

The expression of proliferation marker PCNA increased between days 3 and 21 in control group (7% to 16%) compared with random fluctuations in the test groups. On day 10 and 21, control group had the highest expression level (16% compared with 8-10% in the test groups), however, the difference failed to reach statistical significance on day 21 (Figure 3.13).

The transcription factor RUNX2 was expressed in all groups at all the time points, however no statistically significant differences between groups could be detected at any stage (Figure 3.14).
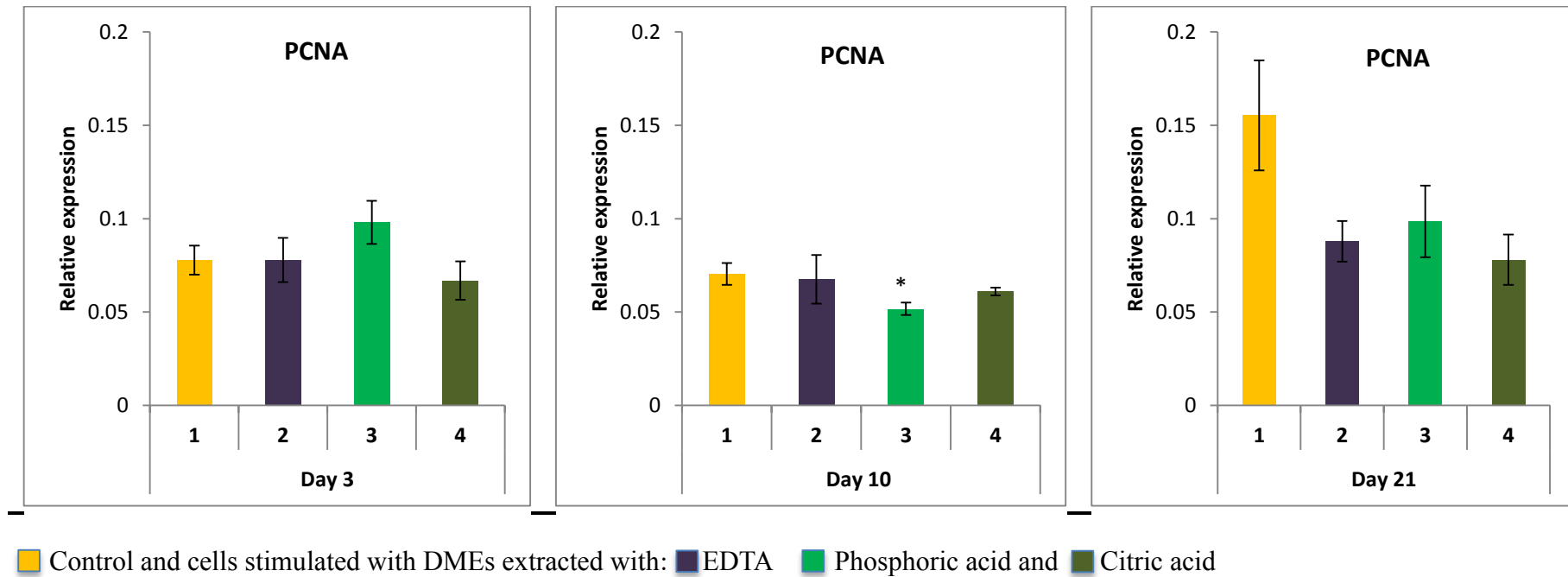
Figure 3.13: Gene expresion of PCNA in 1) control, 2) stimulated with EDTA extracted DMEs 3) stimulated with phosphoric acid extracted DMEs and 4) stimulated with citric acid extracted DMEs on days 3, 10 and 21. Expression levels increased overtime in control group and is significantly higher than the DME stimulated groups on day 21. * represents significant difference with control at P<0.05. No differences were detected between the test groups (P>0.05).
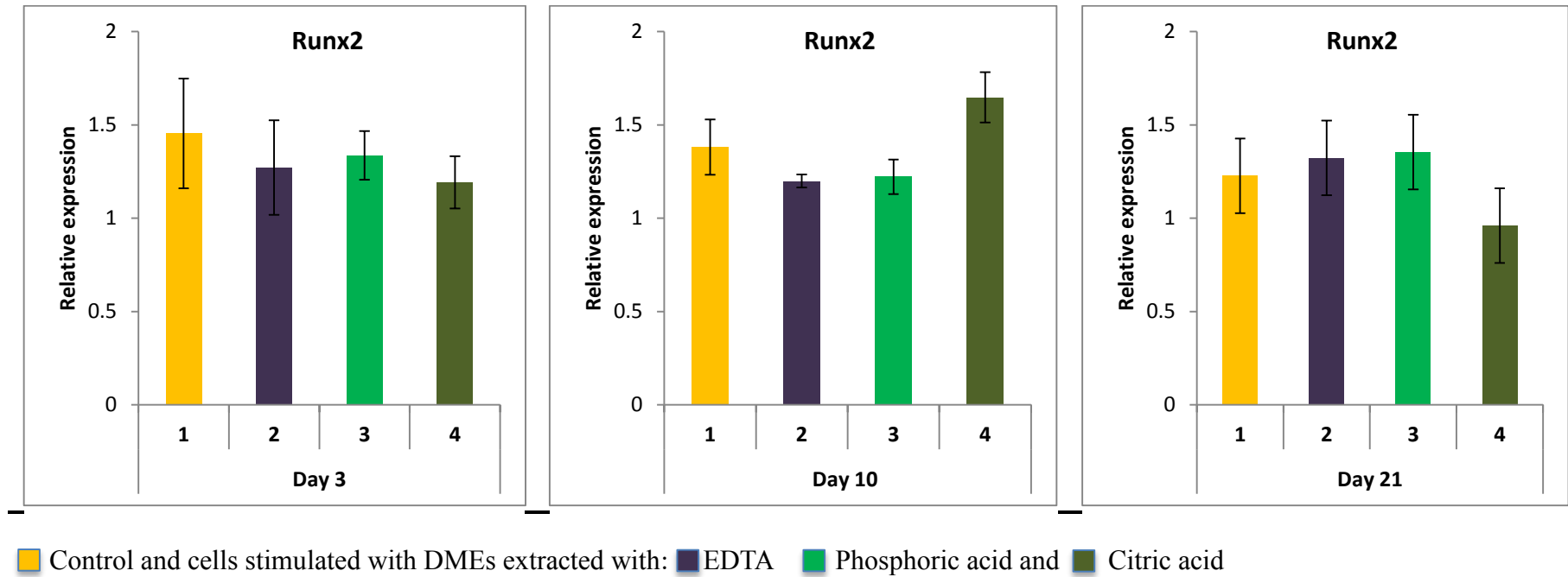
Figure 3.14: Gene expression of RUNX2 in 1) controls and, cells stimulated with 2) EDTA extracted DMEs 3) phosphoric acid extracted DMEs and 4) citric acid extracted DMEs on days 3, 10 and 21. No significant difference (P>0.05) could be detected between groups at any time point.

An upregulation in the expression of differentiation marker osteopontin was observed with DME stimulation of DPSCs (0.13-0.17% in test groups compared with 0.04% in control) on day 3 and 2.6-4.5% in tests compared with 0.2% in controls on day 21, in other words expression for test groups on day 21, was 13-28 times that of control group, ($P<0.01$) (Figure 3.15).

Similarly, the expression of alkaline phosphatise was upregulated with DME stimulation of DPSCs (1.7-3.1% in test groups compared with 1.2% in control on day 3 and 32-43% in tests compared with 27% in control on day 21 ($P<0.01$ in the group stimulated with phosphoric acid extracted DMEs on day 10 and $P<0.05$ for groups stimulated with EDTA and phosphoric acid extracted DMEs on day 21) (Figure 3.16).
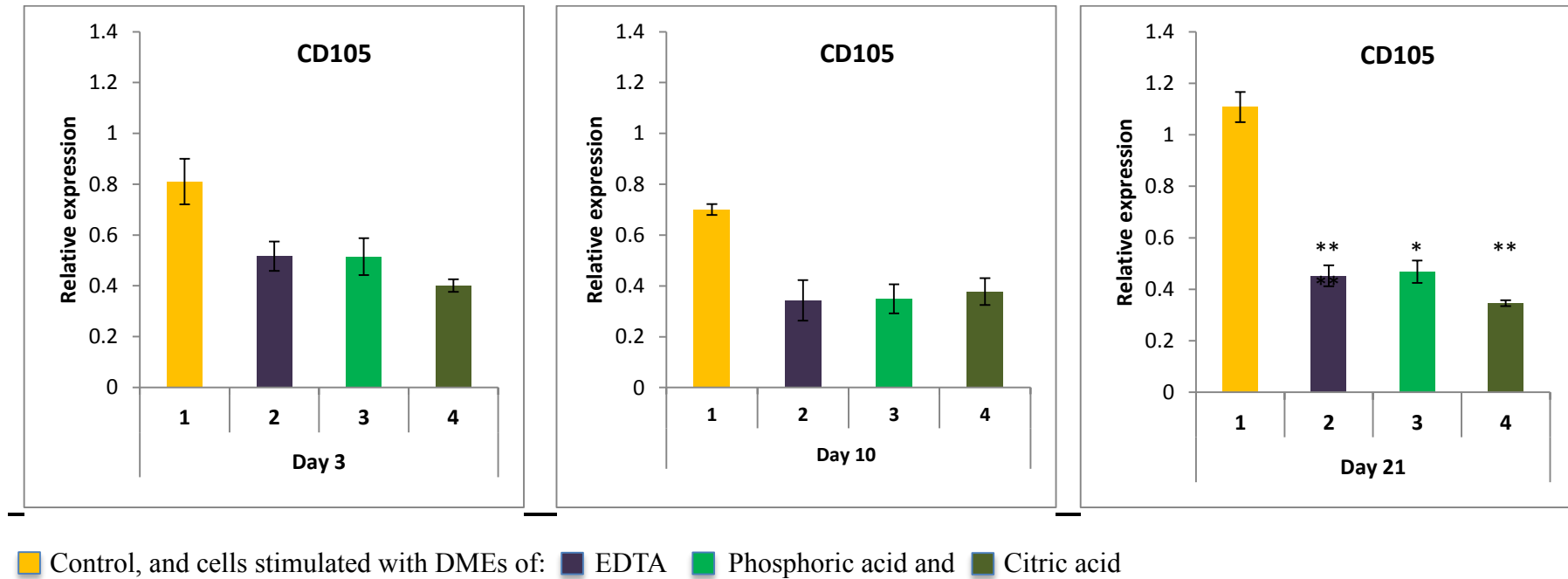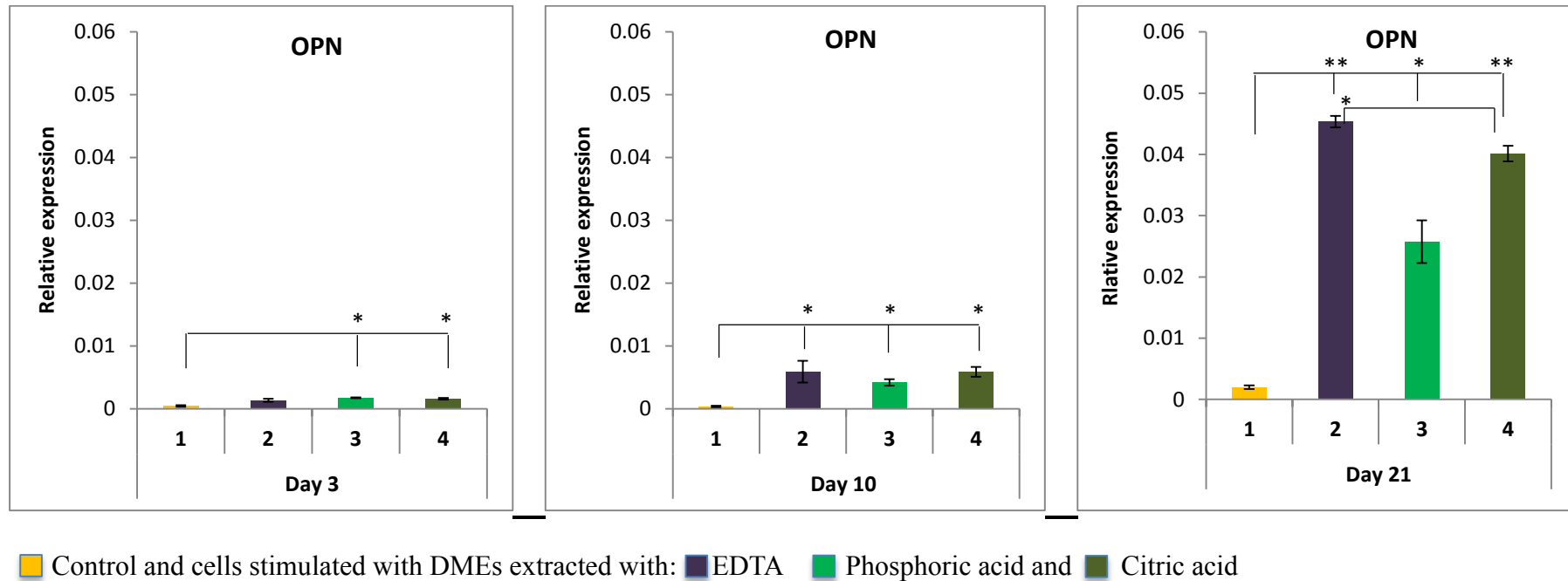
Figure 3.15: Gene expression of Osteopontin in 1) controls and, cells stimulated with 2) EDTA extracted DMEs 3) phosphoric acid extracted DMEs and 4) citric acid extracted DMEs on days 3, 10 and 21. Expression in control group is significantly lower than the DME stimulated groups throughout the experiment. * and ** represent significant difference between groups at P<0.05 and P<0.01 respectively.
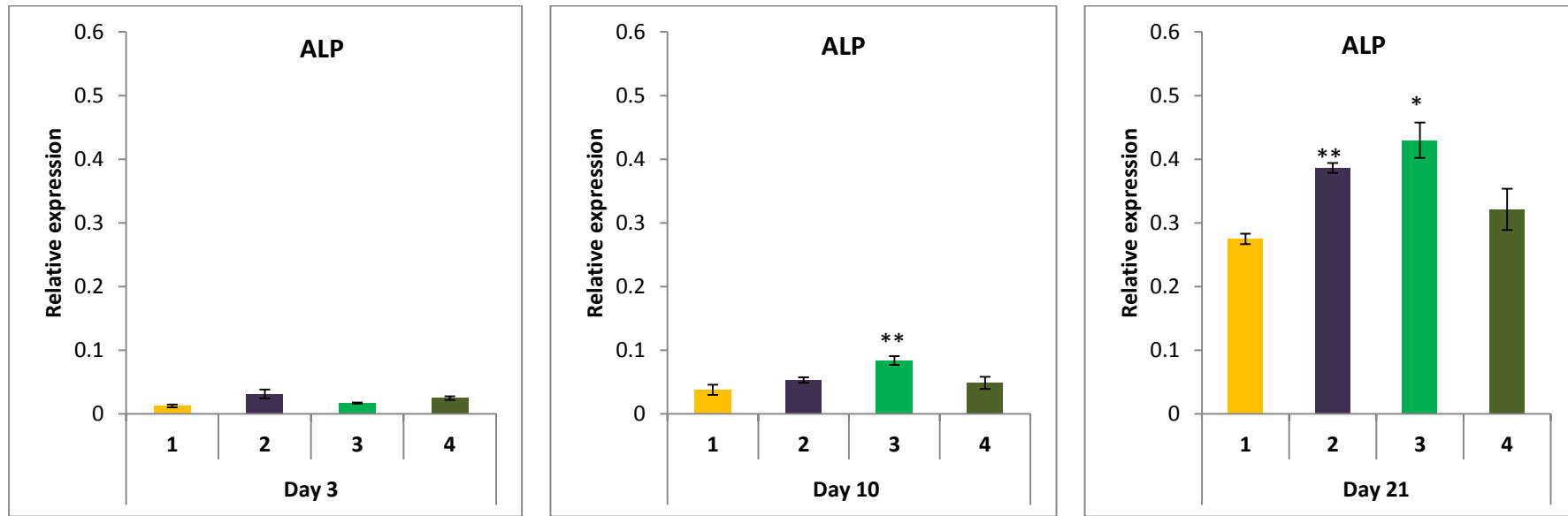
Figure 3.16: Gene expression of Alkaline Phosphatase in 1) controls and, cells stimulated with 2) EDTA extracted DMEs 3) phosphoric acid extracted DMEs and 4) citric acid extracted DMEs on days 3, 10 and 21. Expression in control group was significantly lower than the DME stimulated groups throughout. * and ** represents significant difference with control at P<0.05 and 0.01 respectively. No differences were detected between the test groups (P>0.05).

3.4 Discussion

The Dental Pulp Progenitor clone (A31) used in this study, had previously been characterised and was known to be highly proliferative with population doublings extending beyond 80 PD (over 300 days) and its ability to differentiate onto osteoblast, adipocyte and chondrocyte lineagaes, when cultured in the appropriate media, was demonstrated (Amr Alraies, PhD thesis, 2013, School of Dentistry, Cardiff University).

The results of this chapter demonstated the ability of phosphoric acid and citric acid as well as EDTA extracted DMEs to induce a potent stimulatory response in dental pulp progenitor cells (DPSCs). The MTT assay showed a significant increase in cellular metabolic activity which signifies DPSCs proliferation and increased cell numbers (Loveland et al. 1992). The ability of DMEs to induce DPSC differentiation was also investigated in this chapter. Although the abillity of EDTA extracted DMEs in inducing mineralised phenotype differentiation has previously been reported (Liu et al. 2005; Davies et al. 2014), what is novel in this chapter is the investigation of such ability in relation to the two clinically used acids, phosphoric and citric acid.

In in vitro studies, odontoblastic/osteoblastic differentiation is usually characterised by the ability of cells in producing a number of extracellular proteins including collagen type I and the specific non-collagenous proteins of the ECM, mineralised nodule formation, increased levels of alkline phosphatase activity and expression of differentiation marker mRNA (Kim et al. 2014a; Twine et al. 2014). In this chapter, the ability of DMEs in inducing mineralised tissue phenotype in DPSCs was evidenced by the presence of the ECM proteins, collagen I and DSP, and mineralisation of the matrix as well as the upregulation of mineralisation marker genes, osteopontin and alkaline

phosphatase. Differentiation of stem cells is a gradually progressing process similar to an in vivo situation, starting with the commitment of the progenitor cells to the lineage and differentiation to immature followed by the mature fully differentiated cells (Liu et al. 2013). As such, specific transcription factors induce odontoblast/osteoblast-like cell differentiation in DPSCs to acquire mineralised phenotype and promote the expression of proteins typical of mineralised tissue.

Relating to the stage of development, a marker of early-stage odontogenesis/ osteogenesis is ALP. ALP provides the organic phosphate for hydroxyapetite formation in the ECM (Harris 1990) and has been widely used to evaluate the osteogenic potential of cells *in vitro* (Alonso et al. 2008; Bakopoulou et al. 2011; Wang et al. 2013). The expression of ALP was evaluated by both RT and qPCR in this chapter. RT-PCR results revealed the expression of ALP in all DME stimulated groups on day 14 and 21 while the same method failed to detect ALP expression in control group at all the time points. QPCR supported this data, showing higher expression levels for all the DME stimulated groups at all the time points. For example stimulation by EDTA and citric acid extracted DMEs resulted in respectively 2.5 and 2 fold increased ALP expression (3.1 and 2.5%) in DPSCs compared with the un-stimulated controls (1.2%) as early as day 3 ($P>0.05$). The expression of ALP increased progressively in all the test as well as control groups, and the expression was consistently higher in the stimulated cells compared with controls on day 10 ($P<0.01$ in group stimulated with phosphoric acid extracted DMEs) and day 21 ($P<0.01$ in groups stimulated with EDTA and phosphoric acid extracted DMEs). This finding suggests that stimulation by DMEs, resulted in increased expression of ALP gene which is important in promoting DPSCs differentiation to a mineralised tissue phenotype. Similar results have been reported in another in vitro

study where stimulation of DPSC with EDTA extracted DMEs resulted in an upregulation in ALP, coinciding with initiation of mineralisation in the matrix (Liu et al. 2005).

Runx2 is considered the major transcription factor controlling osteoblast differentiation. It regulates the expression of a number of osteogenic genes such as osteopontin (OPN), osteocalcin (OCN), bone sialoprotein and collagen type I (reviewed in Liu and Lee (2013)). Even a heterozygous loss of RUNX2 is sufficient to result in skeletal deformities in mice similar to cleidocranical dysplasia in humans (Komori et al. 1997) while its homozygous mutation results in death right after birth due to respiratory failure in mice which also suffered a lack of osteoblats and bone (Baba et al. 2004). RUNX2 expression was detected in all groups by qPCR in this study with no statistically significant difference between groups. Expression of RUNX2 in control as well as DME stiumulated groups at all stages of the experiment (day 3-21), may indicate potentiality of the clone to differentiate to mineralised tissue phenotype rather than an actual marker of differentiation, as other authors suggested (Marie 2008; Semeghini et al. 2012). Expression of collagen type I was investigated using RT-PCR and similar to RUNX2, it was shown to be present in in all groups throughout the experiment (day 1 to day 21). QPCR was used to investigate the expression of OPN in this chapter and it was revealed that stimulation by DMEs in all the test groups resulted in a significant upregualtion compared with the unstimulated controls from the early stage (3-4 fold, 0.13-0.17% in test groups compared with 0.04% in control on day 3, $P<0.05$ in groups stimulated with phosphoric and citric acid extracted DMEs) through to the late stage of the experiment (day 21). The expression levels, as well as fold differences between the tests and control group, progressively increased and reached 2.6-4.0% in tests compared

with 0.2% in control group (13-23 fold increase in tests compared with control group, P<0.05 in all DME stimulated groups) on day 21. The up regulation of typical ostoblast/odontoblast genes (such as ALP and OPN), together with specific phenotype changes in DPSCs would be considered as a marker of odontoblast/osteoblast-like cell differentiation as reported by other in in vitro studies (Mori et al. 2011; Qu and Liu 2013).

Further to the investigation of gene expression, immunocytochemistry was performed in this chapter to investigate the presence of the typical ECM protein, type I collagen and the non-collagenous mineralised ECM protein, dentine sialoprotein (DCP) in the cultures. Collagen type I was present in the matrix of DME stimulated cultures as early as day 2, but not in control cultures at this stage. The protein was however detected in control cultures on day 14, suggesting a comparatively delayed protein translation in the unstimulated cells. Similarly, immunocytochemistry revealed DSP to be present in the matrix produced by DME stimulated cells as early as day 2 but could not detect the protein in controls at this stage. Expression of DSP may mark the initiation of mineralisation in ECM as such a role has been defined for it in relation to dentine ECM *in vivo* (Suzuki et al. 2012). On day 14, DPS was detected in control cultures as well in stimulated cultures although the fluorescence was not as widespread. The delayed appearance of ECM proteins in the unstimulated control compared with DME stimulated culture further supports the crucial role of DMEs in inducing differentiation in DPSCs in this chapter. These findings were also supported with histological investigation in conjunction with alizarin red staining.

A change in phenotype was observed after only 5 days in culture in DME stimulated groups where alizarin red staining confirmed the presence of mineralised nodules in the ECM. Upon visual inspection, the nodules appeared to increase in size over time, and by day 21 in DME stimulated cultures, the nodules appeared to be more widespread and covered larger areas in DME stimulated cultures. Although some staining was observed in the control group also, this was not until much later (day 15). The stained structures did not have the distinct nodule morphology as in DME stimulated cultures, and did not appear to grow in size over time. These results reassert the gene expression data suggesting that stimulation by phosphoric and citric acid as well as EDTA extracted DMEs induce DPSCs to what is evidenced as a mineralised phenotype.

With Oil Red O staining, some random and sporadic adipogenic differentiation in control cultures was observed at the late stage of the experiment, day 21. There was no convincing evidence of adipogenic differentiation in the stimulated cultures with Oil Red O staining. The above observation was further supported by RT-PCR results where expression of PPARγ2, the key adepogenic transcription factor, was detected relatively early (day 7) in the control group with only a faint product band appearing in the stimulated group later on in the experiment (day 14). Accordingly, it may be suggested that while stimulation by DMEs drives the cells towards a mineralised phenotype differentiation, in the absence of such stimulation the DPSCs may have some tendency for sporadic adipogenic differentiation under the experimental conditions applied in this chapter.

The evidence of random, sporadic differentiation in control cultures, to both mineralised and adipogenic phenotypes, at the late stage of the experiment (day 21), may be

explained by the increased cell numbers and cellular contact in cultures that have reached confluency for days by this stage of the experiment. It has been demonstrated *in vitro* that cell-cell contact may influence differentiation in mesenchymal stem cells (MSCs) via a number of possible mechanisms involving the conjugation of cell surface molecules in the adjacent cells. This may involve formation of gap junctions allowing the passage of cell signalling molecules, also juxtacrine ligand-receptor pairing which enable communication between cells (Tang et al. 2010). Gap junctions have been shown to exist between DPSCs as evidenced by the presence of gap junction-forming protein Connexin-43 (Zhurova et al. 2010). Furthermore, it has been shown that both osteogenic and adipogenic differentiation may be regulated via cell-cell contact (Tang et al. 2010).

In addition to the mechanisms explained above, cell communications can take place via paracrine signalling which may play a role in inducing differentiation (Hemmingsen et al. 2013). Several cell signalling molecules expressed in DPSCs genome, including bone BMPs (Kim et al. 2011) and insulin growth factor (IGF) (Shi et al. 2001), may display both pro-osteogenic and pro-adipogenic effects (James 2013). Based on the opposite trends observed in the expression of ALP and PPARγ_2 between the DME stimulated groups and controls, as demonstrated by RT-PCR results in this chapter and the observed difference in phenotypes between the stimulated cultures and control group in the period of this study (21 days), it may be concluded that stimulation by DMEs, drives the cells towards mineralised tissue differentiation and reduces their tendency for adipogenic differentiation.

