Endogenous Growth Factor Release for Maxillofacial Tissue Repair

A thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

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Chapters 2, 3 and 4 have been presented at several local and international conferences:

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- **2014: IADR/PER Congress Dubrovnik, Croatia.**

Influence of Calcium Hydroxide on Bone Marrow Stem Cell Behaviour.

- **2014: Cardiff Institute of Tissue Engineering and Repair.**

Certificate of Commendation for “Calcium Hydroxide at pH7 mediates bone marrow stem cells function”.

http://www.cardiff.ac.uk/citer/%5Bhidden%5Dresources/CITER%20News%2023rd%20Edition%20December%202014.pdf.

- **2015: IADR/AADR/CADR General Session & Exhibition, Boston, USA.**

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Summary

The main goal of bone repair is to regenerate pre-existing properties and restore tissue integrity and function. It has been reported that bone contains numerous growth factors which are proposed to be released from the matrix during injury and mediate the repair process. These molecules act in synergistic action causing recruitment of progenitor stem cells to sites of bone injury, which then proliferate and differentiate into mature bone synthesising cells capable to initiate repair processes. It has been demonstrated that combinations of growth factors, such as the combinations found in the bone matrix, may be more effective in promoting bone healing compared with single growth factor therapy.

This project focuses on understanding bone repair processes by stimulating e*x vivo* fractured rat mandible model with either endogenous growth factors released by chemical treatment or by exogenous single growth factor therapy and investigating their effects on cellular behaviour. This project utilised the *ex vivo* mandible model as a promising alternative to current model and fractures were made within the *ex vivo* mandible slices to mimic bone fracture repair scenario. In summary, *ex vivo* experimental models were used successfully to investigate mechanism of bone repair. The results demonstrated that bioactive growth factors, particularly TGF-β1, BMP2 and VEGF successfully released from the bone matrix by EDTA, citric acid and calcium hydroxide. These growth factors found to affect cellular behaviour, by influencing proliferation and differentiation of osteoprogenitor cells. Calcium hydroxide derived endogenous growth factors mediated the repair process of mandibular bone greater than exogenously applied BMP2. Calcium hydroxide may provide a novel therapeutic approaches to utilise the synergistic effect of cocktail growth factors entrapped in bone matrix to stimulate optimal bone regeneration and avoid issues regard single growth factor therapy.

Abbreviations

3D Three Dimensional

α-MEM Alpha -Minimum Essential Media

α-SMA Alpha Smooth Muscle Actin

ALP Alkaline phosphatase

FGF fibroblast growth factor

BMP Bone morphogenetic protein

BMSC Bone marrow stem cell

BSA Bovine Serum Albumin

BSP Bone Sialoprotein

Ca^{2+} Calcium

CBFa1 Core binding factor alpha subunit protein

cDNA Complementary DNA

CO_2 Carbon dioxide

dH_2O distilled water

DAPI 4’6-Diamidino-2-Phenylindole

DMEM Dulbecco's modified Eagle's medium

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

dNTP Deoxyribonucleotide triphosphate

ECM Extracellular matrix

EDTA Ethylenediaminetetraacetic acid

EGF Epidermal growth factor

ELISA Enzyme-Linked Immunosorbent Assay

FGF Fibroblast Growth Factor

FITC Fluorescein Isothiocyanate

FDA Food Drug Administration

GAG Glycosaminoglycan

H_2O_2 Hydrogen Peroxide

HCL Hydrochloric acid

H&E Haematoxylin and eosin

HRP Horseradish peroxidase

IL Interleukin

IGF Insulin-like growth factor

MSCs Mesenchymal stem cells

MMP Metalloproteinase

MSC Mesenchymal stem cell

NFκB Nuclear factor-κB

$Ca(OH)_2$ Calcium hydroxide

OH^- Hydroxyl group

OCN Osteocalcin

OPG Osteoprotegerin

OPN Osteopontin

OSX Osterix

PBS Phosphate buffer saline

PCNA Proliferating Cell Nuclear Antigen

PCR Polymerase chain reaction

PD Population doubling

PDGF Platelet-derived growth factor

qRT-PCR Quantitative real-time polymerase chain reaction

PDL Periodontal Ligament

PDLSCs Periodontal Ligament Stem Cells

RANK Receptor Activator of Nuclear Factor kP

RANKL Receptor Activator of Nuclear Factor Kp Ligand

rhBMP Recombinant Human Bone Morphogenetic Protein

RNA Ribonucleic acid

ROS Reactive oxygen species

RPM Revolution per minute

RT Reverse transciptase

Runx2 Runt-related transcription factor 2 (aka Cbfa1)

SEM Scanning electron miroscope

SMAD SMA and MAD Related Family

SDS Sodium dodecyl sulphate

TBS Tris buffered saline

TMB 3,3',5,5'-tetramethylbenzidine

TNF Tumour necrosis factor

TGF-β Transforming growth factor-β

UV Ultraviolet

VEGF Vascular endothelial growth factor

Unit of measurement

% percentage

bp base pairs

ºC degree celcius

cm centimetre

cm^2 centimetres squared

g gravitional acceleration

M mole

mg milligram

mL millilitre

mm millimetre

mM millimole

nm nanometer

ng nanogram

pg picogram

µg microgram

µL microlitre

µM micrometre

rpm revolutions per minute

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Chapter 1. Introduction

1.1 Basic bone biology

In order to understand the mechanism of bone repair, it is crucial to understand the basic composition of bone and its influence on the process of bone regeneration. Bone is a mineralized dense connective tissue involved in different functions such as providing framework and support to the body as well as protecting internal vital organs (Nanci and Ten Cate 2013). It also plays other roles in providing a reservoir of mineral ions and attachment points for muscles and tendons (Hadjidakis and Androulakis 2006; Deng *et al.* 2008).

Bone includes different tissues such as endosteum, periosteum, neural, vascular and chondoral tissues. Endosteum is thin connective tissue membrane that lines the interior cavities within the bone. It contains haematopoietic stem cells (HSCs) that generate blood cells (Arai *et al.* 2009), osteoblasts, osteoclasts and osteoprogenitor cells (Clarke 2008; Lang and Lindhe 2015). Periosteum, the outer surface of bone, consists of an outer fibrous layer rich in fibroblast cells and inner osteogenic layer containing progenitor cells that develop into osteoblast cells (Kalfas 2001). Blood supply in bone plays an important role in bone nourishment, healing and maintaining homeostasis.

Histological cross sections of bone tissue can be classified into compact cortical bone and spongy trabecular bone (Figure 1.1). Compact bone is a dense bone surrounding the trabecular bone. It consists mainly of haversian systems or secondary osteons. Spongy bone is a cancellous sponge like bone within the medullary cavity. It consists of extensively connected bony trabeculae make room for blood vessels and marrow (Figure 1.2) (Nanci and Ten Cate 2013; Lang and Lindhe 2015). Osseous tissue consists of a complex composite of cells and extracellular matrix (ECM). The cellular components participate in maintaining bone structure and function. The extracellular matrix (ECM) is made of inorganic hydroxyapatite crystals and organic part composed mainly of Type I collagen (Nanci and Ten Cate 2013; Lang and Lindhe 2015).

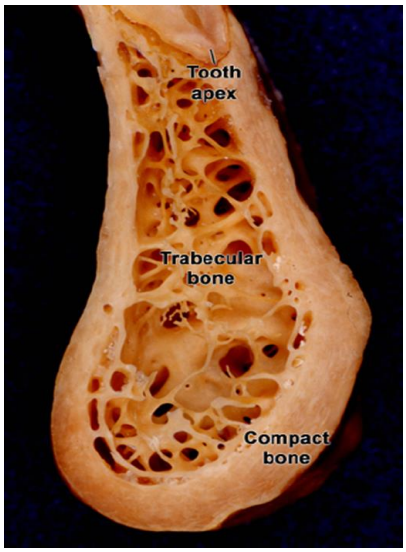


Figure 1- 1: Mandible body. Compact bone is a dense bone surrounding the trabecular bone. (adapted from Nanci and Ten Cate 2013).

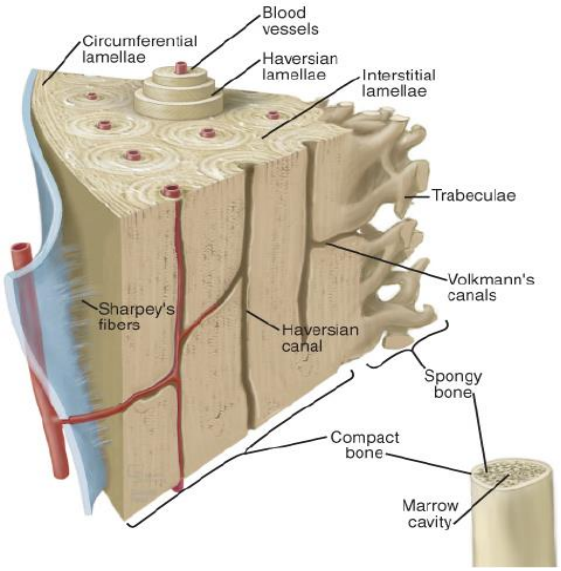


Figure 1- 2: Structure of bone (adapted from Nanci and Ten Cate 2013).

1.1.1 Cellular Components of Bone

The cellular components of bone participate in maintaining bone structure and function. Bone cells include osteoprogenitor cells, osteoblasts, osteocytes and osteoclasts. Each of these cells is involved in different functions. They play an important role in formation, breakdown and general regulation of bone (Kartsogiannis and Ng 2004; Hughes *et al.* 2006a). Imbalances in their actions directly affect bone growth and remodelling.

Several connective tissue cells are derived from mesenchymal stem cells. Mesenchymal stem cells are undifferentiated, self-renewing cells found within the marrow space and periosteum (Bielby *et al.* 2007b). They have the potential to differentiate into several cell types such as adipocytes, chondrocytes,

osteoblasts, osteocytes and myoblasts (Kartsogiannis and Ng 2004; Taichman 2005). The bone marrow space also contains hemopoietic stem cell niche which produce erythrocytes, leukocytes and ultimately osteoclasts (Kartsogiannis and Ng 2004; Taichman 2005). Osteoprogenitor cells are derived from mesenchymal stem cell and able to differentiate into osteoblast cell. During bone regeneration, mesenchymal stem cells are recruited to the site of injury. Numerous growth factors and signalling molecules, detailed later, stimulate the proliferation and differentiation of these mesenchymal cells, until a population of fully mature, active osteoblasts are present at the site of injury (Bielby et al. 2007b).

Osteoblast cells arise from multipotent mesenchymal stem cells. They are found on the surface of bone or osteoid usually as a single layer. They participate in embryonic skeletal development and post-natal bone remodelling (Mackie 2003; Clarke 2008). Osteoblast cells undergo a number of proliferation and differentiation process before reaching a terminal phenotype (Kartsogiannis and Ng 2004; Hughes et al. 2006a). The differentiation process is regulated by a complex system of growth factors which determine the final fate of these cells (Lieberman *et al.* 2002b). Most of the organic components of the bone matrix are produced by the osteoblasts, including type I collagen, proteoglycans, glycoproteins and alkaline phosphatase enzyme. Active osteoblasts have a lifespan of around 3 months, after which they either undergo programmed cell death (apoptosis), become inactive bone lining cells or become embedded in the mineralized matrix and differentiate into osteocytes (Jilka *et al.* 1998; Manolagas 2000b).

Osteoblasts differentiate into osteocytes when the cell is encased by osteoid matrix which subsequently mineralises. Osteocytes are the most abundant cells found in bone tissue, comprising around 95% of the total, 10 times more prevalent than osteoblasts (Tamara A. Franz-Odendaal 2006) and their lifespan are approximately 25 years, exceeds that of the osteoblast (Knothe Tate *et al.* 2004). The processes of adjacent cells contact each other via gap junctions, maintain the vitality of osteocytes by passing nutrients and metabolites between blood vessels and distant osteocytes, regulating ion homeostasis, and transmitting electrical signals in bone (Furlan *et al.* 2007; Jiang *et al.* 2007). It is currently not clear what specific events lead to certain osteoblasts being selected for terminal differentiation to osteocytes and effectively becoming trapped in newly formed bone, although it is probably influenced by a variety of factors, such as cell position and the presence of local signalling factors (Manolagas 2000a). Although osteocytes have reduced synthetic activity and diminished mitotic division, they are actively involved with remodelling of bone. Osteocytes develop a web of dendritic extensions which extend along the bone canaliculi, minute channels running through the compact bone tissue, connecting osteocytes to other cell types (Manolagas 2000a). It has been theorized that osteocytes act as mechanosensors that can respond to mechanical strain and bone microdamage, then transmit signals to osteoblasts and/or osteoclasts for resorption or bone formation (Rodan 1997; Manolagas 2000a; Burger *et al.* 2003). Thus, osteocyte cells responsible for overall control of bone maintenance and communication with surrounding tissues (Owen 1967; Tamara A. Franz-Odendaal 2006).

Osteoclasts are osseous cells that also play important roles during bone regeneration. They are large, multinucleated cells with fine, finger like cytoplasmic processes derived by the differentiation of monocyte / macrophage haemopoietic progenitor cells, found in the bone marrow (Schell *et al.* 2006). Monocyte precursor cells migrate from the bone marrow to the bone surfaces where they proliferate, differentiate into mononuclear preosteoclasts, and fuse with each other to form multinucleated mature osteoclasts, capable to cause resorption of the bone (Chambers 2000; Boyle *et al.* 2003). Osteoclasts present in resorption craters known as Howship lacunae on bone surfaces or in deep resorption cavities called cutting cones. They are specialized in resorbing mineralized bone matrix by binding tightly to the bone surface via the apical membrane and secreting protons and osteolytic enzymes, forming a structure known as a resorption pit (Chambers 2000; Boyle et al. 2003). Osteoclasts are recruited into the osteoclast lineage pools of monocyte precursors at the bone surface (Chambers 2000). Cells that express the full morphologic and functional properties of mature osteoclasts are known to be restricted to the surfaces of bones. Osteoclasts or their committed precursors do not possess receptors for parathyroid hormone. The hormonal signal is regulated by osteoblasts (Li *et al.* 2007). However, osteoclasts do have receptors for calcitonin (Nicholson *et al.* 1986). Calcitonin inhibits osteoclast activity by impeding the motility, cytoplasmic spreading, and bone resorption abilities of osteoclasts (Nicholson et al. 1986; Lee *et al.* 1995) thus it can be used for treatment of different diseases such as: osteoporosis, hypercalcaemia and paget's disease. Osteoclasts have a much shorter life span than osteoblasts and osteocytes, the maximal survival of osteoclasts without fusion of precursors is less than 6 weeks (Marks and Seifert 1985). Osteoclasts are not usually seen in routine histologic sections of normal

bone. An increased number of osteoclasts is characteristic marker of pathological conditions associated with increased bone turnover.

1.1.2 Bone signalling pathways

The study of bone signalling pathways has improved our understanding of cellular behavior during bone remodelling. Different bone signalling pathways are activated during migration, differentiation, and activation of bone cells. Remodelling of bone is regulated mainly by the balanced action of osteoblast and osteoclast cells. Differentiation of osteoblast and osteoclast is regulated by a complex system of growth factors (Lieberman et al. 2002b). The intracellular signals conveyed by these growth factors in turn influence the expression of an array of transcription factors and proteins (Hughes *et al.* 2006b).

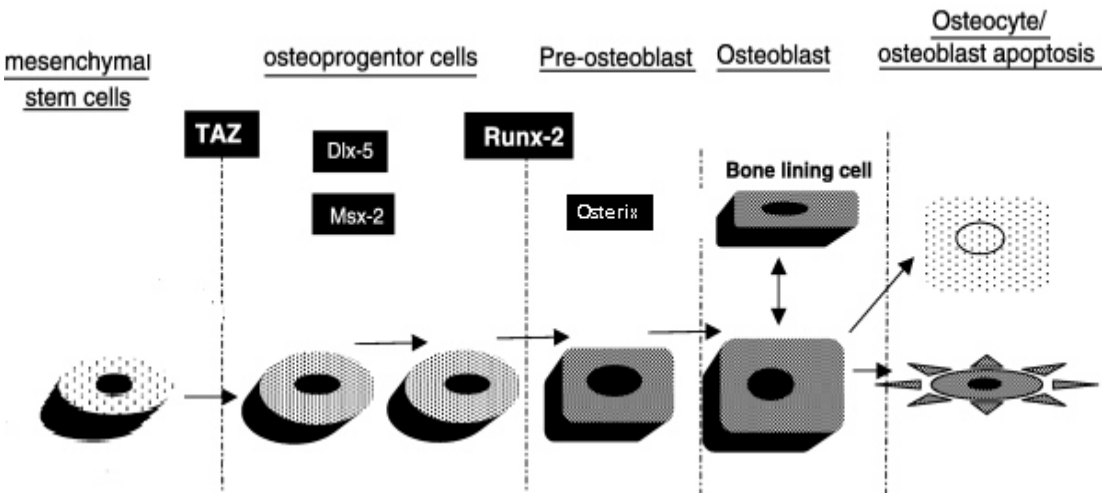


Figure 1- 3: Simplified schematic diagram showing the main stages of the osteoblast differentiation. A host of growth factors act temporally and spatially to drive the stages of differentiation. Intracellular growth factor signalling influences expression of transcription factors which in turn induce expression of osteoblastic proteins. (Adapted from Hughes et al., 2006).

Numerous growth factors exert their influences on different stages of osteoblast differentiation pathway, inducing intracellular signalling processes that in turn affect the expression of transcription factors and proteins within the cells (Hughes et al. 2006b) (Figure1.3). For example, transcription factor TAZ, expressed early in the osteoblast differentiation pathway, associated with the differentiation of mesenchymal stem cells into fully committed osteoprogenitor cells (Hong *et al.* 2005; Hughes et al. 2006b). Runx2/Cbfa1, another transcription factor that is reported to be responsible for differentiation of osteoprogenitor cells into the pre-osteoblast. Komori and his group have reported that Runx2 expression is an absolute requirement for bone formation, as Runx2 knockout mice failed to develop either osteoblasts or a skeletal system (Komori *et al.* 1997). Osterix is transcription factor which is expressed in terminal stage of osteoblasts differentiation (Hughes et al. 2006b). It plays an important role in bone mineralization and It is suggested to be positioned downstream to Runx2 (Nakashima *et al.* 2002a). Osterix knockout mice failed to express osterix as well as to develop a skeleton (Nakashima et al. 2002a). Osterix, is essential for osteoblast differentiation, inducing expression of many osteoblast proteins, including osteocalcin and bone sialoprotein (Kim *et al.* 2004). Runx2 however regulates the expression of osteocalcin, bone sialoprotein (BSP) (Kim et al. 2004), osteopontin and type I collagen (Ducy *et al.* 1997; Hung *et al.* 2011). Bone morphogenetic protein-2 (BMP2) is capable of inducing expression of Runx2 and Osterix, via the upstream regulators Msx-2 and Dlx-5, which are involved in upregulating the expression of Osterix and Runx2 respectively (Kim et al. 2004; Matsubara *et al.* 2008).

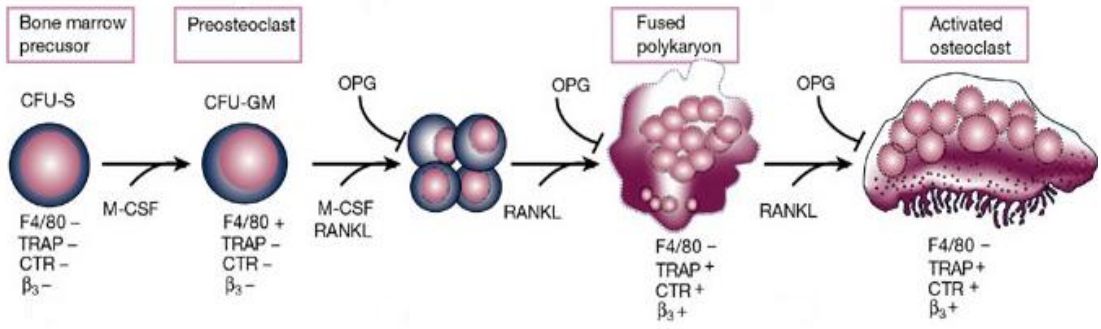


Figure 1- 4: Differentiation pathway of osteoclast from haematopoietic precursor cells. Differentiation is derived via OPG, M-CSF and RANKL, and is associated with expression of phenotypic markers such as tartrate-resistant acid phosphatase, calcitonin receptor (CTR) and the β3 integrin. (Adapted from (Boyle et al. 2003)

Local signalling molecules produced by osteoblast cells play a central role in controlling the process of osteoclastogenesis and bone remodelling (Figure1.4). Differentiation of haematopoietic precursor cells into mature osteoclasts is dependent on the presence of macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B ligand (RANKL). M-CSF and RANKL work synergistically to induce osteoclast differentiation, with M-CSF driving proliferation of osteoclast progenitors, and RANKL inducing differentiation of this expanded population (Quinn *et al.* 1998). During osteoclast differentiation pathway, M-CSF and RANKL induce expression of phenotypic markers that characterise the osteoclast lineage. These include tartrate-resistant acid phosphatase (Bayatian *et al.* 2007), cathepsin K, calcitonin receptor (CTR), and the β_3 integrin (Boyle et al. 2003). TRAP is an enzyme that has been used extensively as a marker for terminally differentiated osteoclasts (Angel *et al.*

2000). The exact role of TRAP in osteoclasts is not fully understood, however TRAP knock-out mice show decreased resorptive activity of osteoclasts (Hayman *et al.* 1996). RANKL is a soluble transmembrane protein, found on the surface of expressing osteogenic cells. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL that inhibits osteoclast differentiation *in vitro* and bone resorption *in vivo* by preventing binding of RANKL to Rank (Simonet *et al.* 1997; Udagawa *et al.* 2000; Schoppet *et al.* 2002). While OPG knockout mice demonstrated osteopenia, OPG-overexpressed transgenic mouse exhibit major increase in bone mass (Simonet et al. 1997) indicating the critical role of OPG pathway in mediating osteoclastogenesis and bone remodelling. OPG is secreted by osteoblasts in response to various osteogenic signals, for example growth factors sequestered in the matrix during its formation, thus preventing bone resorption as required by the physiological situation (Udagawa et al. 2000; Schoppet et al. 2002). The activation and inhibition of osteoclast differentiation is therefore regulated by the receptor Rank, via its interactions with either RankL or OPG produced by osteoblasts.

These identified transcription factors and bone signalling proteins act as key regulatory factors maintaining normal physiological bone remodelling. Any alteration or disruption of these signalling pathways may potentially suppress or delay bone formation and repair process. Therefore the expression of these proteins can be considered biomarkers to assess behaviour of the cells during bone regeneration.

1.1.3 Extracellular matrix components

Extracellular matrix of bone is made of organic and inorganic components which provide bone hardness and resistance to the bone (Nanci and Ten Cate 2013). The organic component is composed of collagen fibers with predominately type I collagen and ground substances which are an aggregate of proteoglycan and non-collagenous glycoprotein (Nanci and Ten Cate 2013). Inorganic part is composed mainly of hydroxyapatite crystals formed from calcium and phosphorus. It includes also smaller amounts of bicarbonate, citrate, magnesium, potassium, sodium and amorphous calcium. Most of these ECM proteins regulate processes of bone formation and resorption by driving osteoblast and osteoclast differentiation. Although each protein has specific roles, they work in synergistic action in order to mediate bone repair process. Expression of these phenotypic proteins during cellular differentiation pathways, make them useful biomarkers for monitoring repair process as well as ideal indicators of cellular behaviour around site of bone injury (Table 1).

Table 1.1: Some of the phenotypic markers seen at different stages of osteoblast differentiation pathway. Compiled from (Katagiri and Takahashi 2002; Hughes et al. 2006a).

Differentiation Stage	Mesenchymal Stem Cells	Osteoprogenitor Cells	Pre-osteoblasts	Osteoblasts
Markers Expressed	-(AlP –ve) -Collagen type I,III,V	-AlP +/–ve -Collagen type I,III,V	- Alkaline Phosphatase - Collagen type I -Bone Sialoprotein -Parathyroid hormone related protein receptor	-Alkaline Phosphatase -Bone Sialoprotein -Parathyroid hormone related protein receptor - Collagen type I -Osteopontin -Osteocalcin -Osteonectin

1.1.3.1 Major proteins expressed throughout osteoblast differentiation pathway

Type I collagen is the primary secretary product of osteoblasts and is the most abundant collagen in the organic part of bone (90%) and the main constituent of the osteoid matrix. Collagen type I molecule is a triple helix composed of three helical polypeptide chains. These collagen helices coil together forming fibrils, which may then further bundle together to form collagen fibres (Allori *et al.* 2008). This arrangement is critical for bone mineralization as the structural network of type I collagen fibres provides gaps between collagen subunit serve as nucleation sites for hydroxyapatite crystal deposition (Buckwalter *et al.* 1995; Baht *et al.* 2008). Since collagen type I is secreted throughout the osteoblast lineage (Seyedin and Rosen 1990; Buckwalter et al. 1995; Kalfas 2001), expression of type I collagen can be a useful indicator of osteoblast activity during bone regeneration.

Another important protein that is expressed throughout osteoblast differentiation are alkaline phosphatases. They are enzymes that also expressed throughout the body in four isoforms; three are considered as tissue-specific enzymes particularly concentrated in liver, intestine and placenta (Schär *et al.* 1997). However the fourth isoenzyme is tissue non-specific alkaline phosphatase (TNAP) that is widely expressed in the body but is particularly concentrated in liver, kidney, and bone (Schär et al. 1997). Bone-specific alkaline phosphatase enzyme is associated with bone mineralisation (Robison 1923), by removing pyrophosphates which inhibit hydroxyapatite formation (Whyte 1994), and releasing inorganic phosphate into the matrix (Houston *et al.* 2004). Alkaline

phosphatase is known to be expressed on the surface of both osteoblasts and osteoblast progenitor cells during bone repair, thus it is commonly used as an indicator of osteoblast differentiation and activity (Katagiri and Takahashi 2002; Sharp and Magnusson 2008). Although serum levels of the enzyme have been shown to be high during fracture repair (Taniguchi *et al.* 2003), it is best to put in consideration that the ubiquity of alkaline phosphatase is not specific indicator of bone formation. However its expression alongside other markers of osteoblast activity would serve as a definite indicator of bone formation at the fracture site.

Bone sialoprotein (BSP) is a non-collagenous matrix protein that is expressed by pre-osteoblasts and mature osteoblasts and present at high levels within mineralised bone tissues (Ganss *et al.* 1999; Gordon *et al.* 2007b). The exact function of BSP remains unknown as it ables to bind to different matrix components such as collagen, matrix metalloproteinases, hydroxyapatite, and integrins (Oldberg *et al.* 1988; Fisher *et al.* 2001; Baht et al. 2008). BSP knockout mice are smaller than normal counterparts with deficient mineralisation in their skeletons and show impaired bone repair (Malaval *et al.* 2008; Malaval *et al.* 2009). This suggests that BSP plays major role in bone mineralisation. In regards to bone repair, BSP has been reported to be expressed during the mineralisation stage (Chen *et al.* 1994; Baht et al. 2008), and also contribute in later stages of osteoblast differentiation (Gordon *et al.* 2007a), therefore its expression is important indicative of bone mineralization during regeneration process.

1.1.3.2 Major proteins expressed by differentiating osteoblasts

Osteocalcin (OCN) is the most abundant non-collagenous protein found in bone matrix (Lian and Gundberg 1988). It is thought to be the most bone specific protein as it is synthesized solely by mature osteoblast cells (Weinreb *et al.* 1990; Ducy *et al.* 1996; Hughes et al. 2006a; Lee *et al.* 2007). Osteocalcin binds tightly to hydroxyapatite, facilitating protein accumulation within the matrix (Lian and Friedman 1978; Kudo *et al.* 1998); thus suggest that osteocalcin involves in bone mineralization and calcium ion homeostasis. Although osteocalcin is mainly incorporated into the bone matrix, it can also be released into the blood circulation where it can function as an indicator of bone turnover in osteoporosis (Price and Nishimoto 1980; Polak-Jonkisz and Zwolinska 1998; Lateef *et al.* 2010). Clinically, osteocalcin can function as a biomarker on the effectiveness of a given drug on bone formation through doing assays for circulating osteocalcin in the serum (Singer and Eyre 2008; Lateef et al. 2010). Recent studies raise the possibility that osteocalcin acts as a hormone that influences energy metabolism by regulating the release of insulin from beta cells in the pancreas (Lee et al. 2007). Osteocalcin knockout mice show no initial skeletal defects and present normal levels of all other bone matrix non-collagenous proteins, but over time reveal increased bone formation rate and a higher overall BMD, indicating a currently undefined regulatory role in skeletal development (Ducy et al. 1996). Although the exact function of osteocalcin is unknown, it is exclusively expressed in bone tissue by osteoblasts and is therefore a good marker of bone formation by osteoblast cell.

Osteopontin (OPN) is another non-collagenous extracellular matrix protein, which is synthesized by a variety of cells including fibroblasts (Ashizawa *et al.* 1996), osteoblasts, osteocytes, odontoblasts, hypertrophic chondrocytes, dendritic cells and macrophages (Murry *et al.* 1994). Osteopontin is reported to modulate different cell action in a variety of manners. It has chemotactic properties, which promote recruitment of inflammatory cells such as neutrophil (Apte *et al.* 2005; Banerjee *et al.* 2006; Koh *et al.* 2007), mast cell (Nagasaka *et al.* 2008) and macrophage (Burdo *et al.* 2007; McKee *et al.* 2011) to inflammatory sites. It also acts as an adhesion protein which associated with cell attachment and wound healing (Merry *et al.* 1993; Yamate *et al.* 1997; McKee et al. 2011). In addition, OPN can function as an anti-apoptotic factor in which it inhibits the activation-induced cell death of macrophages and T cells as well as fibroblasts and endothelial cells exposed to harmful stimuli (Denhardt *et al.* 2001; Standal *et al.* 2004). In case of bone regeneration, expression of osteopontin indicates that osteoblast progenitor cells have migrated to the fracture site, differentiated into osteoblasts and attached themselves to the surrounding tissues in order to initiate osteoid matrix synthesis.

Osteonectin (ON) is a calcium binding glycoprotein that is expressed by osteoblasts in bone undergoing remodelling or repair (Alford and Hankenson 2006; Brekken and Sage 2001; Lane and Sage 1994). It shows affinity to ECM components such as collagen and hydroxyapatite, thus assessing bone matrix organisation, and may be implicated in regulating cell proliferation and cell-matrix interactions (Brekken and Sage 2000; Young et al. 1992). Osteonectin is assumed to be associated with mineralised tissue formation through regulating

the deposition and assembly of other osteoid matrix proteins (Lane and Sage 1994; Brekken and Sage 2001). Interestingly, osteonectin knock-out mice show significant deficient in osteoclasts and osteoblasts numbers, leading to impaired bone formation and display a low-turnover osteoporosis-like phenotype (Delany et al. 2000; Delany et al. 2003). Due to its expression by osteoblasts and association with bone healing, thus its presence in the fracture site would be indicative of existence of osteoblast cells and bone regeneration.

Expression of most of these bone markers has been demonstrated within the *ex vivo* mandible model, within cells residing within the PDL and bone marrow spaces (Smith et al. 2010). These results correlated with previous *in vivo* studies that demonstrate presence of osteoblasts and their progenitors within both the PDL and bone marrow spaces (Isaka et al. 2001; Krebsbach et al. 1999; Lekic et al. 1996). Most of these ECM proteins work in a complex and synergistic way in order to mediate the processes of osteoid deposition and mineralization during bone repair. Although the exact roles of many of the non-collagenous proteins produced by osteoblasts have not been understood, the phase in the osteoblast differentiation pathway at which they are expressed is generally known. This makes them vital indicators in assessing stage of repair processes as well as cellular behaviour around fractured site.

1.2 Physiology and Process of Bone Healing

Regeneration of bone can depend on different factors such as patient's age, fracture location, degree of dislocation and extent of the injury. The process of fracture repair involves a complex cascade of events and is mainly divided into three major phases; reactive phase, reparative phase and remodeling phase. An understanding of these phases is essential to identify cellular behaviour and molecular signals involved during process of bone regeneration.

1.2.1 Reactive Phase

Reactive phase is the inflammatory phase which occurs immediately after initial bone disruption due to fracture and lasts 3–4 days. Bone fractures cause cutting of the blood vessels followed by extravasation of blood. Traumatic disruption of the blood flow causes ischemic necrosis characterised microscopically by the presence of empty lacunae and the presence of blood cells within the tissues adjacent to site of bone injury (Griffon 2008). After injury, a fibrin-rich clot known as hematoma forms at the fracture site, initiating spontaneous fracture healing. Most of the fracture repair process took place around rather than middle of the interfragmentary hematoma (Tecklin 2008). Although bone repair can be obstructed by the presence of persisting unchanged large blood clots at the fracture site(Whittick 1990; Morgan and Leighton 1995), hematoma works as a scaffold for cells, and a spacer guiding the size and shape of the callus (Slatter 1995).

During repair process, presence of hematoma assesses release of different growth factors, thereby stimulating angiogenesis and osteogenesis (Griffon 2008). Transplantation of fracture hematoma has been found to induce endochondral bone formation in ectopic sites, which would be consistent with the presence of osteoinductive growth factors within the hematoma (Mizuno *et al.* 1990; Street *et al.* 2000). Blood clot is rich with thrombocytes which release coagulation factors for angiogenesis and platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1) to stimulate osteogenesis (Lieberman et al. 2002b). Mast cells play a role in formation of new vessels via releasing of vasoactive substances (Heppenstall 1980). The angiogenic properties of fracture hematoma appear to be stimulated by vascular endothelial growth factor (VEGF) (Street et al. 2000) and prostaglandins E1 and E2 which may also mediate early bone resorption by osteoclasts and proliferation of osteoprogenitor cells (Millis 1999). Revascularization of the hypoxic fracture site occurs within hours after the fracture by the transient extraosseous blood vessels emerge from the surrounding soft tissues. Mononuclear phagocytes transported by these new vessels aid in the removal of necrotic bone and construction of the callus.

Macrophages also play a major role in fracture repair by releasing several growth factors, such as fibroblast growth factor (FGF) which initiate fibroplasia both in soft tissue as well as interfragmentary (Leibovich and Ross 1975; Lieberman et al. 2002b). By the end of the first week, blood flow resumes and the blood clot degenerates unless infection, excessive motion, or extensive

necrosis of the surrounding soft tissues persists at the fracture site. Clinically, the end of the inflammatory stage associates with decrease of pain and swelling.

1.2.2 Reparative Phase

Within a few days, mononuclear cells and fibroblast cells begin the transformation of a hematoma into granulation tissue, a loose aggregate of cells interspersed with small blood vessels. This phase is characterised with a slight gain in mechanical strength, since granulation tissue can withstand a tensile force up to 0.1 Nm/mm^2 (Perren and Boitzy 1978). Granulation tissue is rich with collagen fibres types I, II, and III. Collagen are initially deposited, but as the maturation process continues, type I collagen predominates (Lane *et al.* 1979; Remedios 1999). The granulation tissue eventually contains number of mesenchymal stem cells, numerous newly formed blood vessels to provide oxygen and nutrients and macrophages to clean the wound and release cytokines and growth factors (Lang and Lindhe 2015). Mesenchymal stem cells within the cambium layer of the periosteum, the endosteum, the bone marrow, and adjacent soft tissues begin to proliferate during the reactive phase and deposit matrix components in an extracellular location. Several growth factors such as bone morphogenic proteins (BMPs), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) play a major role in chemotaxis, proliferation and differentiation of these stem cells into osteoblasts (Griffon 2008; Lang and Lindhe 2015).

It is unclear the exact time and order of the formation and maturation of tissues within the fracture gap. Days after fracture, osteoprogenitor cells migrate and aggregate prior to their differentiation into osteoblast cells which lay down a matrix of collagen fibres known as osteoid. The osteoblast cells are entrapped inside the matrix and differentiate into osteocyte cells. The osteoid is mineralized by mineral deposition mainly calcium and phosphate (Clarke 2008). The exact mechanism of this process is unclear; however it has been demonstrated that osteoblast cells release a small, membrane-bound matrix vesicles that support nucleation by concentrating calcium and phosphate and enzymatically destroy mineralization inhibitors such as pyrophosphate or proteoglycans (Anderson 2003). It is also assumed that mitochondria in the fracture gap accumulate calcium-containing granules that are released in the hypoxic media created by anaerobic metabolism. Intramitochondrial clusters of calcium phosphate are released in the extracellular matrix and become the source for growth of apatite microcrystallites (Boonrungsiman *et al.* 2012).

At the end of this phase, the fracture gap is bridged by primary bone tissue known as woven immature bone (Sumner-Smith 1982; Whittick 1990) which is transformed into mature lamellar bone. The bony substitution of immature woven bone is represented by trabeculae rod-like features which are later replaced with plate-like trabeculae and fibred lamellar bone and marrow space (Berglundh *et al.* 2003). At this point, bone union is gained however, structure and strength of the fracture site is different from that of the original bone.

1.2.3 Remodelling Phase

The ultimate goal of the healing process is to restore tissue integrity, structure and function. Depending on factors such as age or general condition, the remodelling phase may last for 6–9 years in humans (Wendeberg 1961; Remedios 1999). The remodelling process is influenced by the balanced action between osteoclastic resorption and osteoblastic deposition. At this phase the trabecular bone is substituted with compact bone. Osteoclasts resorb trabecular bone forming a shallow resorption pit known as a "Howship's lacuna". Then compact bone deposited by osteoblasts within the resorption pit.

At the end of the remodelling phase, the fracture callus is remodelled and transformed into a new structure which closely resembles the original bone shape and strength. A key factor for spontaneous fracture bone healing is availability of adequate blood supply together with the gradual increase in stability at the fracture site. Any changes in the vascularity and mechanical support of the fracture site can compromise the healing process causing malunion or non-union which lead to fibrous/cartilage tissue formation. On the other hand an immobilize fracture with sufficient blood supply allow formation of mineralized callus and subsequent good healing (Griffon 2008).

1.3 Growth Factors Involved in Bone Repair

Since growth factors play a ajor role in mediating bone regeneration, evaluation of their precise roles in the healing process is extremely beneficial. These

growth factors act in synergistic way to recruit progenitor cells to sites of bone injury, and stimulate them to proliferate and differentiate into mature bone cells capable of initiating repair processes. Numerous studies have demonstrated their role, expression and application in mediating bone healing (Hughes et al. 2006b; Baker *et al.* 2009; Ehnert *et al.* 2010). A study by Jacobsen *et al* (2008); demonstrated that growth factors regulate bone formation during fracture healing and distraction osteogenesis by stimulating molecular mechanisms and controlling osteoblasts proliferation and differentiation (Jacobsen *et al.* 2008). Among the abundant growth factors available, a number have been identified to be relatively significant in bone healing and repair, such as the following:

TGF-β1 is a secreted protein that belongs to the transforming growth factor beta superfamily. Although TGF-β1 is found in many tissues of the body, it is more prevalent in bone and platelets (Lieberman et al. 2002b; Janssens *et al.* 2005). TGF-β1 has been studied extensively in the field of bone repair (Poniatowski *et al.* 2015), and has been reported to promote fracture healing in several studies by different roles. TGF-β1 is secreted by osteoblasts and osteoclasts and deposited within the bone matrix in a latent inactive dimeric complex termed latency-associated peptide (LAP). During bone turnover, acidification of the resorption lacuna created by osteoclasts is able to activate TGF-β1 (Pfeilschifter *et al.* 1990; Bonewald *et al.* 1997), which should then stimulate the formation of bone. During bone injury, TGF-β1 induces chemotactic recruitment of mesenchymal stem cells and osteoprogenitors from the bone marrow, periodontal ligament and periosteum, and increases their proliferation (Barnes *et al.* 1999; Smith *et al.* 2010). It has been demonstrated that TGF-β1 RNA is

intensively expressed during the whole healing process (Cho *et al.* 2002; Wildemann *et al.* 2004). TGF-β1 also promotes the early stages of differentiation within these cells, increasing levels of type I collagen synthesis and inducing expression of Runx2, a key transcription factor associated with osteoblast differentiation (Bostrom and Asnis 1998; Janssens et al. 2005). During later stages of osteoblast differentiation, TGF-β1 has been observed to inhibit the expression of both Runx2 and osteocalcin, thus acting to inhibit the later stages of osteoblast maturation (Janssens et al. 2005). Therefore, the expression of TGF-β1 around fracture site is a strong indicator of early osteoblast differentiation activity. TGF-β1 has been shown to regulate bone resorption through inhibition of activation, proliferation, and differentiation of osteoclast cells and moreover induces suppression of the recruitment of new osteoclasts (Chenu *et al.* 1988; Pfeilschifter *et al.* 1988; Ehnert et al. 2010).

It has been reported from *in vitro* model that TGF-β1 increases both the proliferation rate of osteoblast cells and the synthesis of type I collagen, indicating its importance in bone matrix production (Centrella *et al.* 1986; Centrella *et al.* 1992). TGF-β1 knockout mice have shown decreased proliferation and differentiation of osteoprogenitor population with disturbed collagen maturation and impaired matrix deposition and mineralisation (Atti *et al.* 2002). Therefore, the role of TGF-β1 during bone regeneration is mainly to maintain recruitment and proliferation of local mesenchymal stem cells and osteoprogenitors, and stimulate some early differentiation stages. Essentially this has the effect of producing a large pool of matrix producing cells ready to be moved on into the later stages of osteoblast differentiation by other growth

factors such as the bone morphogenetic proteins (Centrella et al. 1992; Janssens et al. 2005; Hughes et al. 2006a).

Bone morphogenetic proteins (BMPs) are also members of transforming growth factor β (TGF-β) superfamily. They were identified by Urist and colleagues (1971) where decalcified bone matrix explants in muscle stimulated ectopic bone formation. It has been reported that osteoblast cells synthesize BMPs and sequester them in the bone matrix, which can be released at time of bone resorption, thus maintaining bone mass homeostasis (Mackie 2003). BMP2 and BMP7 are members of the BMP family and have shown to play a major role in bone regeneration, due to their ability to differentiate mesenchymal stem cells and osteoprogenitors into mature fully functional osteoblast cells. BMP2 has been shown to overexpressed at fracture healing site, where it exerts an osteogenic role via direct stimulation of osteoblasts (Bouletreau *et al.* 2002). Several studies have shown that BMP2 is a potent osteogenic factor that controls the osteogenic transcription factors, Dlx5, Runx2 and OSX (Nakashima *et al.* 2002b; Hughes et al. 2006b). BMP7 has been shown to induce cartilage and bone formation as well as increases tissue vascularization and adipogenesis (Lu et al. 2010). BMP7 has also been shown to stimulate alveolar bone regeneration in 5mm mandibular osseous defects through stimulation of osteogenesis, regenerative cementum, and new attachment formation (Giannobile et al. 1998) and in maxillary sinus floor augmentation procedures before placing implant (Bergh et al. 2000). The presence of BMPs around fracture site may indicate the status of the cell differentiation. While BMP2 acts as early and late osteogenic markers and its expression indicates the presence

of mesenchymal stem cells or early osteoprogenitors around the site, the expression of BMP7 may indicates the presence of committed osteoblasts (Cheng *et al.* 2003).

Both BMP and TGF-β influence osteoblast differentiation by signalling through a serine-threonine kinase receptors at the cell surface to initiate complex intracellular signalling cascades resulting in a wide range of effects on the transcriptional and translation activity of the cell (Yamaguchi *et al.* 2000; Chen *et al.* 2012). Intracellular signalling occurs with downstream signalling via phosphorylation of cytoplasmatic R-Smads 1, 5, and 8 in case of BMP and Smads 2 and 3 in case of TGF-β (Chen et al. 2012), which translocate to the nucleus via Smad 4 and regulate transcription of target genes such as Runx2 (Phimphilai *et al.* 2006). It is apparent that BMP and TGF-β are highly involved in skeletal formation and osteoblast differentiation. During bone healing, it is likely that these two growth factors work in a complex and synergistic relationship; in which TGF-β1 stimulates recruitment and proliferation of mesenchymal stem cells and osteoprogenitors, while BMP serves to differentiate these cells into mature osteoblast cells capable of producing new bone.

Vascular endothelial growth factor (VEGF) is a signalling protein that stimulates vasculogenesis and angiogenesis (Yang *et al.* 2012). During healing process, fibrous and granulation tissue are rapidly vascularised through the actions of VEGF, angiogenic growth factors, in order to provide the repair site with the required blood supply (Einhorn 1998; Johnson and Wilgus 2014). Good blood

supply is important for successful osteogenesis, since the vasculature is responsible for providing oxygen, nutrients, soluble factors and cells to the bone tissue (Kanczler and Oreffo 2008; Yang et al. 2012; Johnson and Wilgus 2014). Although the primary target of VEGF is endothelial cells, VEGF has also been shown to directly associated with modulating recruitment, survival, and differentiation of osteoblasts (Mayr-Wohlfart *et al.* 2002; Street *et al.* 2002). Several studies have been demonstrated that there is an interaction between angiogenic and osteogenic factors during bone formation and bone healing. For example, VEGF is able to recruit more mesenchymal stem cells when combined with osteoinductive growth factors such as BMP4 (Peng *et al.* 2002). Although expression of VEGF at fracture site indicates presence of good blood supply, expression of VEGF together with other osteoinductive growth factors is good indicators of both angiogenesis and osseogenesis.

1.4 Impaired Bone Healing

The main goal of bone regeneration is to restore tissue integrity and function, which can be achieved by the integrated complex action of cells, proteins, and growth factors detailed above. In normal bone injuries, a certain amount of time is required before bone fracture can be expected to heal. This normal time may vary according to different factors include age, bone involved, level of the fracture, and associated soft tissue injury. However, there are many factors associated with impaired bone healing in which the normal process of bone healing is interrupted resulting in malunion, delayed union or non-union. A malunion is a fractured bone that has healed but in an unacceptable position.

Nonunion refers to fractures that have healed by forming fibrous or cartilaginous tissue rather than new bone after several months. The differentiation between delayed union and nonunion is sometimes difficult and often based on radiographic criteria and time.

Numerous factors may interfere with bone healing. These include pathological factors such as diabetes (LODER 1988; Gandhi *et al.* 2005), osteomylities, osteonecrosis (Reid 2009) and osteoporosis (Namkung-Matthai *et al.* 2001; Tang *et al.* 2008). As well as lifestyle factors including smoking (César-Neto *et al.* 2003; Balatsouka *et al.* 2005), alcohol and poor nutritional status. Certain mechanical factors also contribute to loss of bone strength and make healing more difficult. These include inadequate reduction and inadequate immobilization which cause excessive motion and loss of bone stability at the fracture site. Other physiological factors can also contribute to poor healing include older age, menopause and reduced blood flow to the fracture site. Certain medications, such as corticosteroid drugs and non-steroidal anti-inflammatory drugs (NSAIDs) may also interfere healing of bone (Harder and An 2003).

Most malunions and nonunions require open reduction and internal fixation surgery to realign and stabilize the fracture fragments into their normal anatomical position. Infection requires surgical debridement followed by intensive antibiotic treatment. Thus, there is much interest in refining and

developing different treatment strategies to use in conditions where healing is impaired or occurs at a significantly reduced rate.

1.5 Conventional clinical approaches to enhance bone regeneration

Fractures are classified into complete and incomplete fractures; open and closed fractures. Most fractures, if left completely alone, would probably heal by normal physiological healing but complete open fracture with large displacement between fracture fragments will need external intervention to promote functional fracture healing. In case of severe trauma, bone resection after ablative surgery or congenital bone deformities, the bone healing is usually difficult or impossible. Current clinical therapies for bone injury are mostly used to aid normal bone healing processes by using distraction osteogenesis and/ or bone graft to restore the bone defects even if there are some limitations in using it.

Different types of distraction devices are currently used for bone regeneration, including external monorail distraction devices (Raschke *et al.* 1993), and intramedullary lengthening devices (Cole *et al.* 2001). They are commonly used to treat bone loss or limb-length discrepancies and deformities. During distraction osteogenesis, bone regeneration is induced between the gradually distracted osseous surfaces. Although these methods are widely used in clinical practice to stimulate or augment bone, they are technically demanding and have several disadvantages, including associated complications, requirement for

lengthy treatment for both the distraction (1 mm per day) and the consolidation period (usually twice the distraction phase), as well as impacts on the patient's psychology (Green *et al.* 1992; Aronson and Rock 1997; Dimitriou *et al.* 2011).

Different types of bone grafts are currently used for bone injury autograft, allograft and xenograft. Autogenous bone graft is bone obtained directly from the patient. It has numerous advantages including its ability to be osteoconductive and osteoinductive. Although it is safe, available and shows no risk of immune rejection, it necessitates additional surgery to the patient increasing the risk of morbidity at the donor site as well as increasing the cost of the surgical operation (Salkeld *et al.* 2001). Allogenic bone graft is constructed from another individual after treating it with different sterilizing techniques. It is an alternative material which provide large amount of bone graft and overcome some problems in autogenous graft. It has a limited supply, limited capacity to incorporate with host bone (Heiple *et al.* 1963) and reduced osteoinductivity (Lutolf *et al.* 2003), When compared to autograft. Xenograft is made from animal bone, most commonly bovine bone, after removing the organic materials from the tissue and leaving the hydroxyaptite. It has an osteoconductive properties acting as a scaffold stimulating bone formation at the recipient site. Furthermore, the xenogenic grafting materials show some degree of osteoinductive potential by stimulate transformation of the patient's mesenchymal cells into osteoblast cells. Xenograft has the ability to be resorped and replaced by the patient's bone. Synthetic materials which called "alloplastic grafts" such as metal, ceramic, composite and polymers also can be used in treating bone defect. They are available in large amounts thus they can be used to reconstruct segmental bone

defect. The problems associated with bone grafts materials continue to drive the refining and development of novel clinical therapies for bone regeneration.

1.6 Bone Tissue Regeneration

Recently within the last 20 years, the idea of regenerating human tissue is rapidly growing. Tissue regeneration in field of orthopedic and maxillofacial surgery gives a great promise in treating bone defects and overcomes the limitations associated with the conventional treatment approaches. Three ideal properties required for successful tissue regeneration; osteoinduction (bone morphogenetic proteins (BMPs) and other growth factors), osteogenesis (osteoprogenitor cells) and osteoconduction (scaffold).

1.6.1 Cells

It has long been recognized that stem cells are potent and have the ability to differentiate into different cell lineages. These characteristics raise the interest of scientists to consider stem cells in bone regeneration. There are two broad types of stem cells commonly used in tissue regeneration studies; embryonic stem cells and adult stem cells. Embryonic stem cells have the ability differentiate into all adult cell lineages (pluripotent), however their applications are limited due to different ethical issues. Adult stem cells can differentiate into single cell line (unipotent) and it can be isolated from different sources such as bone marrow, brain or adipose tissue.

Adult bone marrow stem cells (BMSC) are capable to differentiate into multiple mesenchymal tissues *in vivo* including bone (Friedenstein *et al.* 1987; Pittenger *et al.* 1999). They are commonly used in bone regeneration. They have several characteristic features such as; ease of isolation, ability to adhere and grow on tissue culture plastics. They have also high proliferation rate, can reach up to 50 population doublings in culture, and their osteogenic potential are not affected by freezing conditions. Clinically, surgeons are used to deliver osteogenic cells aspirated from iliac crest bone marrow directly to the regeneration site, which also contains growth factors (Dimitriou et al. 2011). Although this technique provide satisfactory results in improving bone repair with minimal invasive procedure (Pountos *et al.* 2010b), it depend on patient's health statue, aspiration sites and techniques used (Huibregtse *et al.* 2000). It has been reported that bone-marrow aspiration concentrate (BMAC) is considered an effective product to enhance bone regeneration (Hernigou *et al.* 2005; Jager *et al.* 2011), however it is associated with significant issue with regards to the adequate number of MSCs/ osteoprogenitors required to provide optimal repair responses (Hernigou et al. 2005). Another technique used to facilitate bone regeneration by cell harvesting, *in vitro* expansion followed by cells implantation to the defect area (Bianchi *et al.* 2003; D'Ippolito *et al.* 2004). Such technique is already applied for cartilage regeneration (Wakitani *et al.* 2011), however, it adds substantial time, cost and risks of contamination (McGonagle *et al.* 2007).

Adipose tissue is another attractive and accessible source of osteogenic cells. They are characterised by their abundant availability as well as their ability to expand and proliferate (Niemeyer *et al.* 2010). It has been reported that adipose

stem cells (ASC) can undergo differentiation into various lineages, including osteogenic, chondrogenic and endothelial (Gimble *et al.* 2007). Mesenchymal stem cells extracted from adipose tissue are considered to be alternative to MSC from bone marrow. It is hypothesised that ASC have an equivalent osteogenic potential as BMSC. Studies by Niemeyer et al, 2010 have shown that the osteogenic potential of ASC is seem to be inferior to BMSC. However addition of platelet-rich plasma (PRP) can partially compensate the inferior osteogenic potential of ASC which represents a promising therapeutic approach (Niemeyer et al. 2010). Therefore more work is needed to compare the differentiation potential of ASC and BMSC. As well as investigate the osteogenic potential of using PRP together with adipose stem cells during bone repair.

Issues of limited cell quantity and finding alternative sources of MSCs are being extensively investigated. Recently, periodontal ligament has been reported to possess stem cell properties similar to MSCs (Kaku *et al.* 2012). These periodontal ligament stem cells (PDLSCs) are multipotent cells that are able to differentiate into periodontal ligaments, alveolar bone, cementum (Ji *et al.* 2013), peripheral nerve and blood vessel (Seo *et al.* 2004; Huang *et al.* 2010; Park *et al.* 2011). They have been harvested from the periodontal ligament of human impacted third molars (Seo et al. 2004), permanent teeth and deciduous teeth (Silverio *et al.* 2010; Ji et al. 2013), supernumerary teeth (Song *et al.* 2012) and inflamed granulation tissue (Hung *et al.* 2012; Ronay *et al.* 2014). PDLSCs located in the perivascular wall of periodontal ligaments share similarities with pericytes in morphology, differentiation potential, and the ability to form capillary-like structures in vitro (Iwasaki *et al.* 2013). PDLSCs demonstrated better colony-

forming efficiency than BMMSCs and could differentiate into adipocytes and osteoblasts (Silverio et al. 2010; Song et al. 2012; Ji et al. 2013). Several studies have been investigated the effect of growth factors on stem cell properties of PDLSCs. While BMP4 has been shown to enhance the proliferation and maintain the stemness of PDLSCs during a long-term culture (Liu *et al.* 2013), TGF-β1 upregulates type I collagen and accelerates proliferation and fibroblastic differentiation of PDLSCs (Fujii *et al.* 2010; Kono *et al.* 2013). BMP2, BMP7 and VEGF have also shown to promote repair of bony defect in animal models via enhancing osteogenic differentiation of PDLSCs (Lee *et al.* 2012; Hakki *et al.* 2014). All of these studies suggest that periodontal ligament provides a good source of stem cells that can be used to enhance fracture repair. *Ex vivo* fractured mandible model will give better approach to study the characterisation and cellular behavior of periodontal ligament stem cells within the fracture environment that ultimately improve outcomes of impaired fractured healing.

1.6.2 Growth Factor Therapy

Bone regeneration is driven by different growth factors. During regeneration process, it is necessary to give the cells some cues or signals. Several growth factors have been implicated in guiding cells during fracture healing. Most of these growth factors have been used clinically or under investigation to enhance bone regeneration. Studies on animal models have demonstrated that exogenous application of TGF-β1 can increase callus formation and strength, and bone formation, either on its own or in conjunction with IGF-1 (Srouji *et al.* 2004; Lee *et al.* 2006).

BMPs have been extensively investigated, as they are potent osteoinductive factors. Different animal models have been utilised to investigate the effects of BMPs on bone formation. Recombinant BMPs have the ability to repair bone defects when combined with a carrier of collagen, demineralised bone matrix, biodegradable polymers, or hydroxyapatite (Kang *et al.* 2004). Several experimental and clinical trials have supported the safety and efficacy of the use of BMP2 and BMP7 as osteoinductive bone-graft substitutes for bone regeneration. Particular attention has focused on using a biomimetic scaffold or carrier to deliver BMP2 and BMP7 into the site of injury (Gautschi *et al.* 2007). In clinical studies, BMP2 and BMP7 have also been successfully used to treat case of non-union in long bones (Friedlaender *et al.* 2001; Govender *et al.* 2002a) and in the induction of spinal fusion (Boden *et al.* 2000; Vaccaro *et al.* 2003). Studies using an *ex vivo* tooth slice culture system (Sloan et al. 1998) have demonstrated that exogenouse applications of TGF-β1 by using either alginate hydrogels or agarose beads, show mitogenic effects on subodontoblast cells, and is capable of inducing differentiation of odontoblast-like cells within the dentine-pulp complex (Sloan and Smith 1999; Dobie *et al.* 2002). Recent studies have also been shown that exogenouse applications of TGF-β1 by using agarose beads, demonstrated increase in cellular migration and proliferation; as well as expression of bone matrix protein within *ex vivo* fractured mandible model (Smith *et al.* 2011). Although BMPs have been licensed for clinical use in bone-tissue engineering, several issues need to be further examined. These include identification of the optimum dosage required to elicit a therapeutic response (Reddi 1998; Dimitriou et al.

2011) and understanding the synergistic action of using 'cocktail' of growth factors that have shown significant promising results in preclinical and early clinical investigation (Nauth *et al.* 2010; Dimitriou et al. 2011).

ECM bone is known to act as reservoir of endogenous growth factors bound within it (Hauschka *et al.* 1986; Taipale and Keski-Oja 1997). Release of these factors following bone injury allows presentation of these factors to target cells, and subsequent cellular signalling to initiate reparative processes at these sites, leading to optimal regeneration of the bone (Schonherr and Hausser 2000; Ramirez and Rifkin 2003; Hughes et al. 2006b). There are several chemical treatments that have been demonstrated to solubilise growth factors from within the mineralised matrix of dentine, including ethylenediaminetetraacetic acid (EDTA), calcium hydroxide (Zhao *et al.* 2000; Graham *et al.* 2006) as well as citric acid (De-Deus *et al.* 2008b). Recent studies have been shown that such chemical treatment modalities may also release growth factors from within the matrix of *in vitro* bone slab model (Smith et al. 2011). These acids solubilise growth factors by matrix demineralisation, local matrix dissolution or chelating calcium ions present within the mineralised matrix (De-Deus et al. 2008b).It is unclear whether this simply releases bound growth factors, or if a more complex mechanism is responsible for the release of the matrix-bound growth factors.

Currently, numbers of clinical therapies are benefit from utilising endogenous matrix-bound growth factors rather than addition of exogenous growth factors. Topical application of Dycal®, calcium hydroxide-based material such as mineral

trioxide aggregate (Bayatian et al. 2007), is commonly used in clinical dentistry to facilitate formation of reparative dentine after dentine-pulp complex injury, by driving release of growth factors from dentine matrix (Tziafas *et al.* 2000; Hörsted-Bindslev *et al.* 2003; Graham et al. 2006). Emdogain, a purified enamel matrix protein product, is also used in clinical periodontology to increase growth factor release and inducing cementogenesis and osteogenesis (Nakamura *et al.* 2001; He *et al.* 2004). Expansion of such dental-based materials or development of novel treatments that can facilitate growth factor release, for use in bone regeneration may overcome some of the problems associated with current growth factor therapies using exogenously added bioactive molecules. Development of the *ex vivo* mandible organ culture system may provide an ideal model system with which to test such therapeutics.

1.6.3 Scaffolds

Scaffolds are fundamental for successful bone tissue regeneration. It provides an appropriate place for the cells to migrate, proliferate and differentiate. The ideal scaffold should have specific characteristics; including osteoconductivity, osteoinductivity and osteogenic properties. Moreover, the scaffolds materials should be biocompatible, biodegradable, nontoxic, nonantigenic and nonteratogenic. The scaffold should be highly porous which allows vascularization and nutrient delivery. In addition, the mechanical properties of scaffold must keep the structural integrity during first stage of bone formation. The materials that usually used to produce the scaffolds are either natural or synthetic. Natural derived scaffolds are come from natural tissue such as collagen, fibrin, hyaluronic acids and deminarlized bone matrix. It is usually act

as extracellular matrix and has high biodegradable affinity. Synthetic materials are either absorbable or permanent. The synthetic absorbable materials include; poly glycolic acid (PGA), polylactic acid (PLA) and Polyvinyl alcohol (PVA). Metals and alloys are examples of permanent scaffolds. Synthetic bone substitutes are widely used in clinical practice as osteoconductive materials; however they lack osteoinductive or osteogenic properties. They promote the migration, proliferation and differentiation of bone cells for bone regeneration. They are usually used as adjuncts or alternatives to autologous bone grafts, especially for regeneration of large bone defects, where the requirements for grafting material are substantial. Scaffolds can be used in combination with autologous bone graft, growth factors or cells (Giannoudis et al. 2005). Different studies are ongoing to improve scaffold's properties, to enhance cellular adhesion, growth and differentiation, as well as to allow vascular ingrowth and bone-tissue formation (Dimitriou et al. 2011). Three dimensional scaffolds/cells can be added to the *ex vivo* fractured mandible slices to study bone regeneration. The assessment of the 3D scaffolds in the *ex vivo* fractured model can give clear outcome about the synergistic effects of the growth factor on proliferation and differentiation of the resident and seeded cells.

1.7 Modelling of Bone Regeneration

During bone regeneration, suitable model and clinical systems are required to understand the effects of new therapies on bone structure. *In vitro* studies use only a single or two cell system models to investigate the behaviour of cells to varying treatments such as addition of exogenous growth factors (Itonaga *et al.*

2004; Abdelmagid *et al.* 2007). *In vitro* models have been used to co-culture two different cell types such as osteoblasts with chondrocytes to investigate the biological integration of cartilage grafts with subchondral bone (Jiang *et al.* 2005). *In vitro* models have also been used to co-culture of osteoblasts and haematopoietic stem cells to investigate physical interactions between the cell types (Jung *et al.* 2005). Although *in vitro* models can provide information about cellular behaviour in response to varying factors, the information they can yield is limited as they are unable to recapitulate the spatial arrangement of cells *in vivo*.

To explore and investigate mechanism of bone regeneration, large numbers of in vivo models are used such as rabbits, rodents, primates, and genetic knock-out mice. *In vivo* models of bone repair have been used in critical sized defect models developed in the femur (Fini *et al.* 2005); as well as in the calvaria and mandible (SCHMITZ and Hollinger 1986). Although they have yielded considerable information on bone repair, *in vivo* models also have some limitations and problems. Such as the cost of running the experiments and the technical difficulties of obtaining clear data from cell to cell interaction between different cell types that may have effect on the cells behaviour. Furthermore, ethical implications can be raised from the large number of animals that must be used with such experiments. All of that suggests the need of finding a novel precise experimental model.

To minimise the issues associated with the *in vivo* models and *in vitro* cell culture systems, Sloan and his colleague developed a novel *ex vivo* tooth slice models to investigate dentine repair (Sloan *et al.* 1998). Using such *ex vivo* models have significant advantages of reducing the number of animals required for *in vivo* experimentation. It can be used to study a wide variety of developmental, physiological and pathological conditions. By using this model clearer data may be obtained from the cells and tissues cultured as would be found in the *in vivo* situation, but the systemic influences, which often hinder *in vivo* work, are removed. As well as eliminate the need for costly and unnecessarily numerous *in vivo* studies.

Ex vivo culture of incisor rat slices has been used greatly to improve the understanding of a wide range of dental tissue repair processes, such as angiogenesis inside the pulp tissue (Magloire *et al.* 1996; Smith *et al.* 1998; Sloan and Smith 1999). This culture system has also been used to assess the biocompatibility and cytotoxicity of commonly used dental materials (Turner 2002; Waddington *et al.* 2004a; Graichen *et al.* 2005a). Dhopatkar *et al*. (2005) developed a mandibular slice *ex vivo* organ culture model system to investigate the effect of externally applied forces on the dentin-pulp complex (Dhopatkar *et al.* 2005b). *Ex vivo* culture system has also been used to investigate the effects of orthodontic forces on root resorption (Wan Hassan *et al.* 2012). *Ex vivo* organ culture allowed us to develop a system which supports the differentiation, proliferation and activity of different cells such as fibroblasts, osteoblasts, osteoclasts and immune system cells within cultured slices of mouse mandible (Taylor 2008). Through the *ex vivo* mandibular slices culture it is possible to

have a natural tissue environment which can be used to assess biological responses of the tissue as well as monitor the cellular responses much closer than *in vivo* models.

1.8 Aims

Several studies have demonstrated that growth factors play an important role during bone repair process. Using combinations of different growth factors, such as the combinations found in the bone matrix, may be more effective in promoting bone healing compared with single growth factor therapy such as bone morphogenic protein-2, which has found clinical application, but requires high doses to be effective. This thesis seeks to:

- Investigate processes of bone repair within the *ex vivo* fractured mandible culture model for extended cultures time.
- Investigate the responsiveness of the *ex vivo* fractured mandible culture model to exogenously applied BMP2, in particular investigating the effect of BMP2 on proliferation and differentiation of osteoblasts and their progenitor cells present within the model.
- Use the *ex vivo* fractured mandible culture model to stimulate the release of endogenous growth factors from fractured surfaces of rodent mandibular bone and investigate the ability of endogenously released growth factors to mediate the regeneration of mandibular bone during culture.
- Investigate the effect of calcium hydroxide on cellular behaviour of bone marrow stem cells (BMSCs).
- Assess functionality of a low dose of BMP2-2, BMP7-7 and BMP2-7 expressed by HEK cell lines using human bone marrow stromal cell and *ex vivo* fractured mandible model.

Chapter 2.