Since there are no DPSC specific markers currently identified (Kim et al. 2011), the expression of a number of general MSCs marker, CD73, CD90, CD105 and CD146

were assessed using RT-PCR. All were found to be present for duration of the experiment (21 days) however no obvious change in expression levels could be detected using this method (as judged by intensity of the product band). RT-PCR suggeted a weakening in the expression of TGF-β1 in DME stimulated cells, which has been previously reported in DPSCs undergoing mineralised phynotype differentiation (Liu et al. 2007). Furthermore the expression of CD105 (endoglin) which is part of TGF-β1 cell surface receptor complex, was significantly lower in DME stimulated groups compared with control on days 3-21. Combining the results of all the genes investigated in this chapter strengthens the evidence that stimulation by DMEs extracted by the agents used here, results in the loss of stem cell characteristics and a progression towards differentiation in DPSCs.

Quantification of PCNA gene expression with qPCR revealed a progressive increase for control group between day 3 and 21 (7% to 16%). On day 21, PCNA expression for the control group was higher than all the stimulated groups (however the difference did not reach statistical significance), which may suggest ongoing proliferation in the control while the stimulated cells appear to have moved further forward in their stage of development comparatively. RT-PCR did not suggest any change in the expression of the apoptosis marker Bax during the culture period in this study (21 days). With prolonged in vitro culture of stem cells and incresed cell mass, the cells may suffer a lack of nutriotion and hypoxia, which can accelerate apoptosis of the stem cells (Zhu et al. 2006). Our results suggest that cells here had not undergone apoptotic changes in the period of culture in this study (21 days).

The expression of several other genes were investigated using RT-PCR in this chapter. MSX1 is a transcription repressor which has been shown to have a major influence on calvarial growth and patterning during development (Chai and Maxson 2006) and is crucial for tooth morphogenesis (Jernvall and Thesleff 2000). Heterozygous MSX1 mutations in humans are shown to be associated with non syndromic posterior tooth agenesis (Kapadia et al. 2007) and cleft lip and palate (Dixon et al. 2011). RT-PCR in this chapter suggested an increase in MSX1 expression, as evidenced by an increase in intensity of the product band between day 3 and 21. MSX1 has been suggested to promote proliferation but prevent the differentiation of dental mesenchymal cells, in mice tooth germs *in vitro*, through the inhibition of Bmp2 and Bmp4 expression (Feng et al. 2013). However, an animal study in mice embryos has proposed a dual role for MSX1 in promoting proliferation and differentiation of osteogenic cells as well as the suppression of differentiation in cells not supposed to be differentiating into osteogenic lineage in the embryo *in vivo* (Roybal et al. 2010). The apparent increase in the expression over time in this chapter, may relate to the role of MSX1 in DPSCs proliferation and differentiation to pro-osteogenic/ odontogenic cells.

Using qPCR, the expression of CD146 was found to be significantly upregulated with DME stimulation. CD146 is a cell adhesion protein and is key in vascular endothelial cell activity and angiogenesis (Lv et al. 2014). Recent evidence indicates that in addition to being an inter-cellular and cell-matrix adhesion molecule, CD146 plays an active role in processes such as development, signal transduction, cell migration, differentiation, angiogenesis and immune response (Ouhtit et al. 2009; Wang and Yan 2013). Although generally considered as a marker of multipotency in competent MSCs (Sacchetti et al. 2007; Russell et al. 2010), high expression of CD146 is proposed to be associated with

high osteogenic potential and even possible commitment of MSCs to the osteogenic lineage (Pilz et al. 2011; Ulrich et al. 2015). The up-regulation of CD146 in DPSCs when stimulated by DMEs in this chapter, may be a reflection of the multifunctional nature of this cell surface protein in DPSCs which have become committed to a mineralised phenotype.

Although DSP was detected in the ECM produced by the cells, neither RT nor qPCR were able to detect the expression of DSPP in DPSCs during the course of the experiment. Considering that differentiation is a progressive and gradual process, different sets of genes would be activated at each stage of development leading up to the full differentiation (Twine et al. 2014). There are several possible explanations for this finding; it is possible that this gene was expressed for a short duration in the intervals between the time points tested here, and was therefore missed. Alternatively, the clone used here may have lost the expression of this gene in the earlier passages as has been shown in a study by Min et al (2011); when cultured in osteogenic medium the peak of expression of dentinogenic marker genes such as DSPP and DMP-1 in DPSCs occurred at the early passages of around 5 and it decreased with increasing passage number thereafter. This evidence may explain the failure in detection of DSPP in this chapter as cells of passage 13-14 were used here. Another alternative and more feasible explanation may be that since DSPP is considered predominantly as an odontogenic marker, expressed in mature odontoblasts (Chen et al. 2009), lack of expression by differentiating DPSCs in this chapter indicates differentiation to a general mineralised phenotype rather than mature primary odontoblasts. This hypothesis is supported by the fact that reparative dentine, produced by odontoblast-like cells has a structure more similar to bone than that of dentine produce by primary odontoblasts

(Goldberg et al. 2011). With this in mind, the gene expression profile of differentiated cells in this chapter possibly reflects that of odontoblast-like cells rather than primary odontoblasts in clinical situations.

The results of this chapter demonstrate the ability of DMEs extracted by phosphoric and citric acid as well as EDTA in stimulating odontoblast-like cell differentiation in DPSCs. However, in vital pulp therapy the direct effect of cell signalling molecules released from dentine surface (rather than powder) would be more relevant. The exposure and release charactristics of growth factors from dentine slabs treated by the etching/ conditioning agents will be investigated in the next chapter.

Chapter 4: Exposure and Release of Growth Factors from Conditioned Dentine Surfaces

4.1 Introduction

Although the mechanisms involved in tertiary dentine formation are still poorly understood, the sequestrated growth factors within dentine matrix may provide a possible source of cell-signalling molecules for the initiation of dentine regeneration in trauma/ injury situations. Bioactive molecules and growth factors are effective at very low, picogram levels, in eliciting cellular responses such as cell recruitment, migration, proliferation and differentiation as well as angiogenesis during wound healing and repair (Barrientos et al. 2008; Zhang et al. 2011b). Vital pulp therapy procedures rely on exactly these cellular responses, for regeneration of dentine at sites of pulp exposure or the base of deep cavities, to help limit injury to the viable dental pulp.

There is evidence to show that the application of exogenous growth factors to the viable pulp tissue can induce reparative dentine-like tissue formation. For example, application of TGF-β1-containing alginate hydrogels to the cultured human tooth slices induced odontoblast-like cell differentiation and up-regulated matrix secretion in the human dentine-pulp complex *in vitro* (Dobie et al. 2002). The application of BMP7 to the mechanically exposed rat molars *in vivo*, is shown to induce mineralised tissue formation (Goldberg et al. 2001) and in relation to VEGF, it has been shown that its in vitro application to non-carious human tooth slices, results in neovascularisation of the severed dental pulp (Mullane et al. 2008) which is a crucial step in wound healing and repair *in vivo*.

It has been recently proposed that the growth factors released from dentine matrix, by the action of some clinically used materials such as Ca(OH)$_2$ and MTA, might be the key mediators of a reparative response in injury situations (Graham et al. 2006; Tomson et al. 2007). The ability of phosphoric acid, the commonly used etching agent in dental adhesive systems in solubilising the bioactive dentine matrix components, from human powdered dentine, has been investigated and demonstrated more recently (Ferracane et al. 2013). It must however be appreciated that there are limitations in an in vitro investigation of growth factor released from dentine powder which tends to be carried out over a prolonged periods, spanning over few days even weeks, and at the presence of protease inhibitors. In the context of vital pulp therapy, direct exposure and release of growth factors from dentine surface would be more relevant.

Only several studies have investigated exposure and release characteristics of bioactive components from conditioned mineralised tissue slices (rather than powder) which would more closely resemble clinical conditions. These studies have demonstrated the ability of several clinically used conditioning agents such as EDTA and citric acid in exposure and release of growth factors including TGF-β1, FGF2 and VEGF from dentine and bone slices *in vitro* (Zhao et al. 2000; Smith et al. 2011; Galler et al. 2015). The importance of these findings is that the exposed bioactive molecules may actively influence the behaviour of cells in contact with the surface while the released growth factors may be important in directing cellular fate of the progenitor cells at a distance (Huang et al. 2006a; Galler et al. 2011; Pang et al. 2014), in favour of repair and regeneration. The investigation of comparative ability of the contemporary dentine conditioning agents in growth factor exposure and release kinetics, may therefore be important clinically in the context of vital pulp therapy.

Against this background, the aim of this chapter is to investigate the effects of several dentine conditioning and etching agents (EDTA, phosphoric and citric acids with Ca(OH)$_2$ positive and PBS negative controls) on exposure and release of TGF-β1, BMP2 and VEGF from dentine slabs.

4.2 Materials and Methods

4.2.1 Preparation and conditioning of dentine slices

Ten extracted caries free human molar teeth were obtained, immediately post extraction, from Oral Surgery clinics in Cardiff Dental Hospital with ethical approval and patients' consent (Appendix 1). The attached soft tissues were removed from the surface using a sharp scalpel and teeth were stored in 1% sodium azide prior to removing the enamel and cementum using a dental turbine with water coolant. The crowns were then sectioned using a segmented diamond edged rotary saw with water cooling into approximately 1 mm thick slices. The pulp tissue was removed with a scalpel and slabs were washed thoroughly with PBS (with constant agitation for 5 min) and freeze-thawed three times to remove any possible cell residue. Dentine slices (approximately 0.3 gram in weight) were then immersed for 2 min (for TGF-β1 localisation only), 5 min or 10 min in consistent volumes (1 ml) of etching and conditioning agents used in the previous chapters; 10% w/v EDTA (Fisher scientific, UK) pH adjusted to 7.2, 37% (v/v) phosphoric acid (VWR International) pH <1 and 10% (w/v) citric acid (Fisher Scientific, UK) pH 1.5, 0.02M calcium hydroxide as positive control and PBS as a negative control. The supernatants remaining after dentine conditioning in each solution were stored immediately at -20ºC, for detection and quantification of TGF-β1, BMP2 and VEGF by ELISA. The slices were then gently washed in distilled water and stored in sterile condition in 24 well plates to be air-fixed at room temperature for 48 hours prior to further processing for ultrastructural localisation (immunogold labelling) of the growth factors.

4.2.2 Immunogold labelling as a measure of growth factor exposure on dentine surfaces

Treated dentine slabs were immuno-labelled for TGF-β1, BMP2 and VEGF and viewed under scanning electron microscope (SEM) as described by Zhao et al. (2000) and Smith et al. (2011). After fixation, treated dentine slabs were immersed in the blocking solution containing 5% goat serum and 10% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 1 hr followed by wash buffer (1% goat serum, 1% BSA and 0.1% Tween 20 in TBS) for 15 min.

Dentine slabs were then incubated with polyclonal anti-human primary antibodies for TGF-β1 (raised in rabbit), BMP2 and VEGF (raised in mouse) (Santa Cruz, USA, 1:100 dilution in 1% BSA, TBS) for 2 hr. Dentine slabs were then washed twice with wash buffer and incubated in their corresponding gold labelled secondary antibodies (BBI diagnostics, particle diameter 30nm, diluted 1:100 in 1% BSA, TBS) for 1 hr. As negative controls, primary antibody was withdrawn for slabs treated by calcium hydroxide for 5 min, prior to incubation with secondary antibodies. For immunolocalisation of TGF-β1, an additional negative control was used in which immuno-labelling was inhibited by pre-incubation with a 10-fold excess (1:10) of specific blocking peptide (Santa Cruz, USA) diluted in 1% BSA, TBS.

Following two washes in buffer and two further washes in distilled water, slabs were incubated with silver enhancing agent (BBI diagnostics) for 15 min. Slabs were then washed in distilled water for 2×15 min and air dried for 24 hr prior to being examined by an EBT1 scanning electron microscope (SEMTech Ltd, Derbyshire,UK) at a magnification of ×550 (for BMP2 and VEGF) or ×750 (for TGF-β1).

Density of gold labelling in specimens was quantified by counting the particles on photographs of the SEM in 5 random fields of $20\mu m^2$ on each of the 3 dentine slabs used for each time point, for every treatment. Means and standard errors were calculated and data were analysed using one-way analysis of variance (ANOVA) and Tukey's post hoc test using SPSS software. Differences in growth factor exposure were analysed between different chemical treatments at each time point. Significance levels were defined as * = P<0.05 (significant), ** = P<0.01 (very significant).

4.2.3 Identification and quantification of TGF-β1, BMP2 and VEGF from treated dentine surfaces by ELISA

The supernatants were collected after 5 and 10 min immersion of dentine slabs, with consistent surface area and weight, in the same volumes of various conditioning agents (PBS, EDTA, phosphoric acid, citric acid and $Ca(OH)_2$) and the collected supernatants were immediately stored in -20ºC. Commercial ELISA development kits were used to measure the levels of released TGF-β1 (Human/Mouse Ready-Set-Go, eBiosciences), BMP2 and VEGF (Peprotech, NJ, USA). The kits contained specific capture antibodies to the growth factor being investigated, biotinylated detection antibodies, and avidin-HRP. The provided standards contained a known amount of protein of interest.

4.2.4 Preparation of ELISA plates

For TGF-β1, the capture antibody was diluted 1 in 250 with the coating buffer provided in the kit. For BMP2 and VEGF the capture antibodies were diluted to 1 and 0.5μg/ml respectively in PBS. Ninety six well plates (Fisher Scientific, UK) were coated with 100μl of each capture antibody and incubated over night at 4ºC for TGF-β1 and at room temperature for BMP2 and VEGF according to the manufacturers' instructions. The

liquid was then aspirated and the wells washed 4 times with the wash buffer containing 0.05% (v/v) Tween-20 in PBS. Wells were then blocked, for 1 hr at room temperature, with 200µl 1× assay diluent for TGF-β1 and 300µl block buffer containing 1% w/v BSA in PBS for BMP2 and VEGF, as per manufacturers' instructions. Plates were washed 4 times in the wash buffer.

4.2.5 Preparation of ELISA standards and samples

ELISA was carried out as two sets of independent experiments to verify consistency. The supplied standards were diluted in diluent buffer, which was prepared by mixing the 5× ready-made assay diluent, 1 in 5, with distilled water for TGF-β1 ELISA and was made up of 0,05% (v/v) Tween-20, 0.1% (w/v) BSA in PBS for VEGF and BMP2 ELISAs. Standard dilutions ranged from 8, 2 and 1ng/ml to zero for TGF-β1, BMP2 and VEGF ELISAs respectively using 2 fold dilutions. 100µl of each standard concentration was added, in triplicates, to each coated ELISA plate. Triplicate wells loaded with diluent buffers alone were used to represent zero concentration.

100µl of the supernatants were added to the wells also in triplicate, next to the standards, and incubated overnight. The solutions were then aspirated and the wells washed 4 times using the wash buffer. Plates were incubated for a further 1 hr following addition of 100µl of the kit biotin conjugated detection antibody, diluted 1 in 250 with the 1× assay diluent for TGF-β1 to each well. Biotinylated detection antibodies were diluted in diluent buffer to 0.5 µg/ml and 0.25 µg/ml for BMP2 and VEGF respectively and 100µl were added to each well followed by 2 hr incubation, at room temperature. The liquid was aspirated and the wells washed 4 times before adding 100µl of avidin-HRP, diluted in diluent buffer to 1:250 for TGF-β1 and 1:2000 for the remaining two ELISAs, was added to each well. Incubation at room temperature for 30 min followed. The liquid

was aspirated and the well washed 4 times prior to adding 100μl of the provided substrate solutions to each well. For TGF-β1, plates were incubated at room temperature for approximately 15 min before stopping the reactions by adding 50μl of 1M phosphoric acid to each well, as per manufacturer's instructions. For BMP2 and VEGF ELISA plates, after adding the substrate solution and incubation at room temperature for colour development, the absorbency was quantified straight away, with no addition of a stop solution.

4.2.6 ELISA signal quantification

The plate absorbance was read in a Microplate^{TM} reader (BioTek instruments Limited) at 450nm for TGF-β1 and 405nm for VEGF and BMP2 ELISAs. Standard curves were generated by plotting the concentration against absorbency for the standards on the Excel programme, based on a best fit line, which allowed concentrations of TGF-β1, BMP2 and VEGF in samples to be calculated. Regression analysis was used to assess the linearity of the best fit line which was found to have $R^2 > 0.9$, indicating linearity. The experiment was repeated and the results were presented as mean and standard errors from n=6.

ELISAs were repeated twice before combining the data for statistical analysis. Means and standard errors were calculated and data were analysed using one-way ANOVA for independent samples. Taking into account the spread of the data, Bonerroni adjustment used in the previous chapter was considered too conservative for pairwise comparisons, rendering the difference between groups insignificant. To reduce the chances of false negative (type II error), Tukey's HSD post hoc test was used instead on the SPSS package. Differences in growth factor release were analysed between different chemical

treatments at each time point Significance levels were defined as * = P<0.05 (significant), ** = P<0.01 (very significant).

4.3 Results

4.3.1 Immunogold labelling of TGF-β1 on treated dentine surfaces

Comparison of SEM images revealed differences in dentine surface characteristics in slabs treated by various agents. EDTA and the acids appeared to be more efficient in removing the smear layer, revealing dentine tubular pattern after 5 min treatment, while in slabs treated with $Ca(OH)_2$, residues of the smear layer appeared to be present, with the tubules still partially occluded, after 10 min treatment (Figures 4.1, 4.2).

Average counts of immunogold labelled particles, per $20\mu m^2$, on SEM images of treated dentine slabs revealed that all conditioning agents used (10% EDTA, 37% phosphoric acid, 10% citric acid and 0.02M calcium hydroxide), exposed more TGF-β1 on the surface after 2 min treatment compared with PBS ($P<0.01$ for $Ca(OH)_2$). Average counts of gold labelled particles also revealed an upward trend in the exposure of TGF-β1 between 2 and 5 min for all treated dentine slabs (Figure 4.4). $Ca(OH)_2$ was the most effective agent in exposing TGF-β1 after 2, 5 and 10 min dentine treatment ($P<0.01$). When considered as a function of time, after an initial increase between 2 and 5 min, there was a drop in surface levels of TGF-β1 between 5 and 10 min on slabs treated with $Ca(OH)_2$. Treatment with EDTA, phosphoric and citric acids on the other hand resulted in a sharp increase in the surface levels of TGF-β1 between 2 and 5 min but not much change between 5 to 10 min (Figure 4.4).

Figure 4.1. Immunogold labelling of TGF-β1 (indicated by arrows) exposed on dentine surfaces treated with: 0.2M Ca(OH)$_2$ for (A) 2 min, (B) 5 min, (C) 10 min, and EDTA for D) 2 min, E) 5 min and F) 10 min. In contrast to slabs treated with the other conditioning agents, smear layer is still present on the Ca(OH)$_2$ treated slabs after 5 and 10 min treatment which is apparent from the partially covered dentinal tubules (B and C).

Figure 4.2. Immunogold labelling of TGF-β1 (indicated by arrows) exposed on dentine surfaces treated with phosphoric acid for (A) 2 min, (B) 5 min, (C) 10 min and citric acid for D) 2 min, E) 5 min and F) 10 min. The outline of the dentinal tubules can be clearly observed.
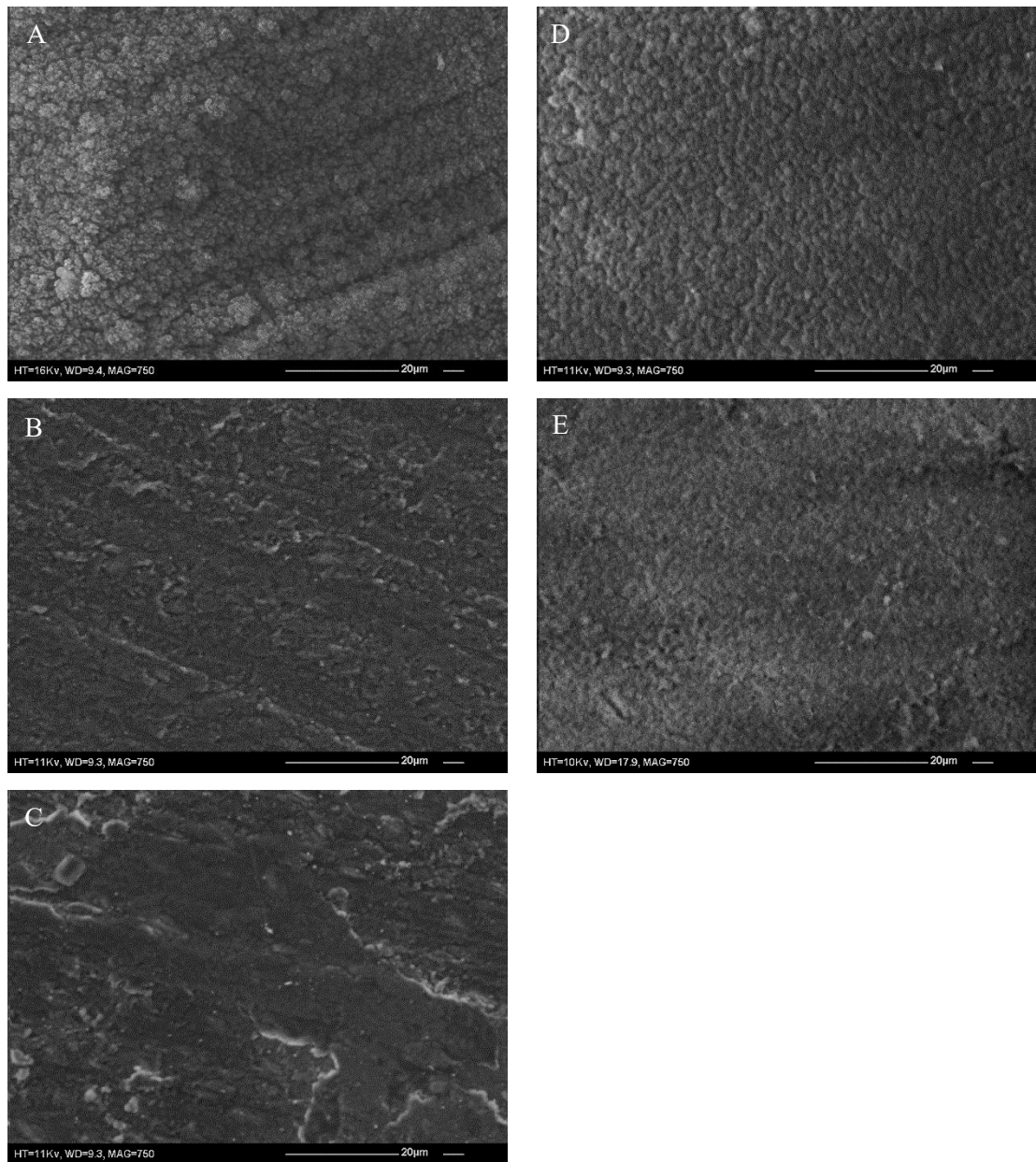
Figure 4.3. Immunogold labelling for TGF-β1, dentine surfaces treated with PBS for (A) 2 min, (B) 5 min, (C) 10 min and the negative controls treated with Ca(OH)$_2$ for 5 min: D) incubated with a 10-fold excess of specific peptide to block TGF-β1 labelling and E) primary antibody for TGF-β1 withdrawn.

Figure 4.4. Average TGF-β1 counts of immunogold labelled particles per 20μm² of treated dentine surface. ** represents significant difference at P<0.01.

4.3.2 Immunogold labelling of BMP2 on treated dentine surfaces

Average counts of immunogold labelled particles on the treated dentine slabs revealed that all conditioning agents used, exposed greater amounts of BMP2 on dentine surfaces as demonstrated by increased gold particle number after 5 ($P<0.01$ for all but phosphoric acid) and 10 min treatment compared with the control solution, PBS. When considered as a function of time, after treatment with $Ca(OH)_2$ between 5 and 10 min the surface levels of BMP2 revealed little change while treatment with EDTA, phosphoric and citric acid during the same time period resulted in a downward trend in the surface levels of BMP2. Amongst the conditioning agents used, phosphoric acid was the least effective agent in exposing BMP2 after 5 ($P<0.05$) and 10 min treatment (Figures 4.5, 4.6 and 4.7).
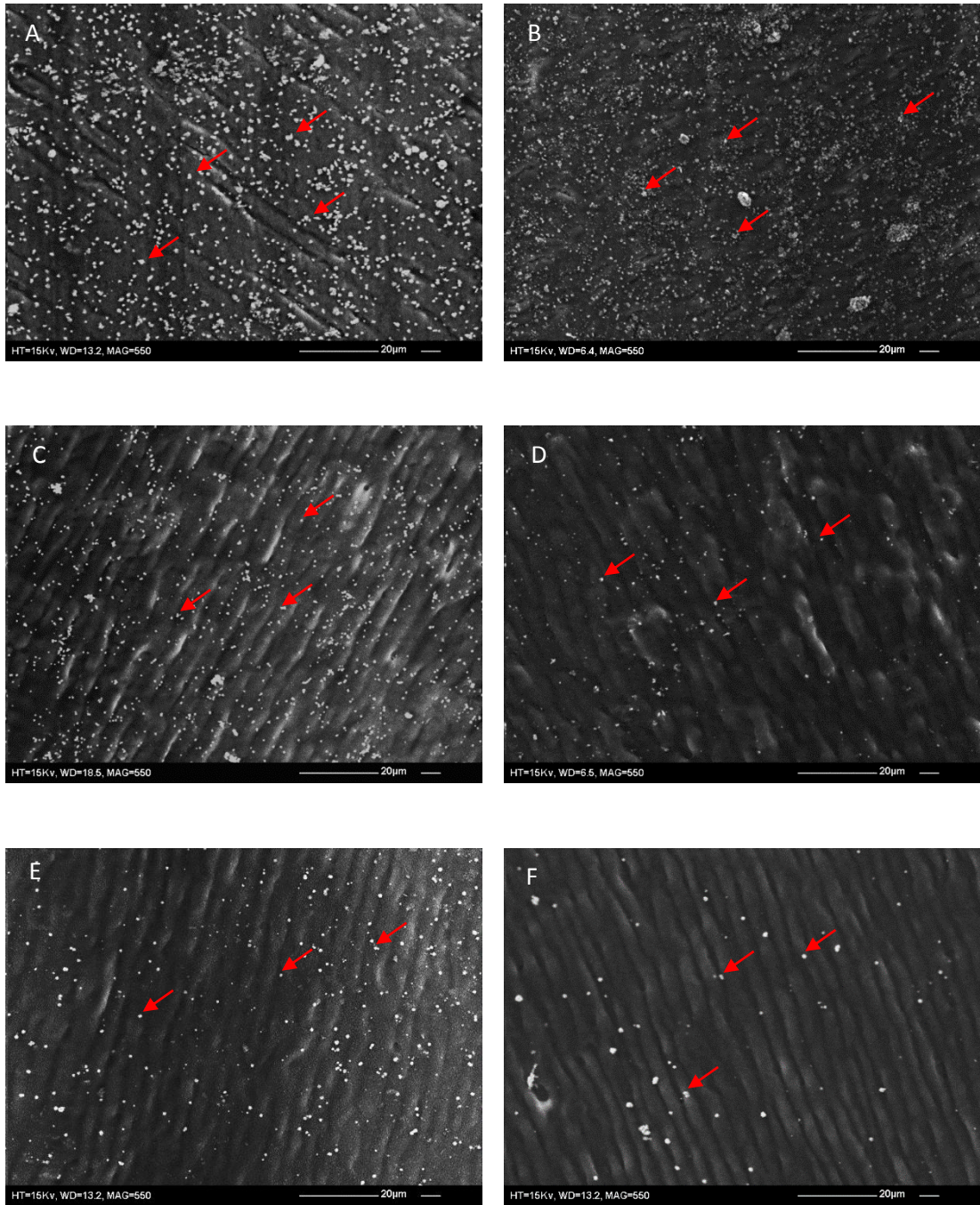
Figure 4.5. Immunogold labelling of BMP2 (indicated by arrows) exposed on dentine surfaces treated with $Ca(OH)_2$ for A) 5 min and B) 10 min, EDTA for C) 5 min and D) 10 min and phosphoric acid for E) 5min and F) 10 min.
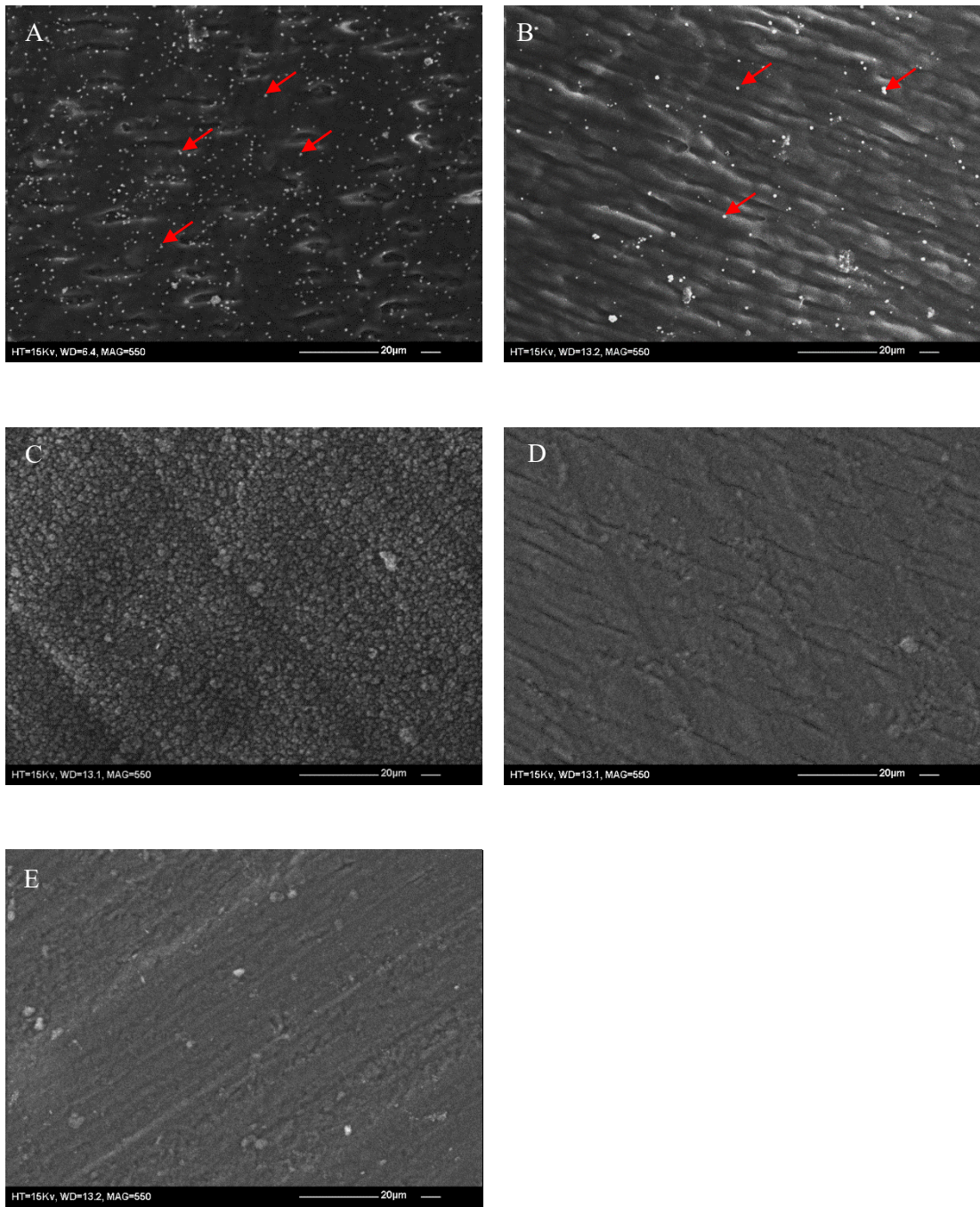
Figure 4.6. Immunogold labelling of BMP2 (indicated by arrows) exposed on dentine surfaces treated with citric acid for A) 5 min and B) 10 min, PBS for C) 5 min and D) 10 min and negative control (E), treated with Ca(OH)$_2$ for 5 min, primary antibody withdrawn.
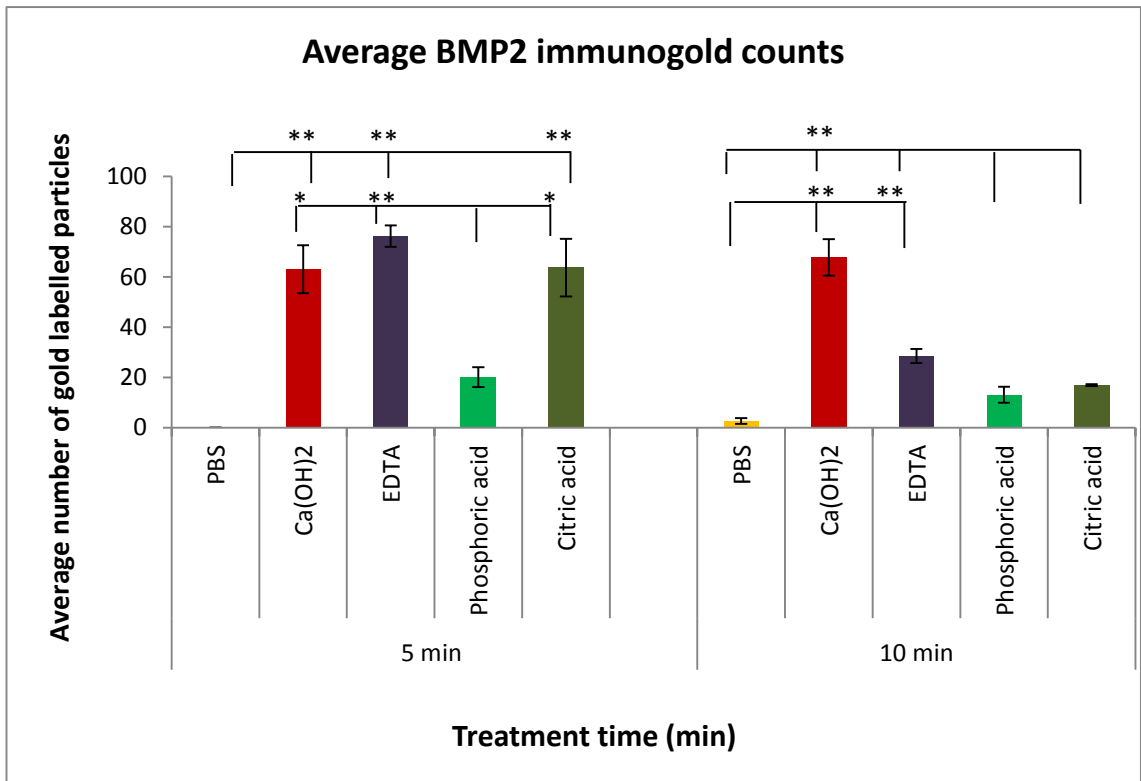
Figure 4.7. Average BMP2 counts of immunogold labelled particles per 20μm^2 of treated dentine surface. * represents significant difference at P<0.05 and ** at P<0.01.

4.3.3 Immunogold labelling of VEGF on treated dentine surfaces

Comparison of SEM images and counts of immunogold labelled particles, identified a similar pattern for BMP2 and VEGF exposure on treated dentine surfaces.

Average counts of immunogold labelled particles on treated dentine slabs revealed that all conditioning agents used, resulted in a significant exposure of VEGF compared with PBS, after 5 ($P<0.05$) and 10 min treatment ($P<0.05$ for all but citric acid) (Figure 4.10). When considered as a function of time, similar to BMP2 data, after treatment with $Ca(OH)_2$ between 5 and 10 min, the surface levels of VEGF revealed little change while treatment with EDTA, phosphoric and citric acid during the same time period resulted in a downward trend in the surface levels of VEGF.

Similar to BMP2 data, phosphoric acid was the least effective agent in exposing VEGF after 5 min treatment ($P<0.05$) (Figures 4.8, 4.9 and 4.10).
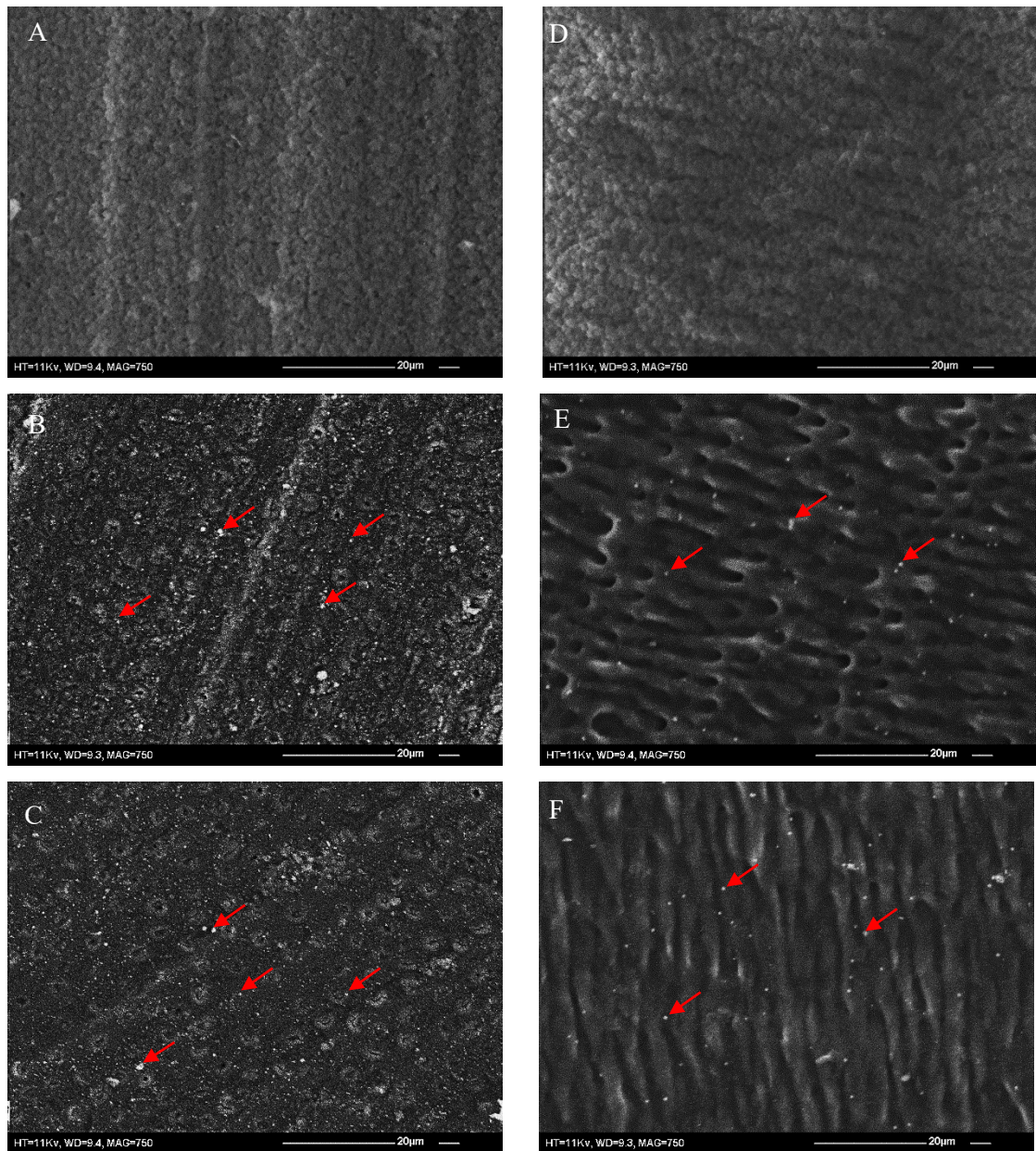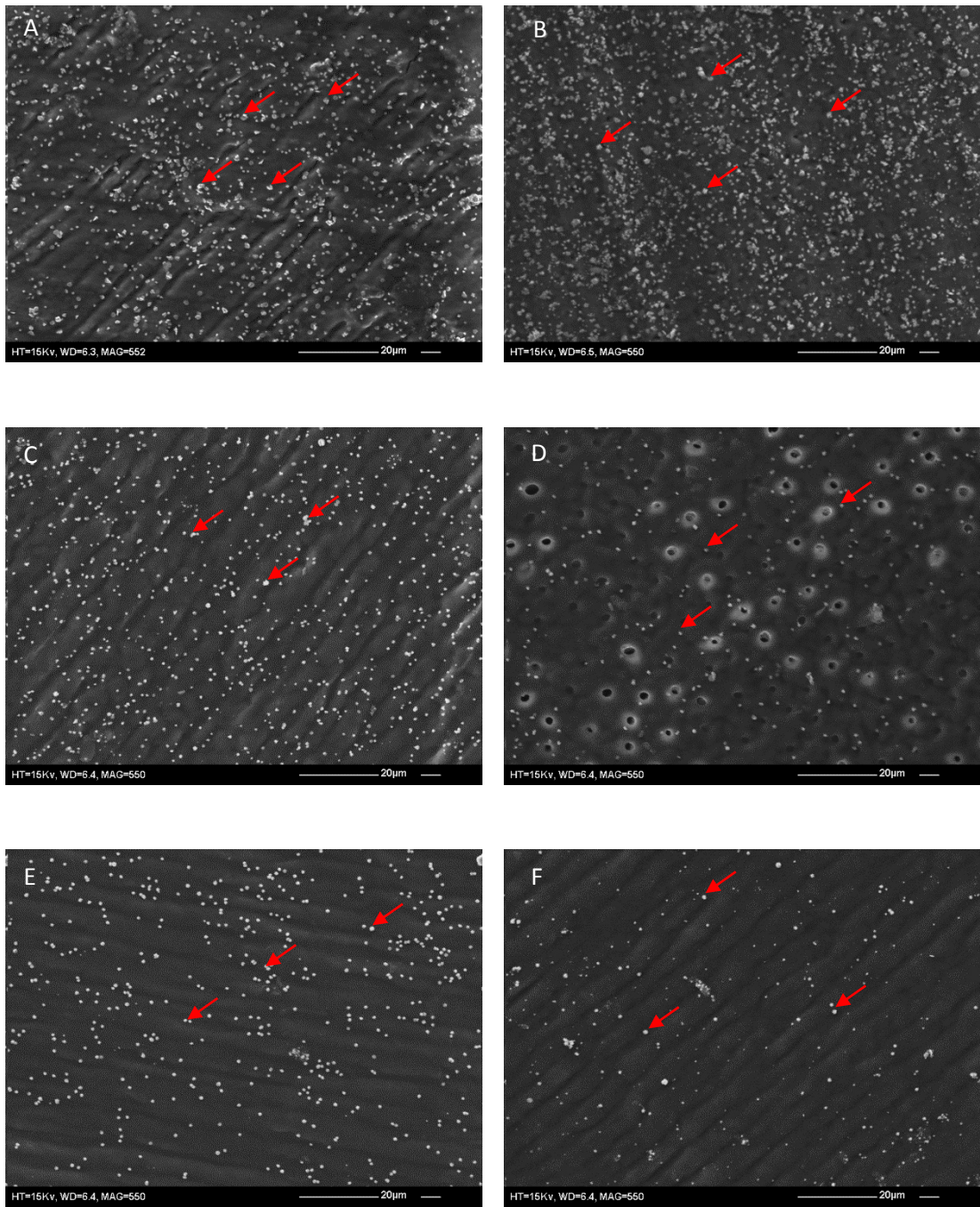
Figure 4.8. Immunogold labelling of VEGF (indicated by arrows) exposed on dentine surfaces treated with Ca(OH)$_2$ for A) 5 min and B) 10 min, EDTA for C) 5 min and D) 10 min and phosphoric acid for E) 5 min and F) 10 min.
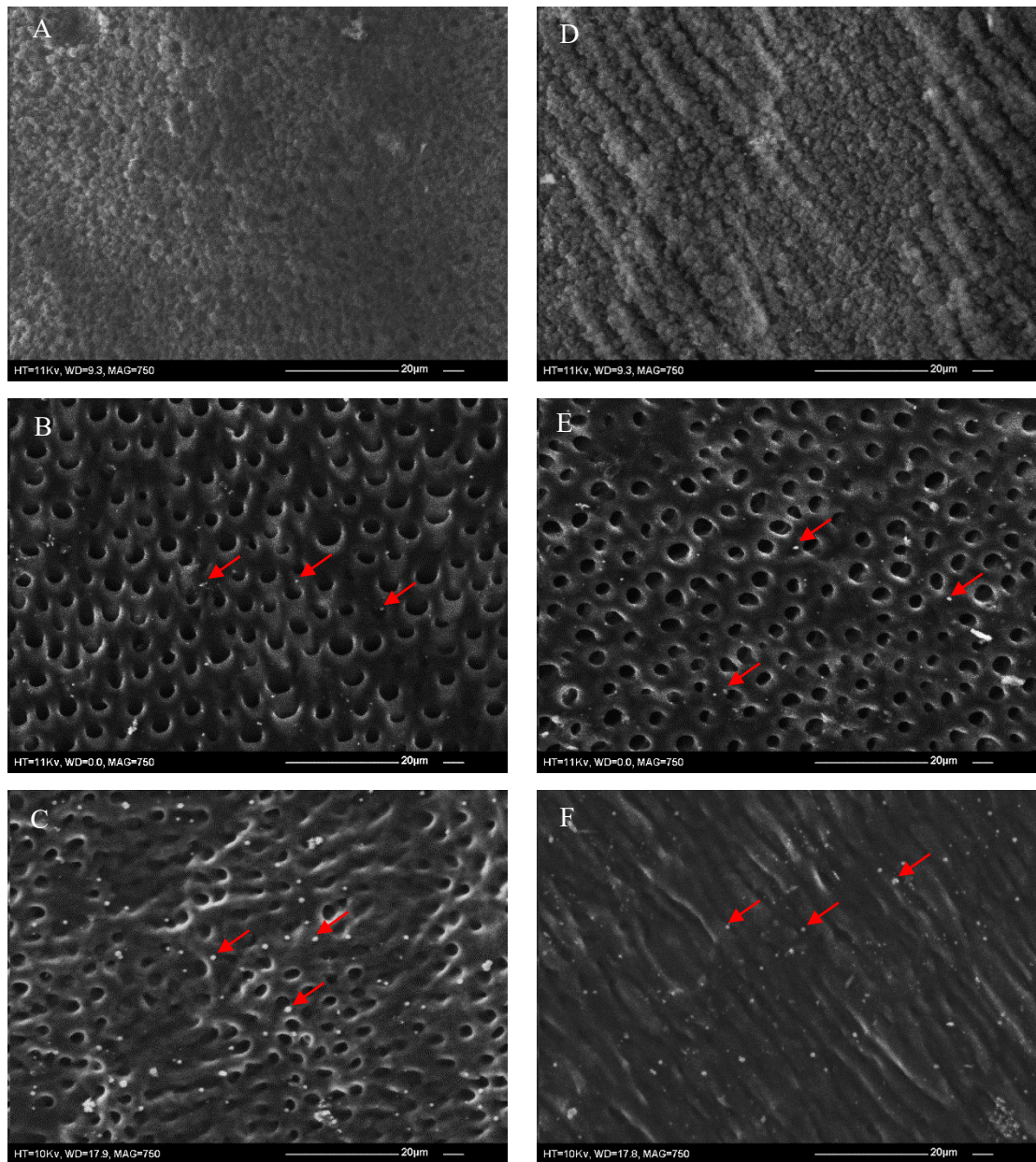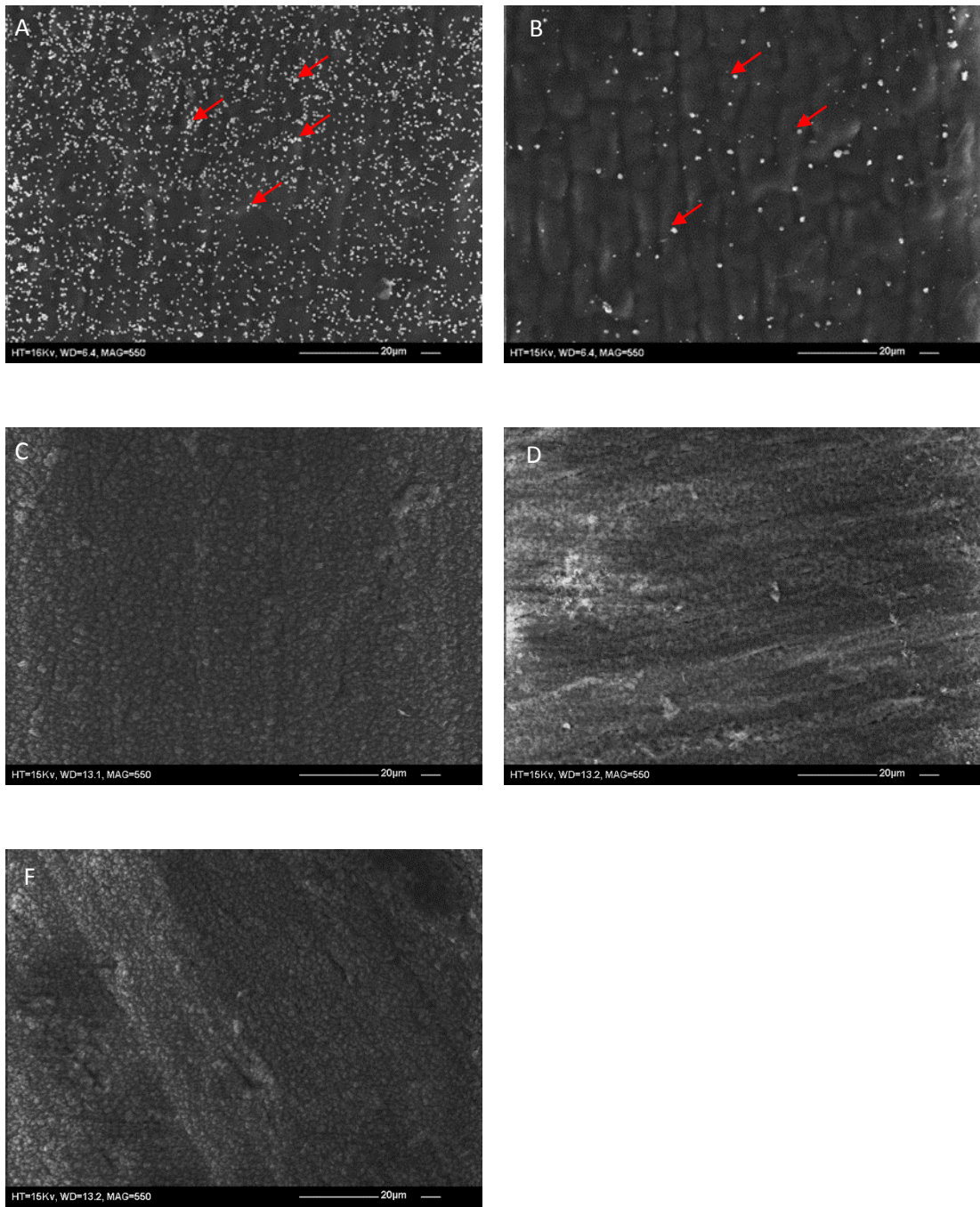
Figure 4.9. Immunogold labelling of VEGF (indicated by arrows) exposed on dentine surfaces treated with citric acid for A) 5 min and B) 10 min, PBS for C) 5 min and D) 10 min and negative control, treated with Ca(OH)$_2$ for 5 min, primary antibody withdrawn.
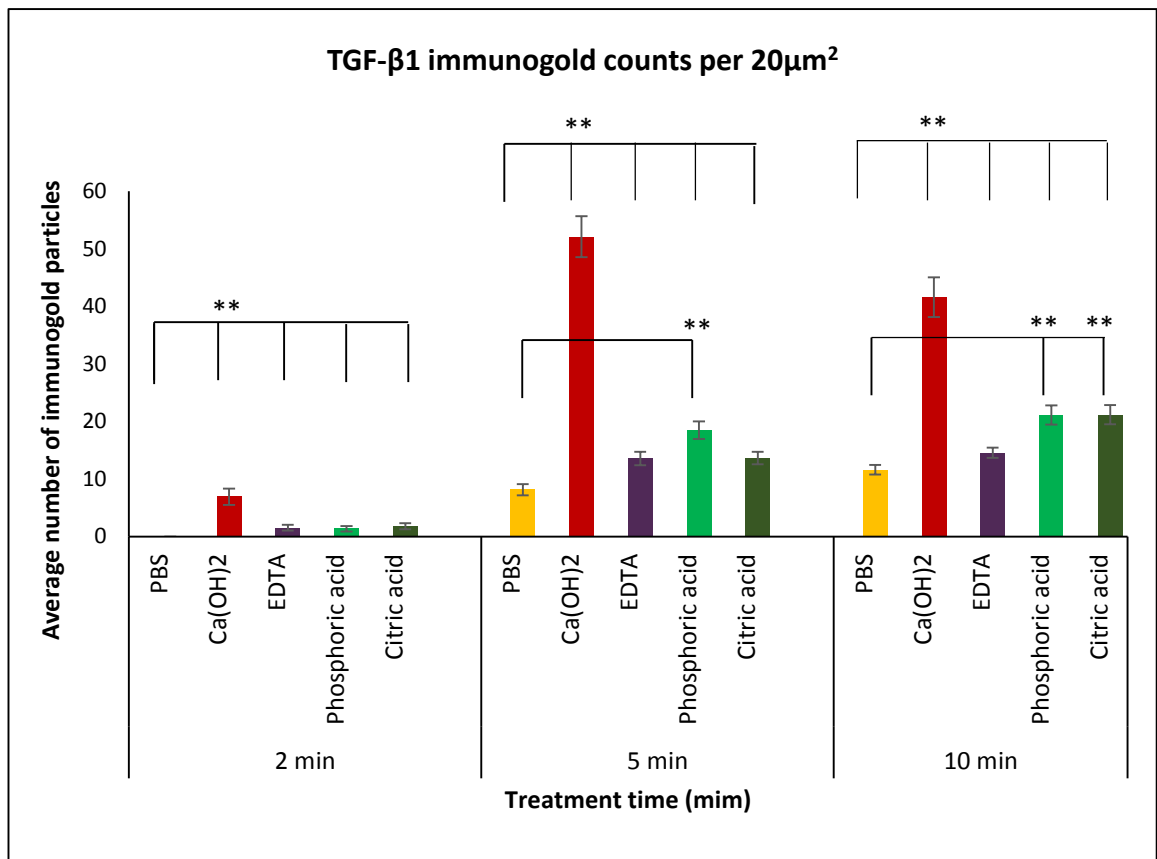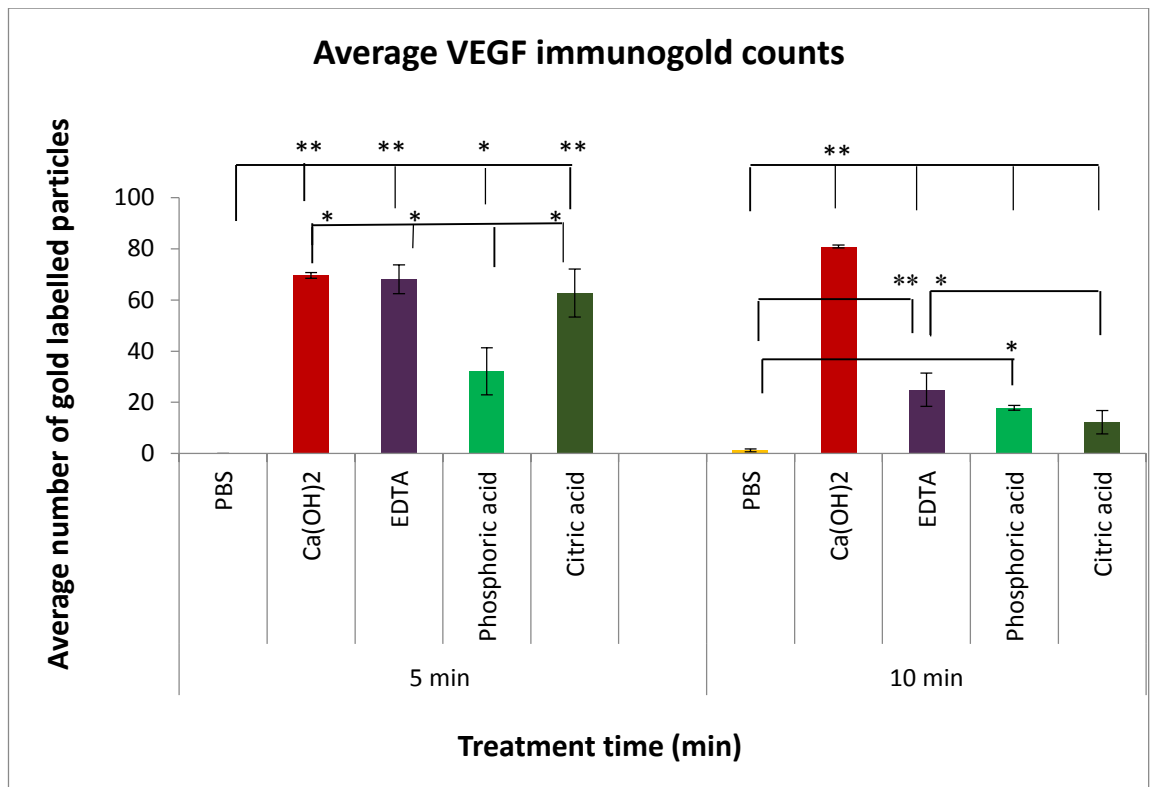
Figure 4.10. Average VEGF counts of immunogold labelled particles per 20μm^2 of treated dentine surface. * represents significant difference at P<0.05 and ** at P<0.01.