STIMULATION OF THE EX VIVO FRACTURED MANDIBLE MODEL WITH EXOGENOUS GROWTH FACTOR

2.1 Introduction

To study bone regeneration, a suitable model is essential to understand and simplify the complex interactions that occur during healing and repair processes. Results from *in vitro* cell culture have some limitation because cells are usually cultured outside their normal environment and away from their surrounding tissues. There are also some concerns associated with the current *in vivo* animal models which include the cost of running the experiments, the systemic influence and the technical difficulties of obtaining clear data from cell to cell interaction between different cell types that may have effect on the cells behaviour. Furthermore, ethical implications can be raised from the large number of animals that must be used to examine the many variables associated with such experiments. All of this suggests the need of finding alternative models.

To minimise the issues associated with the *in vivo* models and *in vitro* cell culture systems, Sloan and colleagues (1998) developed a novel *ex vivo* tooth slice model to investigate dentine repair (Sloan et al. 1998; Bielby *et al.* 2007a). Using such *ex vivo* models ensured significant advantages of reducing the cost as well as the number of animals required compared to *in vivo* experimentation.

It can be used to study a wide variety of developmental, physiological and pathological conditions. By using this model clearer data may be obtained from the cells and tissues cultured as would be found in the *in vivo* situation, but the systemic influences, which often hinder *in vivo* work, are removed. The model involves slicing of rat incisor (Sloan et al. 1998) or mandible (Bielby et al. 2007a; Smith et al. 2010; Colombo *et al.* 2015) into 2mm transverse slices followed by culturing each slice separately in a way that each slice considered as a one experiment.

Ex vivo culture models have been used for different studies such as to improve the understanding of cartilage defects (Graichen *et al.* 2005b) and angiogenesis inside the pulp tissue (Smith et al., 1998, Sloan and Smith, 1999, Magloire et al., 1996). These models have also been used to test biocompatibility and cytotoxicity of drug activity (Turner *et al.* 2002; Waddington *et al.* 2004b) and to assess effect of externally applied forces on the dentin-pulp complex (Dhopatkar *et al.* 2005a). *Ex vivo* culture system have also been used to investigate the effects of orthodontic forces on root resorption (Wan Hassan et al. 2012) as well as assess tissue response to bacterial growth (Roberts *et al.* 2013). The model was responsive also to TGF-β1, applied via agarose beads to tooth slice culture system (Sloan et al. 1998) and to mandible slice culture system (Smith et al. 2010), and results demonstrated that exogenously applied TGF-β1 is capable of stimulating cellular migration, proliferation and expression of bone matrix proteins. Recently the model was used successfully for culture and characterization of transplanted dental pulp progenitor cells (Colombo et al. 2015).

BMP2, as discussed in the introduction to this thesis, is a growth factor that plays an important role during bone regeneration procedures as it is capable of differentiating mesenchymal stem cells and osteoprogenitor cells into mature, fully functional osteoblast cells, capable of producing new bone (Wozney 1992; Lieberman et al. 2002b; Cheng et al. 2003). BMP2 has been approved by food and drug administration (FDA) to be used clinically in the treatment of non-union in long bones (Govender et al. 2002) and in the induction of spinal fusion (Boden et al. 2000). This thesis chapter aims to investigate the validity of the fractured rat mandible slice model when culture for more than 21 days also to investigate the effect of adding BMP2, as a single growth factor therapy, to the fracture site of the mandible model in an environment mimic fracture repair.

2.2 Materials and Methods

2.2.1 Preparation of fractured mandible slices

Twenty eight day old male Wistar rats, housed in the animal house facility of Cardiff University, were freshly sacrificed in accordance with Schedule 1 of the UK Animal (Scientific Procedures) Act 1986. Mandibles were dissected and soft tissue was removed by using a sterile disposable scalpel blade and sterile forceps then placed into sterile washing medium. Mandibles were held by two Mosquito forceps; and the condyle, ramus, and molars were removed with a segmented diamond-edged rotary bone saw (TAAB, Berkshire, UK) (Figure 2.1). Mandibles were sectioned into slices of approximately 2mm thickness and placed immediately into washing medium of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Co Ltd, UK) prepared containing 1% concentration of antibiotics solution (10,000 units of penicillin, 10mg/ml of streptomycin, and 25µg/ml amphotericin B) (Invitrogen, UK) and 200mM L-glutamine (Invitrogen, UK).The rotary disc was sterilized by washing with 70% ethanol prior to use and cooled with washing medium during slicing. Mandible slices were fractured by placing pressure onto the central portion of the lingual plate using a sterile scalpel blade. Fractures were prepared in a similar location in each mandible slice. Fractured mandible slices were cultured using Trowel type cultures (Begue-Kirn *et al.* 1992; Sloan et al. 1998; Smith et al. 2010).

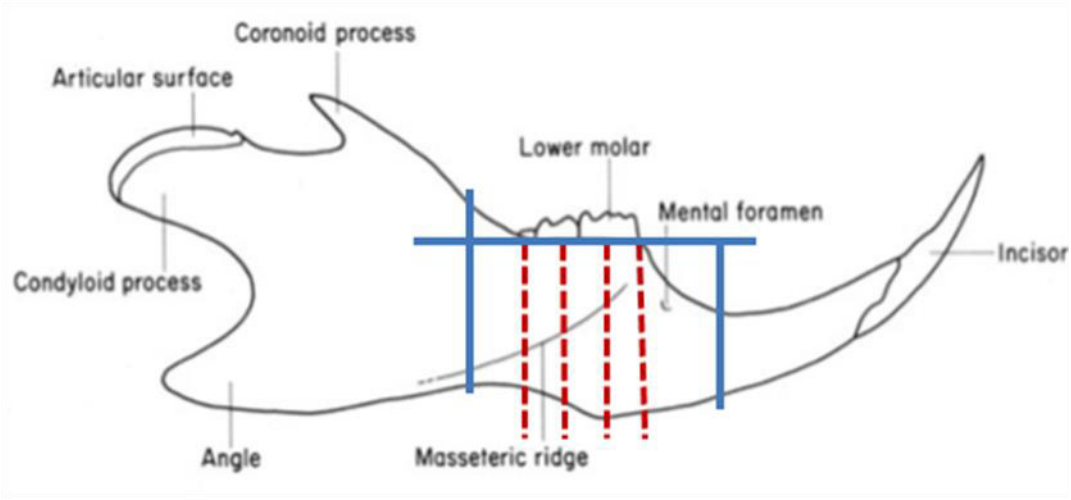


Figure 2- 1: Schematic representation of dissected mandible preparation. The condyle, ramus, molar and incisor teeth were cut using a rotary bone saw and discarded. Two-millimetre-thick transverse slices of mandibles were prepared by cutting through the tissue as indicated by the vertical lines.

2.2.1.1 Trowel type cultures

Mandible slices were embedded into individual wells of a plastic 96-well plate (Greiner Bio-One, UK), which was filled previously with 100µl sterile molten embedding media prepared by mixing 1% low melting point agar (agarose type VII; Sigma Gillingham) 1:1 with culture medium. Culture medium prepared by adding 10% heat inactivated foetal calf serum (Invitrogen, UK), 0.15mg/ml of ascorbic acid (Invitrogen, UK), 200mM L-glutamine (Invitrogen) and 1% concentration of antibiotics solution (10,000 units of penicillin, 10mg/ml of streptomycin, and 25µg/ml amphotericin B) (Invitrogen, UK) into DMEM (Invitrogen). Mandible slices were left at room temperature for approximately five minutes until embedding media became semi-solid.

During agar setting, a sterile plastic ring support and Millipore filter (mixed esters of cellulose acetate and nitrate) were placed in each well of plastic 12-well plate (Greiner Bio-One, UK). Embedded mandible slices were transferred from the multi well dish and placed on a Millipore filter which was floated on the surface of 2 ml of culture medium in Trowel type cultures (Trowell and Westgarth 1959) (Figure 2.2). Mandible slices in Trowel type cultures were incubated at 37°C, in a humidified atmosphere of 5% CO_2 in air and cultured for 35 days. Culture medium was changed every other day.

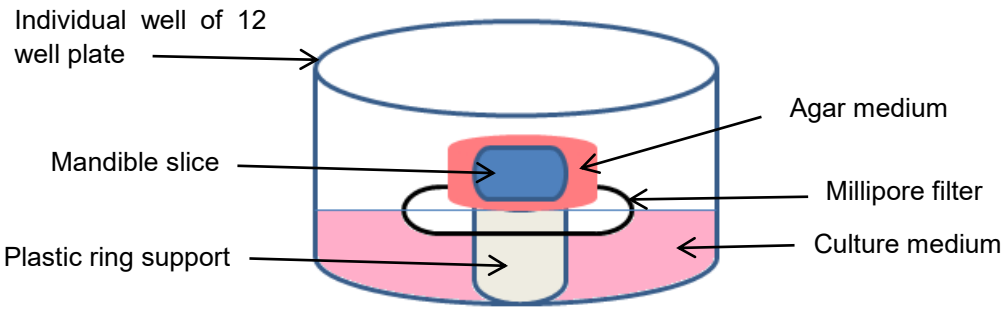


Figure 2- 2: Trowel type culture. Embedded mandible slices were placed on millipore filter floated on culture medium and supported by plastic ring.

2.2.2 Addition of BMP coated agarose beads to fractured mandible slices

Human recombinant BMP2 (Invitrogen, UK) was solubilised in 20mM acetic acid to a concentration of 0.1mg/ml, and then further diluted to a working concentration in 0.1% BSA in TBS and stored in -20°C. 150µm sized Affi Gel Blue agarose beads (BioRad Laboratories, UK) were centrifuged to separate agarose beads from PBS then incubated with 50µl 100ng/ml BMP2 in 0.1% BSA in TBS for 1 hour at 37°C.

Mandible slices were prepared as described above. Prior to embedding in semi-solid agar medium, the fracture site was packed with 3 agarose beads (Figure 2.3) using sterile forceps and perio probe, with the aid of a dissecting microscope (Bausch and Lomb, UK). As a control, fracture sites were left empty without beads or packed with agarose beads that had been previously incubated in PBS. The mandible slices were cultured in Trowel type cultures for 7, 14 and 21 days.

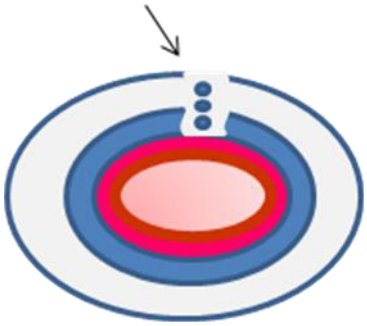


Figure 2- 3: Agarose beads soaked in BMP2 were placed in fractured area (arrow), before embedding in semisolid agar medium.

2.2.2.1 Haematoxylin and Eosin (H&E)

Following culture, mandible slices were fixed in 10% (w/v) neutral buffered formalin at room temperature for 48 hrs. Slices were then demineralised in 10% (w/v) formic acid at room temperature for 72 hrs. Mandible slices were placed in a plastic basket and covered with lid. Mandible slices were processed for histological examination by dehydrating through a series of 70, 90, and 100% graded alcohols, cleared through xylene, and taken to molten paraffin wax (Raymond Lamb, UK) on an automatic tissue processer (Shandon Pathcentre). Slices were embedded in paraffin wax in a metal mould prior to sectioning. Sections of 7µm thickness were cut on a sledge microtome (Leica SM 2400) at different levels throughout the tissue and mounted onto polysine glass slides

(Thermo Scientific, UK). In order to improve adhesion of sections to the slides, sections were dried in an oven at 60°C for 30 minutes. Sections were stained with haematoxylin and eosin using an automatic tissue stainer (Shandon LiniStain, UK) in which sections were taken through a series of solutions containing xylene, series of descending graded alcohols, water, haematoxylin, acid alcohol, Scott's tap water and eosin. After a final wash in tap water, sections were finally dehydrated with alcohol and cleared with xylene. Slides were mounted under a glass cover slip with DPX mounting medium (Raymond Lamb, UK). Stained sections were examined on an Olympus AX70 light microscope. Photographs were taken using a Nikon DXM digital camera and ACT-1 imaging software. Cells within the periodontal ligament (PDL) of H&E stained sections were automatically counted digitally using Image ProPlus software (Media Cybernetics Inc, USA). 100µm^2 areas within five areas around and proximal to the fracture site were counted per section as well as unfractured, contralateral, away and opposite to the fracture site (Figure 2.4).

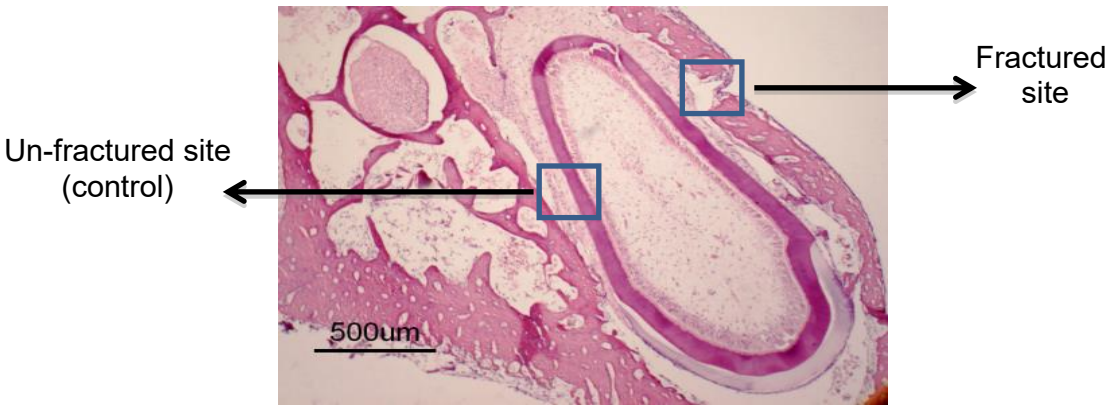


Figure 2- 4: Areas of interest showing number of cells were counted proximal to the fracture site and at un-fracture site which was away and opposite to the fracture site, used as an internal control.

2.2.2.2 Immunohistochemistry

Immunohistochemical staining was carried out using a Vectastain ABC peroxidase kit (Vector Laboratories Ltd). The kit included normal horse serum, a universal biotinylated secondary antibody and the 'ABC' reagent. The horseradish peroxidase was visualised by the development of a peroxidase substrate that produces a colour. Paraffin embedded mandible slices were cut and mounted on polysine glass slides as described above (section 2.2.2.1). Slides were dried at 60°C overnight. Sections were deparaffinised with two washes of xylene for 5 minutes then cleared with 100% of industrial methylated spirits (IMS) twice for 5 minutes and then rinsed twice with tap water for 5 minutes. Sections were incubated in 0.1% triton X 100 for 10 min and washed twice in tris-buffered saline (TBS) buffer for 5 min. Sections were then incubated in 3% hydrogen peroxide solution for 10 min and washed twice in (TBS) buffer for 5 min. Sections were outlined with a paraffin wax marker followed by addition of blocking serum (50µl serum in 2ml TBS) for 20 min. Excess serum was wiped from the slides and sections incubated for 1 hour with appropriate primary antibody.

An indicator of cellular proliferation, proliferating cell nuclear antigen (PCNA) primary antibody, rabbit anti-rat (Santa Cruz Biotech, Santa Cruz, CA,USA), was diluted 1:20 in 1% BSA/TBS. Osteopontin (OPN) primary antibody, bone marker, goat anti-rat OPN (Santa Cruz Biotech, Santa Cruz, CA,USA), was diluted 1:250 in 1% BSA/TBS. Another bone marker, osteocalcin (OCN) primary antibody, goat anti-rat OCN (Santa Cruz Biotech, Santa Cruz, CA, USA), was diluted 1:50 in 1% BSA/TBS. As a negative control, the primary antibody was excluded and

replaced with TBS. Following primary antibody incubation sections were washed twice for 5 min in TBS buffer. Sections were then incubated for 30 min with the universal biotinylated secondary antibody and washed twice for 5 min in (TBS) buffer. Sections were incubated for 30 min with the VECTASTAIN 'ABC' reagent. Following two further TBS buffer washes for 5 min, sections were incubated with DAB peroxidase substrate solution (Vector Laboratories) for 4 min. Sections were rinsed in tap water for 5 min, and counterstained with 0.1% light green for 30 seconds. Finally, sections were dehydrated in IMS for 5 min, cleared in xylene for 5 min, and glass coverslips were applied using DPX mounting medium (Raymond Lamb, UK). Sections were evaluated microscopically on an Olympus AX70 light microscope. Photographs were taken using a Nikon DXM digital camera and ACT-1 imaging software. A total of 5 mandible slices were used per treatment, for each marker.

2.2.3 Statistical analysis

Statistical analysis was carried out using GraphPad InStat 3 software (GraphPad software, Inc, USA). Standard errors of the mean were calculated, and mean values were analysed using one-way ANOVA and Tukey's post hoc test to analyse differences between treatment types and controls. P values below 0.05 at 95% confidence intervals were held to be statistically significant.

2.3 Results

2.3.1 Culture of mandible slices for upto 35 days

Histological evaluation of mandible slices that had been cultured for 35 days demonstrated maintenance of tissue structure throughout the section (Figure 2.5). Cells still appeared viable with round, darkly stained nuclei. Cell and tissue architecture were well preserved adjacent to the fracture site, demonstrating maintenance of cell and tissue structure without sustaining excessive damage to cells and tissues within adjacent areas.

Statistical analysis of average PDL cell numbers (Figure 2.6) proximal to the fracture sites demonstrated that there were a significant differences between uncultured (day 0) fractured mandible slices (48 (±1.5) cells/100μm^2) and cultured fractured mandible slices ($p<0.001$). No significant differences were identified between fractured mandible slices cultured for 7days (28 (±1.3) cells/100μm^2) and 14 days (26 (±1.2) cells/100μm^2) ($P>0.05$). Digital image analysis of cell counts proximal to the fracture sites calculated an average of (14 (±1.2) cells/100μm^2) in 21 days, (12 (±1.2) cells/100μm^2) in 27 days, and (10 (±1.2) cells/100μm^2) in 35 days cultures. Statistical analysis demonstrated that there were a significant decrease in average PDL cell numbers after 7 days ($p<0.001$). No significant differences were identified between average PDL cell numbers of fracture and unfracture sites at each day ($P>0.05$).

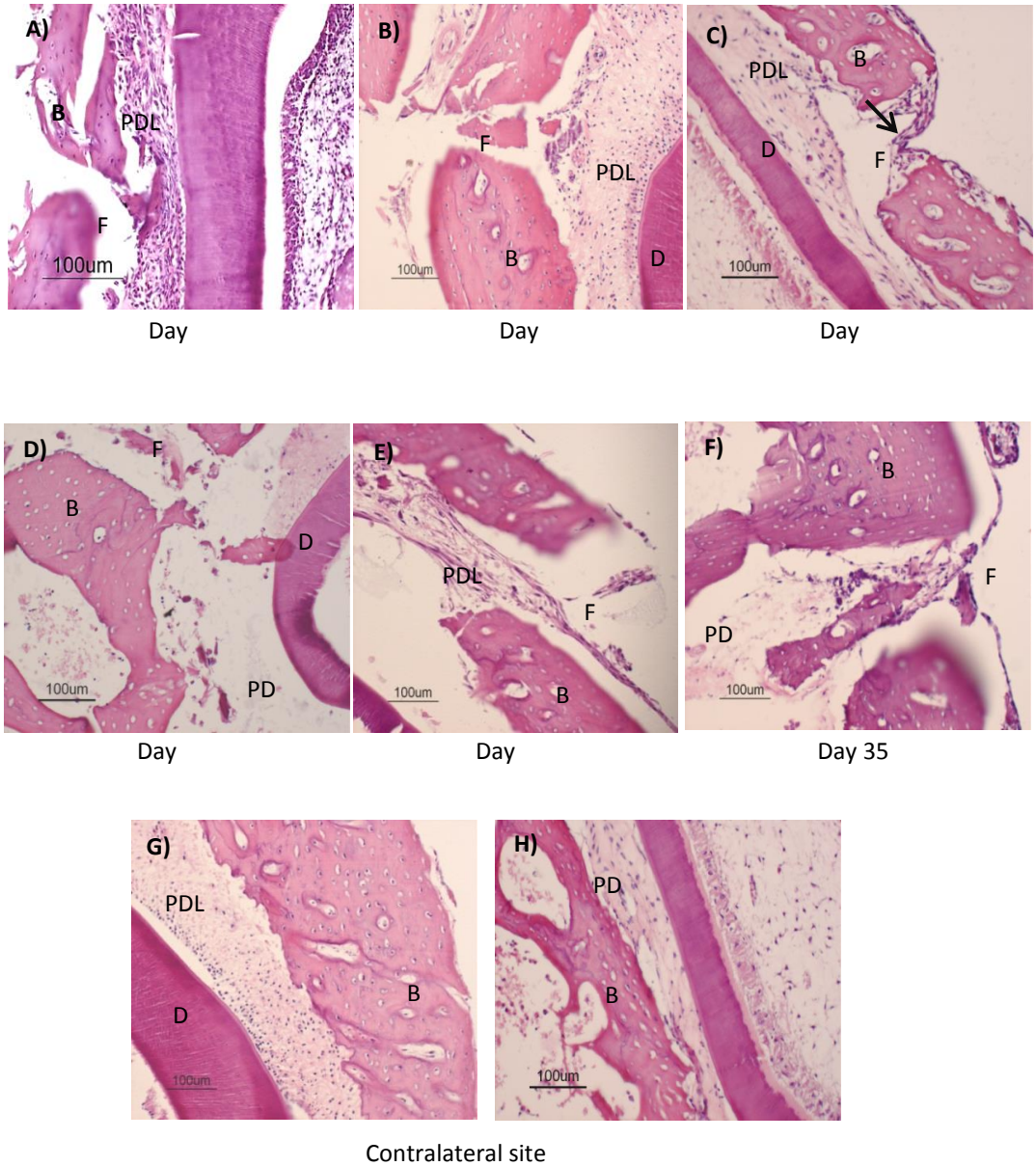


Figure 2- 5: Histological examination of fractured mandible slices cultured for 35 days. [A-F] fractured site, [G, H] contralateral side. PDL: periodontal ligament; D: dentine; B: bone; F: fracture site.

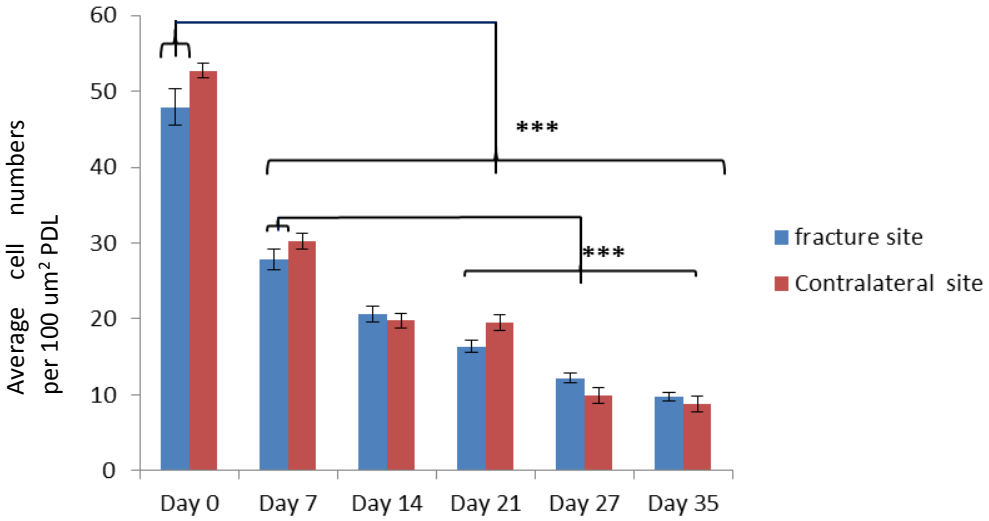


Figure 2- 6: Comparison between fractured mandible slices cultured for 35 days. Average PDL cell numbers per 100 um^2 area were counted proximal to the fracture sites and at unfractured contralateral site. n=5. Error bars indicate standard error of the mean. ***P<0.001.

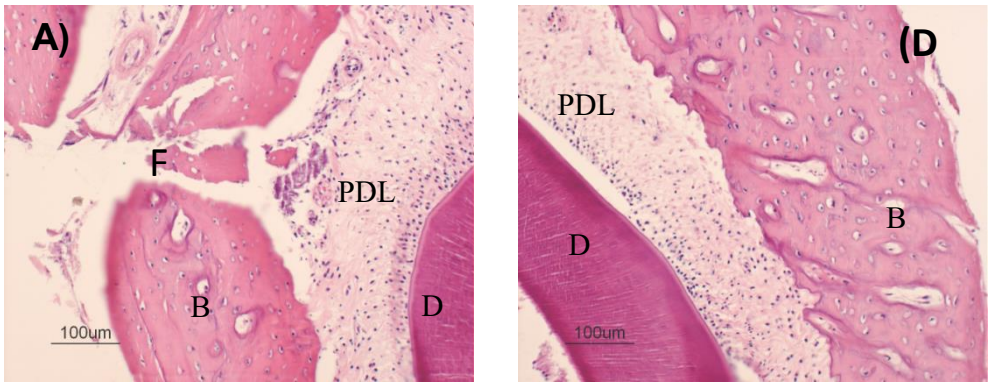
2.3.2 Addition of BMP2 coated agarose beads to fractured mandible slices

2.3.2.1 Histology of BMP2 stimulated mandible slices for 7 days

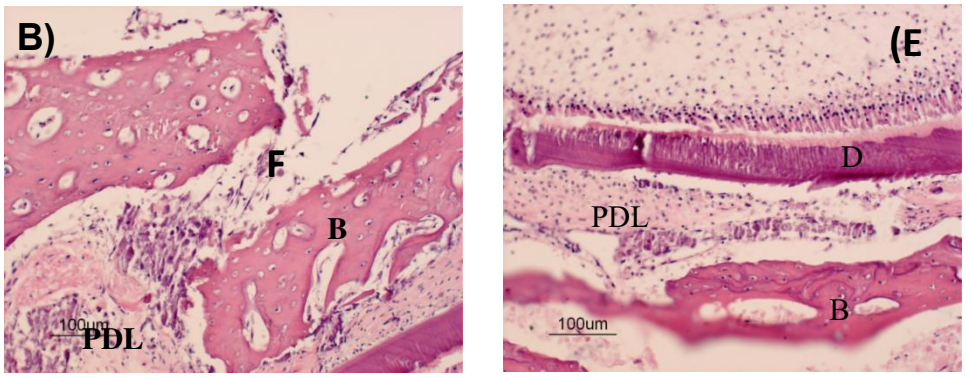
Histological evaluation of fractured mandible slices that had been stimulated for 7 days with BMP2 beads demonstrated maintenance of tissue architecture throughout the section when compared with mandible slices packed with PBS beads and without beads (Figure 2.7). Fractured mandible slices demonstrated aggregation of cells within the PDL in close proximity to the site of fracture and beads placement in the three groups. This aggregation of cells was not observed within regions of PDL opposite the fractured site (Figure 2.7). Minimal loss of tissue architecture appeared within the PDL around area of BMP2 bead placement.

Statistical analysis of average PDL cell counts (Figure 2.8) demonstrated no significant differences between treated and untreated mandible slices at site of fracture ($P>0.05$). Digital image analysis of cell counts at the fracture site calculated an average of (28 (±1.1) cells/100μm^2) in slices with no beads, (24 (±1.2) cells/100μm^2) in slices with PBS beads and (29 (±1.3) cells/100μm^2) in slices with BMP2 beads. Although average PDL cell counts demonstrated high cell number in BMP2 stimulated slices, this increase was not significant ($P>0.05$). There was also no significant difference in average cell numbers within regions of mandible slices opposite the site of stimulation between the three groups ($P>0.05$). Although not significant, there was a slight increase in average cell numbers within regions of mandible slices opposite the site of stimulation when compared with fracture site ($P>0.05$).

a) No Beads



b) PBS Beads



c) BMP2 Beads

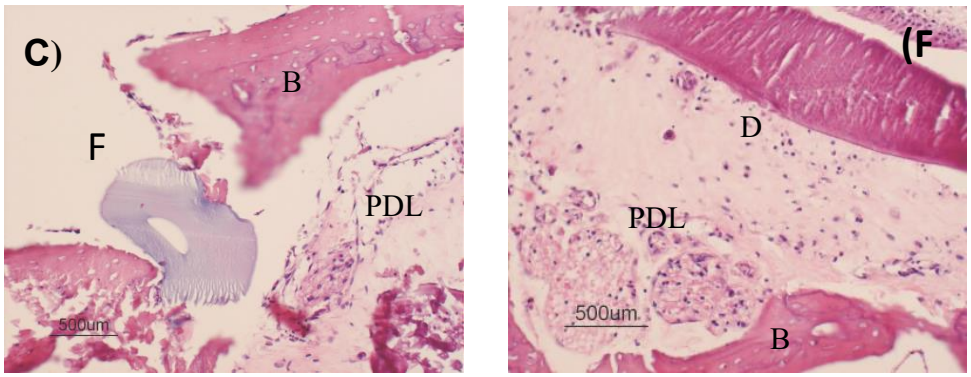


Figure 2- 7: Histological examination of mandible slices cultured with; a) no beads, b) PBS beads, c) BMP2 beads for 7 days. (A-C) fractured site, (D-F) contralateral unfractured side. PDL: periodontal ligament; D: dentine; B: bone; F: fracture site.

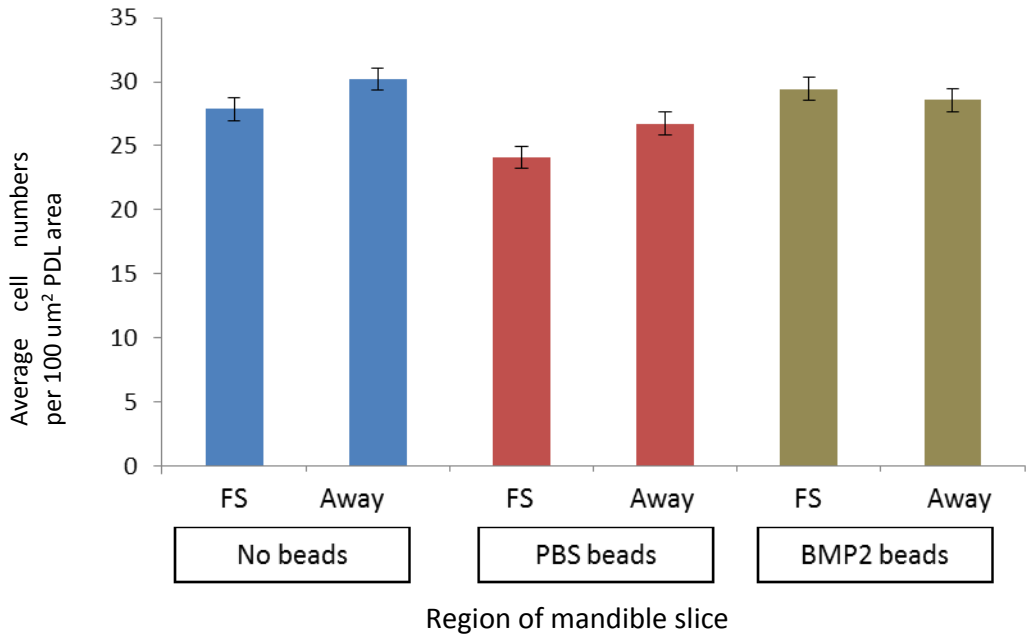


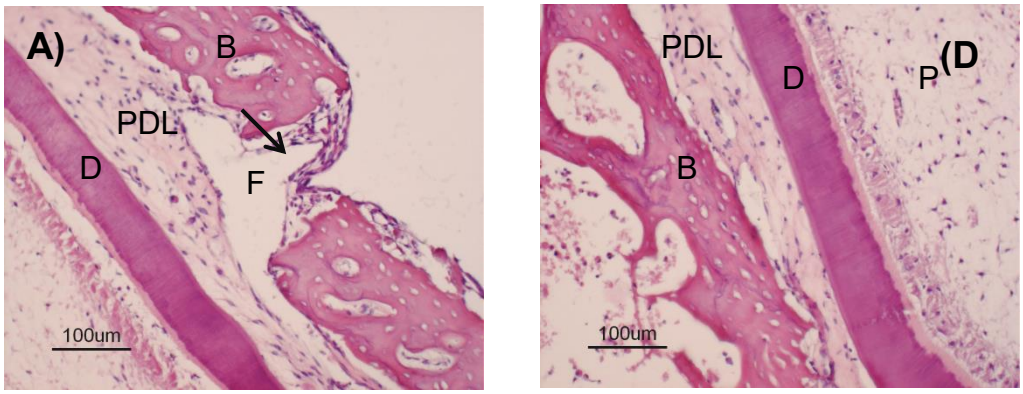
Figure 2- 8: Comparison of average cell numbers per 100 um2 area within the PDL of fractured mandible slices cultured with no beads, PBS beads or BMP2 beads for 7 days. FS: fracture site; Away: contralateral, unfracture site. (n=8), Error bars indicate standard error of the mean.