4.3.4 Release of growth factors from dentine slabs after conditioning

Standard curves for TGF-β1, BMP2 and VEGF ELISAs were generated by plotting the absorbance against concentrations of the growth factors (Figure 4.11).
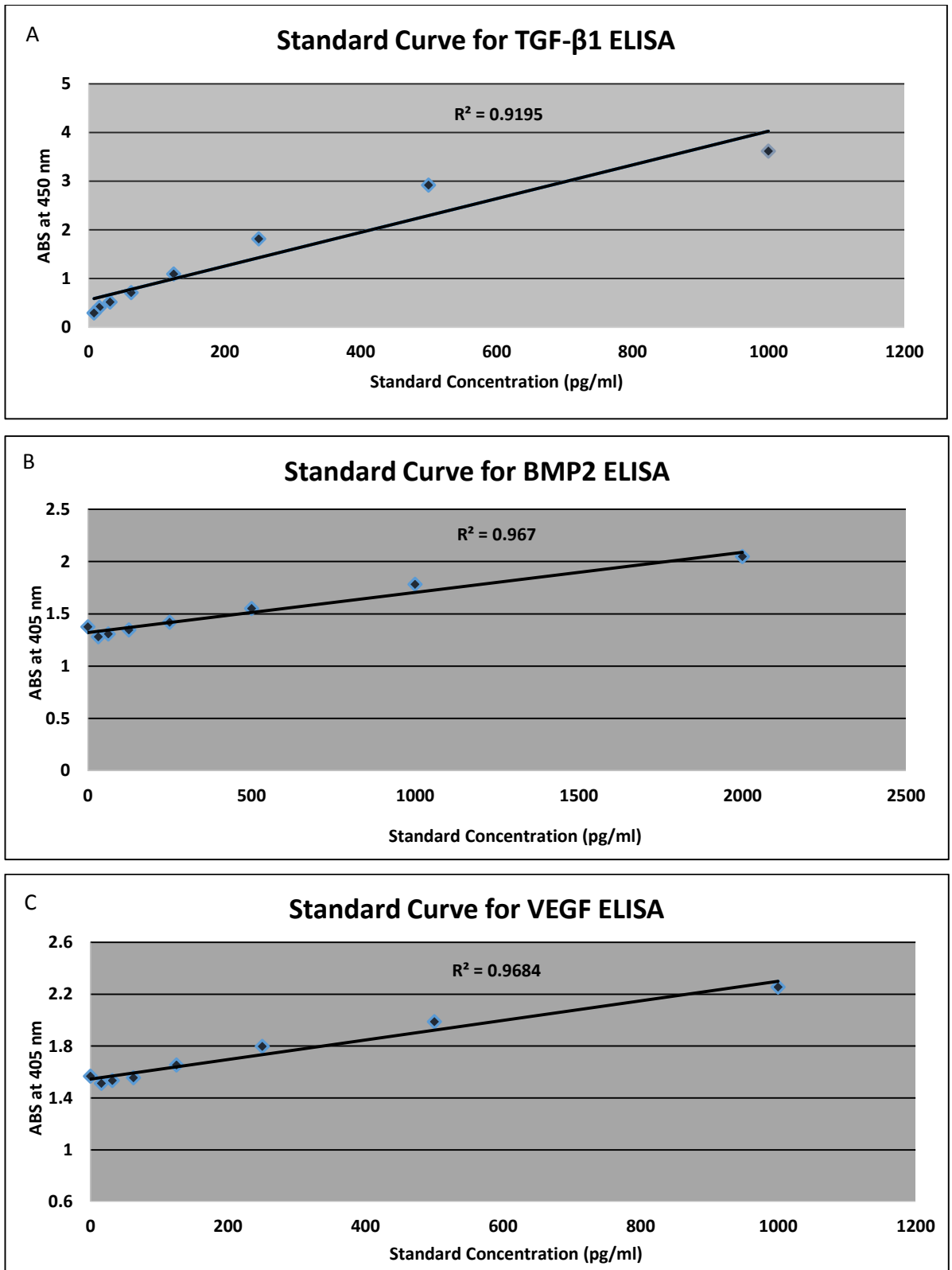
Figure 4.11. Standard curves for A) TGF-β1, B) VEGF and C) BMP2 ELISAs for detection of growth factors in the supernatants of conditioned dentine.

4.3.4.1 TGF-β1 release

ELISA data revealed the presence of TGF-β1 only in the supernatants of EDTA treatment after 5 and 10 min dentine conditioning ($P < 0.01$) (Figure 4.12).
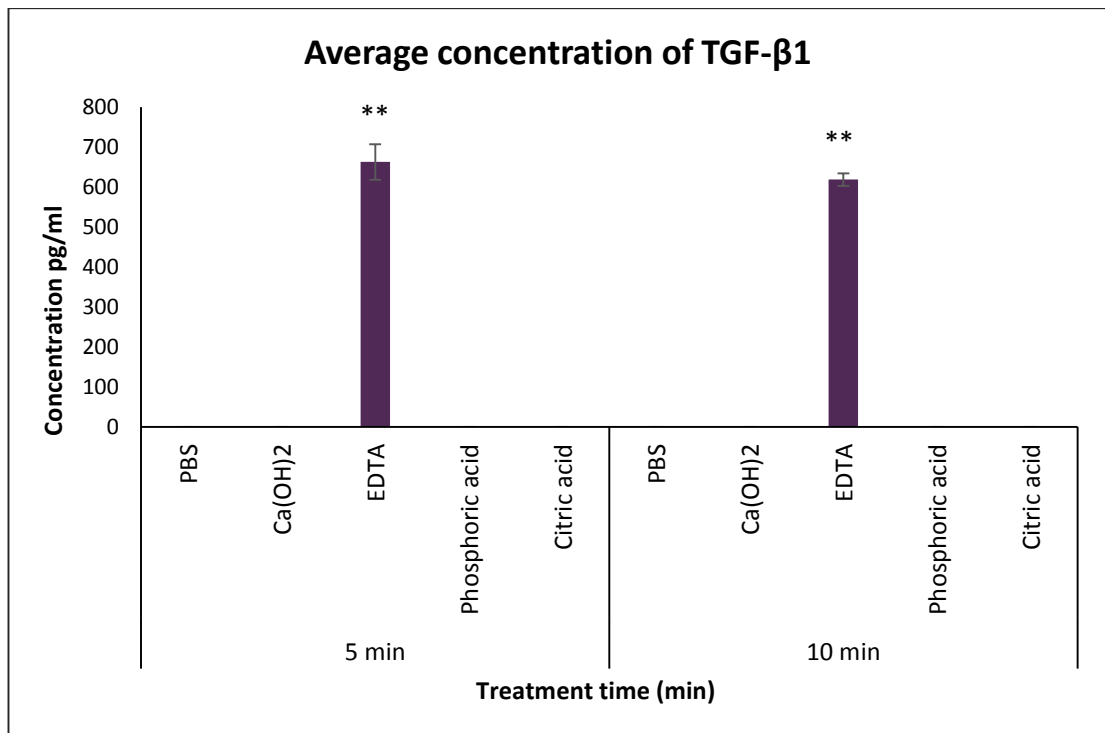
Figure 4.12. Average concentrations of TGF-β1 in the supernatants of conditioned dentine. ** represents significant difference with all other conditioning agents ($P < 0.01$).

4.3.4.2 BMP2 and VEGF release

ELISA data revealed similarities for BMP2 and VEGF release from dentine in that the highest concentrations of both growth factors were detected in the supernatants of citric acid treatment after 5 min conditioning of dentine ($P<0.01$). BMP2 and VEGF were also detected in the supernatants of $Ca(OH)_2$ treatment after 5 min dentine conditioning however the concentrations were too low and did not reach statistical significance (P values 0.78 and 0.12 for BMP2 and VEGF respectively, compared with PBS). BMP2 was also detected in the supernatants of phosphoric acid after 5 and 10 min treatment, although the concentrations did not reach statistical significance (P values 0.32 and 0.23 at 5 and 10 min compared with PBS). After 10 min dentine conditioning, both growth factors were only detected in the supernatants of citric acid ($P<0.01$).

ELISA failed to detect BMP2 and VEGF in the supernatants of EDTA at either of the time points (Figures 4.13 and 4.14).
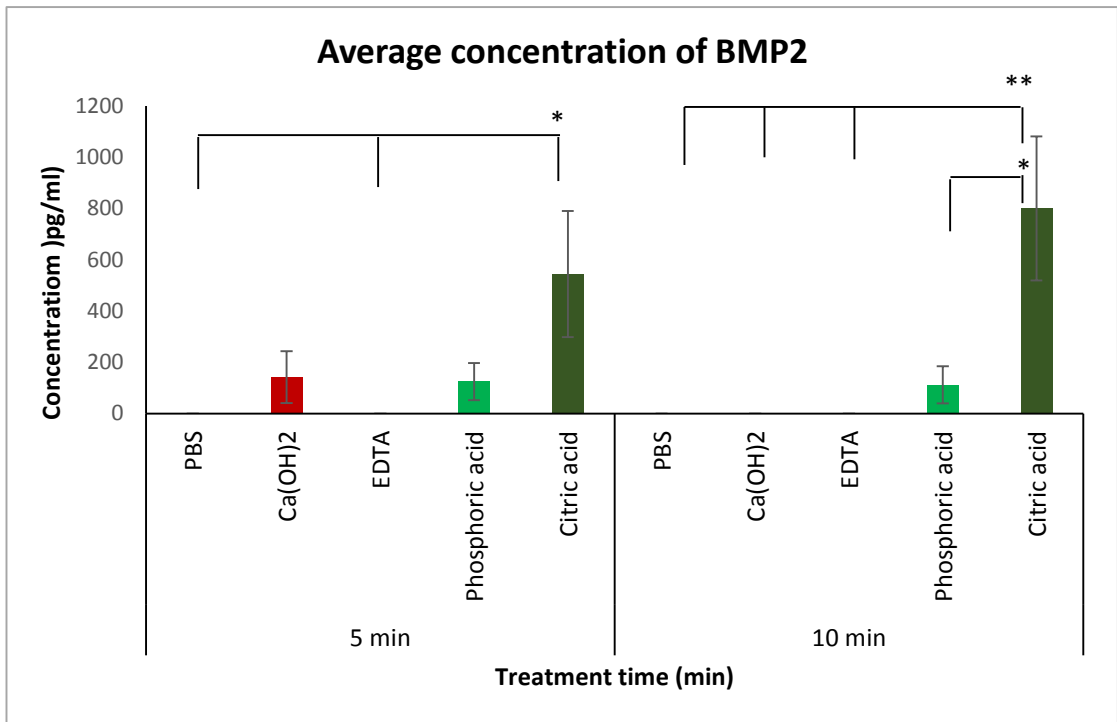
Figure 4.13 Average concentrations of BMP2 in the supernatants of conditioned dentine. * represents significant difference at P<0.05 and ** at P<0.01.
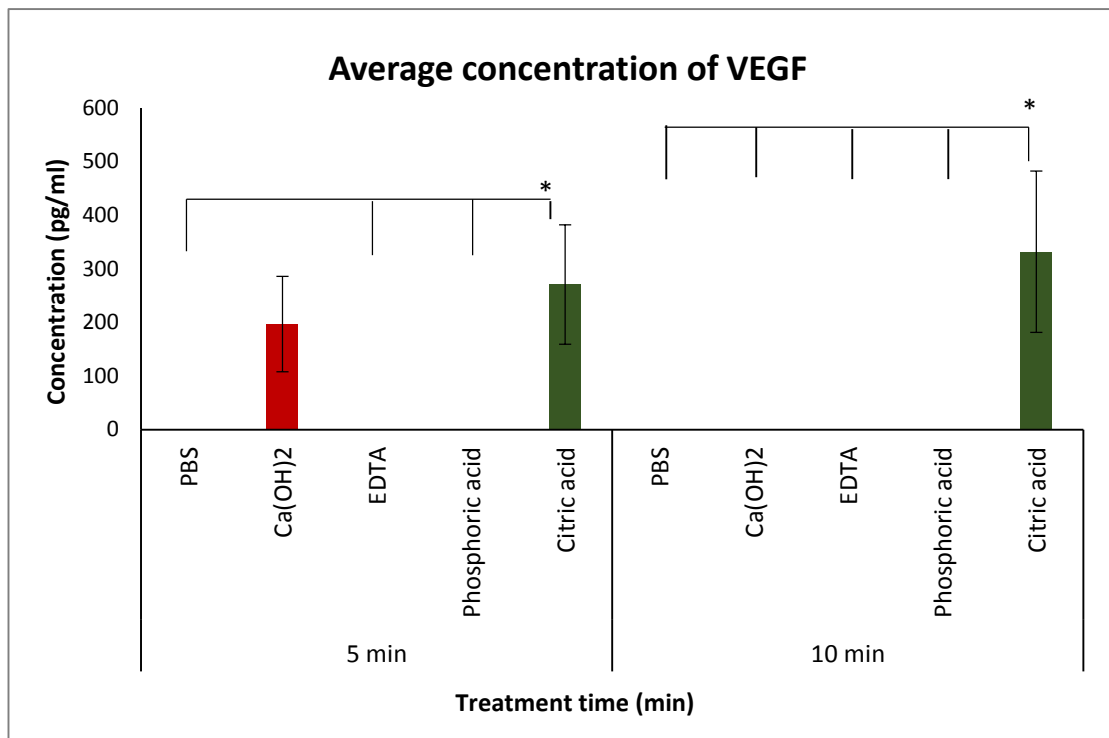
Figure 4.14. Average concentrations of VEGF in the supernatants of conditioned dentine. * represents difference with the marked group at P<0.05.

4.4 Discussion

This study provides evidence that a range of chemicals with alkaline, acidic or calcium chelating properties have the ability to expose and release TGF-β1, BMP2 and VEGF from dentine after short term treatment of the surface, when compared with a physiological solution, PBS. Considering that these growth factors are capable of inducing mineralised phenotype differentiation of stem cells and processes leading to dentine regeneration (Mullane et al. 2008; Yang et al. 2009; Arany et al. 2014), this is a significant finding.

Differences were observed in the investigated growth factors exposure and release kinetics, as demonstrated by immunogold labelling and ELISA data, which may be a reflection of the mechanism involved in exposure and release processes in relation to various agents used. The counts of immunogold labelled particle revealed that although $Ca(OH)_2$ was the most effective agent in exposing TGF-β1 throughout the experiment, a drop in the surface levels of TGF-β1 was detected between 5 and 10 min after treatment with this agent. Over the same period, treatment with EDTA and the acids resulted in an increase in surface levels of TGF-β1. Other studies have reported the effectiveness of EDTA in exposing TGF-β1 on dentine surfaces, however their data quantified surface growth factor presence at a single time point rather than exposure kinetics over time (Zhao et al. 2000; Galler et al. 2015). TGF-β1 exposure kinetics on treated bone surfaces however has been previously investigated. Smith et al (2011) have reported similar TGF-β1 exposure kinetics in bone to what our data shows here, in relation to dentine. The surface levels of TGF-β1 on bone surfaces also progressively increased between 5 and 15 min when treated with EDTA and citric acid while a

downward trend in the levels of the growth factor was observed for Ca(OH)$_2$ treated surfaces during the same time period.

In relation to BMP2 and VEGF increasing treatment time from 5 to 10 min with Ca(OH)$_2$ resulted in little change while treatment with EDTA and the acids resulted in a decrease in surface levels of the growth factor during the same period. The observed similarities in the effects of EDTA and the acids in growth factor exposure in this chapter may be a reflection of similarities in the mechanism of action of these agents in relation to a mineralised tissue as dentine. In short application times *in vitro* (up to 2 min), EDTA removes the inorganic component of dentine, leaving dentine surface rich in collagen (Dogan and Qalt 2001), similarly acid etching demineralises dentine, leaving the surface low in minerals but rich in proteins (Matos et al. 1997). This process may result in progressive exposure of ECM proteins, including the growth factors in short treatment times as observed with TGF-β1. However, longer treatment times (10 mins), may result in the erosion of dentine which involves a breakdown of the inorganic component as well the loss of protein (or release) into the surrounding environment (West et al. 2000; Calt and Serper 2002), as it was observed with decreased surface levels of BMP2 and VEGF in this chapter.

Growth factor exposure after Ca(OH)$_2$ treatment showed a different trend in comparison with the above agents. It has been shown previously that treatment of a mineralised tissue by an alkali, solubilises and degrades matrix proteins (Partridge and Elsden 1961), as opposed to removing the mineral contents in relation to EDTA and acids. Furthermore, the evidence shows that dentine irrigation with an alkaline agents such as NaOCl creates an apatite rich and collagen sparse sub-surface (Mountouris et al. 2004;

Zhang et al. 2010). In this chapter the observed gradual increase followed by a drop of surface growth factors on Ca(OH)$_2$ treated dentine probably represents solubilisation of proteins near the surface, exposing the growth factors initially followed by their subsequent loss into the aqueous environment. This hypothesis was supported by growth factor release data where BMP2 and VEGF were detected in the supernatants after 5 min treatment.

Similar findings to the growth factor exposure data in this chapter were reported by Smith et al. (2011) where exposure patterns of TGF-β1 on treated bone surface differed with various chemicals used. The authors postulated that when treated by a chemical, in addition to the mechanisms explained above, release of components from a mineralised tissue surface is dependent on the electric charges carried by the apatite crystals and the proteins. This will determine whether components attract or repel each other, the later facilitating the release of the growth factors into the surrounding environment. In acidic environment both apetite crystals and proteins are likely to carry positive charges, therefore repulsing each other (Doss 1976; Harding et al. 2005), facilitating their release into the aqueous environment, rather than accumulation on the surface. Conversely, proteins adopt a negative charge in alkaline pH (Doss 1976; Harding et al. 2005) while hydroxyapatite acquires a positive charge in Ca(OH)$_2$ due to specific adsorbance of calcium ions (Doss 1976). This may explain the highest levels of growth factors on dentine treated with Ca(OH)$_2$.

In relation to growth factor release, the results of this chapter revealed that TGF-β1 was only detected in the supernatants of EDTA treatment. Similar results have been reported by Galler et al.(2015) were dentine treatment by a number of chemicals including citrate

buffer, citric acid phosphate buffer and citric acid (10%, pH 2), which was used in this chapter also, resulted in the release of comparatively minute concentrations of TGF-β1 compared with EDTA (10%, pH 7, as in here). BMP2 and VEGF could not be detected in the supernatants of EDTA treatment in this chapter. This finding also was supported by Galler et al (2015) where EDTA treatment for 5 and 10 min released negligible concentrations of VEGF compared with TGF-β1 (average 15-28 pg/ml compared with 300-over 400 pg/ml). Furthermore, this chapter demonstrated that different chemicals released different amounts of (or none at all) BMP2 and VEGF from dentine matrix. Citric acid was the most effective agent in releasing these two growth factors here. This may be a reflection of the mechanisms involved in the release of proteins as well as the pH of the agent used.