2.3.2.2 Histology of BMP2 stimulated mandible slices for 14 days

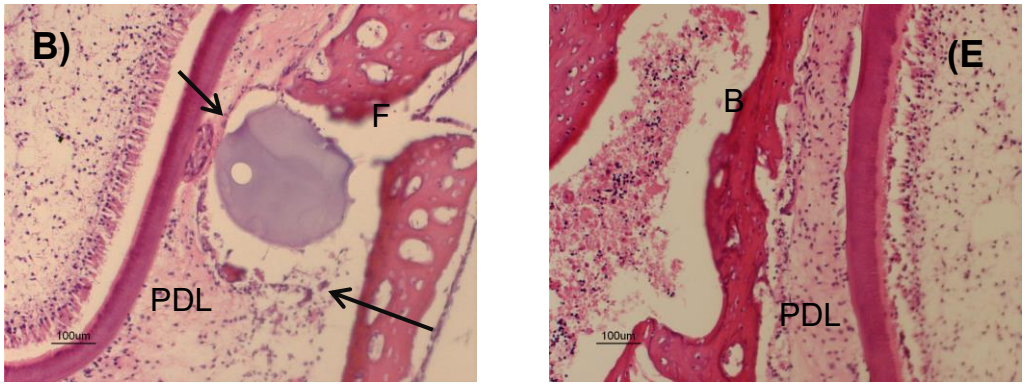
Histological evaluation of mandible slices that had been stimulated for 14 days demonstrated maintenance of tissue architecture throughout the section (Figure 2.9). Fractured mandible slices demonstrated an aggregation of cells within the PDL in close proximity to the site of fracture and bead placement in the three groups. This aggregation of cells was not observed within regions of PDL opposite the fractured site. Minimal loss of tissue architecture appeared within the PDL around area of bead placement.

Statistical analysis of PDL cell counts (Figure 2.10) demonstrated a slight increase in PDL cell numbers at the site of fracture within non stimulated mandible slices (20 (±1.1) cells/100μm^2) when compared with the mandible slices stimulated with PBS-beads (16 (±1.1) cells/100μm^2) and BMP2-beads (19 (±0.8) cells/100μm^2) ($P>0.05$). In area opposite the site of stimulation, digital image analysis of cell counts calculated an average of (19.7 (±1.0) cells/100μm^2) in no beads slices, (19.2 (±1.4) cells/100μm^2) in PBS slices, and (18.2 (±1.0) cells/100μm^2) in BMP2 slices. There was no significant difference in average cell numbers within regions of mandible slices opposite the site of stimulation between the three groups ($P>0.05$).

a) No Beads



b) PBS Beads



c) BMP2 Beads

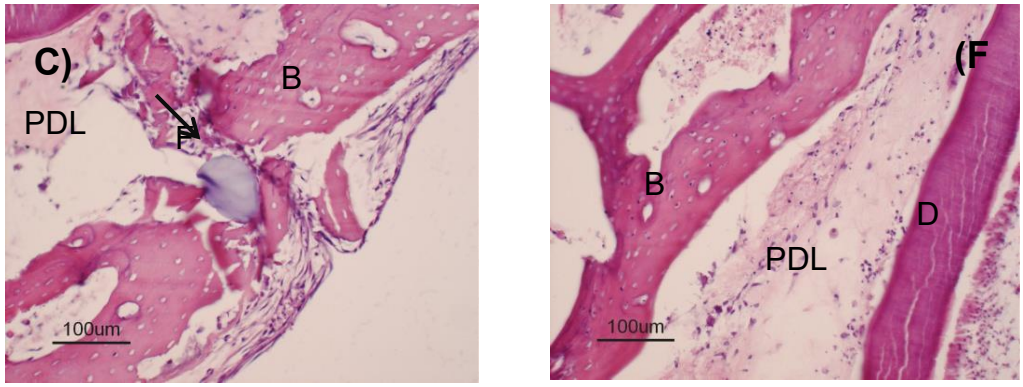


Figure 2- 9: Histological examination of mandible slices cultured with a) no beads, b) PBS beads, c) BMP2 beads for 14 days. (A-C) fractured site, (D-F) contralateral, unfracture site. PDL: periodontal ligament; D: dentine; B: bone; F: fracture site; arrows indicate aggregation of cells.

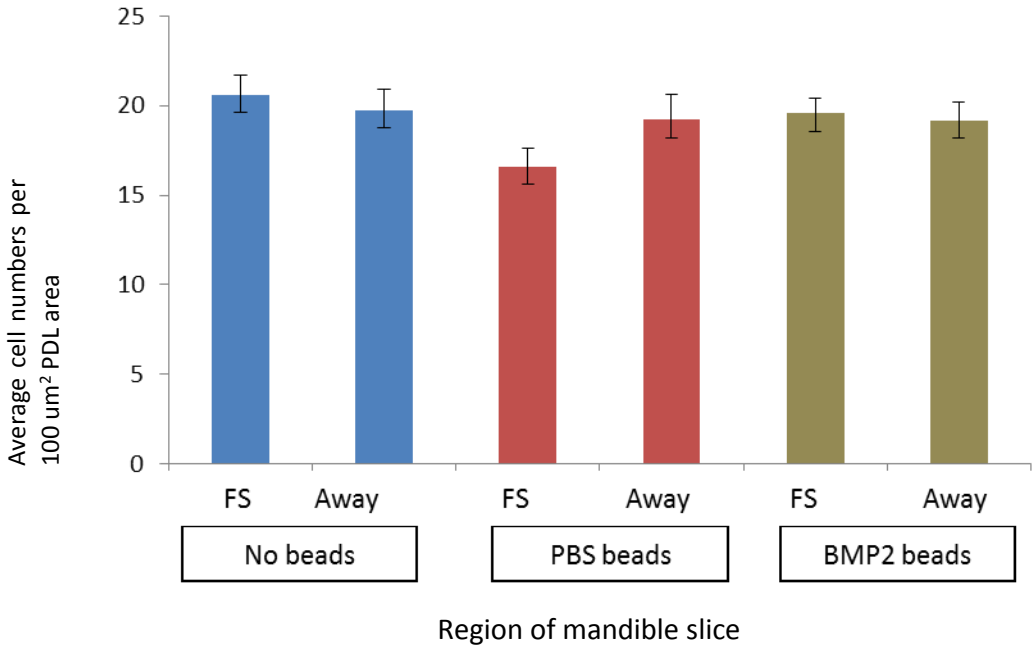


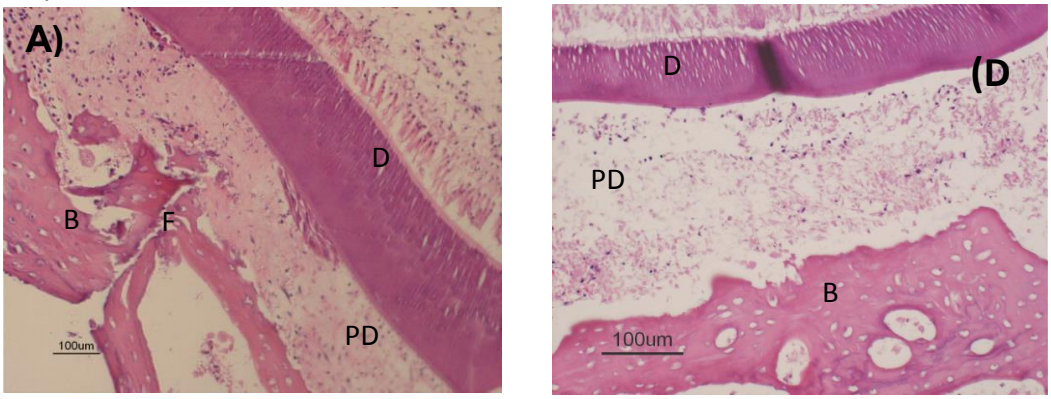
Figure 2- 10: Comparison of average cell numbers per 100 um^2 area within the PDL of fractured mandible slices cultured with no beads, PBS beads or BMP2 beads for 14 days. FS: fracture site; Away: contralateral, unfracture site. (n=8), Error bars indicate standard error of the mean.

2.3.2.3 Histology of BMP2 stimulated mandible slices for 21 days

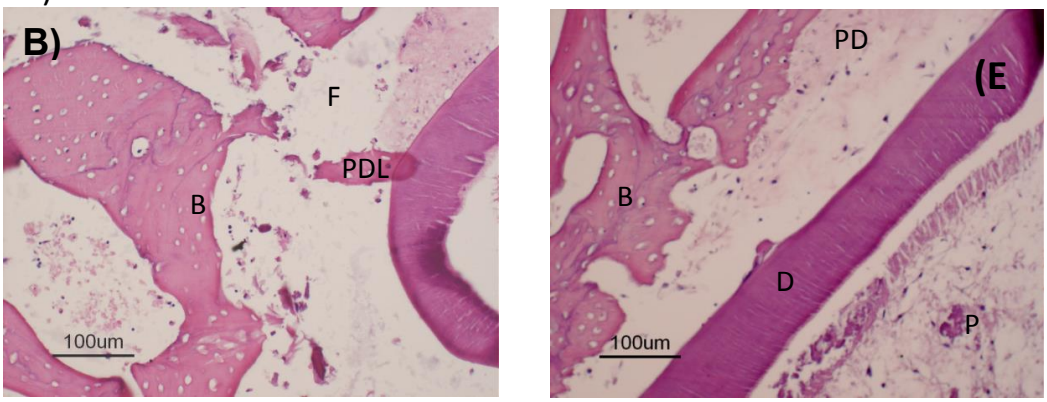
Fractured mandible slices stimulated for 21 days demonstrated minimal loss of cells throughout the section (Figure 2.11). There was no aggregation of cells within the PDL at the site of fracture. Digital image analysis of cell counts (Figure 2.12) at fracture site calculated an average of (16 (±0.6) cells/100μm^2) in no beads slices, (17 (±0.9) cells/100μm^2) in PBS slices, and (15 (±0.7) cells/100μm^2) in BMP2 slices. However digital image analysis of cell counts opposite to the fracture site calculated an average of (19 (±1.2) cells/100μm^2) in no beads slices, (20 (±1.6) cells/100μm^2) in PBS slices, and (21 (±1.0) cells/100μm^2) in BMP2 slices. Statistical analysis, demonstrated no significant difference in average cell numbers within regions of fractured mandible slices ($P>0.05$). There were also no significant differences in average cell numbers between fractured and unfractured sites as well as in areas opposite the site of stimulation between the three groups ($P>0.05$).

Statistical analysis of PDL cell counts (Figure 2.13) of BMP2 stimulated fractured slices demonstrated significant gradual decrease of cell numbers over the 21 days. Results demonstrated that PDL cell counts at BMP2 stimulated fractured sites were significantly higher after 7 days of culture (30 cells/100μm^2) when compared with BMP2 stimulated fractured sites after 14 (20 cells/100μm^2) and 21 days (15cells/100μm^2).

a) No Beads



b) PBS Beads



c) BMP2 Beads

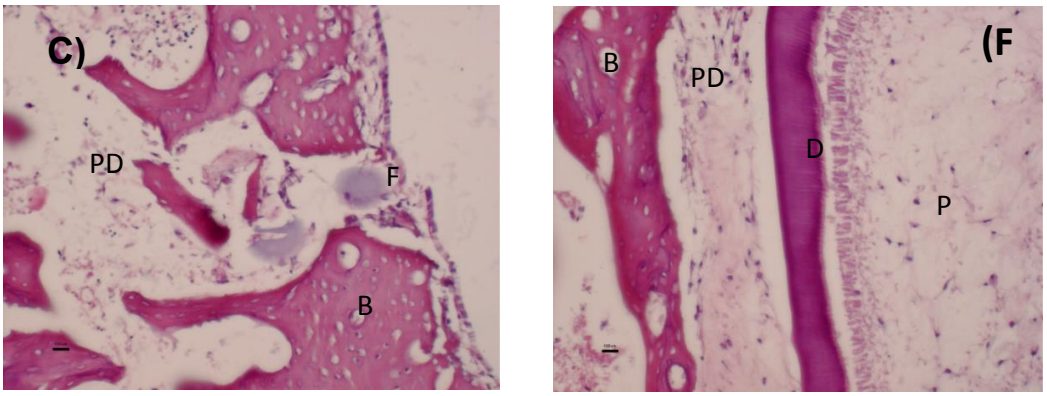


Figure 2- 11: Histological examination of mandible slices cultured with a) no beads, b) PBS beads, c) BMP2 beads for 21 days. (A-C) fractured site, (D-F) contralateral, unfracture site. PDL: periodontal ligament; D: dentine; B: bone; F: fracture site.

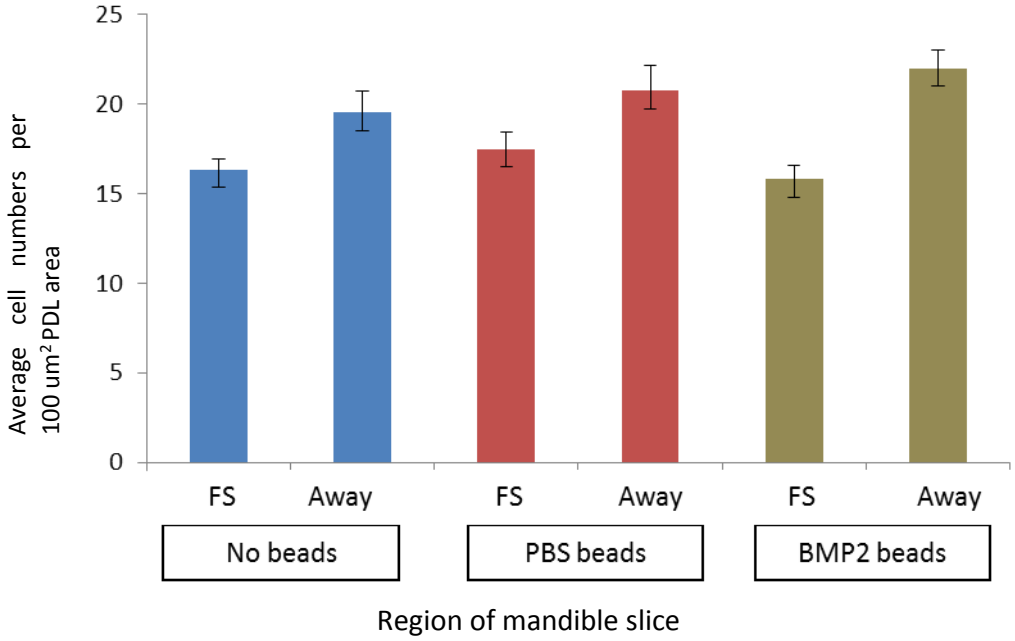


Figure 2- 12: Comparison of average cell numbers per 100 um^2 area within the PDL of fractured mandible slices cultured with no beads, PBS beads or BMP2 beads for 21 days. FS: fracture site; Away: contralateral, unfracture site. (n=8), Error bars indicate standard error of the mean.

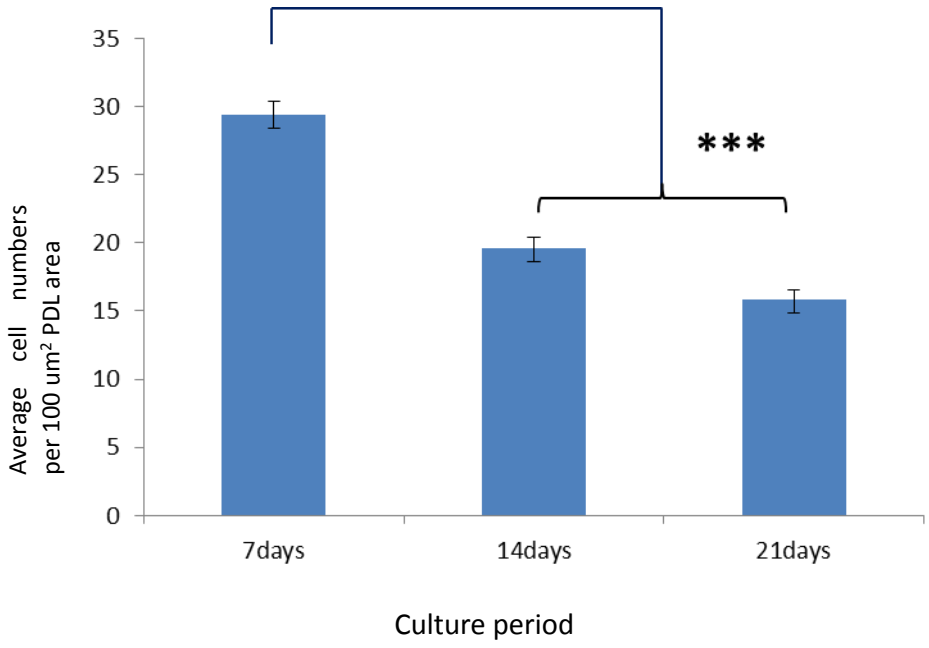


Figure 2- 13: Comparison of average cell numbers per 100 um^2 area within the PDL of fractured mandible slices cultured with BMP2 beads for 7, 14 and 21 days.
*****P<0.01, Error bars indicate standard error of the mean.**

2.3.3 Immunohistochemical characterisation

2.3.3.1 PCNA immunolocalisation

Immunohistochemical staining was used to assess expression of PCNA at fracture site (Figure 2.14). Cells exhibiting positive expression for the proliferation marker PCNA could be observed within the PDL of mandible slices cultured without beads and mandible slices cultured with PBS beads and BMP2 beads at day 7, 14 and 21. Insignificant amount of positive expression could be detected within the cells of the pulp. Negative controls where primary antibodies were omitted demonstrated only the methyl green counterstain with no non-specific staining.

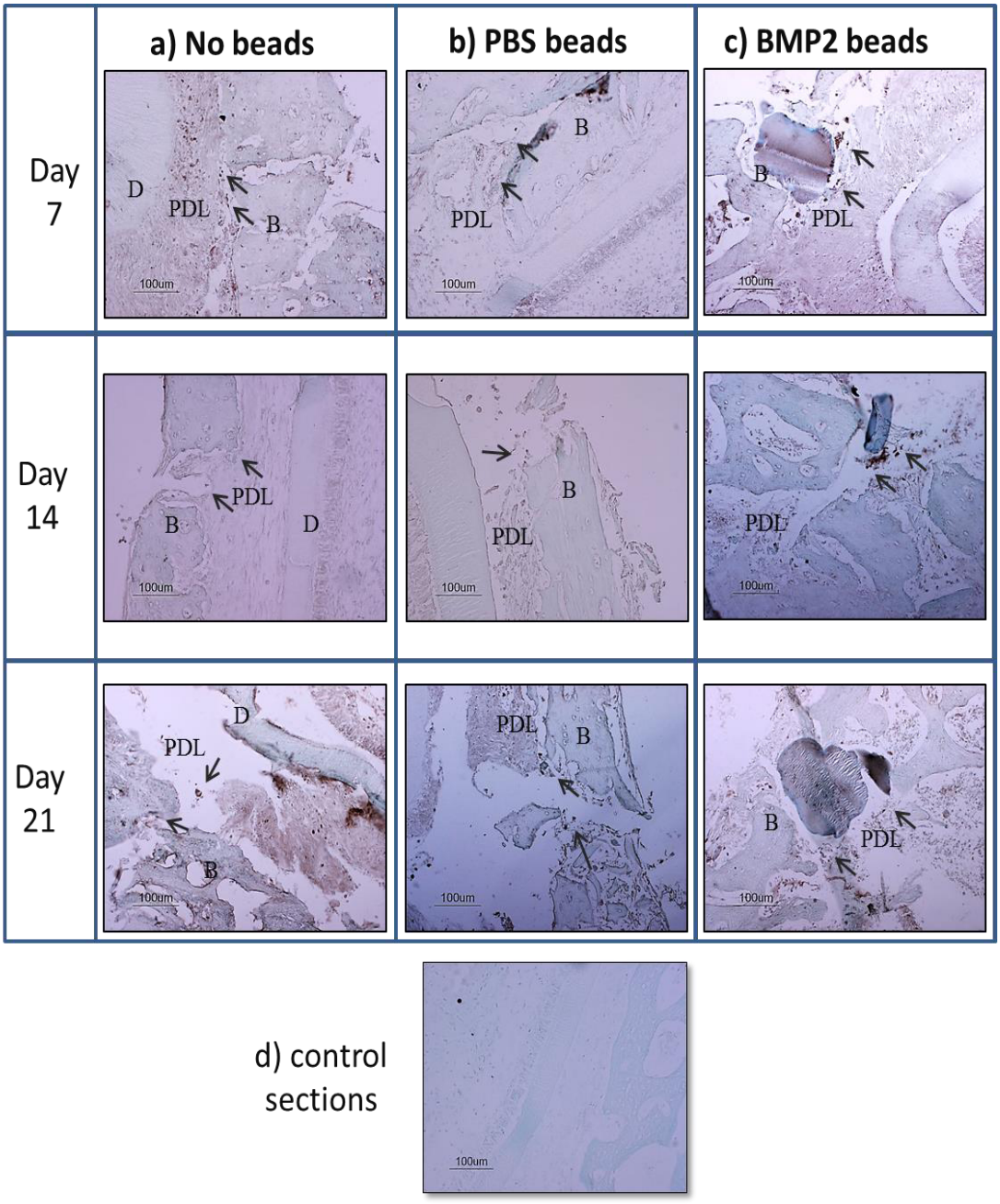


Figure 2- 14: PCNA immunohistochemistry of fractured mandible slices cultured with a) no beads, b) PBS beads, c) BMP2 beads for 7, 14 and 21 days. Arrows indicate PCNA immunopositive cells at fracture site. PDL: periodontal ligament; B: bone; D: dentin. (d) control sections where primary antibody was excluded and replaced with TBS.

2.3.3.2 Osteopontin and Osteocalcin immunohistochemistry

Cells exhibiting positive expression for the bone marker, osteopontin (OPN) (Figure 2.15) and Osteocalcin (OCN) (Figure 2.16) could be observed at fracture site within the PDL of mandible slices cultured without beads and mandible slices cultured with PBS beads and BMP2 beads at day 7. Mandible slices cultured with BMP2 beads demonstrated positive expression of OPN and OCN at day 14 and 21 while control mandible slices that cultured without beads or with PBS beads demonstrated no expression to OPN and OCN at day 14 and 21. Negative controls where primary antibodies were omitted demonstrated only the methyl green counterstain with no non-specific staining (Figure 2.15 and 2.16).

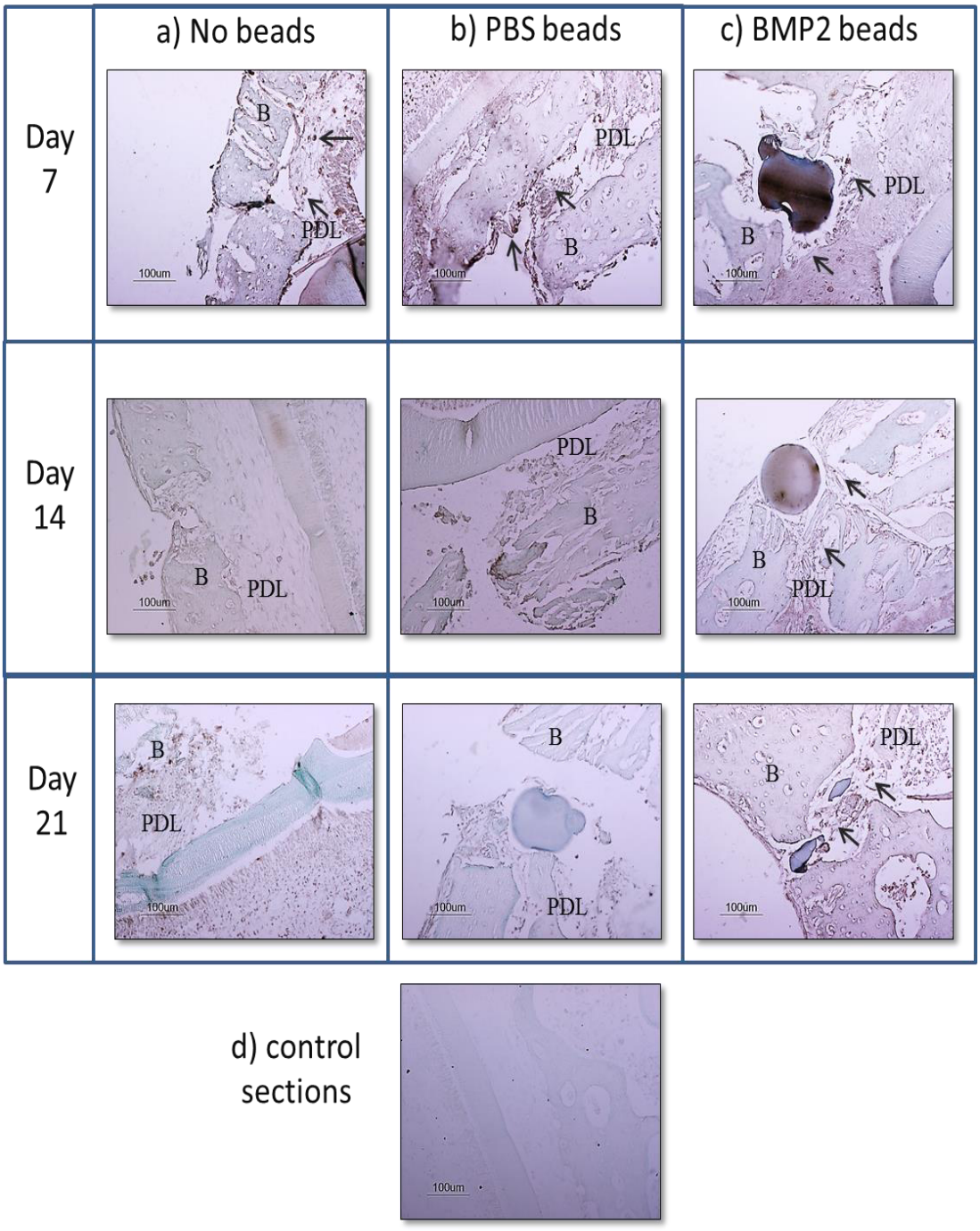


Figure 2- 15: Osteopontin immunohistochemistry of fractured mandible slices cultured with a) no beads, b) PBS beads, c) BMP2 beads for 7,14 and 21 days. Arrows indicate OPN immunopositive cells at fracture site. PDL: periodontal ligament; B: bone; D: dentin. (d) control sections where primary antibody was excluded and replaced with TBS.

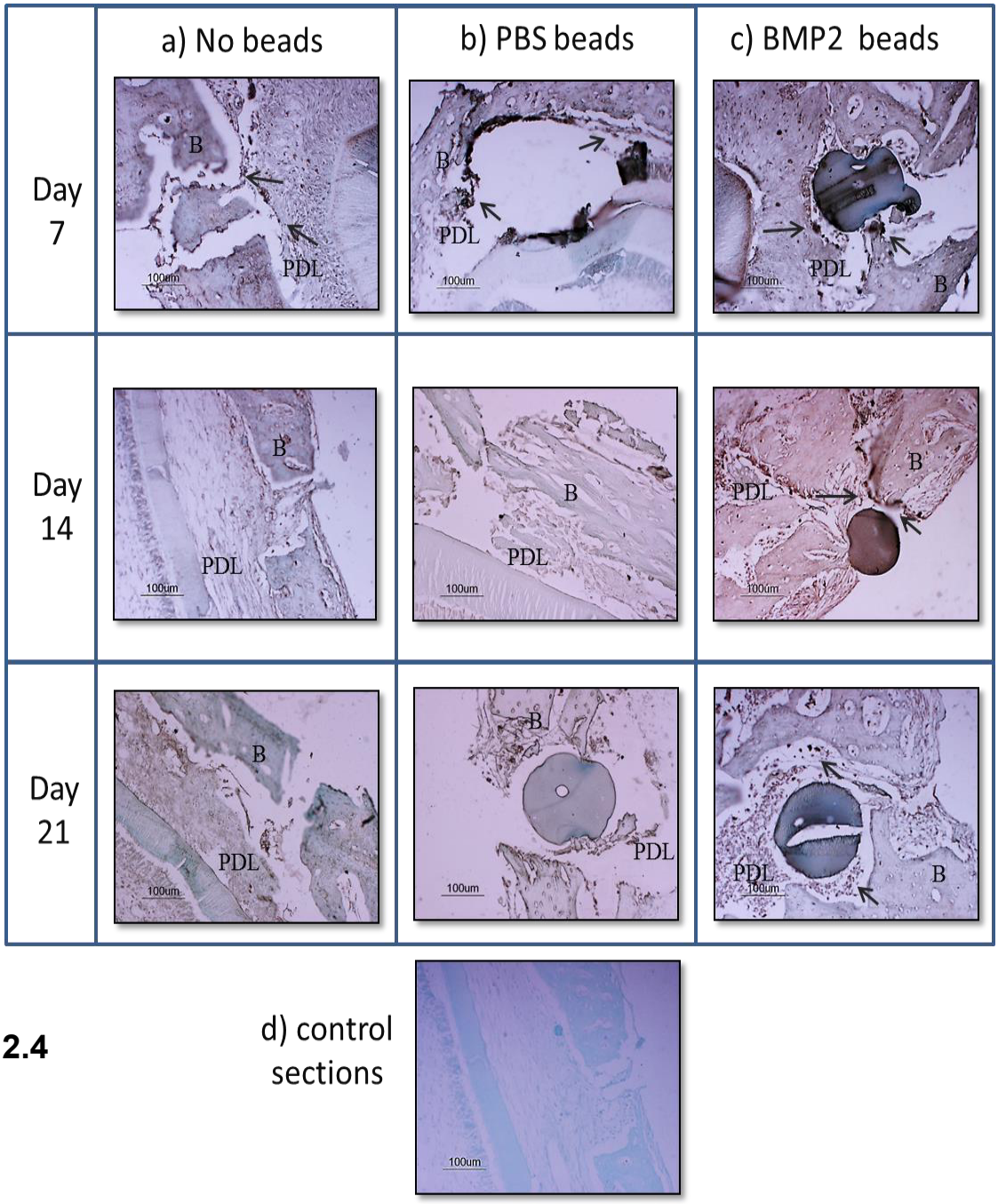


Figure 2- 16: Osteocalcin immunohistochemistry of fractured mandible slices cultured with a) no beads, b) PBS beads, c) BMP2 beads for 7,14 and 21 days. Arrows indicate OCN immunopositive cells at fracture site. PDL: periodontal ligament; B: bone; D: dentin. (d) Control sections where primary antibody was excluded and replaced with TBS.

Discussion

A major problem towards understanding optimal repair processes is the lack of a suitable model system for studying aspects of bone regeneration. An attractive alternative to *in vitro* and *in vivo* models may be *ex vivo* organ culture models (Smith et al. 2010; Wan Hassan et al. 2012; Al-Daghreer *et al.* 2013; Colombo et al. 2015). Data obtained from the *ex vivo* mandible model in this chapter have demonstrated a novel study where mandible slices can be fractured and cultured for up to 35 days without sustaining excessive damage to the cells and tissues adjacent to the site of fracture. Fractured rat mandible slices were also successfully stimulated with topical application of BMP2 via agarose beads and the model demonstrated an osteoinductive response to the applied growth factors.

Fractured rat mandible slices were cultured for up to 35 days with general maintenance of tissue architecture and cellular morphology during this time. However, digital image analysis of cell count of these mandible slices revealed gradual decrease in cell numbers across the culture period. Results demonstrated that the culture model is considered successful for 14 days, suggesting that the model is ideal to study early repair processes, as well as study cells ability to synthesize and secrete matrix during bone regeneration. The decrease in cell numbers which was seen throughout the tissue in both fractured and non fractured site, is probably due to cell death within the PDL across the extended time of culture, a problem often encountered during the culture of cells and tissues for extended time (Arden and Betenbaugh 2004). The gradual decease in cell number across the culture period could be

programmed cell death due to aging of cells over time. Cell viability was tested on *ex vivo* mandible model and results demonstrated that a high level of cellular viability maintained throughout the tissue (Smith et al. 2010). It has been demonstrated that cell survival can be significantly improved by reducing hyperosmolality, while maintaining physiological pH and oxygen homeostasis (Potter and DeMarse 2001). Cell death due to tissue hypoxia may also be a contributing factor to the decrease in cell numbers, which can affect cellular behaviour by reducing cellular proliferation and viability (Park et al. 2002; Utting et al. 2006). It has been reported that hypoxia can affect osteoblast viability (Utting et al. 2006), and osteogenic differentiation by decreasing the expression of the essential transcription factor Runx2 (Park et al. 2002).

Despite the significant loss of PDL cell number after 14 days of culture, previous studies have demonstrated presence of highly viable pulpal cells even after 21 days in the *ex vivo* culture (unpublished work, Cardiff university). This could suggest that PDL cells may be affected with the *ex vivo* culture environment more than other part of the mandible. Cell viability was well maintained after 14 days of culture, therefore the reduction in cell numbers after 14 days of culture might be improved by altering the culture medium, its contents and culture conditions for experiments require long culture period. A study by Potter and DeMarse (2001), demonstrated a way to overcome survival issue of the cells by using sealed culture dishes and a transparent hydrophobic membrane that is selectively permeable to oxygen (O_2) and carbon dioxide (CO_2), and relatively impermeable to water vapour (Potter and DeMarse 2001). Periodontal ligament maintains homeostasis to periodontium via mechanical tensile loading caused

by occlusion and mastication (Davidovitch *et al.* 1988; Lekic and McCulloch 1996). Loss of periodontal ligament function or absence of occlusal stimuli can lead to disuse atrophy of the periodontal ligament (Kanzaki *et al.* 2006). Cyclical tensile force on periodontal ligament cells was shown to increase cell number and induce TGF-β1 stimulation and upregulation of OPG (Kanzaki et al. 2006). Thus stress and strain force are essential to the maintenance of the PDL architecture (Fukui *et al.* 2003; Chiba and Mitani 2004).

BMP2 is a growth factor that plays an important role in stimulating cellular effects during bone formation. It has been demonstrated that BMP2 exerts an osteogenic role via direct stimulation of osteoblasts at the fracture site (Bouletreau et al. 2002). Treatment of mandible slices with BMP2 demonstrated aggregation of cells in fractured sites. This aggregation is likely indicates proliferation of the cells or migration of cells towards site of injury which is probably a cellular response in physiological healing (Lekic and McCulloch 1996; Griffon 2008). Slices cultured in presence of BMP2 beads demonstrated comparable periodontal cell numbers to control mandible culture at fracture sites. This observation suggests that the addition of BMP2 to the fractured mandible could not have role in cell proliferation at site of application. This was confirmed later by the PCNA immunohistochemistry staining which demonstrated expression of PCNA at control and BMP2 stimulated slices. BMP2 has been shown to stimulate proliferation of osteoprogenitors (Yamaguchi *et al.* 1991a; Liu *et al.* 2009). It is possible that the used concentration, the short half-life of BMP2 or the amount of BMP2 released from the beads were not enough to induce cellular effect. However, different studies have been used beads

effectively to deliver a small concentration and a short half-life growth factors such as TGF-β1 (Ganan *et al.* 1996; Sloan and Smith 1999; Smith et al. 2010), FGF-2 (Mori *et al.* 2007), BMP4 and TGF-β2 (Ganan et al. 1996). Although digital image analysis of cell count and PCNA immunohistochemistry demonstrated no specific proliferation effect at BMP2 stimulated slices, immunohistochemistry for the bone markers, OSP and OCN, demonstrated that the main function of BMP2 is to drive the differentiation during repair processes (Lieberman *et al.* 2002a; Hughes et al. 2006b).

BMP2 was delivered to the fractured sites via agarose beads. The beads allow diffusion of the growth factors from within the bead into the surrounding tissue. Such diffusion of growth factors from agarose beads has also been shown in previous studies, both in a tooth slice culture model (Sloan and Smith 1999), and in mandible slice model (Smith et al. 2010). It has been demonstrated that the agarose beads offer a certain degree of protection to the growth factors, keeping the growth factors within the bead itself until they diffuse out into the tissue (Sloan and Smith 1999; Smith et al.2009). Results demonstrated that PDL cell counts at BMP2 stimulated fractured sites were significantly higher after 7 days (Figure 2- 17) when compared with BMP2 stimulated fractured sites after 14 and 21 days of culture. These results may suggest that the BMP2 released from agarose beads elicit a greater response in the first 7 days of culture. This could be due to the short half-life BMP2 (Noh *et al.* 2006; Johnson *et al.* 2009). Growth factors may lose its bioactivity owing to their susceptibility to biodegradation through different pathways, such as denaturation, oxidation or proteolysis (Manning *et al.* 1989; Krishnamurthy and Manning 2002). Previous studies have

demonstrated that the life span of BMP2 is ranging from 4-6 hours (Gruendler *et al.* 2001), which could be the cause of gradual decrease in number of cell over the 21 days. Furthermore, it is possible that the greater effect found at the first 7 days is associated with the biphasic release pattern with an initial burst, followed by a slower release (Yang *et al.* 2002; Ma *et al.* 2015). It has been shown that beads provide rapid diffusion of growth factor after culture (Mori et al. 2007).

Digital image analysis of cell counts demonstrated that there were no significant differences between fractured mandible slices stimulated with BMP2 beads and the control slices. Insertion of beads within the fracture site enabled slight protrusion of the beads into the PDL tissue. It is likely that size of the bead and the space occupied with beads may cause interference during histological examination of the fracture site. It has been demonstrated that due to existent and the size of FGF2- bead, view of the cells was blocked under the microscope (Mori et al. 2007). The authors demonstrated that using polyhedra, which was very small in size, to deliver growth factor provided precise and prolonged growth factor stimulation more than the bead (Mori et al. 2007). Different methods have been studied to deliver growth factor clinically and experimentally all aim to enhance and accelerate functional bone formation. Ideal carrier, as reviewed by Vo *et al,* should be biocompatible, site specific, have local regulation/retention ability and biodegradable for complete tissue regeneration (Vo *et al.* 2012). Affi-gel beads are not biodegradable and occupy adequate size inside the tissue but it was used in this study to deliver and investigate the effect of exogenous application of BMP2 on organotypic fractured mandible model.

Immunohistochemical staining was carried out to characterise the cells residing in areas adjacent to the site of fracture and to assess cellular behaviour after exogenous application of BMP2 beads. Cellular behaviour assessed by investigating the expression of proliferation markers (PCNA) and the bone markers (osteocalcin and osteopontin). Results from immunohistochemical staining demonstrated that cells exhibiting positive expression for the proliferation marker PCNA could be observed within the PDL of mandible slices cultured without beads and mandible slices cultured with PBS beads and BMP2 beads at day 7, 14 and 21. This suggests that the expression of PCNA by resident cells were not directly related to presence of BMP2. It is possible that this may be a physiological repair in response to the fracture alone (Li *et al.* 2002). A fracture can stimulate release of local regulatory factors such as growth factors which can stimulate cellular proliferation and hence express PCNA after fracture (Iwaki *et al.* 1997; Einhorn 1998). Unstimulated fractured mandible model was also shown to express PCNA due to availability of a highly proliferative fibroblasts, osteoprogenitors, and mesenchymal stem cells at periodontal ligament and bone (Smith et al. 2010). The *ex vivo* mandible model lacks of blood supply, and lacks the continuous oxygen supply to the site of injury and therefore it is likely that this hypoxia induced PCNA expression via release of VEGF and TGF-b1 stimulated by hypoxia (Steinbrech *et al.* 1999; Warren *et al.* 2001; Lynam *et al.* 2015). Digital image analysis of cell number at fracture site demonstrated no significant differences in cell number between treated and untreated mandible slices which confirmed also by immunohistochemical staining of PCNA where it detected at treated and control mandible slices.

Osteocalcin and osteopontin were positively expressed within the cells PDL in stimulated and unstimulated fractured mandible slices at day 7 suggesting that this expression was not related to BMP2 stimulation. It had been demonstrated that PCNA, alkaline phosphatase and OPN were expressed within fractured mandible slices without stimulation (Smith et al. 2010). It is likely that the cells expressing OPN and OCN at day 7 are osteoprogenitors, or fully differentiated osteoblast cells remained in the mandible from the start of culture. Furthermore, expression of OPN and OCN observed at day 7 is early to be consider as an active differentiation occurred in response to the fracturing of the mandible alone, as OPN and OCN are not an early osteogenic marker (Ivanovski *et al.* 2001). In contrast, mandible slices cultured with BMP2 beads demonstrated positive expression of OPN and OCN at day 14 and 21 but not in control mandible slices cultured with PBS beads or without beads. It is possible that the fracturing of mandible slices has initiated a reparative response which during extended culture has resulted in increased expression of OPN and OCN, not observed in earlier time points. OPN is up-regulated at sites of inflammation and tissue remodelling (O'Brien *et al.* 1994; Liaw *et al.* 1998), and serves as a chemotactic molecule to recruit inflammatory cells to the site of injury, mediating migration, adhesion, and survival of many cell types (Lund *et al.* 2009; Kahles *et al.* 2014). Moreover, both osteocalcin and osteopontin have been shown to have the ability to form complexes together via calcium bridges (Ritter *et al.* 1992). If the bone lacks either osteopontin, osteocalcin or both, then the bone will have propensity to fracture due to altered micro-damage morphology and reduced crack propagation toughness (Poundarik *et al.* 2012; Nikel *et al.* 2013). Both osteocalcin and osteopontin play critical role in bone mineralization which takes place at later stage of the bone repair process and is enhanced by

osteogenic growth factors such as BMP2 (Luong *et al.* 2012; Sun *et al.* 2015). This could explain expression of osteocalcin and osteopontin at day 7 in control and test groups as an early stage of bone repair process while expressing of osteocalcin and osteopontin at day 14 and 21 in fractured mandible slices stimulated with BMP2 beads.

Although there was significant decrease in cell number after 14 days of culture, the *ex vivo* model proved to be a valid model which enables the culture of more than one cell and tissue types at the same time overcoming the complex interactions that occur in *in vivo* models as well as reducing costs and ethical issues associated with *in vivo* models. In this study BMP2 was applied exogenously to the fractured mandible model, and its effect on bone repair was investigated. Exogenous application of BMP2 induced bone repair by stimulating bone differentiation and mineralization. There are no doubts about the importance of growth factors during bone repair process, especially BMP2 which is approved for clinical therapies. However, there are some concerns associated with growth factor therapy such as the dose, time of application and the agonist and antagonist action between exogenous and endogenous growth factors. Thus investigation the effect of endogenous growth factors bounded within the bone matrix may be essential to improve clinical outcome of bone surgeries.

Chapter 3.

STIMULATION OF *EX VIVO* FRACTURED MANDIBLE MODEL WITH CHEMICALLY RELEASED ENDOGENOUS GROWTH FACTORS

3.1 Introduction

Bone regeneration is driven by different growth factors, most of which have been extensively investigated in demonstrating enhanced bone regeneration. Several experimental and clinical trials have investigated the safety and efficacy of the use of exogenous growth factors as potential supplements for enhancing bone regeneration (Friedlaender et al. 2001; Carragee *et al.* 2011). Although some growth factors have been licensed for clinical use in bone regeneration, several issues need to be further examined to bring the most safe and effective medications into clinical practice. These include identification of the optimum dosage required to elicit a therapeutic response (Dimitriou et al. 2011) and understanding the synergistic action of using 'cocktail' of growth factors that have shown significant promising results in preclinical and early clinical investigation (Nauth et al. 2010; Dimitriou et al. 2011).

Growth factors are natural functional proteins that are released or activated during development and embryogenesis (Hardy and Spanos 2002). They are also essential during wound healing or tissue regeneration (Raja *et al.* 2009). Numerous studies have focused to use different growth factors to accelerate and

mediate the healing process into one that will produce tissue regeneration (Hughes et al. 2006b; Ai-Aql *et al.* 2008; Dinh *et al.* 2015). It has been reported that a combination of different growth factors may enhance tissue regeneration more efficiently and effectively than single growth factors (Huang *et al.* 2005; Kempen *et al.* 2009; Suarez-Gonzalez *et al.* 2014; Dinh et al. 2015). Animal models have been utilised to investigate the effects of the addition of PDGF and IGF on periodontal regeneration *in vivo*. The results demonstrated that the addition of PDGF and IGF into debrided lesions of experimentally induced periodontitis stimulated regeneration of 50% of lost periodontal attachment (Rutherford *et al.* 1992) and significantly increased new bone and cementum formation histologically and clinically (Lynch *et al.* 1991). It has also been demonstrated that exogenous application of TGF-β to bone fractures can increase callus formation and strength, and bone formation, either on its own or in conjunction with IGF-1 (Srouji et al. 2004; Lee et al. 2006). It has also been reported that combination of BMP2 and TGF-β3 co-delivered to critical sized femoral defects in rats via poly(L-lactide-co-D,L-lactide) scaffolds demonstrated an increase in bone formation compared to nontreatment defects (Oest *et al.* 2007). Recent studies have shown that the combination of angiogenic and osteogenic factors may stimulate bone regeneration. Dual release growth factor (VEGF & BMP2) fabricated scaffolds have been shown to enhance the bone regenerative capability in a critical sized femoral defect by releasing VEGF followed by BMP2 from alginate scaffold (Kanczler *et al.* 2010).

Although several studies demonstrated an improvement in the bone regeneration process by a single or combined drug therapy, *in vivo* bone

regeneration is the result of a complex interplay between the various endogenous growth factors. Currently, numbers of clinical therapies are benefit from utilising endogenous matrix-bound growth factors rather than addition of exogenous growth factors. Ethylenediaminetetraacetic acid (EDTA) and citric acid solutions are commonly used in clinical dentistry as detergents to remove the smear layer and expose dentine surfaces (Pitoni et al. 2011) as well as decalcifying agents for calcified narrow roots. It has been reported that these acids are also able to demineralise the human dentine matrix releasing sequestered growth factors such as TGF-β1 from the treated surfaces (Zhao et al. 2000; Tomson *et al.* 2007). Calcium hydroxide, Dycal®, is also commonly used in clinical dentistry as a pulp capping agent to facilitate the formation of reparative dentine after dentine-pulp complex injury (Horsted-Bindslev *et al.* 2003; Smith *et al.* 2008), by driving release of growth factors (Tziafas et al. 2000). Mineral trioxide aggregate (MTA) is also used in clinical dentistry to induce regeneration of dentin, cementum and bone by formation of mineralised tissue in the presence of moisture. Like EDTA and calcium hydroxide, MTA has found to release growth factors, such as TGF-β1 from dentine matrix (Tomson et al. 2007).

Similar to dentin, the extracellular matrix of bone contains a reservoir of different growth factors. These growth factors are synthesized and secreted by cells resident in the bone and become bound to the ECM (Hossner 2005). For example, it has been reported that decorin and biglycan play an important role in regulating the sequestration of matrix-bound growth factors, such as TGF-β1 (Bianco *et al.* 1990). Both decorin and biglycan can bind to TGF-β1 with high

affinity, protecting TGF-β1 from degradation and modulating their sequestration and release from the matrix (Baker et al. 2009). Bound growth factors remain protected in their bio-active state until the matrix is resorbed and degraded during injury (Baker et al. 2009). Following bone degradation, these growth factors are released from their association and may then diffuse to the adjacent cells and participate in the reparative processes leading to optimal regeneration of the bone (Hughes et al. 2006b). The previously mentioned chemicals have been demonstrated to solubilise growth factors from within the mineralised matrix of an *in vitro* bone slab model (Smith et al. 2011). These chemicals solubilise growth factors by matrix demineralisation, local matrix dissolution or chelating calcium ions present within the mineralised matrix (De-Deus *et al.* 2008a). It is unclear whether this simply releases bound growth factors, or if a more complex mechanism is responsible for the release of the matrix-bound growth factors. This chapter aims to investigate the cellular responses of stimulated *ex vivo* fractured mandible model with chemically released endogenous growth factors. Understanding the effect of these chemicals modalities will facilitate development of novel treatments for bone regeneration which may overcome some of the problems associated with current clinical therapies using exogenously added growth factors.

3.2 Materials and Methods

3.2.1 Chemical stimulation of fractured mandible slices

Mandibles slices were prepared and fractured as described in (section 2.2.1). Prior to culture, 10µl of appropriate chemical treatments were applied to the fractured site using microcaps pipettes (Fishersci,UK) (Figure 3.1, A). Slices were treated for 5, or 15 min with either 17% EDTA (pH 7.2), 0.02 M calcium hydroxide (pH 11.7), 10% citric acid (pH 3) or PBS (pH 7.4) as a negative control, followed by washing in PBS twice for 2 min. During treatment times, mandible slices were partially submerged in media, so that the slice remained hydrated. Chemically treated fractured mandible slices were either fixed immediately in 10% (w/v) formalin or cultured for 7 days using Trowel type cultures as described in (section 2.2.1.1). Prior to histological examination, both cultured and uncultured mandible slices were fixed and demineralised for haematoxylin and eosin (H&E) tissue staining as described in (section 2.2.2.1). Cellular characteristics within the fracture site in response to released growth factors were assessed also by immunohistochemistry. Immunohistochemical staining was carried out using a Vectastain ABC peroxidase kit (Vector Laboratories Ltd) on cultured mandible slices treated with 17% EDTA (pH 7.2) and 10% citric acid (pH 3) for 15 min and 0.02 M calcium hydroxide (pH 11.7) and PBS for 5 min as described in (section 2.2.2.2). Proliferating cell nuclear antigen (PCNA) primary antibody, mouse anti-rat (Santa Cruz Biotech, Santa Cruz, CA,USA) diluted 1:20 in 1% BSA/TBS, bone sialoprotein (BSP) primary antibody, bone marker, rabbit anti-rat BSP (Santa Cruz Biotech, Santa Cruz, CA,USA), diluted 1:50 in 1% BSA/TBS and osteopontin (OPN) primary antibody,

bone marker, goat anti-rat OPN (Santa Cruz Biotech, Santa Cruz, CA,USA), diluted 1:250 in 1% BSA/TBS were used as an indicator of cellular proliferation and bone mineralisation respectively. Sections were evaluated microscopically on an Olympus AX70 light microscope. Photographs were taken using a Nikon DXM digital camera and ACT-1 imaging software. A total of 5 mandible slices were used per treatment, for each marker. Number of positive cells for each of the markers was automatically counted within the periodontal ligament using Image ProPlus software (Media Cybernetics Inc, USA). 100μm^2 areas within five areas proximal to the fracture site were counted per section.

3.2.2 Analysis of released growth factors from culture medium supernatants

Supernatants were collected from treated fractured mandible slice cultured for 7 days. Appropriate dialysis tubes were used to diffuse dissolved solutes across a selectively permeable membrane. The dialysis container was kept overnight at 4^oC and water was changed 3 times/day for 3 days. Following dialysis, samples were lyophilized by keeping them overnight at -20^oC then freeze-dried in freeze dryer (Labconco, USA) overnight. Concentrated media were stored at -20^oC prior to use in ELISA. After lyophilisation, samples were diluted with 800 µL PBS. Protein concentration was measured in each sample using a BCA kit (Thermo scientific,Uk). TGF-β1 and VEGF levels were quantified according to the manufacturer's specifications using an ELISA kit from R&D Systems (R&D Systems, Inc., USA). BMP2 levels were also quantified using an ELISA kit from (Antibodies-Online, USA). All assays were carried out in triplicate from 3 individual samples.

3.2.3 Immunogold labelling of released growth factors

Immunogold labelling was carried out to label exposed TGF-β1, BMP2 and VEGF from fractured mandible slices. Mandible slices were prepared as described in (section 2.2.1). Slices were then completely fractured by placing pressure onto the central portion of the lingual and buccal plate using a sterile scalpel blade (Figure 3.1, B) under dissecting microscope (Bausch and Lomb, UK). Fractured mandible slices were then treated individually by dipping in 50µl of either 17% EDTA (pH 7.2), 10% citric acid (pH 3) for 15 min or 0.02 M calcium hydroxide (pH 11.7), PBS for 5 min. After each treatment time, slices were gently washed in PBS twice for 2 min to remove the chemical treatment. After chemical treatment, slices were immunostained for surface exposed TGF-β1, BMP2 and VEGF using an Immunogold silver staining protocol and examined by scanning electron microscope imaging (Bayatian et al. 2007).

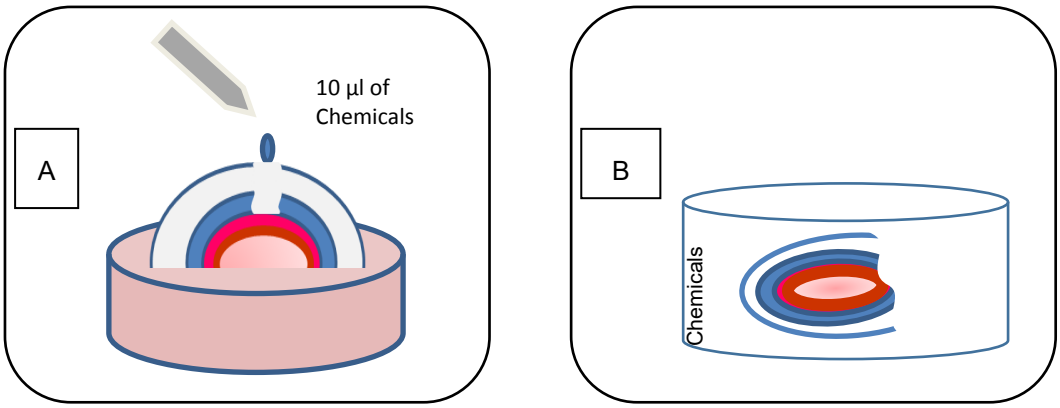


Figure 3- 1: Chemical treatment of fractured mandible slice. (A) Fractured mandible slice treated locally by 10µl of 17% EDTA (pH 7.2), 0.02 M calcium hydroxide (pH 11.7), 10% citric acid (pH 3) and PBS (pH 7.4) as a negative control for 5, or 15 min. (B) Fractured mandible slice treated entirely by dipping in 50µl of the same previous chemical treatments for scanning electron microscope imaging.

Chemically treated mandible slices were blocked for 1 h with 5% goat serum, 10% bovine serum albumin (BSA) in Tris-buffered saline (TBS), followed by buffer wash (1% goat serum, 1% BSA, 0.1% Tween 20 in TBS) for 15 min. Slices were incubated for 2 h with the appropriate primary antibody (Table 3.1) (Santa Cruz Biotech) diluted 1:100 in 1% BSA in TBS followed by twice buffer wash for 5min. As a negative control, the primary antibody was replaced with TBS. Slices were incubated with gold-labelled secondary antibody (BBI diagnostics, Cardiff, UK, particle diameter 30nm, protein 3.6µg/ml; diluted 1:100 in 1% BSA, TBS) for 1 h. Mandible slices were washed with two washes in buffer wash and two washes in distilled water followed by incubation with silver enhancing agent (BBI diagnostics) for 15 min. All mandible slices were then washed in distilled water twice for 15 min and air dried for 24 hrs. Slices were examined by EBT1 scanning electron microscope (SEMTech Ltd, Derbyshire, UK) at a magnification of X550. Using Image ProPlus software, the numbers of immunogold particles were counted within 20µm^2 areas of bone, within five fields of view per slice. A total of 5 mandible slices were used for each time point for each treatment.

Table 3.1: Primary and secondary antibodies used in Immunogold labelling.

Primary Antibody	Source of antibody	Source / species reactivity	Dilution	Secondary Antibody	Source of antibody	Dilution
TGF-β1	Santa Cruz Biotech Sc-146	Rabbit polyclonal antibody	1:100	Gold-labelled anti- rabbit	BBI diagnostics	1:100
BMP2	Santa Cruz Biotech sc-6895	Goat polyclonal antibody	1:100	Gold-labelled anti- goat	BBI diagnostics	1:100
VEGF	Santa Cruz Biotech sc-53462	Mouse monoclonal antibody	1:100	Gold-labelled anti- mouse	BBI diagnostics	1:100

3.2.4 Statistical analysis

Statistical analysis was carried out using the GraphPad InStat 3 software (GraphPad software, Inc, USA). Standard errors of the mean were calculated, and mean values were analysed using one-way ANOVA and Tukey's post hoc test, to analyse differences between treatment types and controls. P values below 0.05 at 95% confidence intervals were held to be statistically significant.

3.3 RESULTS

3.3.1 Histology of fractured mandible slices

3.3.1.1 Histology of uncultured chemically treated fractured mandible slices

Histological evaluation of mandible slices that had not been cultured demonstrated maintenance of tissue architecture throughout the section. The morphological appearance of the mandible slices treated for 5 min (Figure 3.2) were observed to be similar to the mandible slices treated for 15 min (Figure 3.3). The pink eosin staining with the darkly stained round nuclei indicate presence of viable cells within bone, dentine, periodontal ligament and pulp.

Digital image analysis of cell counts (Figure 3.4) of fractured mandible slices treated for 5 min calculated an average of 68 cells/100μm^2 for $Ca(OH)_2$, 52 cells/100μm^2 for EDTA and citric acid and 42 cells/100μm^2 for PBS. However fractured mandible slices treated for 15 min calculated an average of 45 cells/100μm^2 for $Ca(OH)_2$, 60 cells/100μm^2 for EDTA, 69 cells/100μm^2 for citric acid and 46 cells/100μm^2 for PBS. Statistical analysis of average PDL cell counts demonstrated no significant differences between mandible slices treated for 5 or 15 min. Although average PDL cell counts demonstrated high cell numbers in fractured mandible slices treated with $Ca(OH)_2$ for 5 min and with EDTA and citric acid for 15 min, this increase was not significant ($P>0.05$). There was also non-significant difference in average cell numbers within regions of mandible slices opposite the fracture site between all groups, average of 61-72 cells/100μm^2 ($P>0.05$). There was a non-significant increase in average cell

numbers within regions of mandible slices opposite the site of stimulation when compared with fracture sites average of 42-69 cells/100μm^2 (P>0.05).

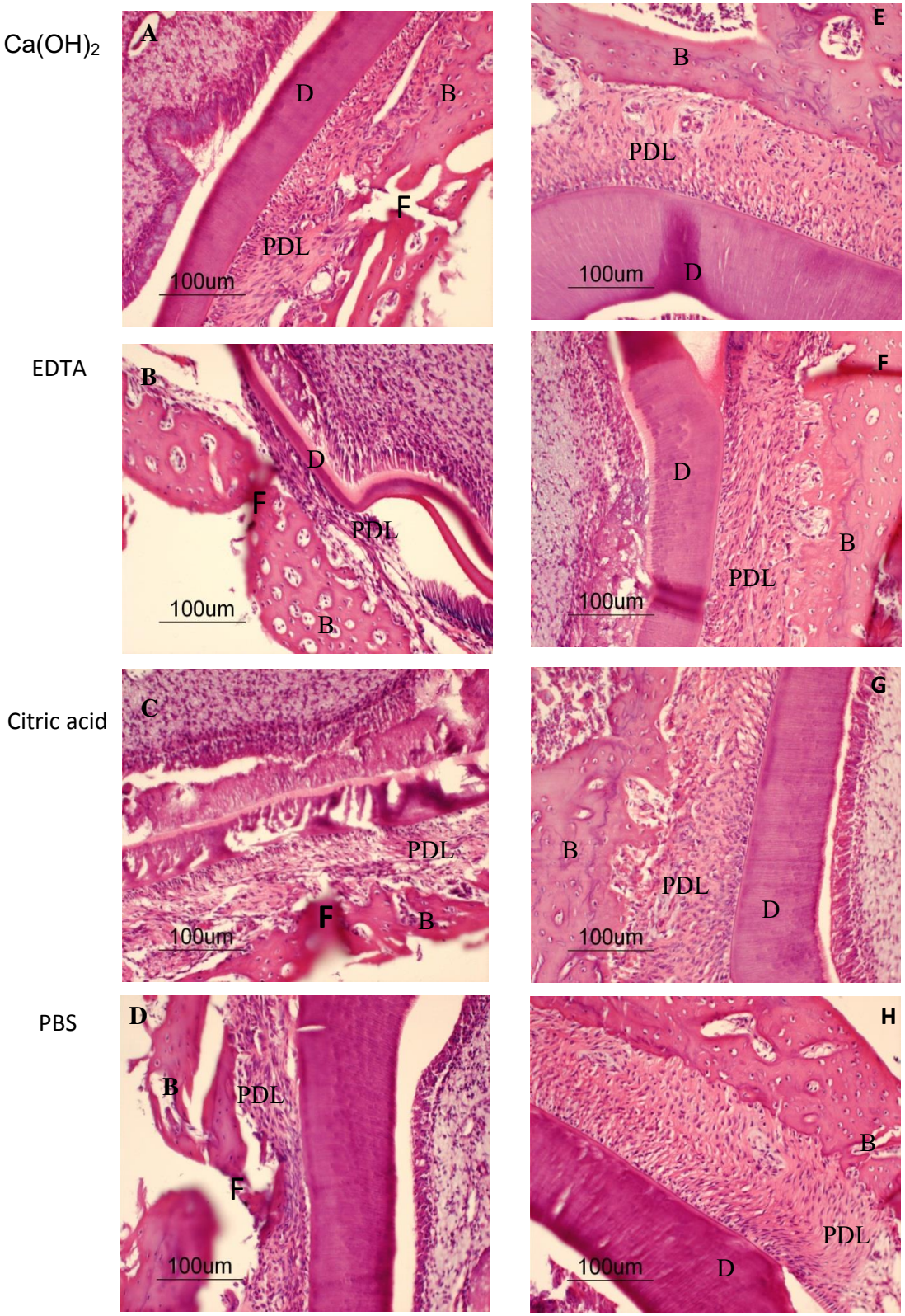


Figure 3- 2: Histological examination of uncultured fractured mandible slices treated with $Ca(OH)_2$, EDTA, citric acid and PBS for 5 minutes. [A-D] fractured site, [E-H] contralateral site. PDL, periodontal ligament; D, dentine; B, bone; F, fracture site.

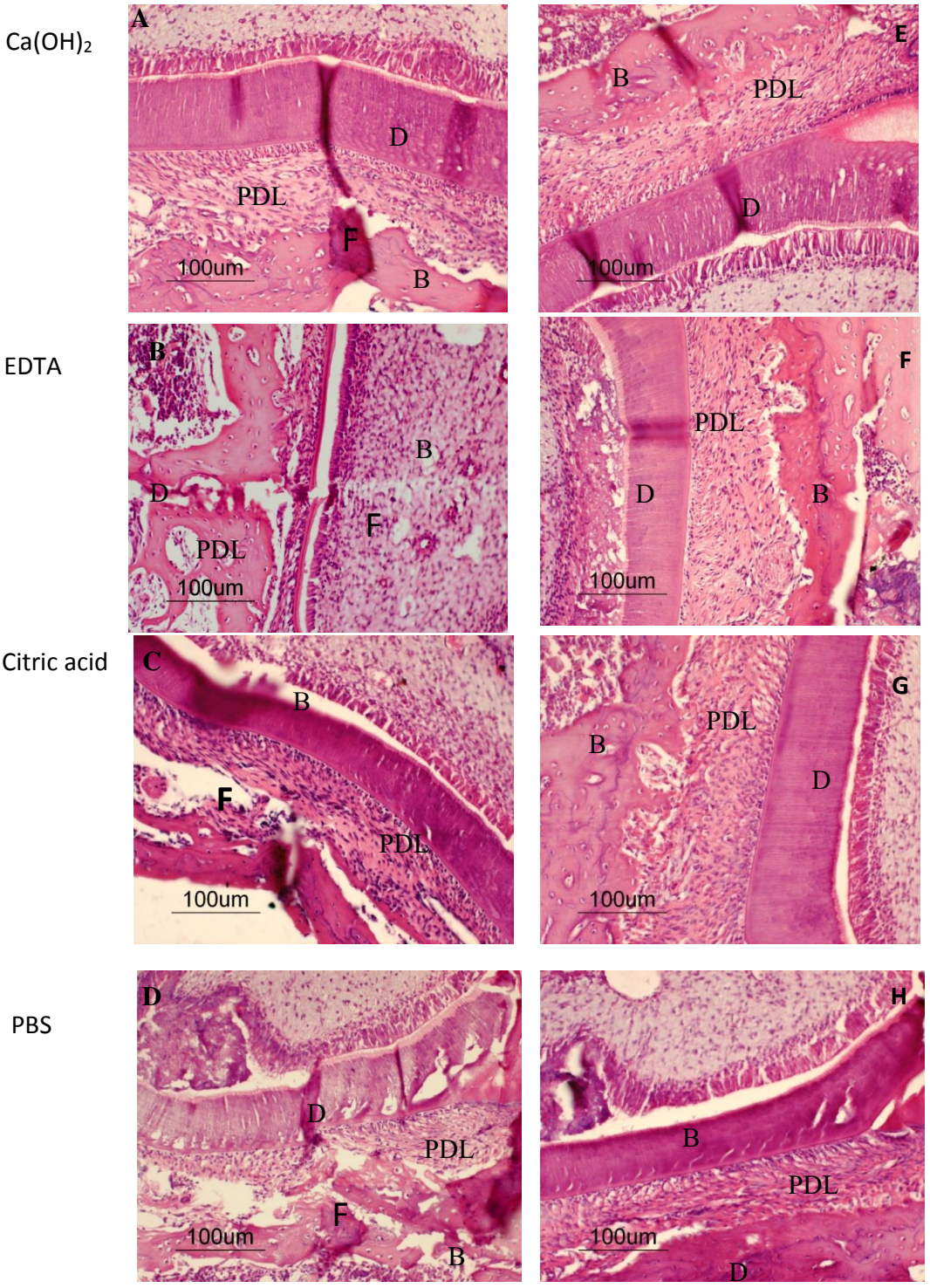


Figure 3- 3: Histological examination of uncultured fractured mandible slices treated with $Ca(OH)_2$, EDTA, citric acid and PBS for 15 minutes. [A-D] fractured site, [E-H] contralateral site. PDL, periodontal ligament; D, dentine; B, bone; F, fracture site.