Amongst the agents used, EDTA is a potent chelator of calcium (at neutral pH) and breaks down the hydroxyapatite lattice by binding calcium ions (De-Deus et al. 2008). Citric and phosphoric acid on the other hand, demineralise dentine due to their acidity. Once released the proteins may be affected by the environment pH, losing their stability and becoming denatured which would impact their detection by a method like ELISA. The quaternary, tertiary and secondary structure of proteins may be affected by the extremes of pH and proteins can lose their stability outside their pH-optimum stability as a result of conformational changes in the protein structure (Goto and Fink 1989; Uversky and Goto 2009; Talley and Alexov 2010). Further to the impact of pH it has been shown that the type of acid is also important in determining the behaviour of different proteins in an acidic environment (Fink et al. 1994). This may explain the varied impact of the agents used on the release of different growth factors into the aquous environment in this chapter. Another important point for consideration is that,

growth factors may be bound to proteoglycans, specific binding proteins and collagen in the ECM (Paralkar et al. 1991; Baker et al. 2009; Dreyfuss et al. 2009). It has been shown, in relation to acid treatment of dentine, that degradation of organic matrix components is a result of the combined effects of acid and dentine proteolytic enzymes and has been suggested therefore that mechanisms involved in solubilisation of various ECM proteins may be different (Dung et al. 1994; Dung et al. 1995).

Amongst dentine ECM proteins are MMPs which may be released when dentine is acid-etched and are capable of cleaving the collagen fibrils (Mazzoni et al. 2012; Mazzoni et al. 2013). Although MMPs are activated and are stable in low pH, they function best at neutral pH (Mazzoni et al. 2015). This means that degradation of organic ECM components may continue, through MMPs activity, for a considerable time after the pH has returned to neutral *in vivo*. Although concerns have been expressed over their possible adverse impact on bond stability of composite restorations (Mazzoni et al. 2015), it has been speculated that MMPs may play a favourable role in proteolytic activation of specific growth factors released from the ECM (Smith et al. 2012a). Contrary to the impact of acidic agents in releasing and activating MMPs, neutral EDTA prevents the release of MMPs (Imbery et al. 2012; Thompson et al. 2012) through chelation of zinc and calcium ions which are necessary for MMPs activity (Tezvergil-Mutluay et al. 2010). It may therefore be speculated that the multitude of mechanisms involved in the solubilisation of mineralised tissue components may result on balance, in the preferential release of one growth factor over another in relation to a specific conditioning agent, as was observed from the results of this chapter.

In clinical procedures involving dentine conditioning and etching, the conditioning agent is washed away prior to restoring the cavity. In that situation, the growth factors released into the solution is removed from the site, and the exposed growth factor may be more important for influencing the behaviour of nearby cells *in vivo*. Therefore, to help inform clinical practice, data obtained from this chapter should be interpreted in combination with that related to the influence of dentine conditioning on the behaviour of DPSCs which will be investigated in the next chapter.

Chapter 5: Differentiation of Dental Pulp Progenitor Cells on Conditioned Dentine Surfaces

5.1 Introduction

With increased popularity of aesthetic tooth-coloured restorations, the interactions between dentine-pulp complex and the constituents of adhesive restorative systems has become an area for intense scrutiny (Lehmann et al. 2009; Nowicka et al. 2012; Li et al. 2014; Kim et al. 2015; Wu et al. 2015). Recent research in this area has extended beyond the simple investigation of physical properties and biocompatibility of materials and turned its focus on understanding the materials potential in contributing to regenerative processes in the tooth.

There is an ongoing effort in developing biologically based therapies. Novel tissue engineering approaches have emerged, involving the use of signalling molecules, matrices and stem cells which pursue the goal of directing tissue events in dentine pulp complex towards dentine/pulp regeneration and ultimately preservation of pulp tissue vitality or stimulation of natural root development in the regenerative endodontics approach (Kim et al. 2012; Obeid et al. 2013; Ravindran et al. 2014). Recently, elaborate and complex tissue engineering techniques have been tested in animal studies and in vivo models with the aim of regenerating de novo pulp and dentine tissues in emptied root canals (Huang et al. 2010; Galler et al. 2012; Yang et al. 2012b; Iohara et al. 2013). Although the results of these studies have been somewhat encouraging, the procedure is practically complex, making the transition from laboratory to clinical practice an ambitious step, not to mention the significance of careful case selection for improved chances of success.

Comparatively, a cell-free approach in which the resident dental pulp progenitor cells in a viable dental pulp are recruited for dentine regeneration, is simpler and technically more manageable. If the pulp progenitor cells are to be induced to differentiate to odontoblast-like cell phenotype, a morphogenic signalling source, as an alternative to the odontogenic epithelium during tooth development would be required (Mitsiadis and Rahiotis 2004). The stimulatory effect of bioactive dentine matrix components on various progenitor cell populations of dental origin is well established (Chun et al. 2011; Smith et al. 2012a; Lei et al. 2013). The cascade of events in the stimulation of dental pulp progenitor cells, leading to dentine regeneration, may be initiated by the release of bioactive dentine matrix components (Smith et al. 2012b). This implies that application of certain chemicals with the ability to release bioactive components from dentine, may trigger the regenerative processes in teeth with viable pulps. Recent work has demonstrated the ability of demineralising agents such as EDTA (clinically used for removal of smear layer in endodontics) and acidic agents (common constituents of adhesive restorative systems), in solubilising non-collagenous proteins from dentine (Dung et al. 1995; Zhao et al. 2000; Ferracane et al. 2013; Galler et al. 2015). Further to this, dentine conditioning with EDTA and acidic agents is shown to impact cell fate of various populations of progenitor cells of dental origin in few recent studies; EDTA treatment of dentine has been shown to promote cell survival of the stem cells from the apical papilla (SCAP) *in vitro* (Trevino et al. 2011). In a study by Li et al. (2011b), dentine treatment with EDTA supported cell growth and induced cell differentiation in dental follicle cells (DFCs) *in vivo* in mice, resulting in dentine regeneration. Huang et al. (2006a) demonstrated odontoblast-like cells differentiation of DPSCs on dentine treated with EDTA and citric acid *in vitro*. Pang et al. (2014) also investigated the behavioural changes of DPSCs when cultured on dentine and demonstrated that EDTA

treatment of dentine, induced cell attachment and odontoblast-like cell differentiation as demonstrated by the expression of differentiation genes in and the formation of mineralised nodules on treated dentine. Furthermore, odontoblast-like cell differentiation of DPSC and intimate adhesion of cells to EDTA treated dentine has been observed *in vivo* in immune compromised mice (Galler et al. 2011). This evidence suggests that dentinogenesis may be stimulated through the use of appropriate conditioning agents. This information may have implications for a number of clinical applications including vital pulp therapy, which is the focus of this study, as well as revascularisation in regenerative endodontics and periodontal tissue regeneration.

Although the effects of acidic agents in solubilisation of bioactive proteins from mineralised tissue slices has been demonstrated (Zhao et al. 2000; Smith et al. 2011; Galler et al. 2015), the impact of clinically used etching agents on the behaviour of dental progenitor cells has not been previously investigated. The aim of this chapter is to investigate the effect of dentine surface treatment by dentine conditioner and etching agents on DPSC behaviour, specifically cell morphology and gene expression.

5.2 Materials and Methods

5.2.1 Preparation and conditioning of dentine slabs

Extracted caries free human molars (15 teeth) were obtained immediately post extraction, from the Oral Surgery clinics in Cardiff Dental Hospital with ethical approval and patients' consent (Appendix 1). The attached soft tissues were removed from the surface using a sharp scalpel and teeth were stored in 1% sodium azide for disinfection prior to removing enamel and cementum using a dental turbine with water coolant. The crowns of the teeth were sectioned using a segmented diamond edged rotary saw with water cooling into approximately 1 mm thick slabs. For SEM observation of cell morphology on treated dentine surface, only slices through the crown above the pulp chamber were used. The pulp tissue was removed with a scalpel. Slabs were washed thoroughly with sterile distilled water (with constant agitation for 5 min) and freeze-thawed three times to remove any possible cell residue. They were then immersed for 5 min in the conditioning agents used in the previous chapters; 10% w/v EDTA (Fisher Scientific, UK) pH adjusted to 7.2, 37% (v/v) phosphoric acid (VWR International) pH <1 and 10% (w/v) citric acid (Fisher Scientific, UK) pH 1.5, 0.02M calcium hydroxide as positive control and PBS as a negative control. The slabs were then rinsed three times in distilled water in a well of 24 well plate (Sarstedt, Leicester, UK), dabbed sideways on sterile towel to remove any excess liquid and placed individually in each well of sterile 24 well plates. The plates were placed in incubator for 30 min to dry completely prior to seeding the cells.

5.2.2 Culture of DPSCs on treated dentine surfaces

A single-colony derived population of DPSCs was obtained from the Mineralised Tissue Group Cell Bank, School of Dentistry, Cardiff. The clone (A31) was previously isolated, via enzymatic digestion and fibronectin adhesion selection (Appendix 2), from human caries free third molars. The clone was characterized and known to be highly proliferative with population doublings extending beyond 80 PD (over 300 days) and its ability to differentiate onto osteoblast, adipocyte and chondrocyte lineages, when cultured in the appropriate media, was demonstrated (Amr Alraies, PhD thesis, 2013, School of Dentistry, Cardiff University). Cells were obtained in accordance with ethical approval and patients' consent. After removal from liquid nitrogen storage, cells were rapidly thawed by transferring to a 37°C water bath. Once thawed, cells were immediately re-suspended in 5ml medium and centrifuged at 1800rpm. The cell pellet was washed with medium and cells expanded in culture in T-75 flasks.

5.2.3 Culture media and conditions

Culture media composed of α-modified Minimum Essential Medium (α-MEM) (containing ribonucleosides, deoxyribonucleosides, L-glutamine and phenol red), with 1% antibiotics (100 units/ml penicillin G sodium, 0.1μg/ml streptomycin sulphate and 0.25μg/ml amphotericin), 10% heat deactivated foetal bovine serum (FBS) (all Invitrogen, Paisley, UK) with addition of 100μM L-ascorbate 2-phosphates (Sigma-Aldrich, UK). Flasks containing cells were incubated in aseptic class II tissue culture hoods (Astec Microflow, Andover, UK) at 37°C with 5% CO_2 until approximately 80% confluent. Cells were then washed with 0.1M PBS, (pH 7.4) before being treated with 2-3ml of pre-warmed accutase (Invitrogen, UK) and placed in incubator at 37°C until cells became detached from the flask (approximately 3-5min). 5ml serum containing

culture medium was added to each flask subsequently to inactivate the accutase and centrifugation at 1800rpm followed. The resulting pellet was re-suspended in culture medium before cell counting and reseeding on treated dentine slabs. Cells (passage 13) were seeded at a density of 3.5×10^4 per slab, in 50µl of culture media, spread evenly over each slab, in individual wells of a 24 well plate. Three slabs were used for SEM and 5-6 slabs for analysis of gene expression per condition, and the experiment duplicated to ensure reproducibility. The plates were placed in incubator at 37°C with 5% CO_2 and left undisturbed for 1.5 hr before adding more culture media (as described above) to each well. From this point onwards, the serum (FBS) concentration in media was reduced to 7% for duration of the experiment. The media was gently squeezed out of the pipet, in one corner of each well to ensure cells seeded on dentine slabs earlier were not disturbed. After 24 hr, dentine slabs were transferred to new wells and media was changed 48 hourly until ready for SEM observation or the analysis of gene expression with Q-PCR.

5.2.4 Ability of conditioned dentine in supporting DPSCs growth, SEM observation of cell morphology

On day 2 and 8 after start of culture, slabs were washed in PBS prior to fixation with 2% paraformaldehyde and 2.2% gluteraldehyde in 0.1M cacodylate buffer for 1hr and subsequently washed 3 times in cacodylate buffer. The fixed samples were dehydrated in 70% ethanol for 15 min prior to being air dried in individual wells of 24 well plates, with the lid ajar, for 48 hr. Qualitative investigation of relative cellular morphology and surface area covered by DPSCs was carried out by visualisation of cells on dentine at ×500 and up to 9k magnification to visualise details under a Vega 3 SEM (Tescan-UK Ltd, Cambridge, UK).

5.2.5 mRNA extraction from cells cultured on conditioned dentine surfaces

RNA extractions were performed using the RNeasy mini Kit (Quigen, Manchester, UK), following the manufacturer's protocol, on days 2, 5, 10 and 14. The kit contains various buffers namely RLT lysis buffer and RW1 and RPE wash buffers. Spin columns in the kit contained membranes for binding RNA. To lyse the cells cultured on treated dentine slabs, three slabs of each condition were placed into one well of 24 well plate and washed with PBS. Three hundred and fifty microliters of buffer RLT with addition of 1% β-mercaptoethanol (Sigma-Aldrich, UK) was poured over the slabs onto each well and for better lysis of the cells repeatedly aspirated and poured into the well again over the dentine slabs. The resulting lysates from each group were placed into QiaShredder columns and centrifuged at 12,000×g for 2 min (Spectrafuge 24D, Jencons, UK) in order to shred cellular components, including DNA, and separate debris. Subsequently, 350µl of 70% molecular grade ethanol (Sigma-Aldrich, UK) was thoroughly mixed with the cell lysate by pipetting and the sample was transferred into an RNeasy Mini Kit spin column (Qiagen, UK) and centrifuged at 10,000×g for 15s and the flow-through discarded. The column was washed with 350 µl of RW1 buffer and centrifuged again at 10,000×g for 15s. The flow-though was discarded, 10µl of DNase1 stock was mixed with 70µl of buffer RDD (both Qiagen, UK) and added to each column and columns incubated for 15 min at room temperature in order to digest any genomic DNA contamination. A wash with 350µl buffer RW1 and 15s spin at 10,000×g followed. Two further washes with 500µl of buffer RPE and spins at 12,000×g for 20 sec and 2 min were followed and the flow-through discarded each time. The column inserts were transferred into a clean RNase free 1.5ml eppendorf (Fisher Scientific, Loughborough, UK) and centrifuged again for 1 min. The RNA was eluted by adding 30µl of RNase

free water before the last spin at 12,000×g for 1 min. The amount of RNA in each sample was quantified using 2µl of each sample by a Nanovue spectrophotometer (GE Healthcare, Amersham, UK). Prior to use, the NanoVue was standardised with 2µl of DNase free water as a blank. After quantification of RNA in samples, they were stored in -80°C prior to being used for reversed transcription.

5.2.6 Reverse transcription

Appropriate volumes of RNA samples containing 0.5µg of RNA were treated with 1µl of Random Primers (Promega) and the volume made up to 15µl with the addition of nuclease free water before incubating the tubes in a G-storm GS1 thermal cycler (Genetic Research instrumentation LTD, Braintree, UK) for 5 min at 70°C to unravel the RNA. The tubes were then removed and immediately cooled by placing on ice. Five microletres of 5×M-MLV (Moloney murine leukaemia virus) buffer, 1.25µl of 10mM dNTPs, 0.6µl of RNasin and 1µl of M-MLV reverse transcriptase (all Promega) and 2.15µl water was added to each tube. Each reaction volume was therefore 25µl in total which was mixed thoroughly. RT-negative control was made by exclusion of reverse transcriptase. The tubes were then placed in the thermal cycler and incubated at 37°C for 1hr followed by 95ºC for 5 min. The generated cDNA was stored at –20°C until use in PCR reaction.

5.2.7 Quantitative Real-Time PCR (qPCR)

A SYBR® Green JumpStart™ Taq ready mix™ (Sigma- Aldrich, UK) was used for the qPCR reactions. The pack contains a ready master mix and a reference dye. Each reaction was set up by mixing 12.5µl of the 2×mastermix, 0.25µl of reference dye, 2µl of 3µM forward primer, 2µlof 3µM reverse primer, 3.25µl sterile water and 5µl of 1 in

10 cDNA. Reaction were set up in Bright White 96-well plates (Primer Design Ltd), in triplicates. Quantitative real-time polymerase chain reactions (Q-PCR) were performed using an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Carlsbad, USA) and the data was processed and analysed by the software.

The cycles started with an initial denaturing cycle for 2 min at 94°C followed by 40 cycles of 15 sec at 94°C, 30 sec at annealing temperature (Table 5.1) and 30 sec at 72°C extension step. The output from the real-time PCR reactions was provided in the form of graphs showing the number of cycles against the increasing fluorescence (amplification plot). The gene expression levels were quantified by setting the Ct (cross threshold) value within the geometric phase for the housekeeping gene GAPDH, against which genes of interest were compared. The expression was considered specific and reliable for analysis only when a dissociation curve peak at a single melting temperature was observed. Using relative quantification, gene expression levels were presented as bar graphs as a percentage of reference gene, GAPDH Ct value. Data were presented as the mean and standard deviation values of percentages and used for the statistical analysis. Statistical analyses were conducted using one-way ANOVA for matched samples followed by pairwise multiple comparisons using Bonferroni adjustment on statistical package SPSS (as explained in chapter 3). Significance levels were defined as * = P<0.05 (significant), ** = P<0.01 (very significant).

Gene	Product size (bp)	Annealing Temp. (°C)	Sequence 5' 3'	Source
ALP	137	55	GGACCATTCCCACGTCTTCAC CCTTGTAGCCAGGCCCATTG	XQ Wei Cardiff University
Bone sialoprotein		62	TTCTCTGCCCTCTCACTCCCTTGAGCCT AAGCACAGGCCATTCCCAAAATGCTGA G	XQ Wei Cardiff University
CD 90	142	55	AATCTGTCCTCGTGTCCA GCGTTGCTACTAAGTGGTT	L Sadaghiani Cardiff University
CD105	186	55	AACAGTCCATTGTGACCTTC TTTTGCTTGGATGCCTGG	L Sadaghiani Cardiff University
GAPDH	253	55	GGTCGGAGTCAACGGATT ATCGCCCCACTTGATTTTG	XQ Wei Cardiff University
GAPDH	231	62	TCCCGCTTCGCTCTCTGCTCCTC GACCAGGCGCCCAATACGACCAAAT	XQ Wei Cardiff University
Osteopontin	430	55	ATCACCTGTGCCATACCA CATCTTCATCATCCATATCATCCA	L Sadaghiani Cardiff University

Table 5.1: Human primer sequences and PCR annealing temperatures for qPCRs

5.3 Results

5.3.1 Morphology of DPSCs on treated dentine surface

SEM images of DPSCs on dentine surface revealed presence of cells in abundance on dentine surface treated by all the conditioning agents on days 2, although the cells did not cover the surface uniformly. Due to the large number of cells settling on treated dentine surfaces and difficulty in identifying individual cells it was not possible to reliably count the numbers for a quantitative comparison. However it was clear that cells on PBS treated dentine were far fewer and sparser compared to dentine treated by the conditioning agents. The dentine surface was covered with a smear layer and large volume of debris was observed on PBS treated surfaces, making it not possible to identify the outline of cells (Figure 5.1a). However, the outline of cells were clearly visible against the open dentinal tubules in the background on EDTA and acid treated surfaces which is a reflection of the ability of the agent in removing the smear layer (Figure 5.1b-d). On calcium hydroxide treated surfaces, the presence of a smear layer on the surface made it more difficult to visualise the outline of cells, although a great number of cells were apparent (Figure 5.1e). A comparison of cell density and morphology could be made at magnification ×500 (Figure 5.1a-e).
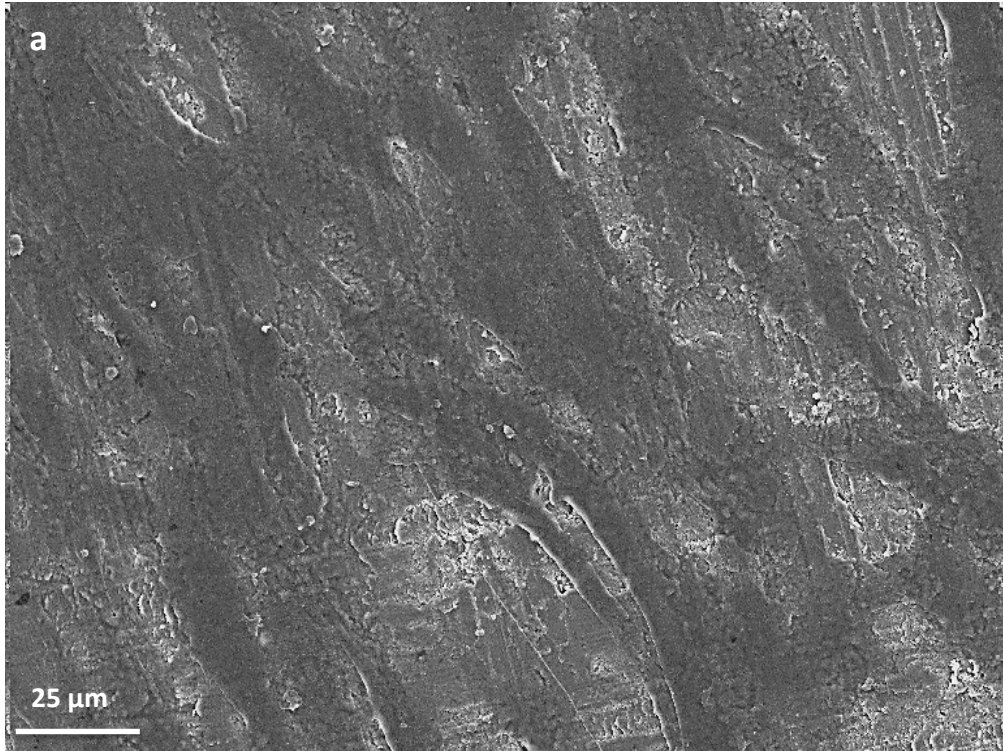
Figure 5.1(a): Representative SEM image of cells on dentine treated with PBS at magnification ×1K on day 2. The outline of individual cells cannot be identified on dentine surface.
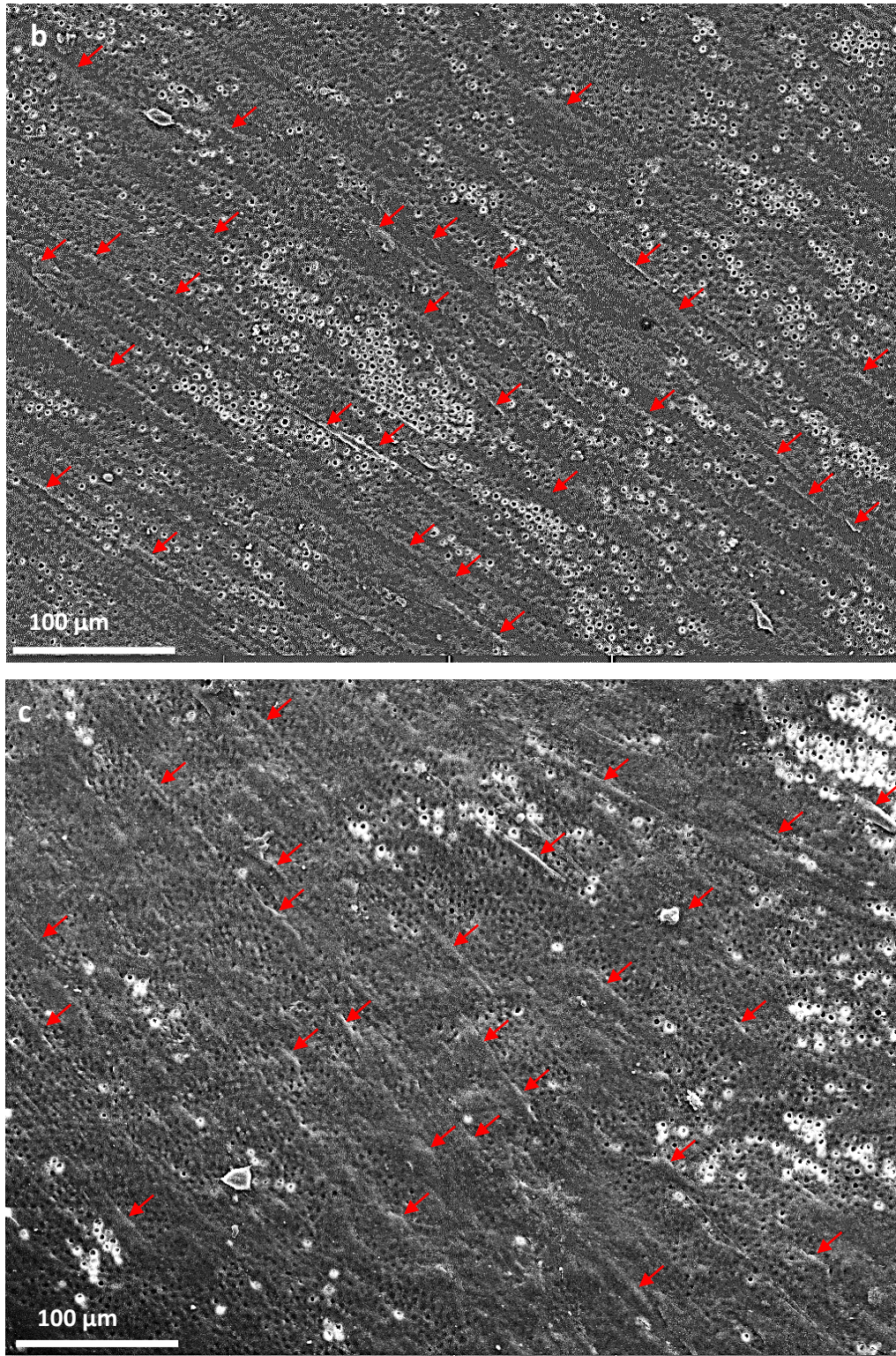
Figure 5.1 (b-c): Representative SEM images of cells on dentine treated with b) EDTA and c) phosphoric acid on day 2 at magnification ×500. The outline of cells can be identified against the open dentinal tubules in the background. Areas where cells have settled (arrows), the tubules cannot be visualised.
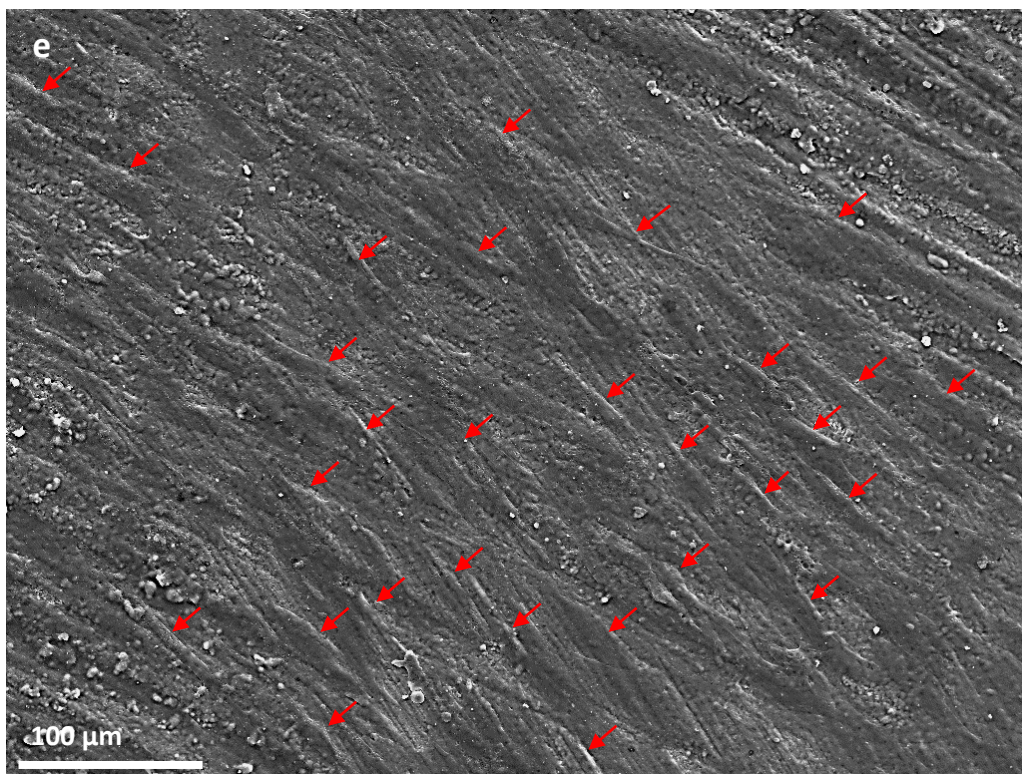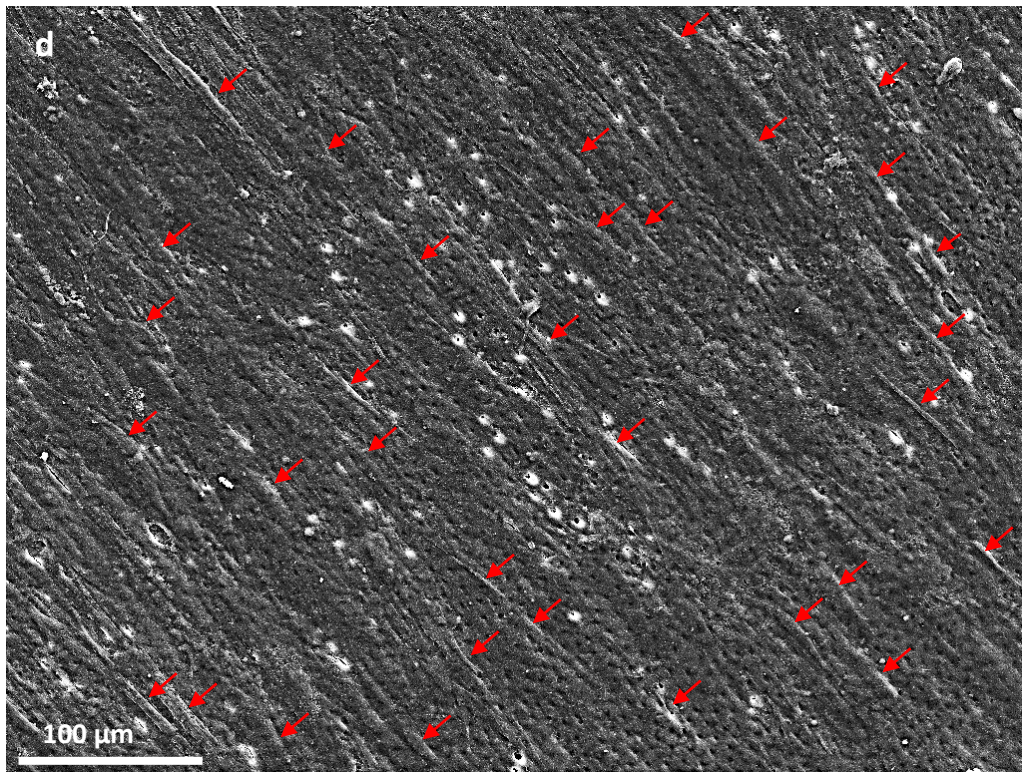
Figure 5.1(d-e): Representative SEM images of cells on dentine treated with d) citric acid and e) Ca(OH)$_2$ on day 2 at magnification ×500. The outline of cells are more obvious on citric acid compared with Ca(OH)$_2$ treated dentine. Areas where cells have settled (arrows), the tubules cannot be visualised (d).