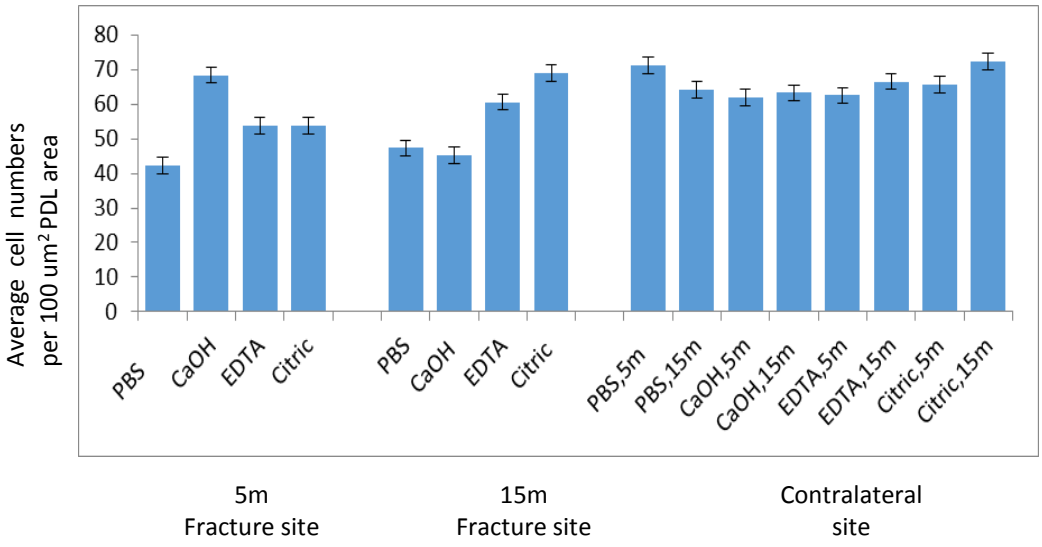


Figure 3- 4 Comparison of average cell numbers per 100 um² area within the PDL of uncultured fractured mandible slices treated with $Ca(OH)_2$, EDTA, CITRIC ACID or PBS for either 5 or 15 minutes. (n=4), Error bars indicate standard error of the mean.

3.3.1.2 Histology of cultured chemically treated fractured mandible slices

Histological evaluation of mandible slices that had been cultured for 7 days demonstrated maintenance of tissue architecture throughout the section (Figure 3.5) and (Figure 3.6). The pink eosin staining with the darkly stained, round nuclei indicate presence of viable cells within bone, dentine, periodontal ligament and pulp. The morphological appearance of the mandible slices treated for 15 min (Figure 3.6) were observed to be similar histologically to the mandible slices treated for 5 min (Figure 3.5).

Digital image analysis of cell counts (Figure 3.7) of fractured mandible slices treated for 5 min calculated an average of 42 cells/100μm^2 for $Ca(OH)_2$, 20 cells/100μm^2 for EDTA and 19 cells/100μm^2 for citric acid. However fractured mandible slices treated for 15 min calculated an average of 26 cells/100μm^2 for $Ca(OH)_2$, 28 cells/100μm^2 for EDTA, 29 cells/100μm^2 for citric acid and 18 cells/100μm^2 for control PBS treated mandible slices. Statistical analysis of average PDL cell counts demonstrated significant differences between mandible slices treated for 5 or 15 min (Figure 3.7). Average PDL cell counts demonstrated significant increase in cell number at fractured mandible slices treated with $Ca(OH)_2$ for 5 min and EDTA and citric acid for 15 min ($P<0.05$). There was significant increase in average cell numbers at fracture sites of mandible slices treated with $Ca(OH)_2$ for 5 min when compared with mandible slices treated with PBS, EDTA and citric acid ($P>0.001$).There was also no significant difference in average cell numbers within regions opposite the fracture site in all groups ($P>0.05$).

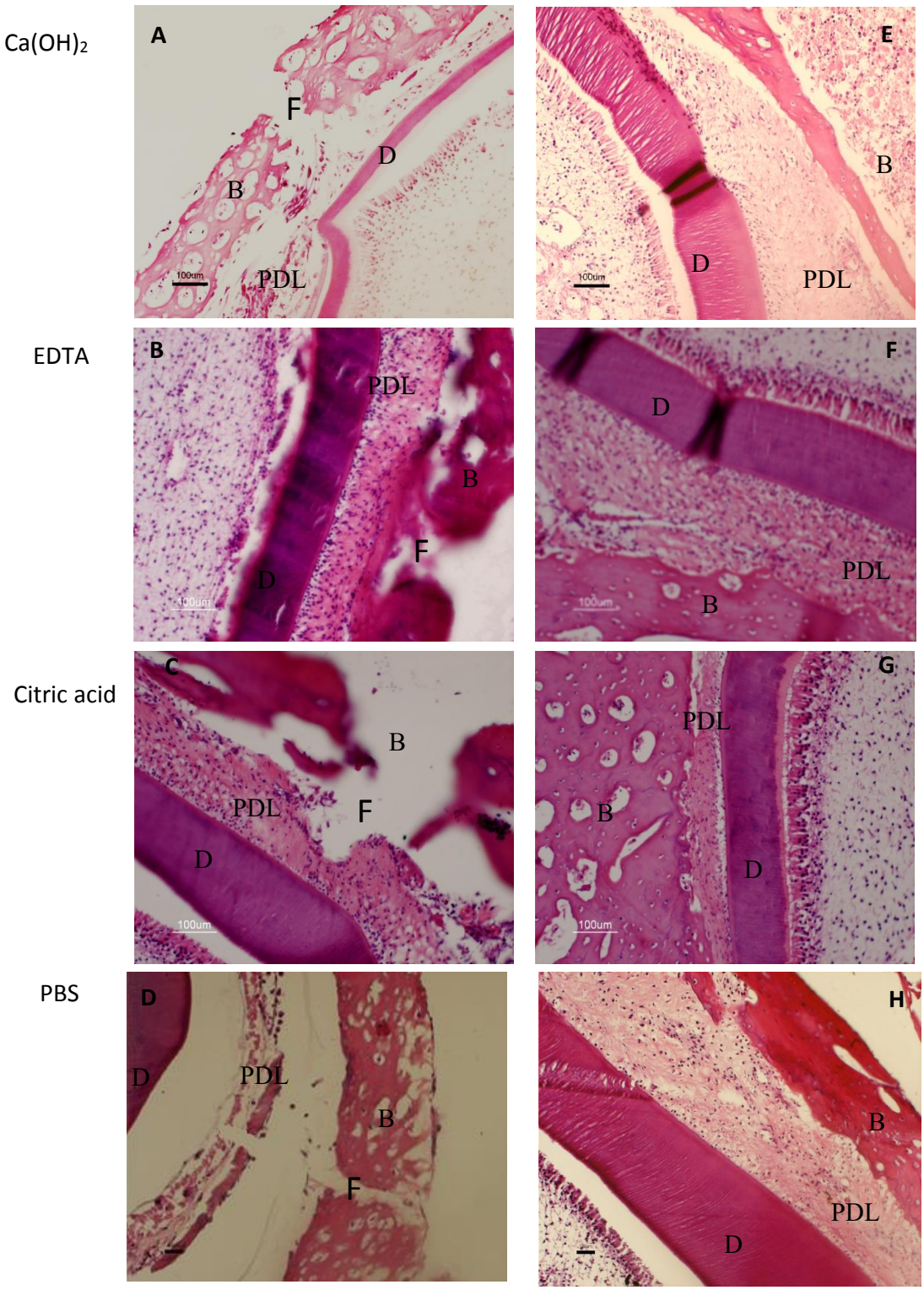


Figure 3- 5: Histological examination of fractured mandible slices treated with $Ca(OH)_2$, EDTA, citric acid and PBS for 5 minutes and cultured for 7 days. [A-D] fractured site, [E-H] contralateral site. PDL, periodontal ligament; D, dentine; B, bone; F, fracture site.

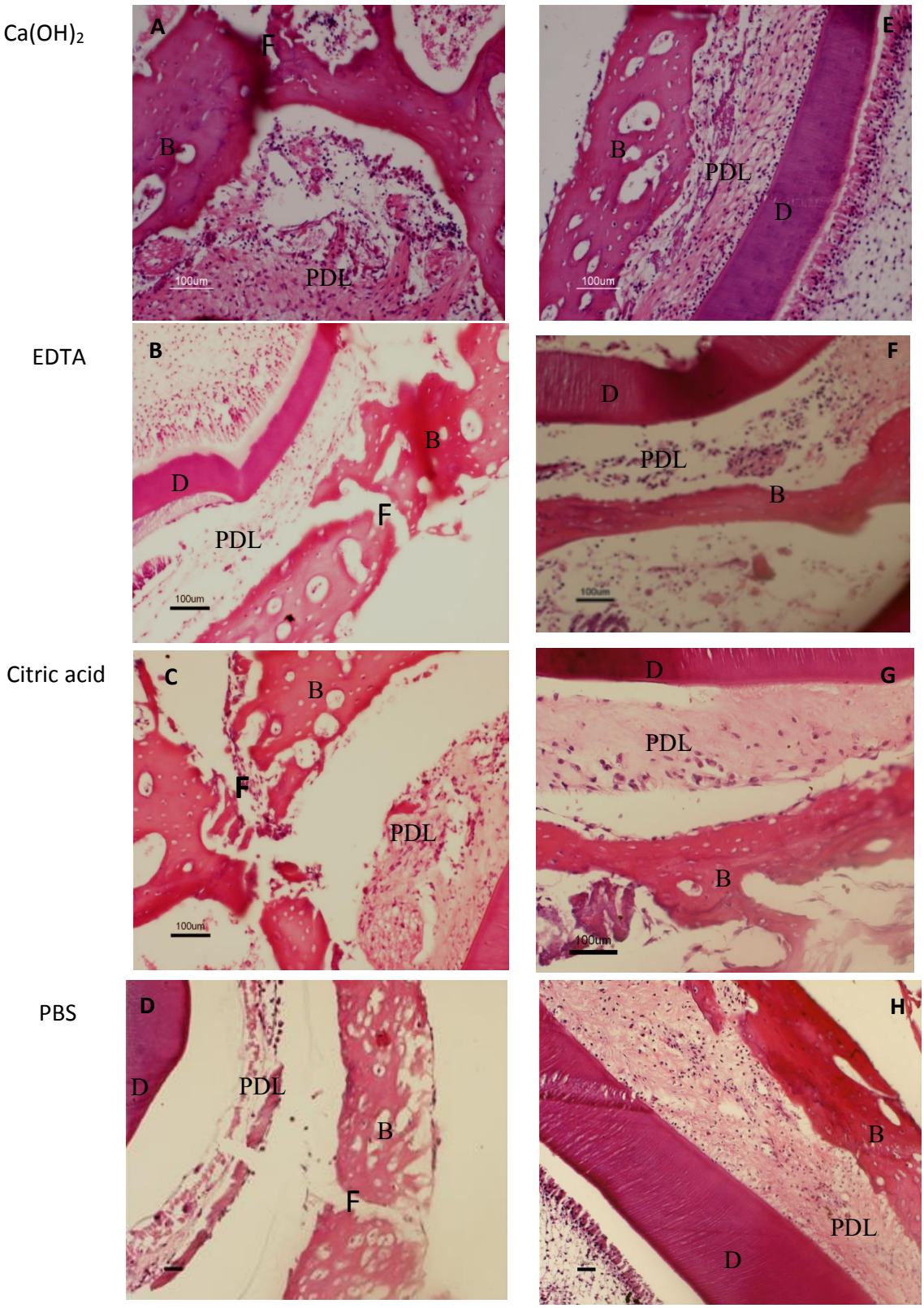


Figure 3- 6: Histological examination of fractured mandible slices treated with $Ca(OH)_2$, EDTA, citric acid and PBS for 15 minutes and cultured for 7 days. [A-D] fractured site, [E-H] contralateral site. PDL, periodontal ligament; D, dentine; B, bone; F, fracture site.

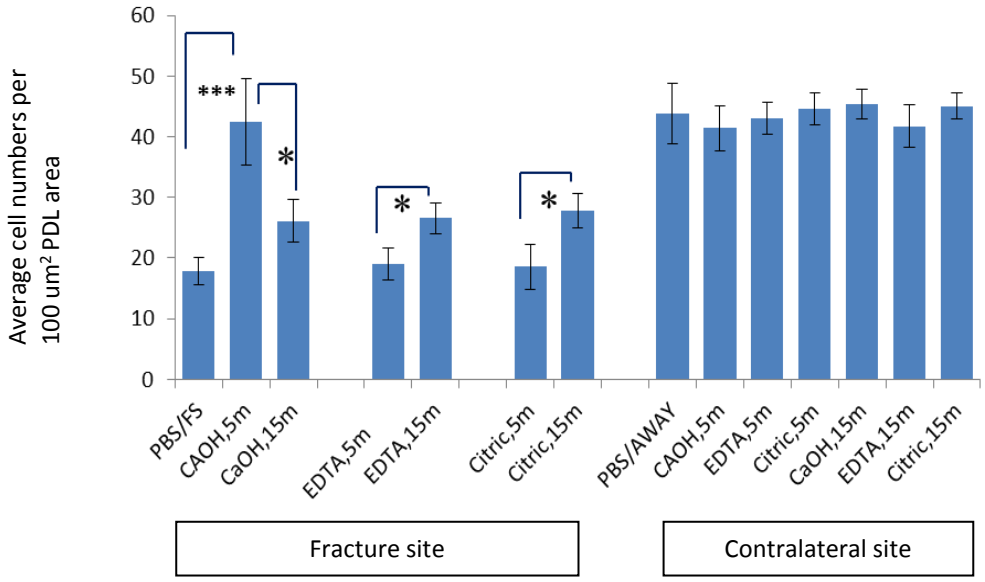


Figure 3-7: Comparison of average cell numbers per 100 um^2 area within the PDL of fractured mandible slices treated for 5 or 15 minutes with $Ca(OH)_2$, EDTA, CITRIC ACID or PBS and cultured for 7 days. ***P<0.001. * P<0.05. n=8, Error bars indicate standard error of the mean.

3.3.2 Immunohistochemical characterisation

Cells exhibiting positive expression for the proliferation marker PCNA (Figure 3.8) and for the bone marker, bone sialoprotein (Figure 3.9) and osteopontin (Figure 3.10) were observed within the PDL of mandible slices treated with calcium hydroxide, EDTA or citric acid, when compared with control PBS treated slices. Negligible positive expression was detected within the cells of the pulp. Negative controls where primary antibodies were omitted demonstrated only the methyl green counterstain with no non-specific staining.

Digital image analysis of immunopositive positive cell counts (Figure 3.11) calculated an average of (6-9 cells/100μm^2) for mandible slices treated with $Ca(OH)_2$ and average of (2-3 cells/100μm^2) for mandible slices treated with EDTA, citric acid or PBS. Statistical analysis of average positive cells demonstrated significant increase in the number of PCNA, BSP and OPN immunopositive cells within the fracture site of the mandible slices treated with calcium hydroxide, when compared with fractured mandible slices treated with EDTA, citric acid or PBS ($p<0.001$).

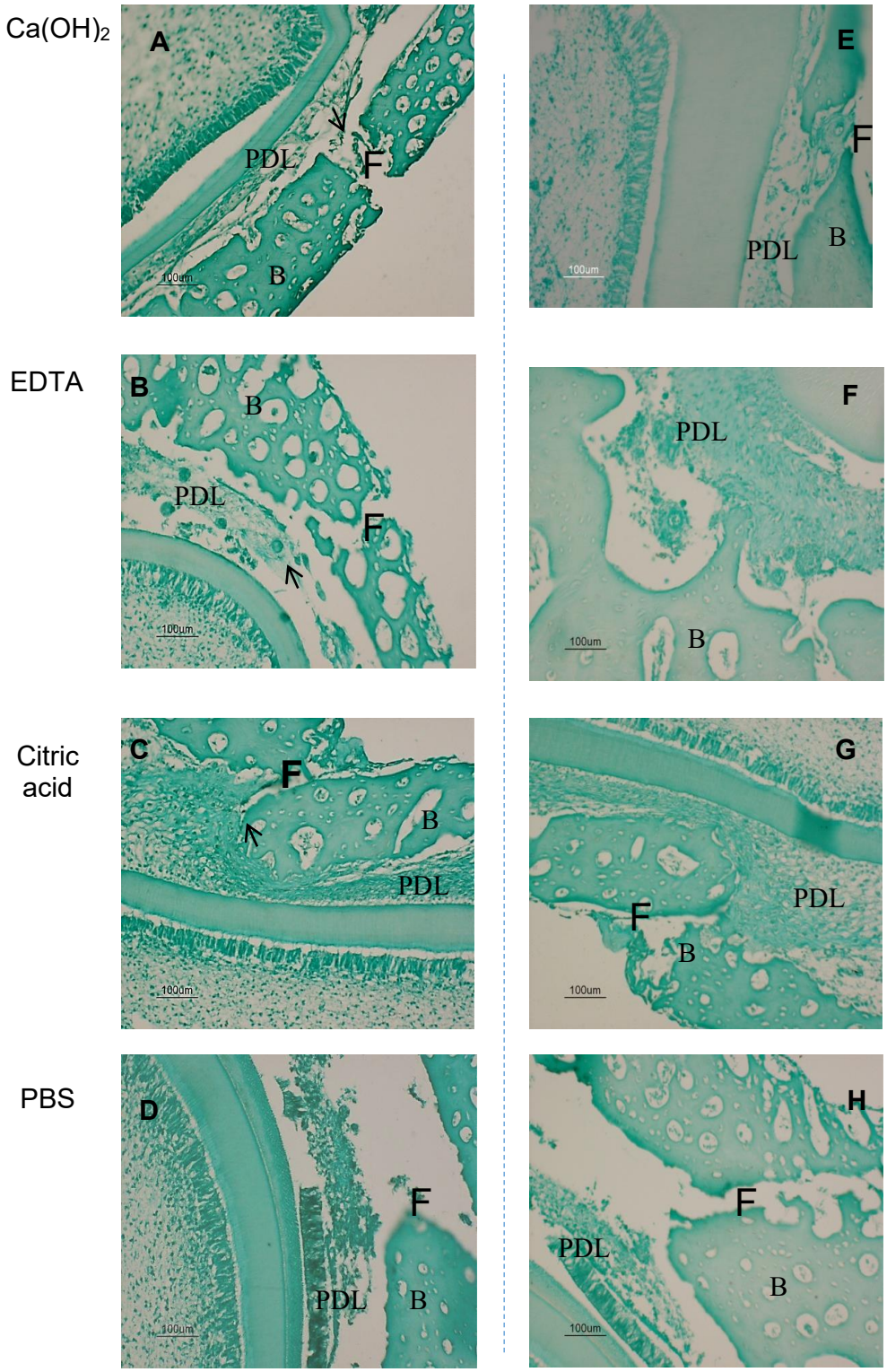


Figure 3-8: Fractured mandible slices treated with $Ca(OH)_2$, EDTA, citric acid and PBS and stained with IHC for expression of PCNA [A-D]. Control sections where primary antibody was excluded and replaced with TBS [E-H]. PDL, periodontal ligament; D, dentine; B, bone; F, fracture site.

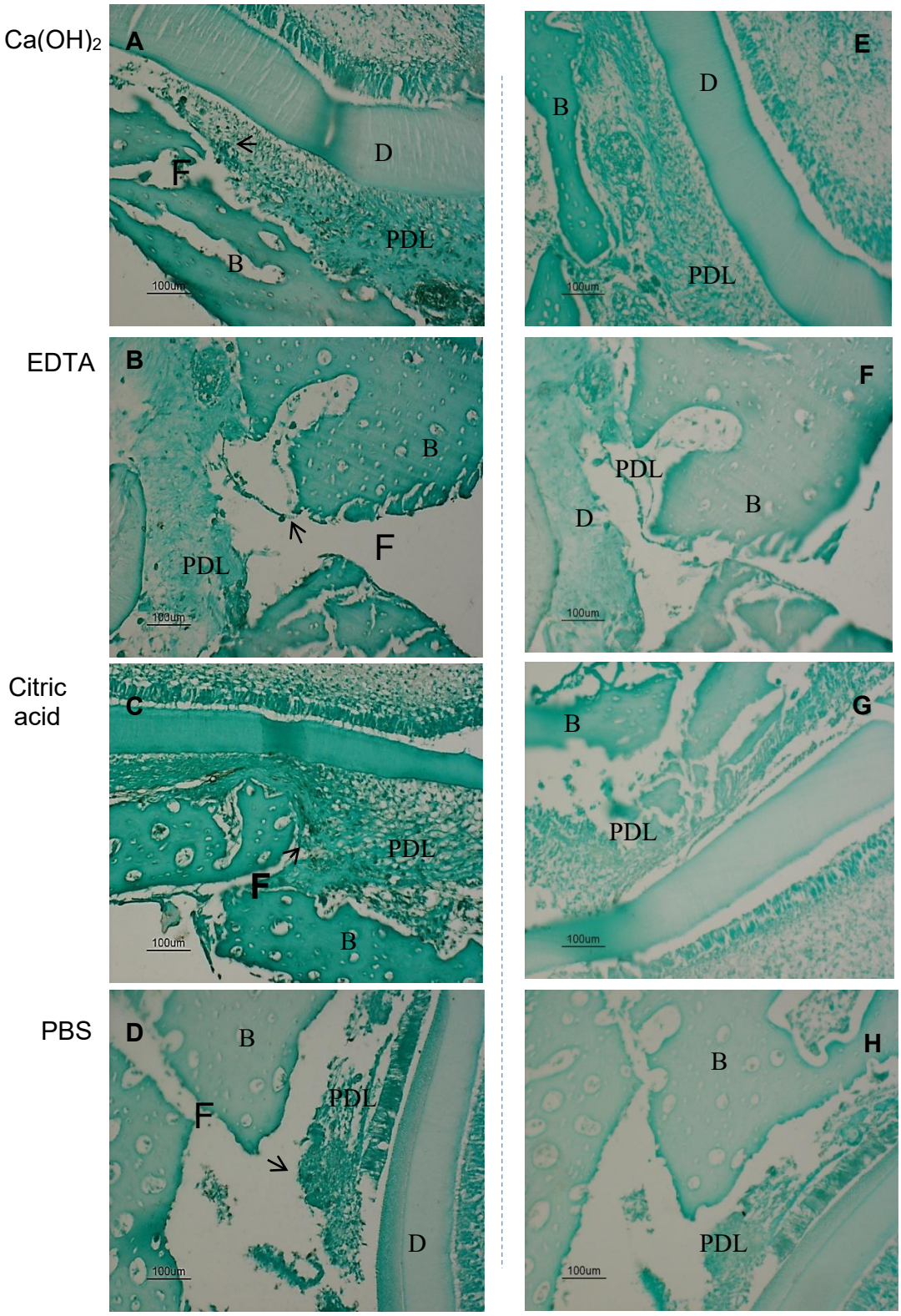


Figure 3- 9: Fractured mandible slices treated with $Ca(OH)_2$, EDTA, citric acid and PBS and stained with IHC for expression of BSP [A-D]. Control sections where primary antibody was excluded and replaced with TBS [E-H]. PDL, periodontal ligament; D, dentine; B, bone; F, fracture site.

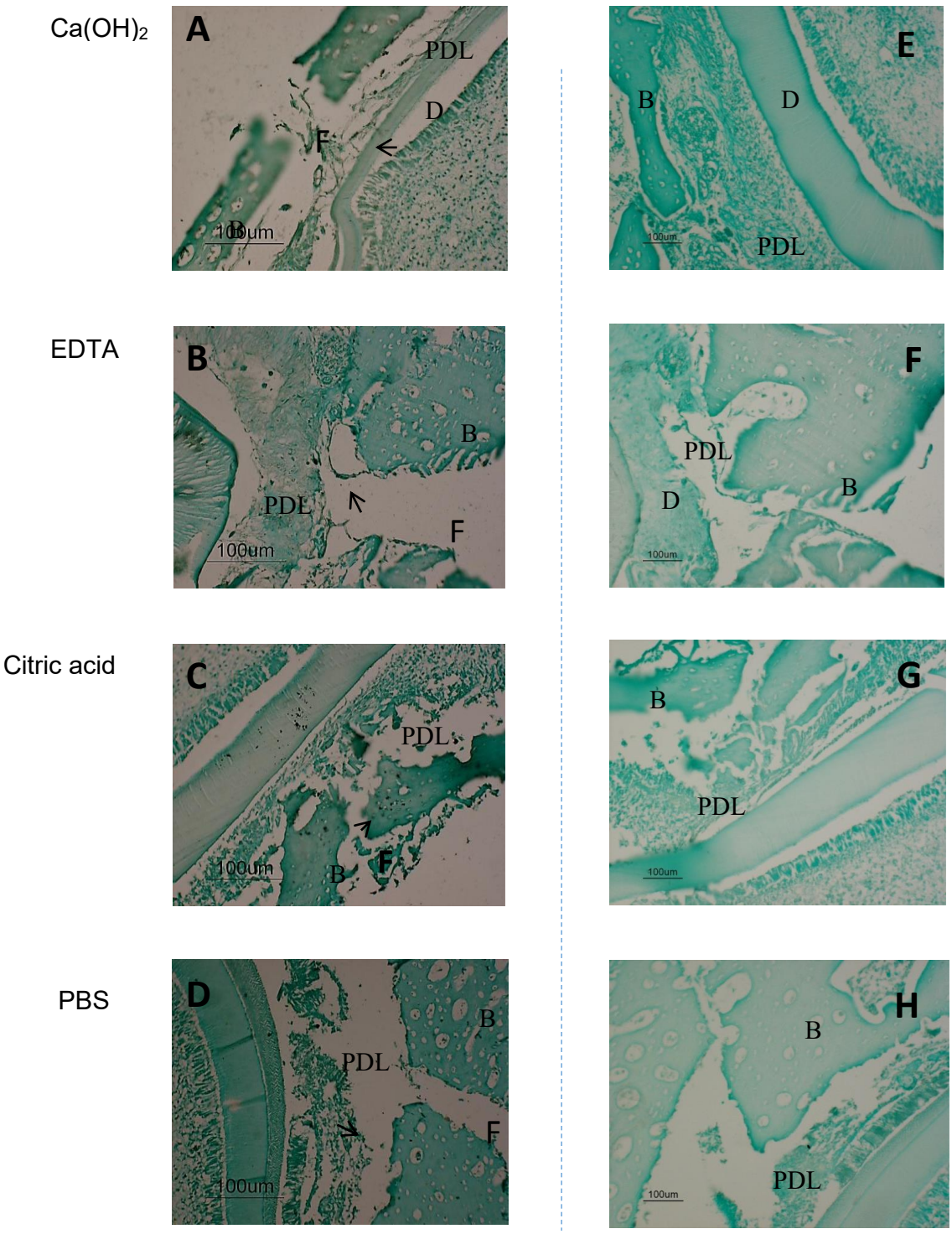


Figure 3- 10: Fractured mandible slices treated with $Ca(OH)_2$, EDTA, citric acid and PBS and stained with IHC for expression of OPN [A-D]. Control sections where primary antibody was excluded and replaced with TBS [E-H]. PDL, periodontal ligament; D, dentine; B, bone; F, fracture site.

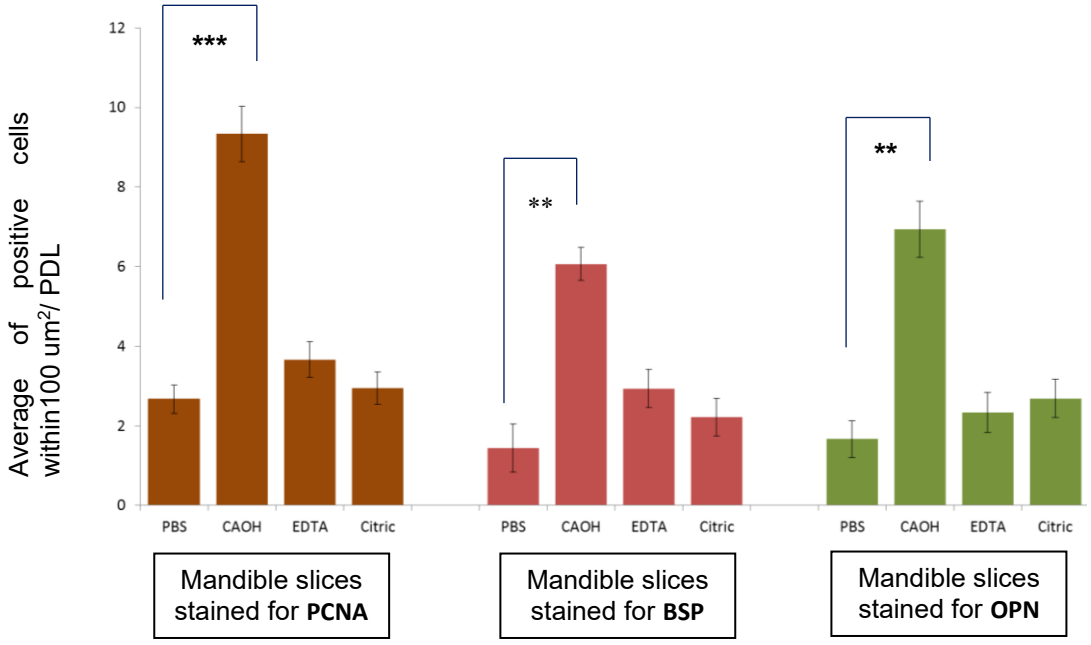


Figure 3- 11: Comparison of average positive cells number within 100 um^2/PDL of fractured mandible slices treated with $Ca(OH)_2$, EDTA, citric acid or PBS at the fracture site and stained immunohistochemically for PCNA, BSP or OPN at fracture site.
*****P<0.001. ** P<0.01. Error bars indicate standard error of the mean.**

3.3.3 Quantification of released growth factors from mandible slices following chemical treatment

All chemically treated mandible slices demonstrated significant increases in TGF-β1, BMP2 and VEGF concentrations when compared with mandible slices treated with PBS (Figure 3.12). In the samples analysed, there were no significant differences between the treatment groups. An average concentration of 5000pg/mg of TGF-β1, 1000pg/mg of BMP2 and 15000pg/mg of VEGF were released from mandible slices treated with calcium hydroxide, citric acid and EDTA. Statistical analysis also demonstrated that the amount of VEGF concentration released from fractured mandible slices was significantly greater than the amount of BMP2 and TGF-β1 ($p < 0.001$).