On day 2, increased magnification revealed large number of cells lining up on the surface, parallel with each other (Figure 5.2a). Cytoplasmic extensions resembling odontoblast processes had developed which extended over dentine surface (Figure 5.2b-c). In areas with less cell density, a uniform substance resembling the extracellular matrix could be visualised around the cells, covering the dentinal tubules (Figure 5.2c).
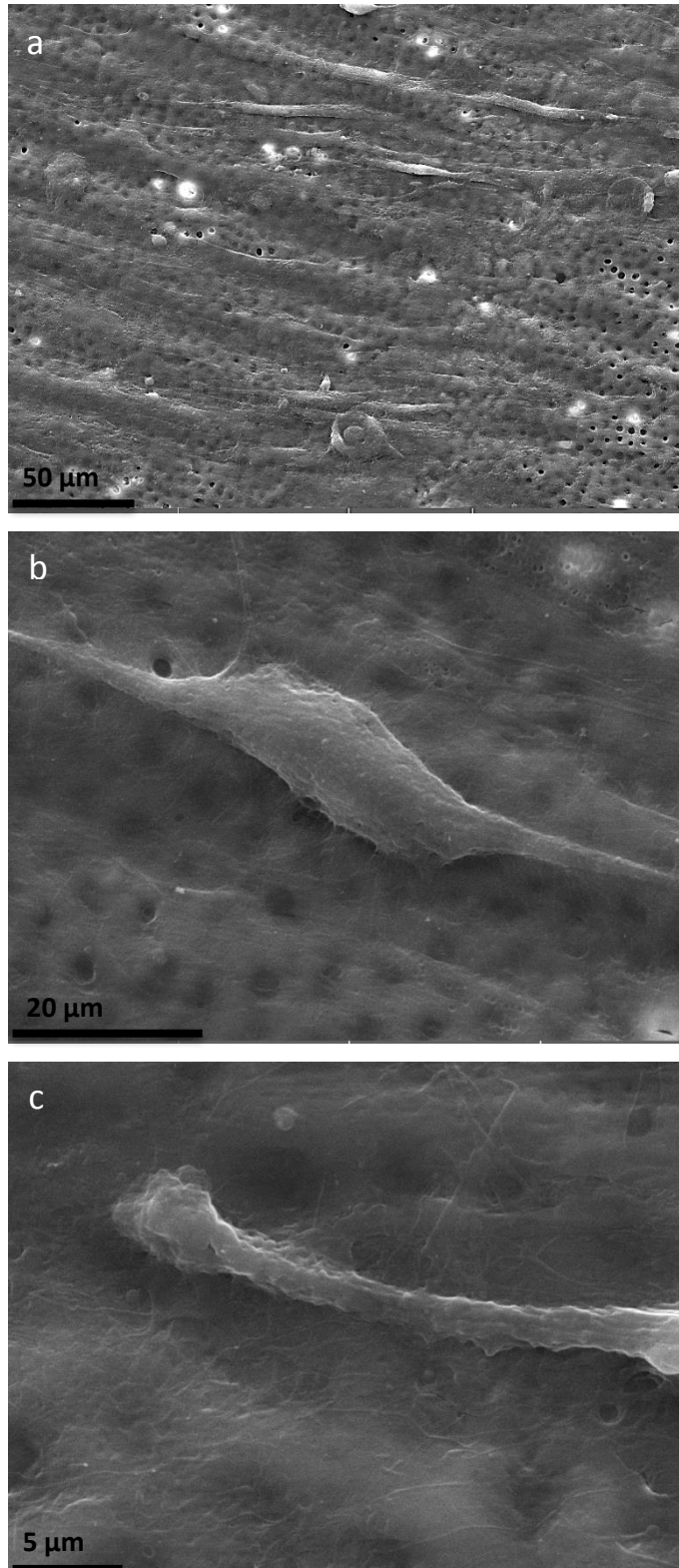
Figure 5.2 (a-c): Representative SEM images of cells on EDTA treated dentine surface on day 2; a) ×1k, b) ×2.3k and c) ×8.9k. Cells are overlapping and the outline of individual cells cannot be identified in some areas. Larger magnification shows a uniform substance, around the cells covering the tubules.

On day 8, comparison of cell density at ×500 magnification revealed very low cell density on PBS and phosphoric acid treated surfaces compared to the other groups (Figures 5.3 and 5.4). On phosphoric acid treated dentine surfaces, large areas of exposed tubules could be visualised in between the sparse cells. In terms of cell morphology, the cells on phosphoric acid treated dentine appeared more elongated and less spread, compared with cells on surfaces treated with the other agents. Furthermore the cells did not appear to have produced matrix compared to cells cultured on surface treated by the other agents, leaving the surface comparatively bare.
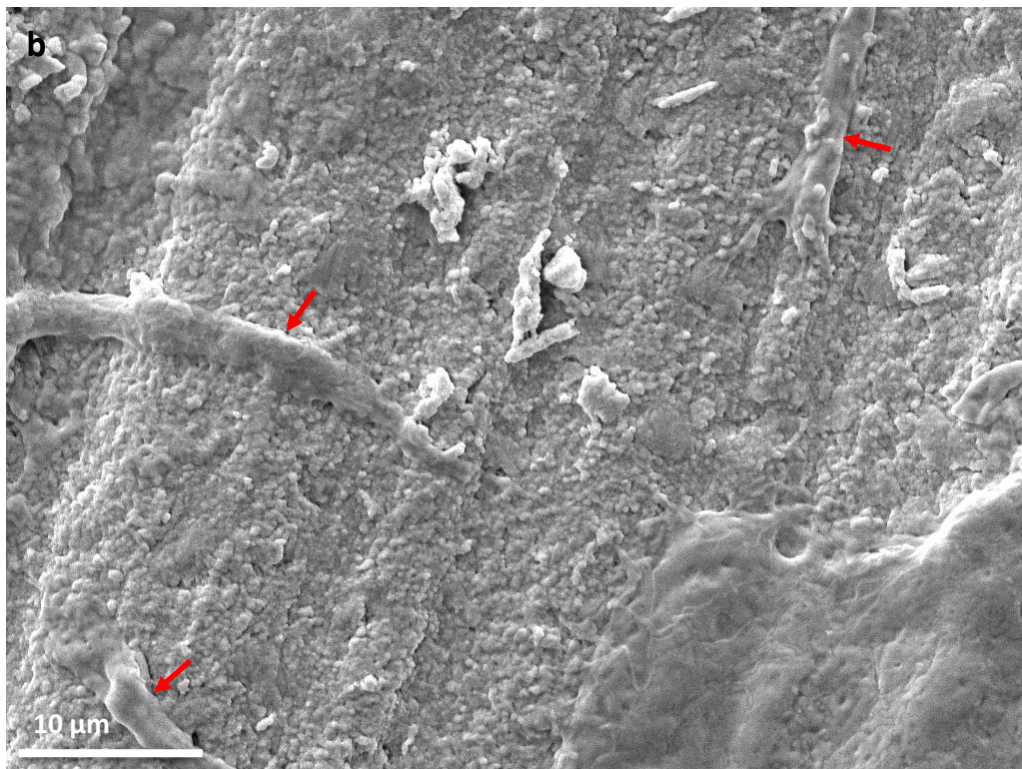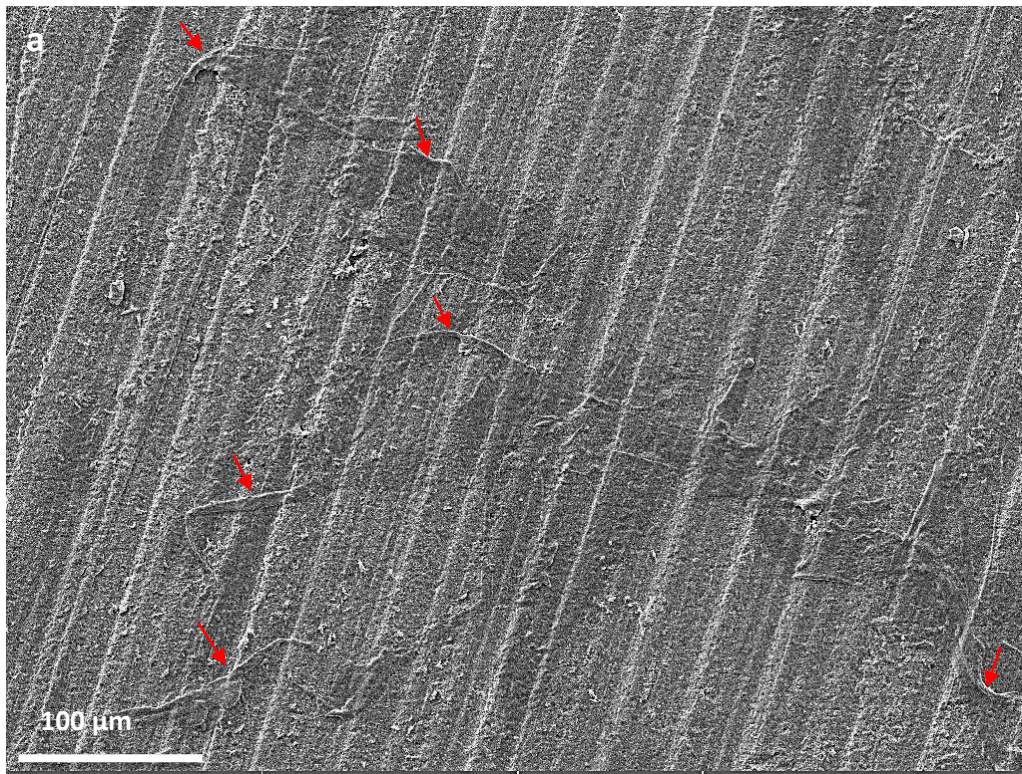
Figure 5.3: Representative SEM images of cells on PBS treated dentine on day 8. a) Magnification ×500 shows cells are few and far in between (arrows). b) Magnification ×5k shows the smear layer-covered surface with few cellular processes extending on the surface (arrows).
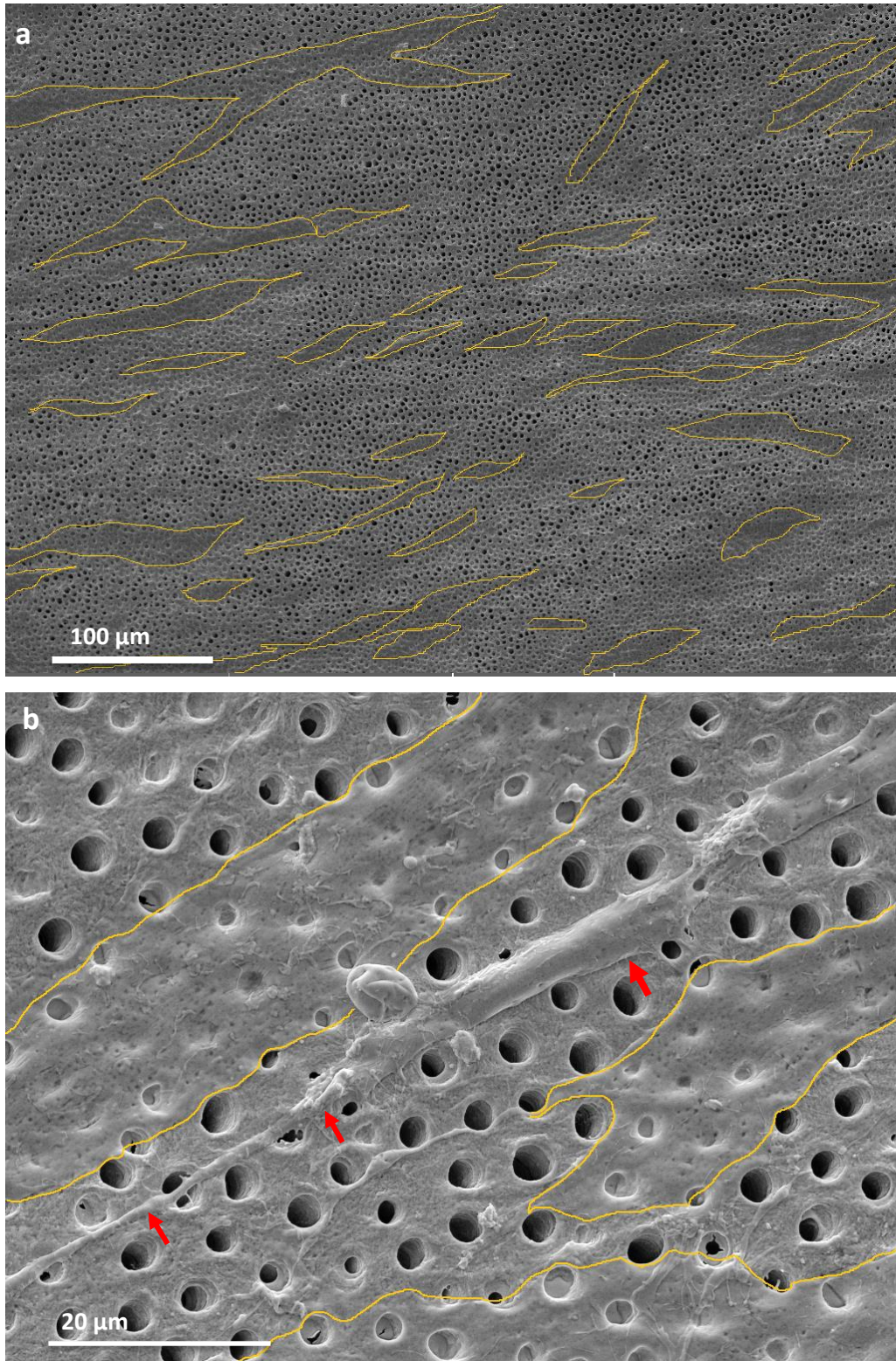
Figure 5.4: Representative SEM images of cells on phosphoric acid treated dentine on day 8. a) Magnification ×500, areas within yellow lines are covered by cells/ extracellular substance b) Magnification ×3.5K shows scarcity of cells. In the centre of image a cell with a long process extends on dentine (red arrows). Tubules are covered with what appears like a thin layer of extracellular material (within yellow lines).

In contrast, cell on dentine surfaces treated by EDTA and citric acid appeared dense with a thick uniform substance covering dentine, hiding the dentine tubular structure (Figures 5.5 and 5.6). In addition to long cellular extensions resembling odontoblast processes, cells seemed to have developed a multitude of short extensions from their bodies reaching out to the extracellular substance on dentine surface. Cells on $Ca(OH)_2$ treated dentine also appeared dense (Figure 5.7). A network of fibrils within the matrix around the cells could be visualised at larger magnification (Figure 5.7b).
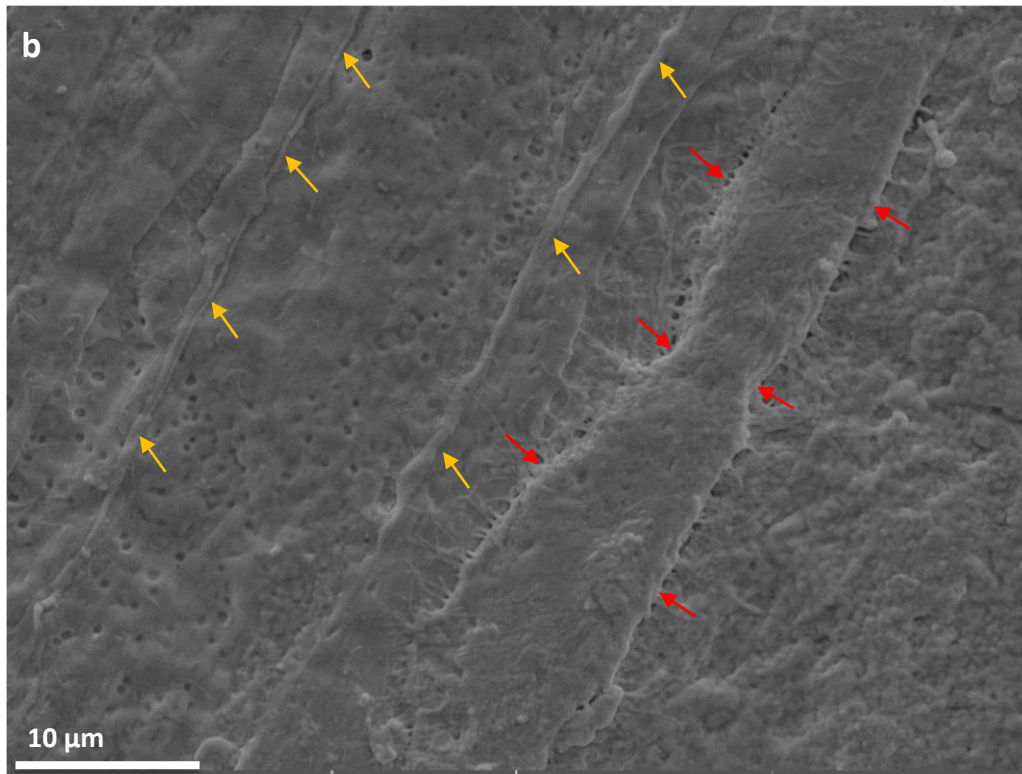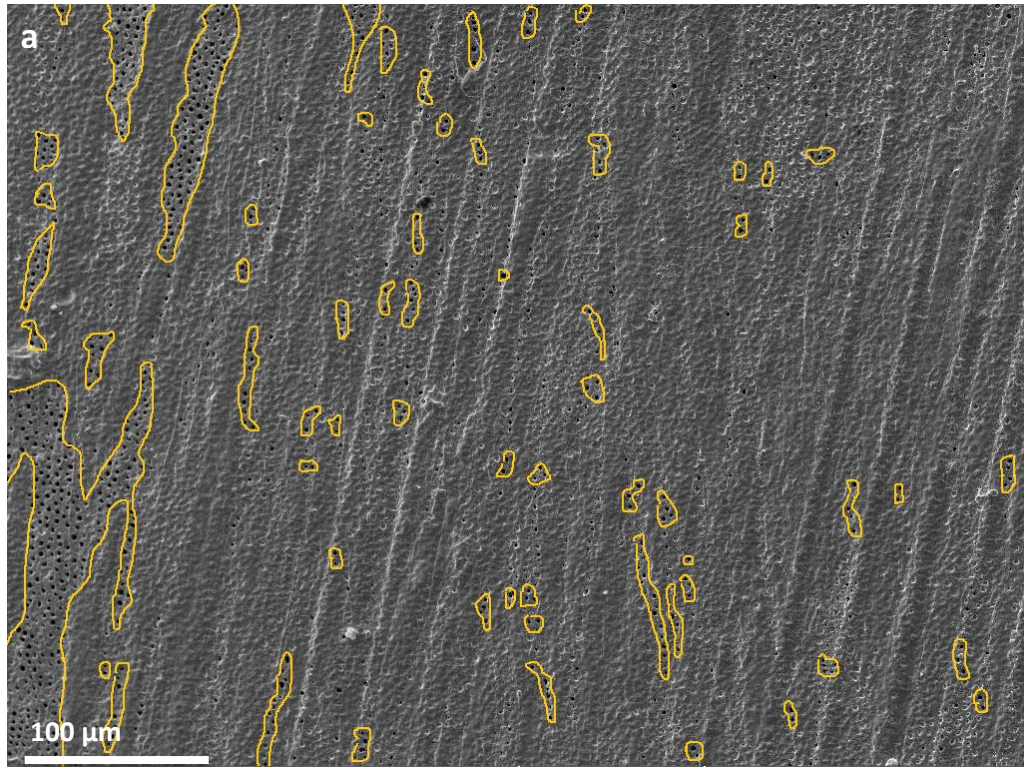
Figure 5.5: Representative SEM images of cells on EDTA treated dentine on day 8. a) Magnification ×500 shows few exposed dentinal tubules (within yellow lines). b) Magnification ×5K shows a cell body (within red arrows) with multiple short extensions reaching out from it, long processes of other cells (yellow arrow), and what appears to be a thick extracellular substance can also be visualised.
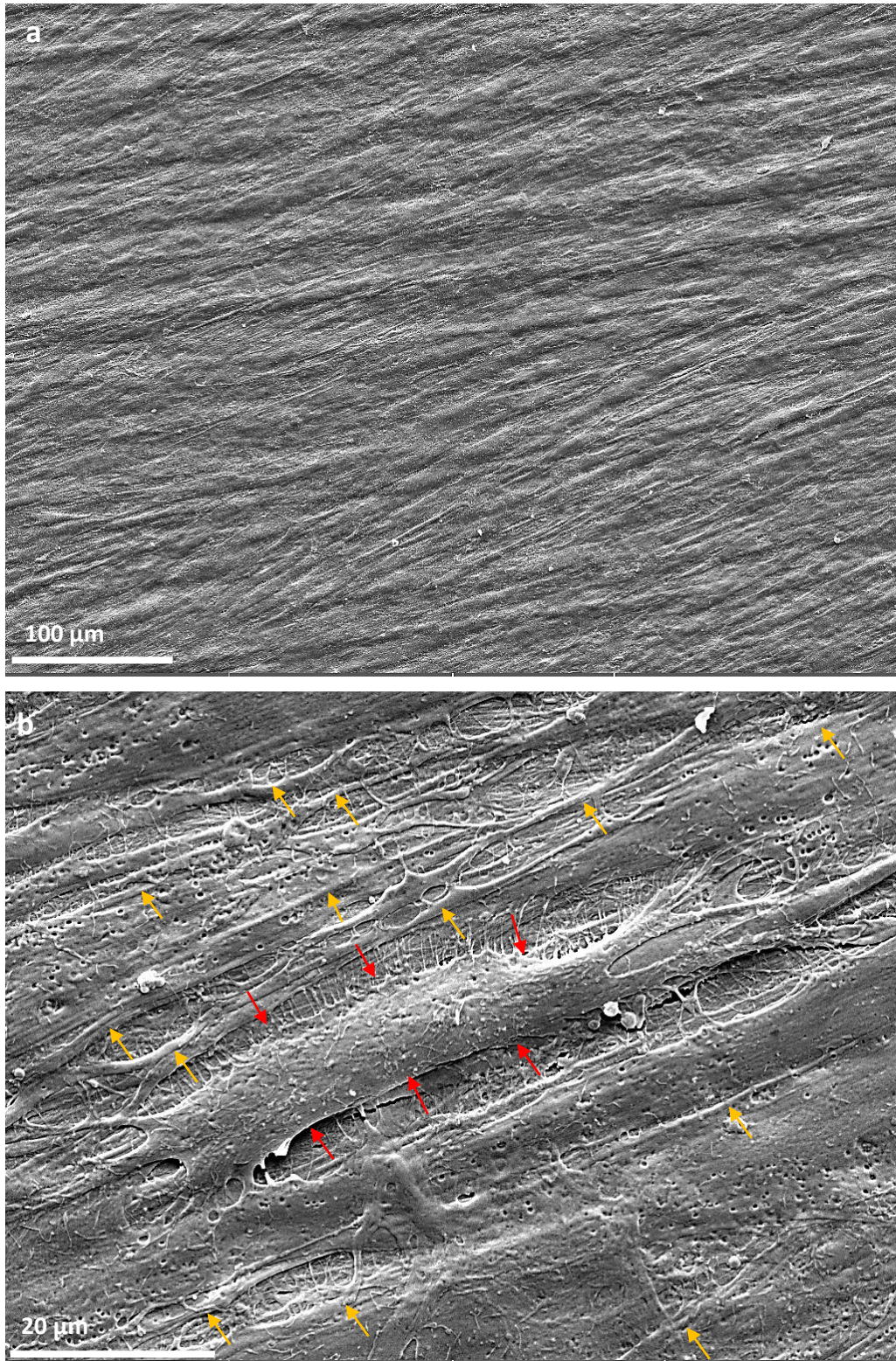
Figure 5.6: Representative SEM images of cells on citric acid treated dentine on day 8. a) Magnification ×500 shows no exposed dentinal tubules indicating a thick coverage. b) Magnification ×3.3K shows a cell body (within red arrows) with multiple short extensions reaching out from it. Long processes of other cells (yellow arrow) and a thick extracellular substance covering dentine tubules can also be visualised.
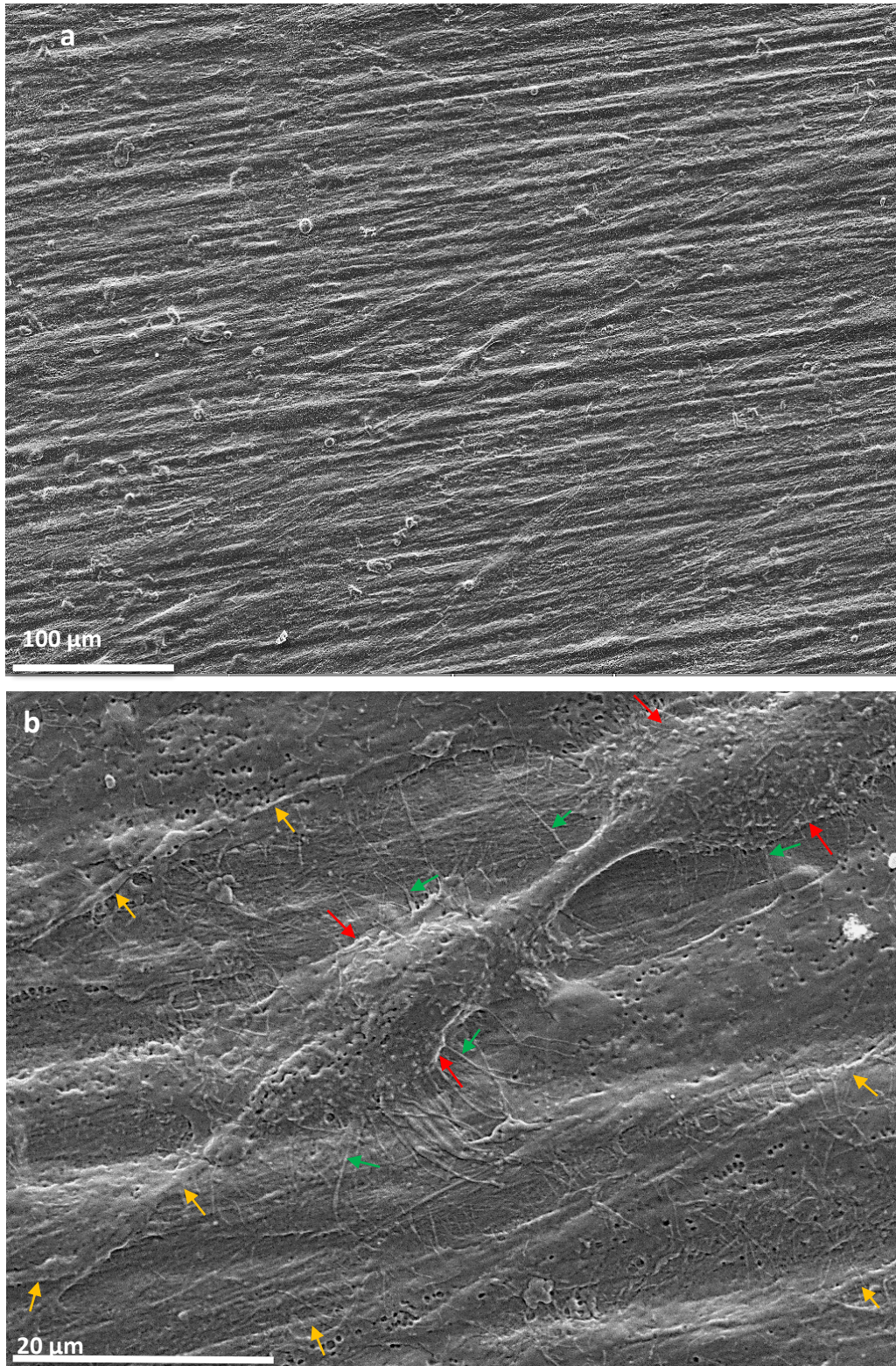
Figure 5.7: Representative SEM images of cells on Ca(OH)$_2$ treated dentine on day 8. a) Magnification ×500 shows prominent outline of multiple cells. b) Mag. ×4K shows two adjacent cells (within red arrows) with multiple short extensions reaching out from them (green arrows), long processes of other cells (yellow arrow) and a thick extracellular substance can also be visualised.

5.3.2 Gene expression of DPSCs cultured on treated dentine surface Quantitative real-time PCR

At an early stage of the experiment (day 2), Q-PCR data revealed a significantly lower level of expression of mesenchymal stem cell markers CD105 and CD90 in cells cultured on plastic compared to those cultured on dentine. Over the period of the experiment, the expression of the above mentioned genes were down regulated in all groups cultured on dentine surfaces while an up regulation between day 2 and 5 for cells cultured in well plates was observed. The sharpest decline for CD105 expression between days 2 and 5 was observed in cells cultured on $Ca(OH)_2$ and citric acid treated dentine where a significantly lower expression compared to all other groups was observed. The expression of CD105 continued to decrease in other groups between day 5 and 10 when no significant differences was observed between groups. A similar trend in relation to CD90 expression was observed where after an initial up regulation in cells cultured in well plates between day 2 and 5 (significantly higher expression compared to the groups on cultured on treated dentine on day 5 (P<0.05)). On day 5, the lowest CD90 expression was detected in cells cultured on Ca(OH)2 treated dentine. CD90 expression was down regulated in all groups between day 5 and 10 (Figures 5.8-9).
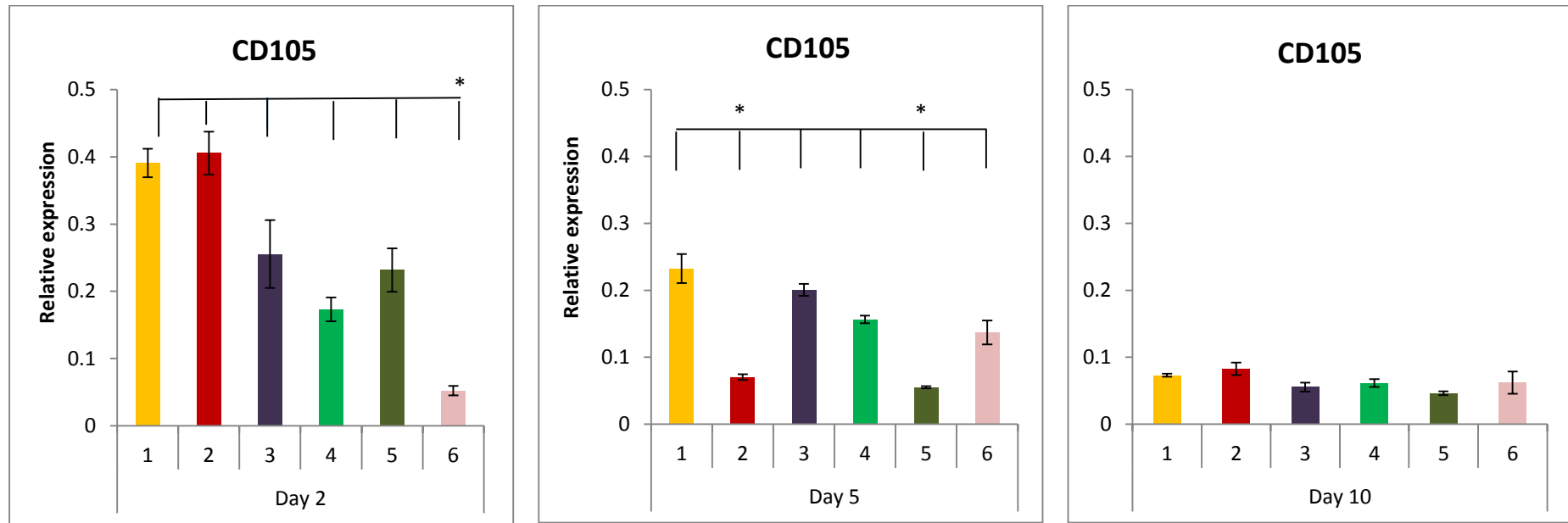
Figure 5.8: Gene expresions of CD105 in cells culture on dentine treated with 1) PBS 2) Ca(OH)$_2$ 3) EDTA 4) phosphoric acid 5) citric acid and 6) unstimulated cells cultured on plactic on days 2, 5 and 10.
* indicates significant difference between groups (P<0.05).
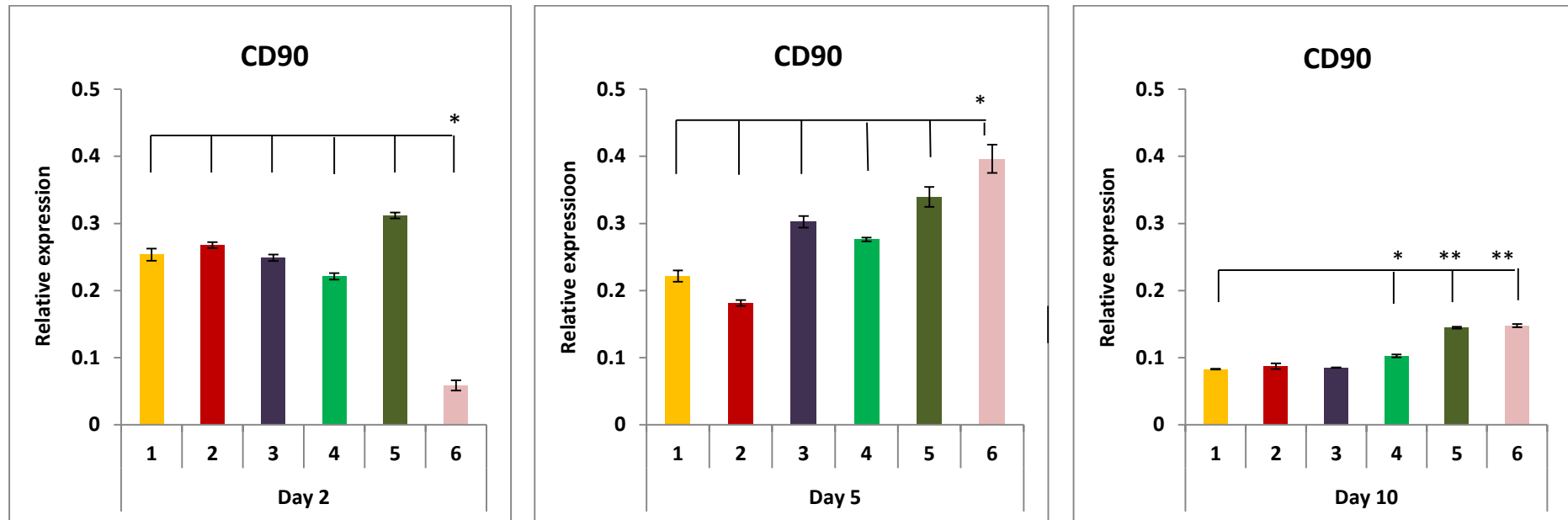
Figure 5.9: Gene expresions of CD90 in cells culture on dentine treated with 1) PBS 2) Ca(OH)$_2$ 3) EDTA 4) phosphoric acid 5) citric acid and 6) unstimulated cells cultured on plactic on days 2, 5 and 10.

* indicates significant difference between groups (P<0.05).

An up regulation for differentiation marker genes ALP and OPN was detected between days 5 and 14 in all groups with a sharp increase in gene expression between day 10 and 14. At early stage of the experiment (day 5) no significant difference between groups was observed for the expression of ALP. On day 10, expression of ALP in cells cultured on $Ca(OH)_2$ treated dentine became significantly higher than all other groups. By day 14, cells cultured on EDTA and citric acid treated dentine showed the highest level of ALP expression compared to all other groups ($P<0.01$ and 0.05 respectively). For OPN, the expression levels in cells cultured on EDTA and $Ca(OH)_2$ treated dentine were significantly higher than all other groups on day 5. On days 10 and 14, the highest expression level was detected in cells cultured on EDTA treated dentine. Expression of OPN was significantly lower in cells cultured on plastic compared to all those cultured on dentine surfaces throughout the experiment ($P<0.05$) (Figure 5.10-11).

BSP expression was not detectable on day 5 in any group. The expression was detected on day 10 in the groups other than those cultured in well plates and PBS treated dentine. By day 14, expression of BSP was still not detected in the cells cultured on plastic or PBS treated dentine and it appeared that cells on $Ca(OH)_2$ treated dentine had lost the expression of this marker. The highest level of expression on day 14 was detected in cells cultured on citric acid treated dentine (Figure 5.12).
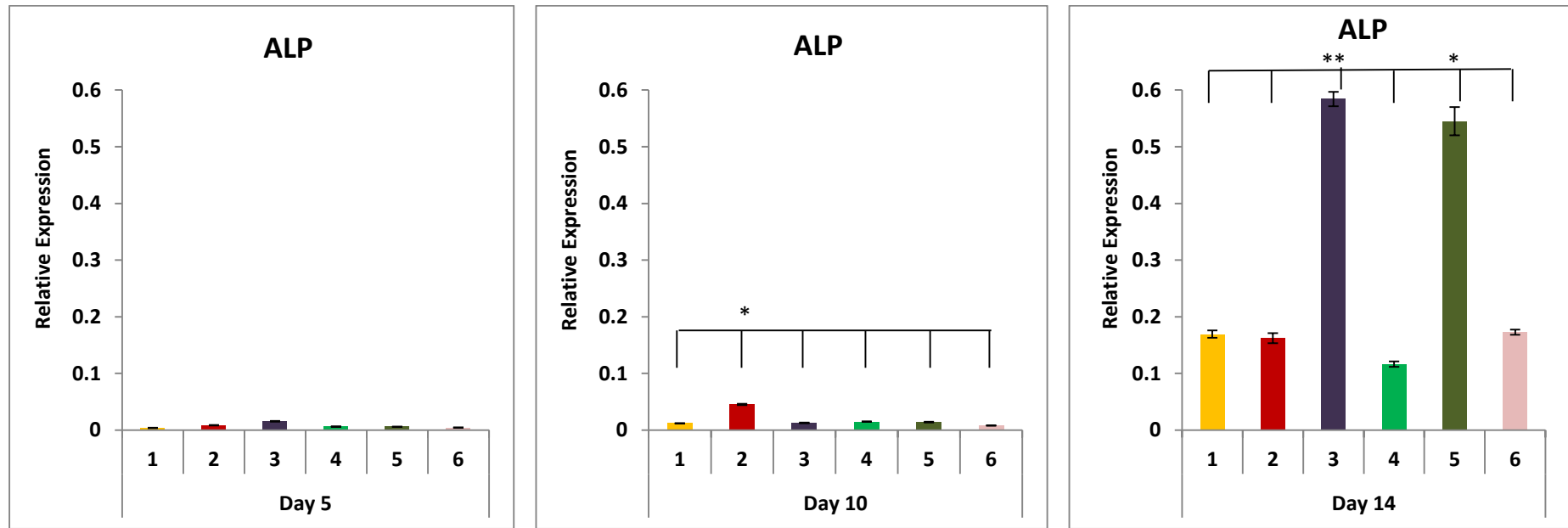
Figure 5.10: Gene expresions of alkaline phosphatase in cells culture on dentine treated with 1) PBS 2) Ca(OH)$_2$ 3) EDTA 4) phosphoric acid 5) citric acid and 6) unstimulated cells cultured on plactic on days 5, 10 and 14.

* and ** indicate significant difference between groups at $P<0.05$ and $P<0.01$ respectively.
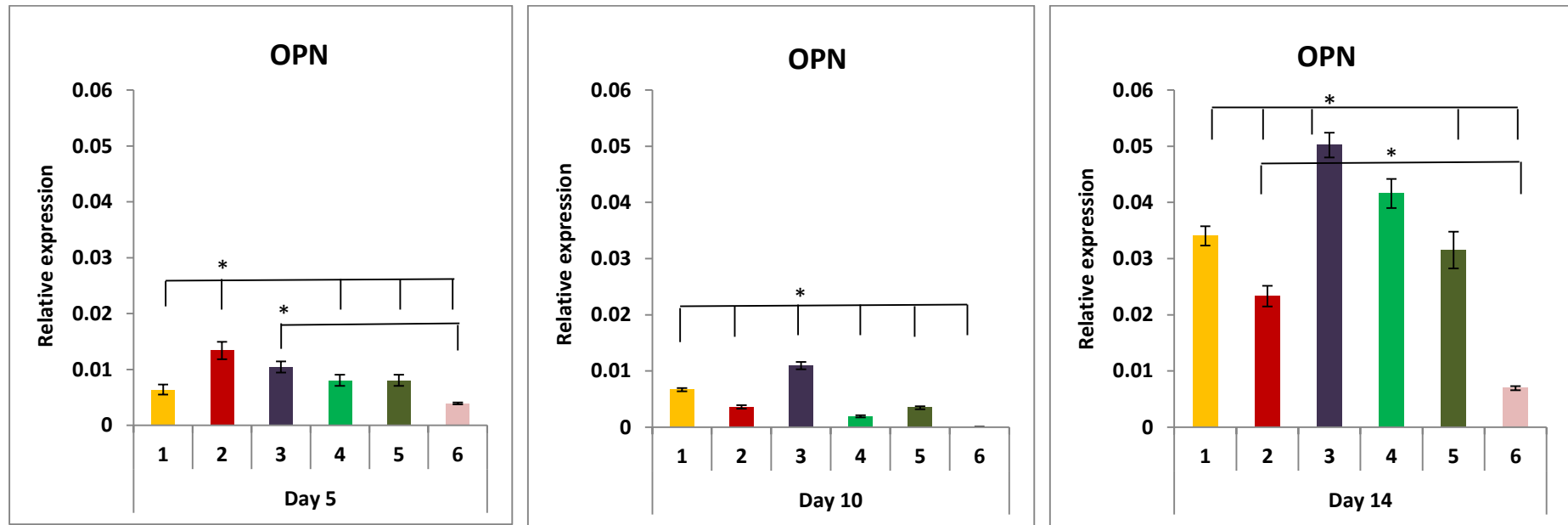
Figure 5.11: Gene expresions of ospetopontin in cells culture on dentine treated with 1) PBS 2) Ca(OH)$_2$ 3) EDTA 4) phosphoric acid 5) citric acid and 6) unstimulated cells cultured on plactic on days 5, 10 and 14.
* indicates significant difference between groups (P<0.05)
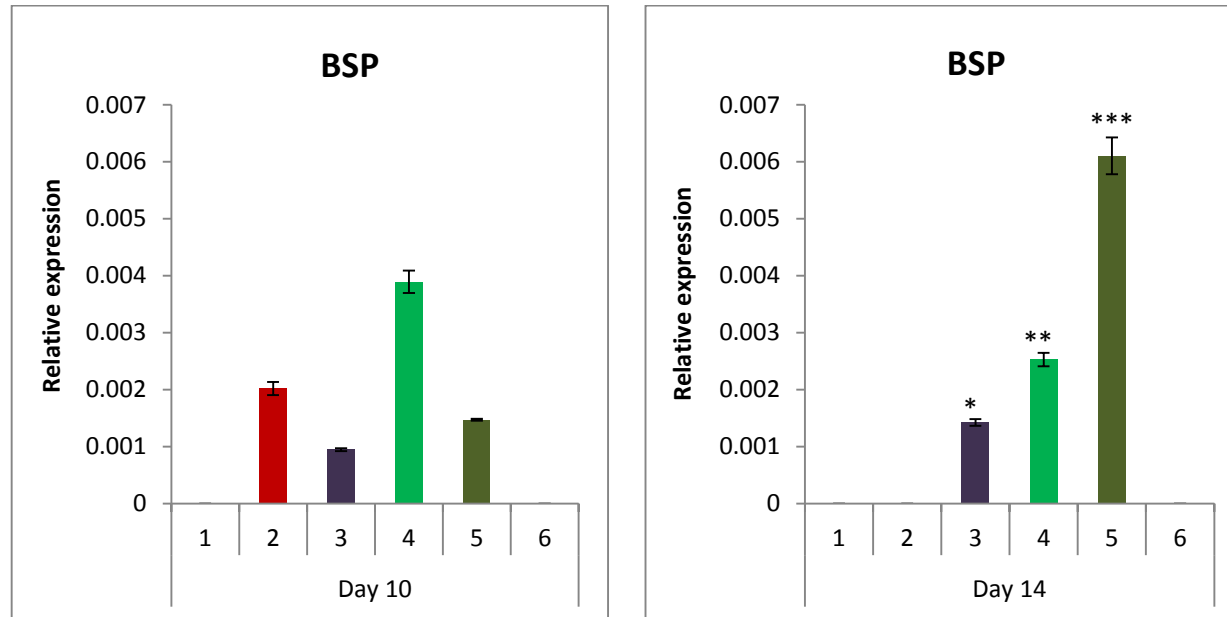
Figure 5.12: Gene expresions of bone sialoprotein in cells culture on dentine treated with 1) PBS 2) Ca(OH)2 3) EDTA 4) phosphoric acid 5) Citric acid and 6) unstimulated cells cultured on plactic on days 5, 10 and 14.

* and ** indicate significant difference between groups at P<0.05 and P<0.01 respectively.

5.4 Discussion

This chapter evaluated the effects of dentine treatment with EDTA, phosphoric acid and citric acid on DPSCs behaviour. Dentine treatment with $Ca(OH)_2$ and PBS served as positive and negative controls respectively. The clone used in this study was characterised and was known to be multipotent, with the capacity to differentiate to a mineral tissue forming phenotype. The potential of a dentine surface treated by various agents in supporting DPSC growth was investigated by SEM imaging at the early stage of day 2 and later on at day 8. No striking differences in terms of coverage of the surface by cells were evident by visual inspection under low magnification between groups on day 2. On day 8 however, the apparent denser cell coverage on EDTA, citric acid and $Ca(OH)_2$ treated dentine suggested those surfaces were more supportive of cells compared to phosphoric acid treated dentine. Scarcity of cells and bareness of the surface on phosphoric acid treated surface may indicate that the surface was not as supportive to cells as those treated by the other agents. In addition to lower cell density, the morphologies of DPSCs were different on phosphoric acid treated dentine. SEM images with larger magnification revealed that the DPSCs were narrower and more spindle shaped compared to the cells on $Ca(OH)_2$, citric acid and EDTA treated surfaces where cells appeared more stretched and anchored on the surface by fibres extending out from the cell bodies onto what appeared like ECM surrounding the cells. Data in the previous chapter showed phosphoric acid was able to expose growth factors on the dentine surfaces, the finding in this chapter in relation to cell scarcity and different morphology of DPSCs cultured on phosphoric acid treated dentine suggests that stimulation by growth factors is not the only mechanism facilitating DPSC growth and expansion. Other potential factors affecting cell behaviour may be the physical properties of treated dentine surfaces.