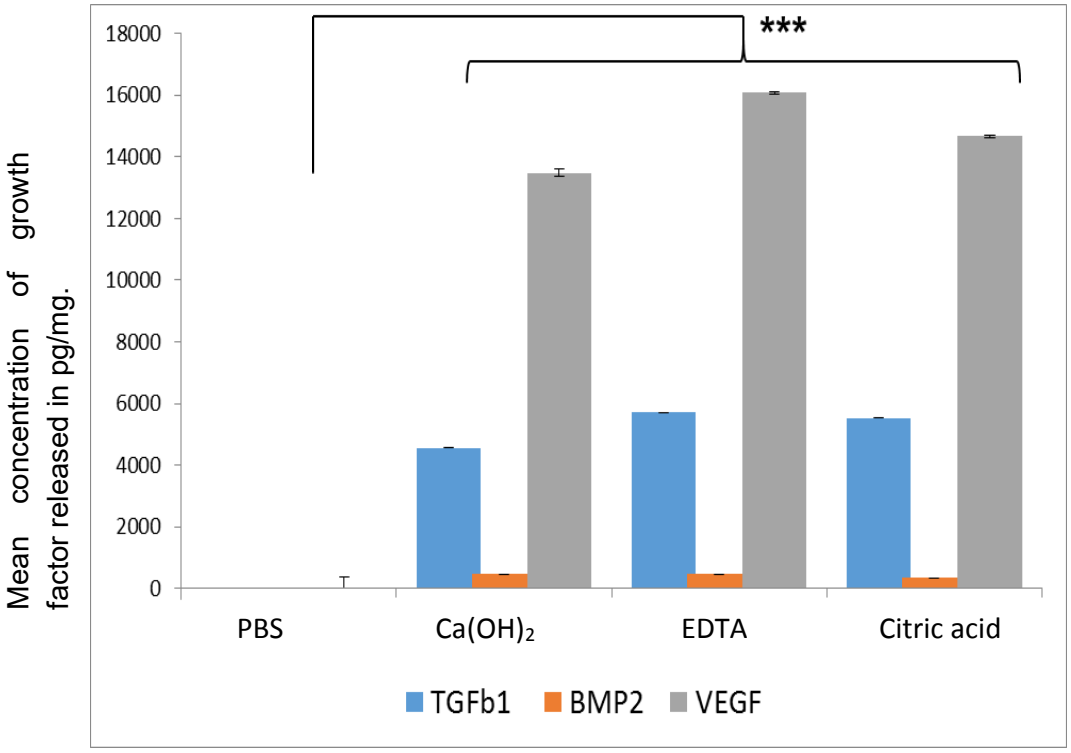
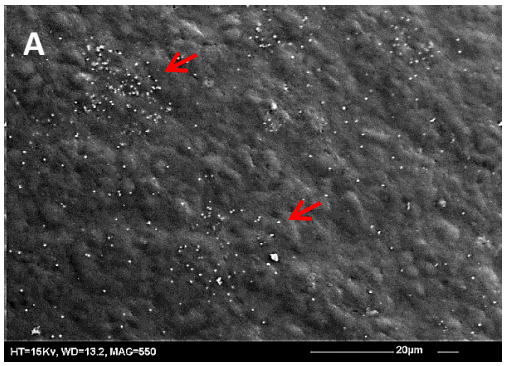


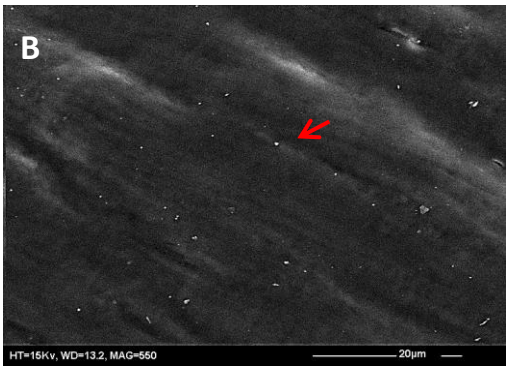
Figure 3-12: Mean concentration of TGF-β1, BMP2 and VEGF released in pg/mg from mandible slices treated with PBS, $Ca(OH)_2$, EDTA or citric acid and cultured for 7 days.
(n=6), All assays were carried out in triplicate. ***P<0.001. Error bars indicate

3.3.4 Immunogold labelling of surface exposed growth factors in scanning electron microscope

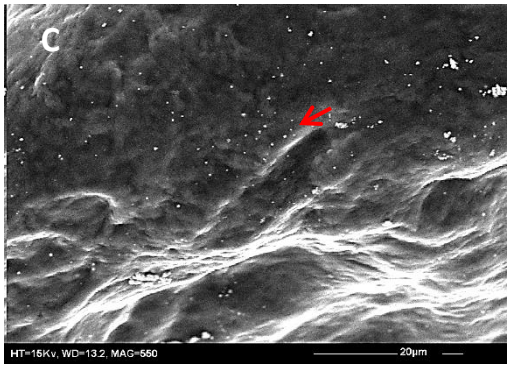
SEM examination revealed that all chemically treated mandible slices demonstrated a significant increases in immunogold labelling of surface exposed TGF-β1 (Figure 3.13), BMP2 (Figure 3.14) and VEGF (Figure 3.15) when compared with mandible slices treated with PBS. Fractured mandible slices treated with calcium hydroxide exposed an average number of 9 TGF-β1, 15 BMP2 and 46 VEGF labelled immunogold particles per 20μm^2 area of bone surface. Fractured mandible slices treated with either citric acid or EDTA exposed an average number of 2 TGF-β1 and BMP2 and 12.5 VEGF labelled immunogold particles per 20μm^2 area of bone surface. Statistical analysis (Figure 3.16) demonstrated that the number of immunogold particles labelling to VEGF exposed from all chemically treated fractured mandible slices were significantly greater than the amount of immunogold particles labelling to BMP2 and TGF-β1 ($p < 0.001$). No significant amount of immunogold labelling to TGF-β1, BMP2 and VEGF were observed on fractured mandible slices treated with PBS as well as negative control slices.



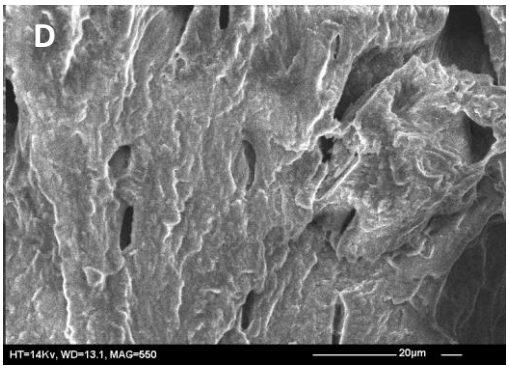
$Ca(OH)_2$



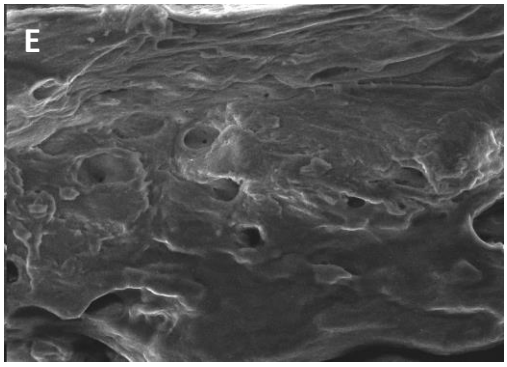
EDTA



Citric acid

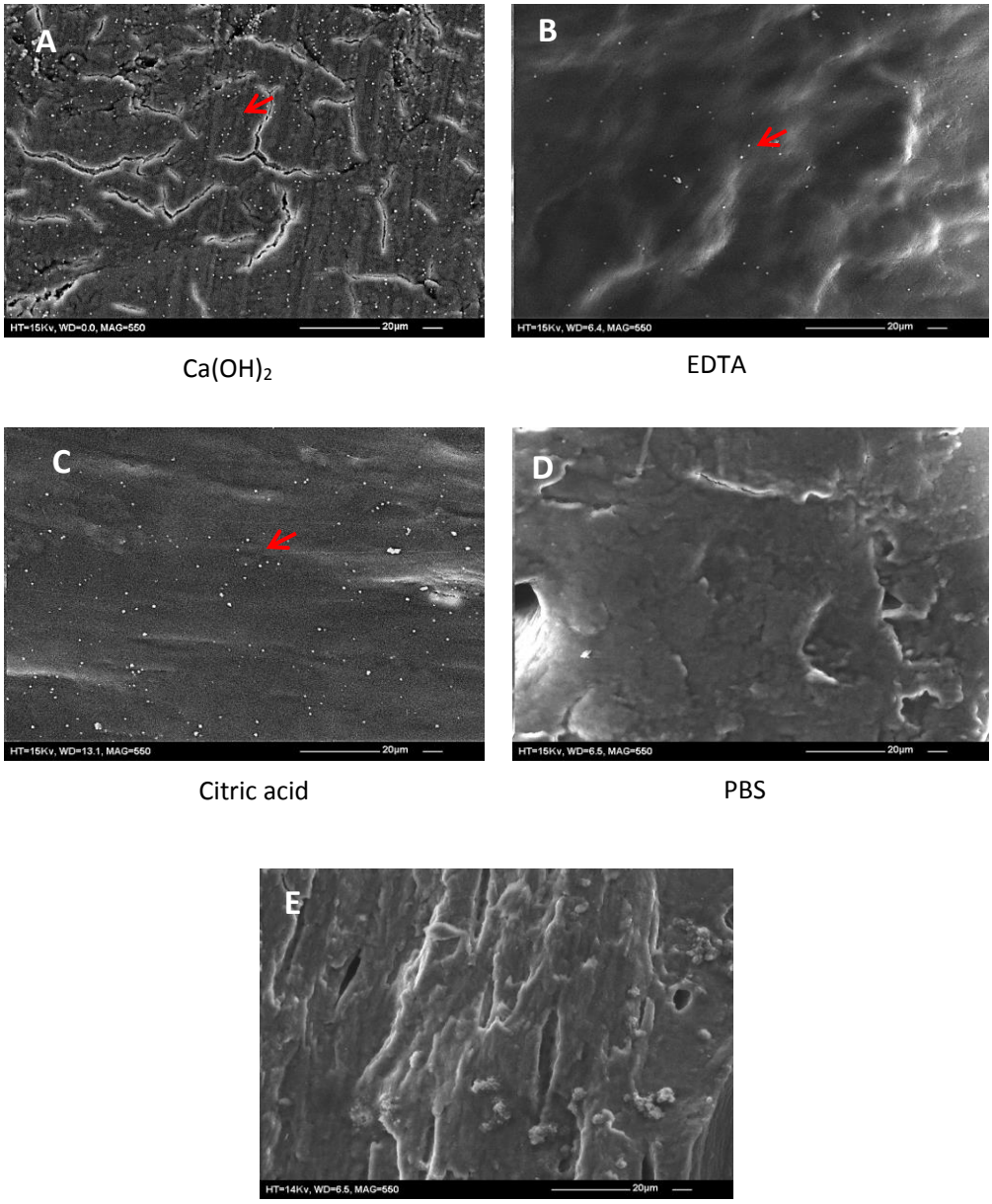


PBS



Control

Figure 3-13: Immunogold labelling of released TGF-β1 (arrows) from fractured mandible slices treated with (A) $Ca(OH)_2$, (B) EDTA, (C) Citric acid and (D) PBS. (E) control where primary antibody was excluded and replaced with TBS.



$Ca(OH)_2$ EDTA

Citric acid PBS

Control

Figure 3-14: Immunogold labelling of released BMP2 (arrows) from fractured mandible slices treated with (A) $Ca(OH)_2$, (B) EDTA, (C) Citric acid and (D) PBS. (E) control where primary antibody was excluded and replaced with TBS.

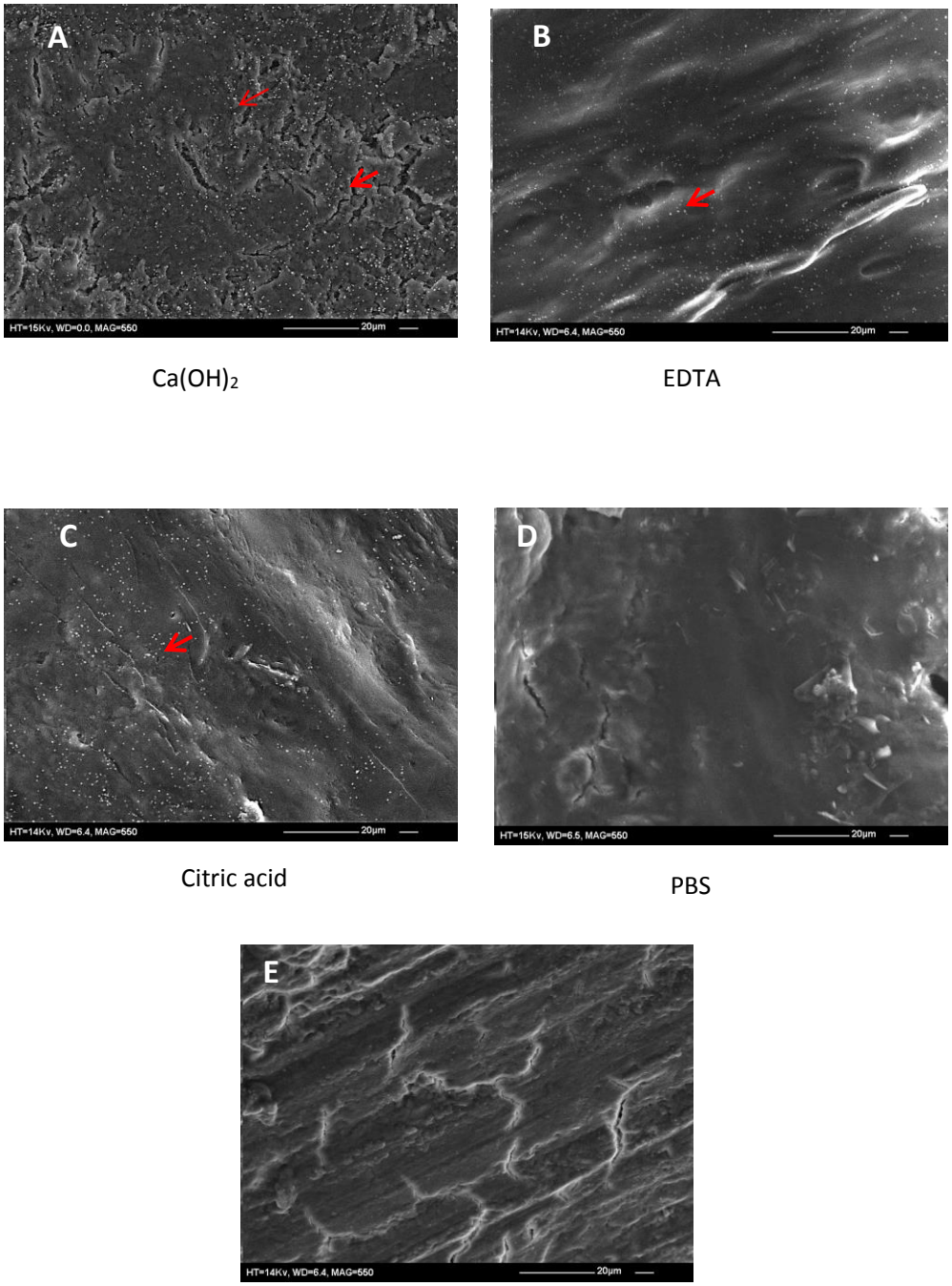


Figure 3-15: Immunogold labelling of released VEGF (arrows) from fractured mandible slices treated with (A) $Ca(OH)_2$, (B) EDTA, (C) Citric acid and (D) PBS. (E) control where primary antibody was excluded and replaced with TBS.

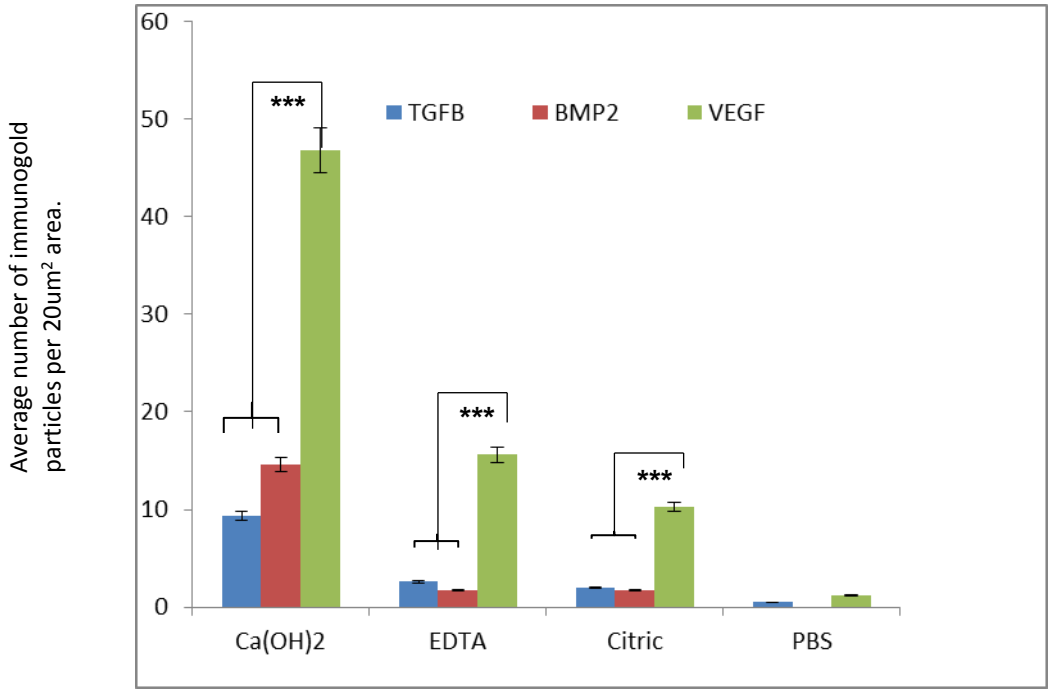


Figure 3-16: Comparison of mean number of (TGF-β1, BMP2, VEGF) immunogold particles per 20um² area of fractured mandible slices treated with $Ca(OH)_2$, EDTA, citric acid and PBS. ***P<0.001. n=5. Error bars indicate standard error of the mean.

3.4 Discussion

Bone matrix contains a reservoir of different growth factors which can be released following bone injury. It has been demonstrated that a combination of different growth factors may improve tissue regeneration more efficiently and effectively than single growth factors (Huang et al. 2005; Kempen et al. 2009; Suarez-Gonzalez et al. 2014; Dinh et al. 2015). Results of this chapter represent a novel study where *ex vivo* mandible slices can be fractured and stimulated with calcium hydroxide, EDTA and citric acid to drive matrix bound growth factors without sustaining excessive damage to the cells and tissues adjacent to the site of fracture and chemical treatment. *Ex vivo* fractured mandible slices treated with calcium hydroxide, EDTA and citric acid successfully demonstrated a significant increase of the key growth factors during bone repair, VEGF, TGF-β1 and BMP2, in both immuno-gold particle expressed on fractured treated surfaces and within conditioned media. These growth factors demonstrated ability to induce cellular proliferation, migration and differentiation during fracture repair.

By using *ex vivo* mandible model, this chapter investigated the effect of chemically released endogenous growth factors on facilitating bone regeneration. Results from histological examination demonstrated that there were no significant differences in cell number between mandible slices treated for 5 or 15 min at D0. These suggest that there is no immediate cellular response or delay in the cells ability to respond to the slicing, fracturing as well as chemical stimulation of the mandible slices. Treated fractured mandible slices

were cultured for up to 7 days, with maintenance of tissue architecture and cellular morphology during this time.

EDTA and citric acid treated slices displayed a similar cell count, with the majority of the increase in cell number occurring only after a treatment time of 15 min. There were a small cell number counted after 5 min treatment, which may indicate an initial gradual increase of the chemicals effects. The number of cells counted after 5 min treatment were similar to those observed from PBS treated slices, and may therefore be original resident cells, rather than a cellular response to the applied chemicals. Calcium hydroxide however demonstrated increase in cell number after a treatment time of 5 min, with significant decrease in the cell counts after 15 min. Calcium hydroxide has cytotoxic properties due to its high alkaline pH and causes formation of necrotic layer thus it could cause apoptosis of the cells and denatured of the protein with the extended treatment time (Al-Awadhi *et al.* 2004).

Slices treated with chemicals demonstrated higher cell number over the control slices. This observation suggests that the increases in cell counts are due to the effects of the applied chemicals, rather than just physical response to the fracture alone. These suggest that the mandible model can respond to exogenous cofactors such as acids and calcium hydroxide to facilitate and accelerate the healing process during bone regeneration. The aim of treating the fractured surfaces with acids and calcium hydroxide was to demonstrate that the release of endogenous growth factors in response to chemical treatments could

influence cellular behaviour in an *ex vivo* mandible model system. Release of endogenous growth factors using chemical treatment has been shown in different studies. For example, EDTA and calcium hydroxide have been shown capable to stimulate growth factors release, particularly TGF-β1, from dentine (Zhao et al. 2000; Graham et al. 2006) and from the matrix of the femur bone slab (Smith et al. 2011).

EDTA and citric acid are known to demineralise both bone (Smith et al. 2011) and dentine matrix (De-Deus et al. 2008a). They accomplish this by dissolution of mineralised matrix or by acting as chelating agents capable of binding and sequestering calcium ions within the mineralised matrix, thus reducing their activity (De-Deus et al. 2008a). The similar solubilisation patterns observed with EDTA and citric acid treatment suggests that both chemicals release growth factors by a similar mechanism. However, previous studies have demonstrated that EDTA is a strong acid (Inoue et al. 1995) and potent chelating agent (Barnett and Uchtman 1979). It is possible that citric acid, at a 10% concentration and a pH 3 may be more efficient or equal to 17% EDTA at a physiological pH of 7.2. Calcium hydroxide, an alkaline agent with pH range 9.2-11.7 is commonly used in clinical dentistry as pulp capping, apexification and apexogenesis. The growth factor release observed with calcium hydroxide was in contrast to the pattern of release observed with EDTA and citric acid treatment. Calcium hydroxide dissociates into calcium ions (Ca^{2+}) and hydroxyl ions (OH^{-}). Scientists suggest that the mechanism of growth factor solubilisation by calcium hydroxide is mainly pH dependent and its efficiency may result from lower solubility. It may be hypothesised that calcium hydroxide has a greater

efficacy for altering protein charge and thus growth factor release by its alkaline pH and the two hydroxyl groups.

ELISA and immunogold labelling results demonstrated that chemical treatments used in this study were capable of releasing TGF-β1, BMP2 and VEGF. The amounts of TGF-β1 and BMP2 released from mandible slices were less than VEGF. Previous studies have shown that TGF-β1 and BMP2 appeared to play a critical role in early stages of fracture healing, whereas VEGF have a greater effect at later stages of fracture healing (Dimitriou *et al.* 2005). Culture supernatants were collected after 7 days of culture, thus it could be that the high peak of TGF-β1 and BMP2 release was missed. It has also been reported that VEGF inhibits expression of BMP2 in rat MSCs (Schonmeyr *et al.* 2010). The mandible is a highly vascularized tissue thus the matrix could have high concentration of VEGF than TGF-β1 and BMP2. TGF-β1 and BMP2 are found in the matrix modulated by decorin and biglycan which affect release of these growth factors (Miguez *et al.* 2011). VEGF however presented in the matrix as a soluble or matrix-bound form which could be released more quicker and easier than TGF-β1 and BMP2 by the chemical treatment (Chen *et al.* 2010). Studies have shown that VEGF is highly expressed in angioblasts, osteoprogenitor and osteoblast cells during the first 7 days of healing but decreases after eleven days (Uchida *et al.* 2003) thus day 7 could be the peak of angiogenesis but not osteogenesis.

Fractured mandible slices treated with calcium hydroxide, EDTA and citric acid exhibited a significant increase in VEGF, TGF-β1 and BMP2, in both immuno-gold particle expressions on fractured treated surfaces and within conditioned media. The observed increases in number of PDL cells close to the site of fracture following chemical stimulation, may indicate stimulation of some early reparative processes within the tissue in response to the applied chemical. These growth factors could cause stimulation of migration, proliferation as well as maintaining survival of cells within the PDL (Zhao et al. 2000; Graham et al. 2006). Since mandible slices were treated locally by the chemicals and were subsequently washed off from the chemicals, it is likely that the findings from this study are a result of the released growth factors influencing cell behaviour within the tissue, rather than a direct effect of the chemical treatment itself.

Acids and calcium hydroxide have differential effects on behaviour of cells within the mandible model due to their different mechanism of action. Immunohistochemistry data demonstrated that in mandible slices treated with calcium hydroxide there was an increased number of PCNA immunopositive cells adjacent to the site of fracture, suggesting that proliferation was being influenced by the endogenous growth factors. SEM images of immunogold-labelled bone surfaces revealed that calcium hydroxide was capable of releasing TGF-β1, BMP2 and VEGF from within the matrix of the bone. TGF-β1 shown in previous studies to be involved in stimulating migration of bone forming cells and macrophages (Tsiridis *et al.* 2007; Dimitriou et al. 2011) and stimulating proliferation of mesenchymal stem cells and osteoprogenitors (Bostrom and Asnis 1998; Barnes et al. 1999). Expression of the bone marker, bone

sialoprotein and osteopontin, were also significantly increased at the fracture site in response to calcium hydroxide treatment. Both bone sialoprotein and osteopontin are mineralized tissue-specific protein expressed in differentiated osteoblasts that appear to function in the initial mineralization of bone (Hughes et al. 2006b; Wang *et al.* 2011). Calcium hydroxide has been reported to increase the levels of BSP and Runx2 mRNA in human osteoblast-like cells (Wang et al. 2011). This increase in the number of immunopositive cells to bone sialoprotein and osteopontin could be due to increase in protein expression by cells differentiated as a result of growth factors released by treating mandible slices with calcium hydroxide. The evidence from the SEM images, which shows release of BMP2 from mandible slices in response to calcium hydroxide treatment, may suggest that the increase in immunopositivity for bone sialoprotein and osteopontin could be due to BMP2 inducing differentiation of committed cells to the osteoblast lineage (Hughes et al. 2006b).

There was a difference in the expression of PCNA and osteogenic bone markers between chemically treated mandible slices, with a higher number for PCNA immunopositive cells. This suggests that more than one cell participates in the proliferation at the fracture site. It could be proliferation of cells residing within the PDL or proliferation of migrated inflammatory cells to subside inflammation and phagocytic uptake of the fracture residue. Recent unpublished data however demonstrated that macrophage and neutrophil significantly decreased after 7days of culture (Waddington et al.). Thus it is most likely that the increase in the cells could be due to migration and proliferation of periodontal ligament cells as well as endothelial cells by TGF-β1 and VEGF (Smith et al. 2010; Zhang *et al.*

2013) and differentiation by BMP2 and VEGF (Lee et al. 2012; Oortgiesen *et al.* 2014).

Ex vivo fractured mandible slices were demonstrated an ability to drive matrix bound growth factors by calcium hydroxide more than EDTA and citric acids. Fractured mandible slices treated with calcium hydroxide exhibited a significant increase in VEGF, TGF-β1 and BMP2. These growth factors have the ability to induce cellular proliferation, migration and differentiation during fracture repair. In large bone fracture, it is difficult to achieve bone union without use of external grafts. *Ex vivo* fractured mandible slices were further tested to investigate effect of endogenous growth factors derived by calcium hydroxide on cells carried by scaffold. Poly vinyl alcohol (PVA) scaffolds and green fluorescent protein (GFP) cells were used to investigate the effect of growth factors released by calcium hydroxide on the scaffold and non-resident cells. Confocal microscope was demonstrated that PVA scaffolds and GFP cells were not detected at fracture sites after 7 days of culture. The method was not finalised because the scaffold was dissolved and the histological processing lead to fade and loss of the green fluorescent of the GFP cells. Another venue of investigation would be reusing *ex vivo* fractured mandible model to study the effect of endogenous growth factors derived by calcium hydroxide on cells carried on a scaffold. This would allow a greater understanding of the ability of the model to carry exogenous cells and scaffold and the ability of endogenous growth factors derived by calcium hydroxide to enhance bone healing for promising clinical treatment of large bone defect.

Chapter 4.

Effects of Calcium Hydroxide on Bone Marrow Stem Cells

4.1 Introduction

Calcium hydroxide has been used extensively in clinical dentistry as a direct pulp capping agent to induce dentine regeneration. During the reparative process, calcium hydroxide shows the potential to regenerate damaged dentine by formation of dentine bridges at sites of calcium hydroxide application through sequential steps of proliferation, migration, and differentiation of progenitor cells. The previous chapter demonstrated that the use of calcium hydroxide on mineralised tissue such as mandibular bone may be effective in inducing hard tissue formation by releasing growth factors from bone matrix and hence facilitating bone repair.

Calcium hydroxide is considered as a gold standard dental material because of its mineralization capacity as well as its effective anti-microbial and anti-inflammatory action. Most of the physical and biological properties of calcium hydroxide are based on dissociation of calcium hydroxide into calcium and hydroxyl ions (Apte et al. 2005). Free calcium ions are reported to be essential for cellular migration, differentiation, and mineralization (Schröder 1985). An increase of local calcium concentration has been shown to activate cells such as stem cells (Adams *et al.* 2006) and osteoblasts (Yamaguchi *et al.* 1998). Calcium ions have been also demonstrated to play a critical role in regulation of

cellular proliferation, differentiation and mineralization (Torneck *et al.* 1983; Kulesz-Martin *et al.* 1984; Zayzafoon 2006). Extracellular calcium has also been reported to up-regulate the gene expression of BMP2 and osteopontin in human pulp cells which induce mineralization in dental pulp tissue (Rashid *et al.* 2003).

Several studies have demonstrated that the mineralisation ability of calcium hydroxide is due to its alkaline pH, leading to release of free hydroxyl ions after dissociation of calcium hydroxide (Glass and Zander 1949; Schröder and Granath 1971; Stanley and Lundy 1972). Influence of the alkaline pH was investigated on human dental pulp cells and the study confirmed that alkaline pH caused increased alkaline phosphatase and BMP2 expression and calcified nodule formation which enhanced cellular mineralization (Okabe *et al.* 2006). Calcium hydroxide can provide an optimal healing environment by the rise of alkaline pH from the free hydroxyl ions. Antimicrobial properties of calcium hydroxide are also based on its ability to release hydroxyl ions and to raise pH to about 12.5 at which very few bacteria can survive (Byström *et al.* 1985; Jhamb and Chaurasia 2014). Endogenous inflammatory mediators such as interleukin-1α and tumour necrosis factor-α play an important role in regulation of inflammation and associated tissue destruction (Cochran 2008; Gabay *et al.* 2010). High alkaline pH has been reported to cause denaturation of such proinflammatory cytokines (Khan *et al.* 2008) and neutralize the acidic inflammatory pH produced by inflammatory reaction, which is harmful to the healing process (Heithersay 1975).

Hard tissue such as bone and dentine, contain numerous growth factors entrapped inside the matrix. The previous chapter demonstrated that growth factors such as TGFß1, BMP2 and VEGF can be released from the matrix after chemical treatment with EDTA, citric acid and calcium hydroxide. In vitro studies demonstrated that calcium hydroxide demonstrated ability to solubilize sequestered bioactive molecules like TGFß1 from dentine matrix (Smith and Smith 1998; Graham et al. 2006) and bone matrix (Smith et al. 2011). Several studies demonstrated that calcium hydroxide has stimulatory effect on TGFß1 from different ways. TGFß1 is secreted mostly in latent form, which restricted its biological activity (Barcellos-Hoff 1996) however it can be activated in alkaline culture medium (Lyons *et al.* 1988). Calcium hydroxide provides an alkaline environment through its hydroxyl ions, which in theory promote the bioactivity of TGFβ through releasing it from the latent complex. It has been reported in *in vitro* study that dentine matrix components such as TGFß1 can be dissolved from the matrix by mineral trioxide aggregate and calcium hydroxide (Tomson et al. 2007).

It has been reported that oral administration of calcium hydroxide caused significant increase in serum inorganic phosphorus concentration and femoral-diaphyseal calcium content and alkaline phosphatase activity of rats with skeletal unloading (Yamaguchi *et al.* 1991b). The authors concluded that oral administration of calcium hydroxide can prevent partly skeletal unloading-induced disorder of bone metabolism (Yamaguchi et al. 1991b). Mineralization induced by calcium hydroxide was shown to be regulated also by bone sialoprotein in rat calvarial osteogenic cell cultures (Da Silva et al. 2008) and

human osteoblast-like Saos2 cells (Wang et al. 2011). It has also been reported that calcium hydroxide enhanced bone regeneration by influencing the differentiation of osteoblasts cells by increasing expression of collagen I and osteocalcin (Han *et al.* 2013) and enhancing osteoblast ALP secreting activity (Liang *et al.* 2000; Kaskos 2013). Oily calcium hydroxide suspension has been shown to direct bone regeneration of bone tissue cultures through inhibiting bone resorption via inhibition of the proteolytic enzymes such as collagenases (Stratul and Sculean 2004). It has also been demonstrated that calcium hydroxide promotes attachment, proliferation and mineralization of human alveolar osteoblasts (Eick *et al.* 2014).

Calcium hydroxide has demonstrated an ability to release bioactive molecules, either through direct stimulation of cells or by solubilisation of dentine extracellular matrix (Sangwan *et al.* 2013). Results from the previous chapter have demonstrated that growth factors can be released from the bone following chemical treatment with calcium hydroxide but further studies are required to investigate cellular responses to the applied calcium hydroxide. This chapter thus aims to investigate the effect of calcium hydroxide on cellular behaviour of bone marrow stem cells. Is calcium hydroxide able to improve viability, proliferation, and mineralization of BMSCs?

4.2 Materials and Methods

4.2.1 Preparation of calcium hydroxide test solution

Calcium hydroxide test solution was prepared by dissolving 0.01875 g of calcium hydroxide in 12.5 mL of distilled water at 23 °C. This solution was stirred for 30 min and centrifuged at 1800 rpm for 5 min. The solution filtered and titrated against the medium to make sure that the pH of the final media (pH 7 or 11) would not be significantly altered (Torneck et al. 1983). A total of 2 mL of the $Ca(OH)_2$ solution was added to osteogenic and culture medium making 0.4mM $Ca(OH)_2$ concentration. Culture medium consisted of αMEM serum (Invitrogen Life Technologies, UK), 20% heat inactivated foetal calf serum serum (Invitrogen Life Technologies, UK), 1% L-ascorbate 2-phosphate (Sigma, UK) and 1% concentration of antibiotics solution (10,000 units of penicillin, 10mg/ml of streptomycin, and 25µg/ml amphotericin B) (Invitrogen, UK). Osteogenic medium consisted of αMEM serum (Invitrogen Life Technologies, UK), 10% heat inactivated foetal calf serum serum (Invitrogen Life Technologies, UK), 10 µM dexamethasone (Sigma, UK), 10 mM β-glycerophosphate (Sigma, UK), 1% L-ascorbate 2-phosphate (Sigma, UK) and 1% concentration of antibiotics solution (10,000 units of penicillin, 10mg/ml of streptomycin, and 25µg/ml amphotericin B) (Invitrogen, UK). A positive control was prepared where BMP2 (0.12ng/ml) (Gibco Life Technologies, UK), was supplemented to the osteogenic or culture medium.