It has been suggested that while cell proliferation and differentiation in MSCs cultured on protein based membranes seems to be largely dependent on the biomolecular signalling from the membrane substrate, effect on cell morphology is mostly dependent on surface topography (Tejeda-Montes et al. 2014). Treatment of dentine by various agents used in this chapter is expected to have resulted in variation in the surface topography as well as physical properties. It has been shown that conditioning of primary dentine with 35% phosphoric acid results in complete removal of smear layer and not only opening but enlargement of the entrances to the dentinal tubules (Osorio et al. 2010). Funnelling of dentinal tubule orifices after treatment of dentine by 37% orthophosphoric acid was also observed in a study by Bolanos-Carmona et al., (2006). SEM images of $Ca(OH)_2$ treated dentine in this chapter, suggested incomplete removal of the smear layer which may result in difference in surface roughness compared with surfaces treated with EDTA and acidic agents which revealed complete opening of the dentinal tubules. Previous studies have described more spread cell morphologies on smooth surfaces compared with elongated and irregular shapes on surfaces with channels and holes (Mata et al. 2002; Tejeda-Montes et al. 2014). The findings in this chapter where cells cultured on $Ca(OH)_2$ surface adopted a more spread morphology compared to those on phosphoric acid treated dentine are in agreement with this evidence.

A number of other studies have also demonstrated the impact of substrate topography on stem cell behaviour; it has been shown that the nanotopographical pattern of a biomaterial on which stem cells are cultured, may influence adhesion, proliferation and differentiation of cells to osteogenic lineage (Dalby et al. 2007; Oh et al. 2009; Kolind et al. 2014). These studies showed a weak cellular adhesion and differentiation

in association with highly ordered topographies and emphasized the importance of substrate physical characteristics (size and distance between the holes or surface projections in these studies) in cell fate. In terms of the biological mechanisms involved, it has been proposed that the effect of topography on cells may be excreted by altering integrin clustering, focal adhesion assemblies, cytoskeletal organization and nuclear mechano-sensing which may ultimately result in adaptive gene and protein-level changes in response to biophysical signals from the extracellular microenvironment (Hamilton and Brunette 2007; Li and Xie 2007; Kokubu et al. 2009; Teo et al. 2010; Yim et al. 2010).

Dentine treatment by chemicals may impact other physical properties of the surface, for example prolonged acid etching with strong acids can result in deep demineralisation of dentine and renders the surface almost devoid of minerals (Hashimoto et al. 2002; Wang and Spencer 2004) which will affect the hardness (Angker et al. 2004). Stiffness of the surface is shown to influence differentiation of stem cells cultured on it (Fu et al. 2010), providing another explanation for differences observed in cell behaviour related to different conditioning agents used in this chapter.

Further to this, strong acids breakdown proteins as well as removing the mineral contents (West et al. 2000; Calt and Serper 2002). It has been shown that increasing treatment time by phosphoric acid from 15 to 30 and 60 sec can result in damaging of the ultrastructure of collagen type I, resulting in reduced detection of the protein on dentine surface (Breschi et al. 2003). The same study has shown that dentine treatment with a mineral chelating agent such as EDTA for the same duration as above, increases detection of dentine surface collagen compared with no treatment (Breschi et al.

2003). Exposure of collagen fibrils in the organic matrix by a chemical, may facilitate cellular adhesion by providing adhesion motifs that enable cell binding via integrin receptors (Heino and Kapyla 2009). Comparatively, an alkaline agent like $Ca(OH)_2$ extracts the matrix proteins (Partridge and Elsden 1961) (rather than minerals), resulting in a different surface nanotopography/ultrastructure compared to the demineralising agents, EDTA and the acids. The findings in this chapter in relation to the phosphoric acid treated dentine apparently being less supportive of cell growth may be a reflection of a combination of surface physical and biochemical characteristics of the treated dentine surface, resulting in a comparatively poorer conditions to support cells cultured on dentine surface.

In addition to the impact of the conditioning agents on dentine, the direct effect of the agent on the cells should not be overlooked. Although dentine slabs were washed vigorously in distilled water after conditioning, presence of any residue may have had an adverse impact on cell survival; culturing rat pulp cells in the presence of 100 times dilution of phosphoric acid (to 0.37%) is shown to cause marked decrease in cell viability compared with another diluted etching agent (0.1% phytic acid) *in vitro* (Nassar et al. 2013). In relation to calcium hydroxide, substance leached from a clinically used cement (Life, Kerr, USA) is shown to be biocompatible for human dental pulp fibroblasts in culture (Cavalcanti et al. 2005). The observed difference in cell density on dentine treated with phosphoric acid compared with the other agents in this chapter may be a reflection direct cytotoxic impact of the agent on DPSCs.

To assess the impact of dentine treatment on DPSC differentiation, expression of two mesenchymal stem cell marker and three mineralization related genes were

investigated in this chapter. The expression of mesenchymal stem cell marker genes, CD105 and CD90 were much lower in the cells cultured in well plates compared to those cultured on dentine surfaces at the very early stage of day 2. This may be a reflection of different material the cells were cultured on, plastic compared to dentine. Similar findings have been reported in relation to rat MSCs where the expression of osteogenic differentiation markers differed significantly in cells cultured on protein-based polymer membranes, in comparison with those cultured on fibronection coated glass (Tejeda-Montes et al. 2014). With increased time in culture up to day 10, the expressions of both CD105 and CD90 were significantly down regulated in cells cultured on all dentine surfaces, while an up regulation for cells cultured on plastic was observed. The sharpest decline in the expression of CD105 between day 2 and 5 occurred in the cells cultured on $Ca(OH)_2$ and citric acid treated dentine and on day 2 these groups revealed the lowest expression of CD105 amongst all groups (P<0.05).

The expression levels of ALP, OPN and BSP in DPSCs were investigated as a marker of odontoblast/ osteoblast-like cell differentiation. Opposite trends in the expression of stem cell and differentiation markers suggests loss of stem cell characteristics and coincides with progression of differentiation process in DPSCs in this chapter. For ALP, significantly higher expression were detected in cells cultured on $Ca(OH)_2$ treated dentine on day 10. By day 14, cells cultured on EDTA and citric acid showed the highest level of expression (P<0.01). Similarly, for OPN, the gene expression was found to be significantly higher in cells cultured on $Ca(OH)_2$ treated dentine on day 5 which combined with OPN data, suggests a comparatively earlier start to differentiation in this group. On day 10 cells cultured on EDTA treated dentine demonstrated the highest OPN expression. By day 14 a significant up regulation of

OPN in all groups cultured on dentine was observed compared to those in well plates. Particularly on day 14, OPN expression appeared to be lower in cells cultured on citric acid compared with those on PBS treated dentine, suggesting that the peak of expression for the former may have been missed. This data suggests that prolonged contact with dentine *in vitro* may result in eventual odontoblast/osteoblast differentiation under the experimental conditions used in this study. Therefore data on gene expression at earlier time point (between day 10 and 14), may have provided a more meaningful comparison.

The expression of BSP was much lower compared to the other mineralisation marker genes investigated in this chapter and undetectable on day 5 for all groups. The expression was detected on day 10 for all groups apart from the cells cultured in well plates and on PBS treated dentine. On day 14, the highest expression was detected in cells cultured on citric acid treated dentine. Overall, the higher expression levels of mineralised tissue genes in cells cultured on EDTA and $Ca(OH)_2$ treated dentine over the period of this study (14 days) suggests that treatment of the dentin surface by these agents promotes odontoblast/osteoblast differentiation of DPSCs. Furthermore, compared with phosphoric acid, dentine treatment with citric acid resulted in a marked down regulation and significantly lower expression of stem cell marker CD105 ($P<0.05$) on day 5 as well as significantly higher expression of differentiation marker ALP on day 14 ($P<0.01$). Combined with SEM observation of cell density and morphology it may be suggested that citric acid is biologically a more plausible option for dentine treatment as far as DPSCs are concerned. These results may be applicable to clinical scenarios related to vital pulp therapy as well as regenerative endodontics and periodontal tissue regeneration.

Chapter 6: General Discussion

The present study evaluated the effect of dentine treatment by several etching and conditioning agents, the constituents of adhesive restorative system, on dental pulp progenitor cells (DPSCs) behaviour. The study focused on the indirect effect of agents, mediated through the released bioactive molecules from dentine, on DPSCs which may provide an important mechanism for inducing a dentinogenic response in the pulp (Smith et al. 2001a; Smith et al. 2012b). The aim was to inform clinical practice in relation to vital pulp therapy where inducing a dentinogenic response is desired and may help limit injury to viable dental pulps. This may help resolve inflammation and restore pulp health enabling preservation of vitality rather than embarking on root canal treatment or extraction (Tjaderhane 2002).

The effects of dentine conditioning by EDTA has been previously investigated; EDTA solubilised dentine matrix extracts have been shown to induce odontoblast-like cell differentiation, at sites of mechanical exposure in dogs, and stimulate reactionary dentinogenesis in ferrets (Tziafas et al. 1995; Smith et al. 2001b). As well as this, studies investigating the impact of dentine matrix extracts (DMEs) on DPSCs behaviour have shown stimulation by EDTA extracted DMEs, results in odontoblast-like cell differentiation and mineralisation *in vitro* and *ex vivo* (Liu et al. 2005; Chun et al. 2011). Furthermore, EDTA treatment of dentine has been shown to induce cell attachment to dentine surface and odontoblast-like cell differentiation *in vitro* and *in vivo* in immune compromised mice (Galler et al. 2011; Pang et al. 2014). At the time the present study was ongoing, no reports were available in the literature of the effects

of phosphoric, citric or poly acrylic acid treatment of dentine on DPSCs behaviour, which was one of the key aspects this study sought to address.

DMEs extracted by phosphoric and citric acid in this study, were shown to contain important dentine ECM growth factors and were found to be capable of influencing DPSCs behaviour, leading to cell differentiation and mineralised nodule formation. To simulate more closely the clinical situation, growth factor exposure and release kinetics from dentine slices (rather than powder) were investigated. Dentine treatment with citric acid resulted in TGF-β1, BMP2 and VEGF exposure in levels comparable with that of EDTA. Citric acid was significantly more effective than phosphoric acid in exposing surface growth factors. Citric acid was also significantly more effective than both EDTA and phosphoric acid in releasing BMP2 and VEGF from dentine. These findings are highly relevant to clinical practice as the growth factors solubilised by these agents, may diffuse through dentinal tubules and induce a behaviour change in the pre-existing odontoblasts in contact with dentine or affect cell fate of progenitor cells residing within the pulp, in favour of a reactionary or reparative dentine formation (Tziafas et al. 1995; Smith et al. 2001b).

In contrast, the failure of polyacrylic acid to solubilise proteins from dentine powder in this study and the observed acid/ protein precipitation might mean immobilisation of ECM proteins at sites of clinical application of this conditioner and glass ionomer restorations (which also contain polyacrylic acid). This finding is relevant to the clinical dilemma of "to bond or to base" as it suggests polyacrylic acid may be unable to induce a biological response in the pulp while opposite was observed for the other conditioning agents. Caution must however be exercised in interpreting this finding as

other properties of restorative materials also contribute to the success of treatment in vital pulp treatment clinically. For example, a key factor for success, is an effective seal to help eliminate bacterial microleakage along the restoration margin and ultimately control of the pulp inflammation (Cox et al. 1987; Sasafuchi et al. 1999; Murray et al. 2002a). Current evidence is contradictory and inconclusive in relation to the comparative superiority of the sandwich technique versus incremental composite resin restorations for microleakage control (Fourie and Smit 2011; Kasraei et al. 2011; Gungor et al. 2014; Moazzami et al. 2014). This is partly a reflection of the huge variety of materials commercially available as well as the lack of standardised research and clinical protocols which render meaningful and conclusive comparisons impossible.

Another consideration with regard to the choice of adhesive restorative technique is concerns over biocompatibility of phosphoric acid and adhesive bonding agent applied to deep cavities. Acid etching with phosphoric acid followed by the application of bonding agents prior to restoring cavities with composite in deep dentine is shown to cause an intense inflammatory pulpal response in teeth scheduled for orthodontic extractions (de Souza Costa et al. 2002; Costa et al. 2003). In line with this evidence, the results of this study showed (chapter five) that surfaces treated by phosphoric acid appeared strikingly less populated by cells compared with those treated by citric acid and EDTA, suggesting a comparatively less attractive surface for cell attachment and growth. For dentine regeneration, cell attachment to the surface needs to take place prior to proliferation and differentiation to odontoblast-like cells and production of new dentine (Ring et al. 2008; Pang et al. 2014). The scarcity of cells on phosphoric acid treated dentine surfaces in this study suggests that such treatment may invoke an

adverse cellular response in DPSCs which would be detrimental for inducing a dentinogenic response in vital pulp therapy.

As for the implications of this finding for clinical practice, considering the apparent desirable biological outcome related to 10% citric acid and EDTA compared with 37% phosphoric acid, considerations may be given to replacing phosphoric acid by milder acids as etching agents in adhesive restorative systems. Ten percent citric acid and 17% EDTA have been previously shown to be capable of etching dentine and to be effective in removing the smear layer, smear plugs, demineralising intertubular dentine and exposing a stable collagen matrix (Saeki et al. 2001; Breschi et al. 2002; Bogra and Kaswan 2003). In relation to enamel, phosphoric acid etching has proven to be a successful procedure for increasing bond strength between enamel and composite restoration (Goes et al. 1998). Concerns have been expressed regarding the lower composite to enamel bond strength in the self-etch adhesive systems, attributed to the lower etching capacity of milder acids in these systems, compared with the etch-and-rinse systems where phosphoric acid is used as the etching agent (Ernest et al. 2004; Goracci et al. 2004; Brackett et al. 2006). However, a recent in vitro study concerning the possible long term detrimental effects of MMPs released from dentine following dentine etching on dentine-composite bond interface, proposed EDTA at neutral pH as a phosphoric acid substitute for etching dentine and enamel (Imbery et al. 2012). Imbery et al. 2012 demonstrated comparative bond strength for specimens treated with EDTA and phosphoric acid-etched followed by chlorhexidine treatment to inhibit the activation of released MMPs. This suggests that beside a desirable biological response, additional long term advantage of bond stability may be gained when etching by EDTA rather than phosphoric acid. However, further investigation

is required to bridge the gap between laboratory and clinic for optimising dentine treatment times as well as concentrations of the conditioning agent in a commercial product for achieving desirable physicho-chemical properties for the dental tissues to ensuring a reliable and durable bond interface (Walshaw and McComb 1996).

Several limitations related to the present study need to be acknowledged. The DPSC clone used here was isolated from caries free impacted molar teeth, with presumed healthy dental pulp tissue. Clinically, vital pulp therapy is employed in the management of caries affected or traumatised teeth in which pre-existing pulpal inflammation is expected to be present. It has been suggested that while stem cells derived from inflamed pulps possess typical stem cell characteristics, they may show varied properties (Zhou et al. 2015). The possibility of DPSCs behaviour observed in this study not strictly reflecting that of cells in clinical scenarios should be considered.

The impact of inflammation in the local microenvironment on stem cells has been widely investigated [reviewed in Kizil et al (2015)] and the general belief of detrimental effect of pro-inflammatory signalling and beneficial effect of anti-inflammatory signalling for stem cell activity has been challenged. With specific reference to DPSCs it has been reported that inflammatory process did not affect stem cell properties such as morphology, proliferation rate and differentiation potential of cells isolated from the inflamed dental pulps (Pereira et al. 2012), but chronic exposure of cells to pro-inflammatory markers *in vitro* promoted proliferation, while inhibiting differentiation in DPSCs (Boyle et al. 2014). In contrast to this, initial inflammatory reaction is suggested to favour odontoblast-like cell differentiation and promote healing in experimental studies of pulp capping the mechanically exposed healthy

pulps (Jegat et al. 2007; Goldberg et al. 2008). Overall, evidence suggests that low levels of pro-inflammatory mediators promote tissue repair, while long standing chronic inflammation may inhibit healing in dentine-pulp complex (Cooper et al. 2014). This means that response to vital pulp treatment may vary based on the inflammatory status of the pulp tissue in clinical situations, which the current in vitro study has not accounted for.

Further to this, the influence of age on the pulp stem cells regenerative potential must be considered. The pulp tissue volumes decreases with age due to gradual deposition of physiological secondary and pathological tertiary dentine (Murray et al. 2002d). Fewer colonies of human DPSCs can be isolated from the older than younger patients (Iida et al. 2010). Furthermore, human DPSCs isolated from older individuals have been shown to lose their proliferation and differentiation capabilities after repeated passaging *in vitro* (Takeda et al. 2008; Bressan et al. 2012). However, it has been demonstrated that the low proliferative ability of DPSCs isolated from the aged donors is observed in monolayer culture *in vitro*. When cultured on 3D nanostructured scaffolds and used *in vivo* to repair critical size defects, the cells demonstrated the same ability in terms of time taken for repair and the quality of extracellular matrix as the younger donors (Bressan et al. 2012). Furthermore, comparison of mobilised DPSCs (MDSCPs) from aged donors to young donors demonstrated that there was a modest age-related decline in stem cell properties including migration and differentiation potential but the regenerative potential of aged MDPSCs was similar to that of young MDPSCs in ex vivo models (Horibe et al. 2014). Although evidence suggest minimal alteration in human DPSCs with aging, the pulp tissue response to vital pulp treatments clinically would depend on not only the materials and techniques

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applied but also on crucial host related factors which cannot be strictly replicated in a laboratory based study. The true effect of dentine conditioning on inducing a dentinogenic response in patients should be investigated in clinical trials.

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Dr Alastair James Sloan
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7 November 2007

Dear Dr Sloan

Full title of study:	**The effect of adhesive restorative materials on solubilisation of bioactive dentine matrix components and dentine regeneration**
REC reference number:	**07/WSE04/84**

Thank you for your letter of the 2 November 2007, responding to the Committee's request for further information on the above research, and for submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised].

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA).

There is no requirement for [other] Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document.

You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Application	Parts A and B	17 July 2007
Investigator CV		06 March 2007
Protocol	1	06 March 2007
Covering Letter		17 July 2007
Peer Review	Cardiff and Vale NHS Trust	10 July 2007
Compensation Arrangements	UMAL - Cardiff University	
Participant Information Sheet: Information Sheet under 16 years old	3	18 September 2007
Participant Information Sheet: Parent/Guardian Information Sheet	2	18 September 2007
Participant Information Sheet	1	06 March 2007
Participant Consent Form	1	06 March 2007
Participant Consent Form: Parent/Guardian	2	18 September 2007
Response to Request for Further Information		02 November 2007
Memo from Cardiff University		20 March 2007
Email from Cardiff University	Trust Research Governance Officer	01 November 2007

R&D approval

All researchers and research collaborators who will be participating in the research at NHS sites should apply for R&D approval from the relevant care organisation, if they have not yet done so. R&D approval is required, whether or not the study is exempt from SSA. You should advise researchers and local collaborators accordingly.

Guidance on applying for R&D approval is available from http://www.rdforum.nhs.uk/rdform.htm.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

Here you will find links to the following
   a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service on the application procedure. If you wish to make your views known please use the feedback form available on the website.
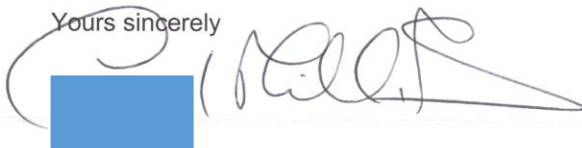   b) Progress Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

c) Safety Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

d) Amendments. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

e) End of Study/Project. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nationalres.org.uk .

| 07/WSE04/84 | **Please quote this number on all correspondence** |

With the Committee's best wishes for the success of this project

Yours sincerely

Executive Officer
South East Wales Research Ethics Committees

Email: ████████@bsc.wales.nhs.uk

Enclosures: Standard approval conditions SL-AC2 for other studies

Copy to: R&D office for Cardiff University

                     R&D office for Cardiff & Vale NHS Trust

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Version1

06/03/2007

Patient Information Sheet

Effects of Dental materials on Dentine and Pulp

The School of Dentistry is planning to carry out a research project. You are invited to contribute to this project. Before you decide please take time to read the following information. You will have the opportunity to ask for more information if you wish to do so.

What is the research about?

Tooth decay is a very common problem in most societies. Removal of decay and restoration (filling) of a decayed tooth is the usual treatment option. When a filling fails or the decay is too deep in the first place, more complex and expensive forms of treatment such as root canal treatment and crowns may be needed and if these treatments are not possible or fail, the tooth may need to be extracted.

Recent evidence has shown that certain dressing and filling materials used in dentistry can stimulate the cells in the centre of a tooth to produce extra layers of dentine. The dentine covers the delicate and sensitive cells in the centre of a tooth and gives the tooth the ability to protect itself from further damage. To an individual this can mean reduced pain and sensitivity following placement of fillings and possibly reducing the need for root canal treatment and extraction of teeth.

In our research we will look at many different materials commonly used for filling teeth and identify those with this positive effect. Results of this study will be

published in scientific journals and could have significant effects on the way dental treatment is planned in the future.

How can I contribute and what will happen if I take part

Your dentist has decided that you require a tooth (teeth) to be extracted. You could help us by giving permission for your extracted tooth to be used in this study. The extracted teeth will be anonymised and can not be traced back to you.

In the laboratory the teeth will be used to test the effects of different filling materials on the tooth substance including the cells within the pulp. These procedures **will not** involve any DNA testing. As the teeth are anonymised, we will not contact you after this.

Do I have to take part?

You do not have to give your permission for your extracted tooth to be used in this study and it is entirely up to you to decide. However please be informed that the Hospital policy does not encourage patients to take away the extracted teeth for health and safety reasons. In the event that you do not wish to allow your extracted tooth to be used in this study, the Hospital will keep it for safe disposal.

If you wish to give your permission please sign the attached consent form.

Contact details

If you feel that you need more information before giving your consent please contact:

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Version1
06/03/2007

PARTICIPANT CONSENT FORM

Title of Project:

Effects of Dental Materials on Dentine and tooth pulp

Name of Researchers:

Leili Sadaghiani, Christopher Lynch, Alastair Sloan, Rachel Waddington

Please initial box

1. I confirm that I have read and understand the information sheet Version1 dated 06/03/2007

   for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time,

   without giving any reason.

3. I agree to take part in the above study.

_____   _____   _____
Name of Participant   Date   Signature

_____   _____   _____
Name of Person taking consent Date   Signature
(if different from researcher)

Isolation of Dental Pulp Progenitor Cells

(Amr Alraies PhD Thesis, School of Dentistry, Cardiff University, 2013, Pages 37-8).

Normal human wisdom teeth were collected from three adult patients at the School of Dentistry, Cardiff University with informed patient consent and ethical approval by the South East Wales Research Ethics Committee of the National Research Ethics Service (NRES), UK. Teeth were sterilised by soaking in 70% ethanol and the attached soft tissue removed using a paper towel, prior to dissection. Teeth were grooved in the centre of three areas (mesial, distal and occlusal) with a rotary bone saw. On a dissection board, a chisel and hammer were used to separate each tooth into two halves and the pulp removed with a forceps and placed into universals filled with culture medium to maintain hydration. Pulp tissues were minced on a glass slide and cells were placed into 5ml bijoux tubes containing 1ml of 4μg/μl collagenase/dispase enzymes (Roche, Welwyn Garden City, UK). Pulps were incubated for 1 h, with regular agitation every 20 min, to increase cell dissociation. Pulpal tissues from individual patients were prepared separately. The contents of each bijoux were passed through a 70μm mesh cell strainer into a 50ml Falcon tube (BD Falcon, UK), to obtain a single cell suspension. The cell strainer was washed in 10ml medium. Cells were centrifuged at 1,800rpm (Labofuge400, Thermo Scientific, USA) for 5 min and further resuspended in 1ml medium. Cell numbers were calculated using 0.4% trypan blue vitality stain (Sigma, UK) for monolayer cell culture. A 10μl aliquot of each cell suspension was diluted in a known amount of the 38 stain and cells counted in a Neubauer Haemocytometer by light microscopy (Nikon eclipse TS100, UK). Cells were subsequently seeded in fibronectin coated 6 well plates (Sarstedt, Leicester, UK) at 4000/cm2.

Fibronectin Adhesion Assay

Following the protocol of Waddington et al., (2009)*, 10μg/ml fibronectin, derived from human plasma (Sigma) was reconstituted in 0.1M PBS at pH 7.4 containing 1mM Ca^{2+} and 1mM Mg^{2+}. Fibronectin (1ml/well) was pre-coated onto wells of 6-well

plates overnight. The fibronectin pre-coated wells were seeded with the cell suspension at 4,000 cells per cm2 (40,000 cells/ well). Cells were allowed to adhere at 37ºC, 5% CO_2 for 20 min. Following adhesion, the culture medium and non-adherent cells were removed and the wells refilled with 2ml fresh culture medium. Cells were fed every 2 days with 2ml of fresh working medium each time.

During the culture, the isolated DPPCs were monitored using an inverted light microscopy (Nikon Eclipse TS100, Tokyo, Japan) at x10 magnification and the number of cells per colony recorded. Jones and Watt (1993) defined a colony as a cluster with a minimum of 32 cells. By day 12, identifiable colonies were isolated by using 1ml sterilised pipette tips with the ends trimmed off leaving a cylindrical tube; with its edges smeared with petroleum jelly; and placed onto the wells to form an isolated water-tight seal around the marked colonies. Pre-warmed accutase (100µl, PAA, Velizy-Villacoublay, France) was added at 37ºC, 5% CO_2 and the isolated clones were then expanded until the numbers were sufficient for seeding into T-75 flasks.

* Waddington, R.J, Youde, S.J, Chi, P.L, and Sloan, A.J. 2009. Isolation of distinct progenitor stem cell populations from dental pulp. *Cells Tissues Organs* 189, pp. 268–274