4.2.2 Bone marrow stem cell culture

Rat bone marrow stem cells (BMSCs), kind gifts from J Harrington (Harrington *et al.* 2014), were seeded at 4000 cells/cm^2 and cultured with culture medium. Cells were incubated at 37^{o}C in a 5% CO_2 atmosphere. Medium was changed every two days and mycoplasma testing was routinely conducted every time cells were passaged. When cells reached approximately 80% confluence, they were passaged with accutase (Sigma), counted with a haemacytometer using Trypan Blue solution and reseeded at 4000 cells/cm^2. Cells were continually propagated and underwent multiple passaged until sufficient cells were gained. Numbers of cells were recorded in proportion to original number of cells to calculate population doubling level over the passaged period. Cells were seeded in 6-well plates and culture slides in triplicate at 1 × 10^4 cells/cm^2 and cultured with culture medium for 24 hours before adding the treatment medium. Cells in 6-well plates were used for RT-PCR and cells in culture slides were used for staining analysis. Cells were cultured with eight different medium (Table 4.1). Cells were incubated at 37^{o}C in a 5% CO_2 atmosphere for 1, 3, 7 and 14 days during which images were taken.

Table 4. 1: Different medium used for the culture of BMSCs. Cells were challenged with BMP2, $Ca(OH)_2$ at (pH 7 or 11) supplemented culture or osteogenic media.

Culture medium	Culture medium +$Ca(OH)_2$ at pH 7	Culture medium +$Ca(OH)_2$ at pH 11	Culture medium +BMP2
Osteogenic medium	Osteogenic medium +$Ca(OH)_2$ at pH 7	Osteogenic medium +$Ca(OH)_2$ at pH 11	Osteogenic medium +BMP2

4.2.3 Characterisation of Cell Marker Expression

4.2.3.1 Extracting mRNA from cell cultures

RNA Extraction was performed using the Qiagen RNeasy Kit (Qiagen, Manchester, UK) following the manufacturer's protocol. Cells were initially cultured in 6-well plates until 80% confluence. Cells were washed with PBS then lysed by adding 600 µL of Buffer RLT lysis buffer (Qiagen, UK) supplemented with 10 µL/mL of β-mercaptoethanol (Sigma-Aldrich, UK). Cells were disrupted using a syringe plunger. The lysate was transferred to a QiaShredder column (Qiagen, UK) and centrifuged at 13,300g for 2min. Cell lysates were treated with 1:1 volumes of 70% molecular-grade ethanol (Sigma-Aldrich, UK) and mixed well by pipette action. The mixtures were then transferred into an RNeasy Mini spin column (Qiagen, UK) and centrifuged at 10,000 g for 15 seconds. The flow-through was discarded and the column was washed with 350 µL of Buffer RW1 (Qiagen, UK) and centrifuged again at 10,000g for 15secs and the flow-though was discarded. The column was treated with a mixture of 10 µL of DNase I stock and 70 µL Buffer RDD (both Qiagen, UK) and incubated at room temperature for

15 min. The column was then washed with 350 µL of Buffer RW1 (Qiagen, UK) and centrifuged again at 10,000g for 15 seconds and the flow-though was discarded. A further 2× 500 µL of Buffer RPE was added and centrifuged for 15 seconds then 2 min and the flow-through discarded after each spin. The column insert was then transferred into an RNase-free 1.5mL eppendorf (Fisher Scientific, Loughborough, UK) and 40 µL RNA-free water (Promega, Southampton, UK) was added for eluting the RNA by centrifuging at 10,000 ×g for 1 min. The eluate was pipetted back into the column and centrifuged again to maximise yield. RNA amount in each sample was quantified using NanoVue (GE Healthcare, Amersham, UK). Samples were kept on ice during quantification procedure. The NanoVue was standardised with double reading of 2 µL of RNA-free water as a blank before using 2 µL of RNA sample for quantification at 260/280nm absorbance. Total RNA collected in the eppendorf was stored at -80ºC.

4.2.3.2 Reserve Transcription (RT) PCR

RT-PCR was performed using the Moloney murine leukaemia virus (M-MLV) reagents from Promega to generate cDNA from RNA. 500ng of total RNA was treated with 1 µL of Random Primers (Promega) with the addition of DNase free water to adjust final volume to 15 uL. This mixture was then incubated in a G-storm™ GS1 thermal cycler (Genetic Research Instrumentation Ltd, Braintree, UK) at 70 ºC for 5 min to unravel RNA. Samples were immediately cooled and stored on ice. A reaction master mix was made from 5 µL of 5xM-MLV reaction buffer, 0.6 µL of RNasin, 1.25 µL of 10mM dNTPs, 1 µL of M-MLV and 2.15 µL DNase free water to adjust final volume to 10 µL. A 10 µL of Master-mix was

added to 15 µLof RNA/random primer mix and incubated at 37 ºC for 1 hour. The newly generated cDNA was stored at -20 ºC until further used for PCR reaction. A reverse-transcription negative control (RT-ve) was prepared without sample RNA.

4.2.3.3 End-Point Polymerase Chain Reaction (PCR)

PCR was set up by adding 1 µL of cDNA generated by reverse transcription to 5 µL 5x Green GoTaq™ Flexi Buffer (Promega), 1 µL 25mM magnesium chloride, 0.5 µL 10mM PCR Nucleotide Mix, 0.25 µL GoTaq™ DNA Polymerase, 1.25 µL 10µM Forward Primer and 1.25 µL 10µM Reverse Primer (Table 4.2). All reaction volumes were adjusted to a total volume of 25µl with nuclease free water. Reactions were run on a G-storm™ GS1 thermal cycler (Genetic Research Instrumentation Ltd) with an initial denaturing step of 95ºC for 5 min, followed by thirty five cycles of a 1 min 95ºC denaturing step, a 1 min 55ºC annealing step (as it stated in table) and a 1 min 72ºC extension step. A final extension step at 72ºC for 10 min was run, ending the reaction. Primers sequences were obtained from related studies and using National Centre for Biotechnology Information (NCBI), Primer-BLAST search. β-actin was used as a control reference gene. RT-negatives and PCR-negatives were used as negative controls. All PCR products were loaded in agarose gels to visualise bands by the Gel Doc 2000 (BioRad, Hemel Hempstead, UK).

4.2.3.4 Quantitative Polymerase Chain Reaction (qPCR)

qPCR was carried out to quantify level of expression of PCNA, CASP-3, OPN and BSP. It was set up by designing the layout for samples and genes to be tested. cDNA was prediluted to 1:5 in nuclease free water. A mastermix reaction for primer design SYBR green was made up from 10 µL of 2x Mastermix (Primer Design), 2 µL 3um Forward Primer, and 2 µL 3um Reverse Primer and 1 µL nuclease free water to adjust final volume to 15µl. In a bright white 96 well Q-PCR plate (Primer Design Ltd), 5 µL of cDNA were added to each well followed by the addition of 15 µL of MasterMix to the corresponding wells. All samples were analysed in triplicate. 96 well plates was sealed with a clear adhesive qPCR seal sheet prior spun at 5000rpm for 5 min. Plate was loaded into the qPCR machine, ABI Prism 7000 Sequence Detection System and ABI Prism 7000 SDS Software V1.0, (Life Technologies Ltd., UK). Reaction conditions were one cycle of an initial denaturing step of 95ºC for 10 min, followed by 40 cycles of a denaturing step at 95ºC for 15 seconds, an annealing step at 55ºC for 30 seconds and an extension step at 72ºC for 30 seconds. Primer sequences were obtained from previous related studies that follow criteria of qPCR. Results of qPCR was analysed using the 2–ΔΔCt method (Livak and Schmittgen 2001). Expression of β-actin, a housekeeping gene, was used as a control reference.

4.2.3.5 Agarose Gel Preparation

To visualise PCR products a 10 µL of sample was loaded on to 1-2 % agarose gels. To prepare a 2% agarose gels, 1.4g of agarose powder (Invitrogen, UK) was mixed with 70mL of 0.5xTBE buffer and heated by microwave for 20 seconds. The mixture was swirled in between until the agarose solution was clear, prior to the addition of 1% Safeview DNA Stain (NBS Biological Ltd., UK). The gels were poured into a casting tray containing comb and allowed to set at 4ºC, for 15 min. Gel was then placed in an electrophoresis tank containing 0.5x TBE buffer. The comb was carefully removed and then 10µl of PCR reaction added to wells, in addition to a 10 µL 100 and 50 base pair (bp) DNA step ladder (Promega). Loaded gels were subjected to electrophoresis, at 80mV for approximately 45 min, in 1x TBE running buffer. The gel was visualised using UV light by GelDoc™ Scanner (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and images were captured using Quantity One Image Analysis Software (Bio-Rad).

Table 4. 2: Forward and reverse primer sequences for gene markers used for PCR analysis. B-actin was used as a housekeeping gene

Gene Marker	Primer sequence (5'->3') F, Forward; R,Reverse	Annealing Temperature	Source
β-actin	F:TGAAGATCAAGATCATTGCTCCTCC	65 °C	(Harrington *et al.* 2014)
	R:CTAGAAGCATTTGCGGTGGACGATG	65 °C	
PCNA	F:TGA AGT TTT CTG CGA GTG GG	55 °C	(Guo *et al.* 2012)
	R:CAG TGG AGT GGC TTT TGT GAA	55 °C	
Casp-3	F:CAT GAC CCG TCC CTT GAA	55 °C	(Cheng *et al.* 2008)
	R:CCG ACT TCC TGT ATG CTT ACT CTA	55 °C	
BSP	F:CAGAAAGAGCAGCACGGTTG	55 °C	(Li *et al.* 2012)
	R:CCTCGTAGCCTTCATAGCCA	55 °C	
OPN	F:TCC AAG GAG TAT AAG CAG AGG GCC A	55 °C	(Kawashima *et al.* 1999)
	R:CTC TTA GGG TCT AGG ACT AGC TTG T	55 °C	

4.2.4 Cells proliferation, osteogenic and apoptotic staining

4.2.4.1 Immunocytochemical staining

Immunocytochemical staining was carried out on cells cultured in culture slides. Cells were washed with TBS for 5 minutes, then incubated with 4% paraformaldehyde for 30 minutes. Cells were suspended in PBS and stored at 4°C until required. Before starting staining procedure TBS was removed from

wells and cells were washed three times in TBS. For detection of intracellular proteins, cells were incubated in 1% Triton-X 100 for 30 minutes followed by incubation at 1% BSA bovine serum albumin (BSA, Fisher Scientific, UK) for 1 hour at room temperature. Serum was removed from the wells and cells incubated with appropriate primary antibody diluted in 1% BSA in TBS at 4^{o}C for overnight. Proliferating cell nuclear antigen (PCNA), bone sialoprotein (BSP), cleaved caspase-3 (casp-3) and osteopontin (OPN) primary antibodies, all were rabbit anti-rat except OPN was goat anti-rat (Santa Cruz Biotech, USA) diluted 1:50 in 1% BSA/TBS. Isotype control antibodies, goat (Santa Cruz Biotech, USA) and rabbit (Cell Signaling Technology, UK), used in the same primary antibody concentrations, and primary antibody exclusion were used as a negative control antibody. Following primary antibody incubation cells were washed with 0.5% Tween-20 in TBS 3 times for 5 minutes each wash. Cells were then incubated for 1.5 hour under darkness with the appropriate fluorescent marker conjugated secondary antibody, goat anti-rabbit or rabbit anti-goat, (Santa Cruz Biotech, USA), diluted 1:250 with 1% BSA/TBS. Cells were washed with 0.5% Tween-20 in TBS 3 times for 5 minutes. The cells chamber was removed using ethanol and slide tool. Cells were stained with Vectashield® mounting medium with DAPI (Vector Laboratories Ltd, UK). Slides were covered by cover slips prior to sealing with nail polish. Cells were evaluated microscopically on an Olympus AX70 fluorescent microscope at 373nm/456nm (FITC). Photographs were taken using a Nikon DXM digital camera and ACT-1 imaging software.

4.2.4.2 Alizarin red Staining

Alizarin red Staining (Sigma-Aldrich, Inc) was used to determine the presence of calcific deposition by the cells. As before, fixed cells were washed twice with PBS prior alizarin red S staining. Cells were stained with 0.5 ml/well alizarin red S, prepared by dissolving 20g/L in double distilled water, pH 4.2, for 3 min at room temperature with gentle shaking. Wells were subsequently washed with distilled water until the wash solution was clear of red. Samples were left to dry at room temperature and viewed by light microscopy (Nikon Eclipse TS100).

4.2.5 Statistical analysis

Statistical analysis was carried out using the GraphPad InStat 3 software (GraphPad software, Inc, USA). Standard errors of the mean were calculated, and mean values were analysed using one-way ANOVA and Tukey's post hoc test, to analyse differences between treatment types and controls. P values below 0.05 at 95% confidence intervals were held to be statistically significant.

4.3 Results

4.3.1 Cellular morphology

After 24 hours of initial culture, cells were further cultured for 14 days in culture medium (Figure 4.1) or osteogenic medium (Figure 4.2) supplemented with either BMP2 or calcium hydroxide at pH 7 or 11 to investigate the effect of calcium hydroxide on BMSCs. During the culture period, cells for all groups were observed to increase in number and demonstrated a fibroblast like morphology with the characteristic long, spindle, bipolar shape. After day 7 there were marked variations in the proliferative capacity between the groups. Cells cultured in calcium hydroxide at pH 7 and BMP2 demonstrated high proliferative capacity similar to the control medium. In contrast, cells treated with calcium hydroxide at pH 11 began to change in their morphology at day 7 such that the cells became wider and larger in size, although still maintaining their spindle and bipolar shape. At day 14, cells treated with calcium hydroxide at pH 11 demonstrated significant decrease in cell number and tended to become round and started losing their shape. The cells became detached from culture walls and floated in the medium.

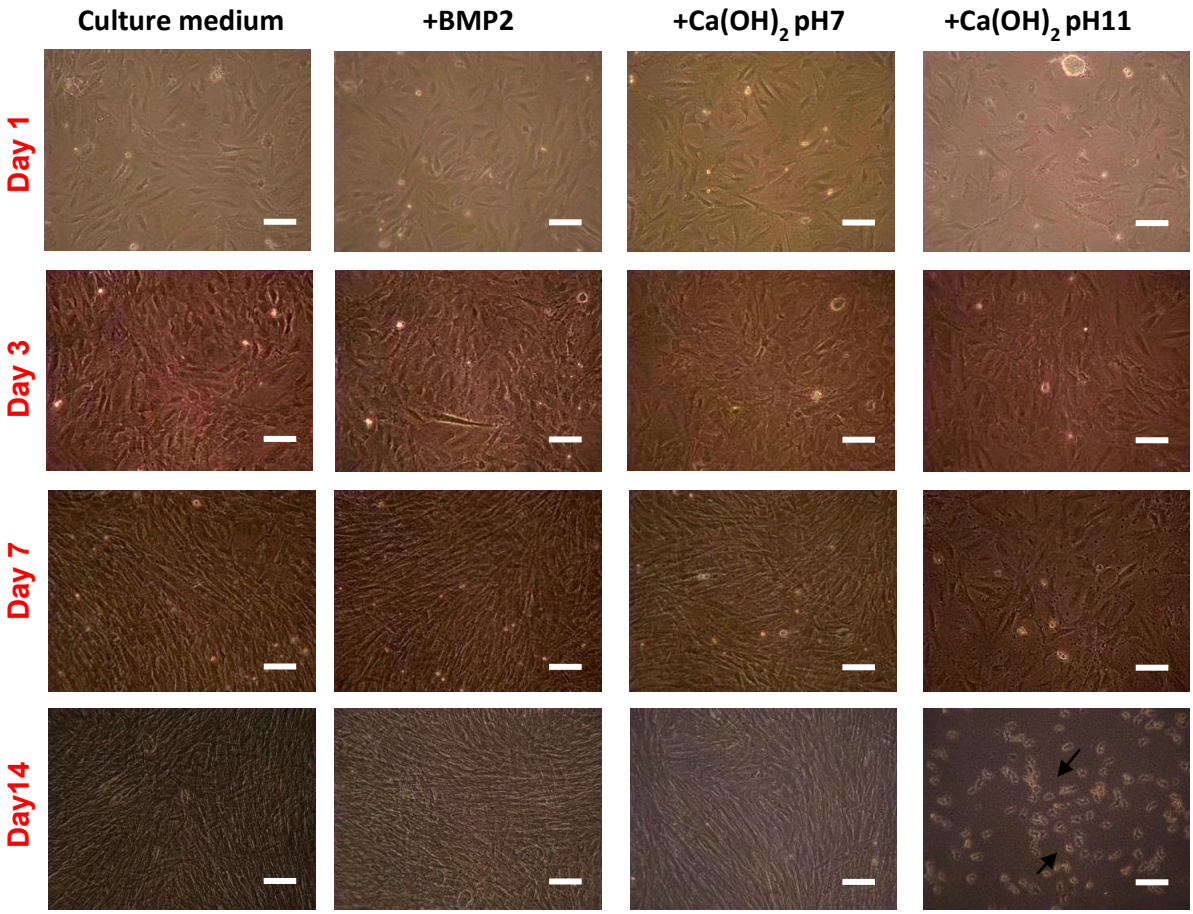


Figure 4- 1: Comparison of the morphological appearance of cells challenged with culture medium supplemented with either BMP2, $Ca(OH)_2$ at pH7 or pH 11 and cultured for 1, 3, 7 and 14 days. Cells treated with calcium hydroxide at pH 11 were detached from the well and floated in the medium (arrow). Scale bar is 100um.

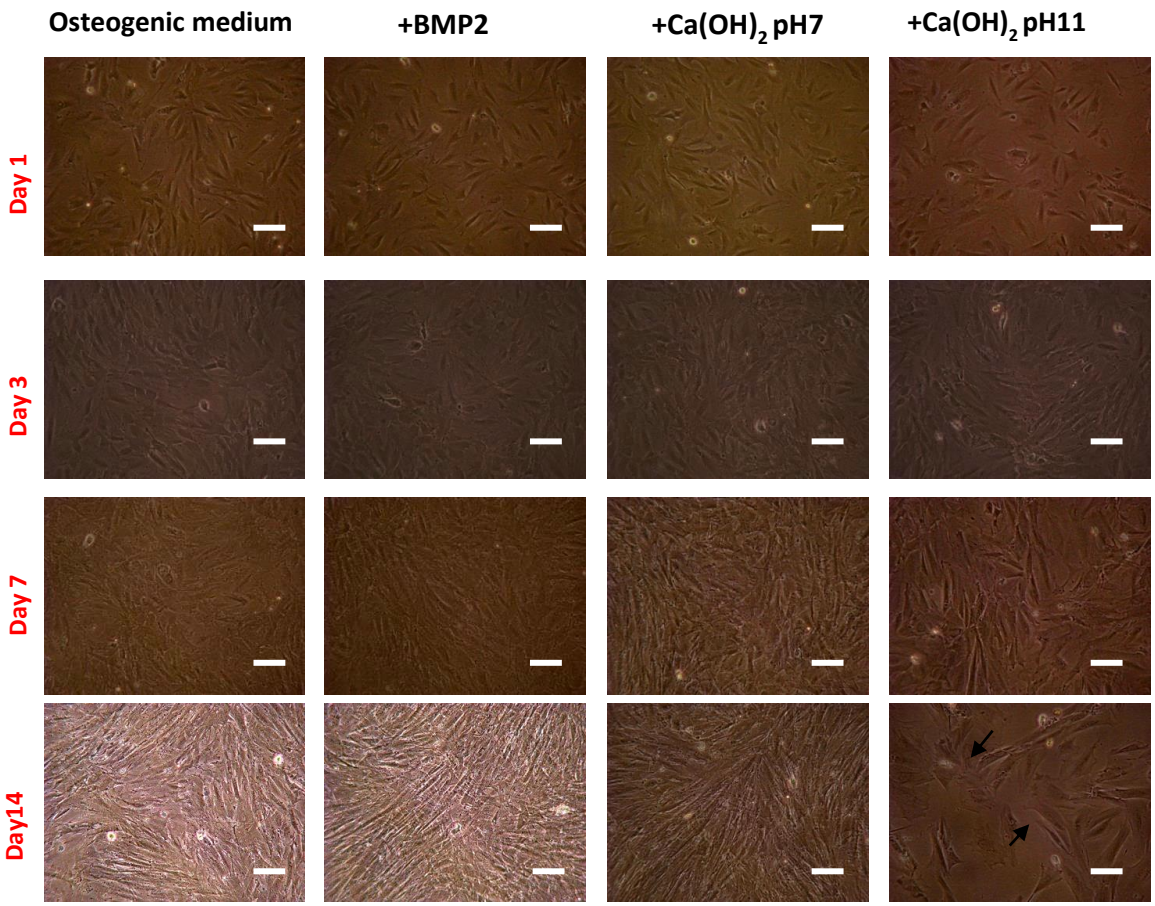


Figure 4- 2: Comparison of the morphological appearance of cells challenged with osteogenic medium supplemented with either BMP2, $Ca(OH)_2$ at pH7 or pH 11 and cultured for 1, 3, 7 and 14 days. Cells treated with calcium hydroxide at pH 11 were decreased in number looked more flattened than other cells (arrow). Scale bar is 100um

End-point PCR Gene Expression Level of PCNA, CASPASE-3 and BSP

Expression of PCNA and CASPASE-3 were investigated at early and late culture time points to investigate the effects of $Ca(OH)_2$ on cellular proliferation and apoptosis during culture while using β-actin as a housekeeping gene (Figure 4. 3). PCNA was expressed in all cell groups at day 1, 3 and 7. At day 14, PCNA was expressed in all cell group except cells treated with culture medium supplemented with $Ca(OH)_2$ at pH11. Casp-3 was expressed in all cell groups at day 1, 3, 7 and 14 except at cells treated with culture medium supplemented with $Ca(OH)_2$ at pH11 at day 14 where it demonstrated strong expression. Expression of BSPII gene was investigated in cells cultured for 14 days to investigate the effects of $Ca(OH)_2$ on cellular differentiation (Figure 4.3). BSPII was expressed only in cells treated with osteogenic medium, osteogenic medium supplemented with $Ca(OH)_2$ pH 7 and osteogenic medium supplemented with BMP2. The PCR-ve and RT-ve experimental controls demonstrated no expression of PCNA, CASPASE-3 and BSP genes.

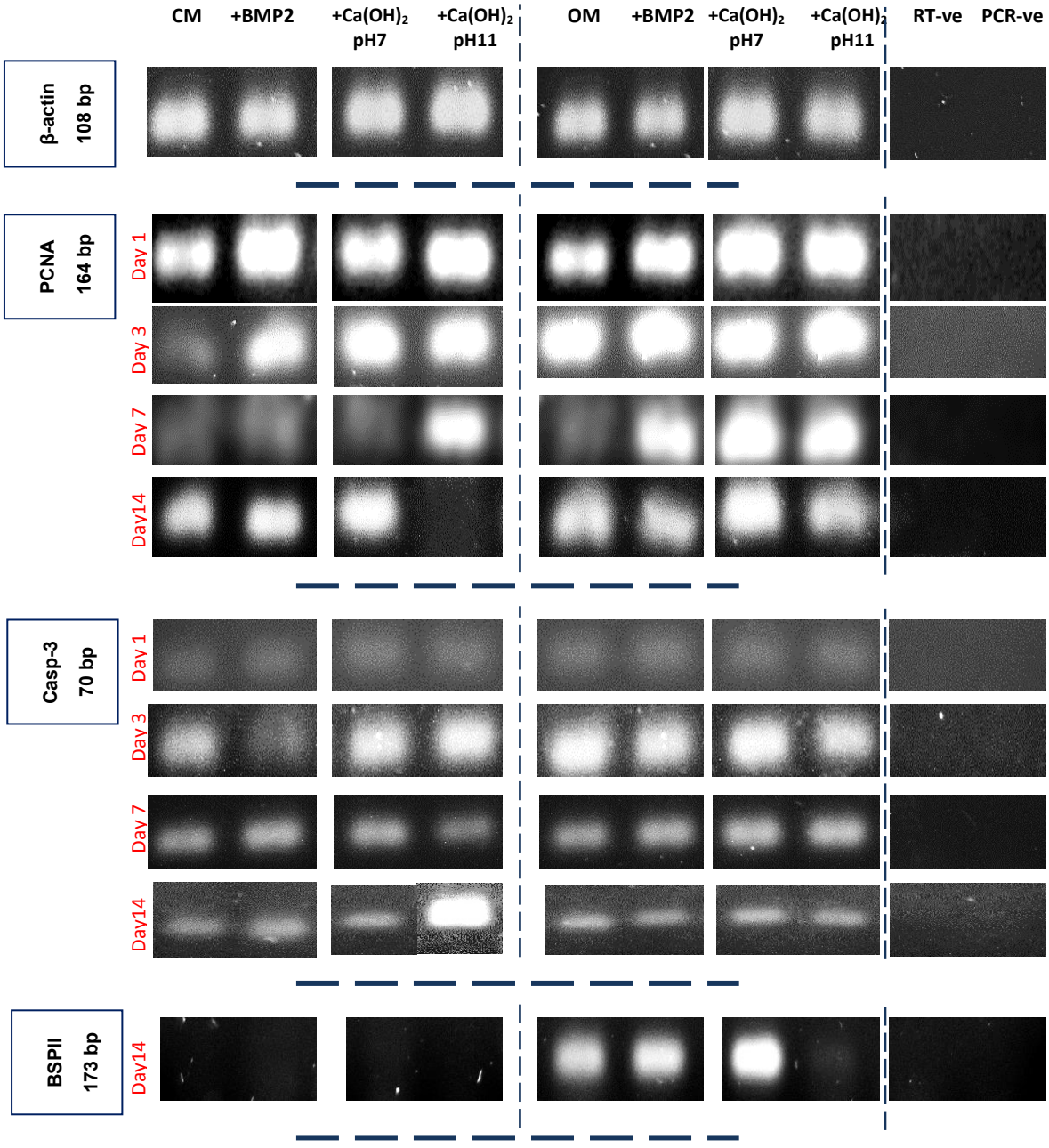


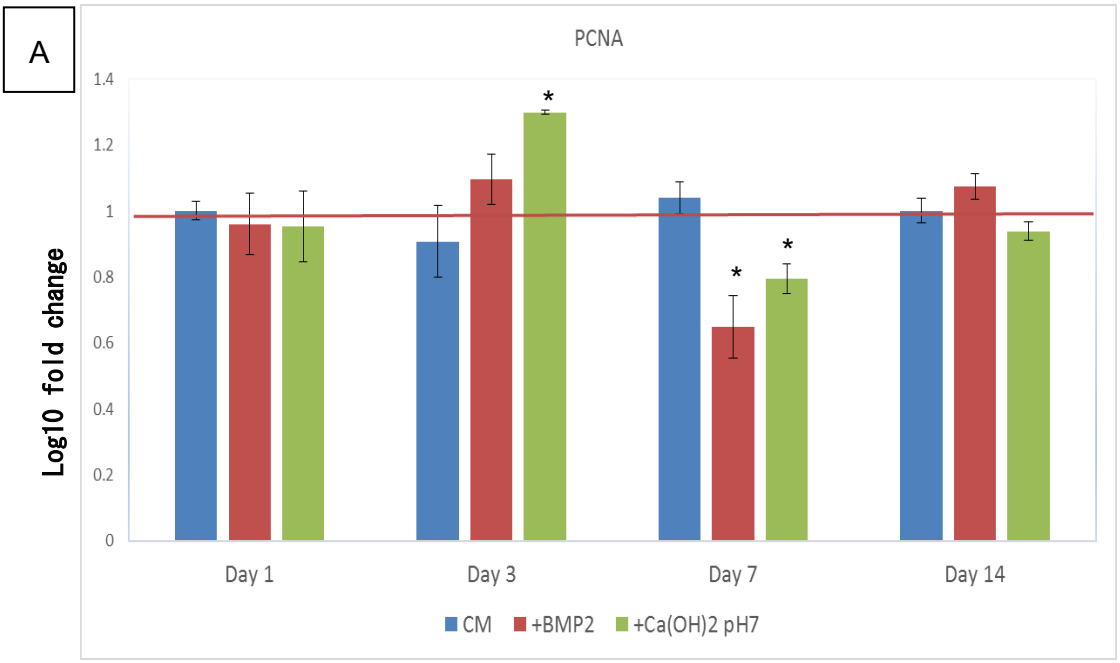
Figure 4- 3: End-point PCR showing expression of PCNA, CASP-3 and BSPII gene markers from cells challenged with culture or osteogenic medium supplemented with either BMP2, $Ca(OH)_2$ at pH7 or pH 11 and cultured for 1, 3, 7 and 14 days. β-actin was used as a housekeeping gene.

4.3.2 qPCR Gene Expression

4.3.3 qPCR Gene Expression Levels of PCNA and CASPASE-3

In culture medium supplemented group, PCNA and CASPASE-3 expression were analysed by qPCR to quantify expression of PCNA and CASPASE-3 genes and compare proliferative and apoptotic effect of different treatment at different culture time. PCNA (Figure 4.4.A) was expressed similarly at all groups at day1. At day 3, cells treated with $Ca(OH)_2$-pH7 demonstrated significant over expression of PCNA greater than BMP2 and control group ($p<0.05$). However, cells treated with BMP2 and $Ca(OH)_2$-pH7 expressed significantly less PCNA than control group at day7 ($p<0.05$). No significant differences were observed between cells treated with BMP2 and $Ca(OH)_2$-pH7 when compared with control group at day 14 ($p>0.05$). Among all groups, the highest expression of PCNA was observed in cells treated with $Ca(OH)_2$-pH7 after 3 days of culture ($p<0.05$). However for expression of CASP-3 at culture medium supplemented group (Figure 4.5.A), there was significant decrease in expression of CASP-3 at all groups when compared with the control group at day 1, 3, 7 and 14 ($P<0.05$). Among all groups, the lowest expression of CASP-3 was observed in cells treated with $Ca(OH)_2$-pH7 at all culture days ($p<0.05$).

When examining the osteogenic medium supplemented group, cells treated with $Ca(OH)_2$-pH7 demonstrated less expression of PCNA than control and BMP2 groups at day 1 (Figure 4.4.B) ($p<0.05$). When compared with the control group, cells treated with BMP2 and $Ca(OH)_2$-pH7 demonstrated less expression of PCNA at day 3, 7 and 14 ($p<0.05$). In contrast (Figure 4.5.B), cells treated with BMP2 demonstrated higher expression of CASP-3 at day 1 greater than control and $Ca(OH)_2$-pH7 groups ($p<0.05$). CASP-3 demonstrated similar expression at all groups at day 3, 7 and 14. Day 1 demonstrated the highest expression of CASP-3 among all groups ($p<0.05$).



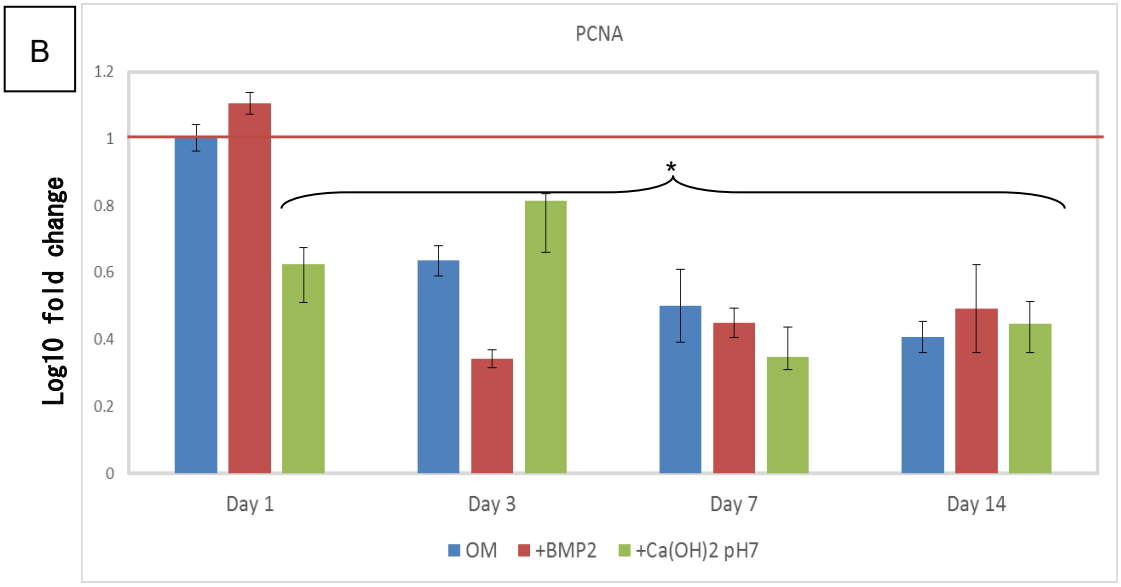


Figure 4- 4: Gene expression of PCNA of cells challenged with (A) culture medium (CM) or (B) osteogenic medium (OM) supplemented with either BMP2 or $Ca(OH)_2$ at pH7 and cultured for 1, 3, 7 and 14 days. * $p<0.05$. Error bars indicate standard error of the mean.

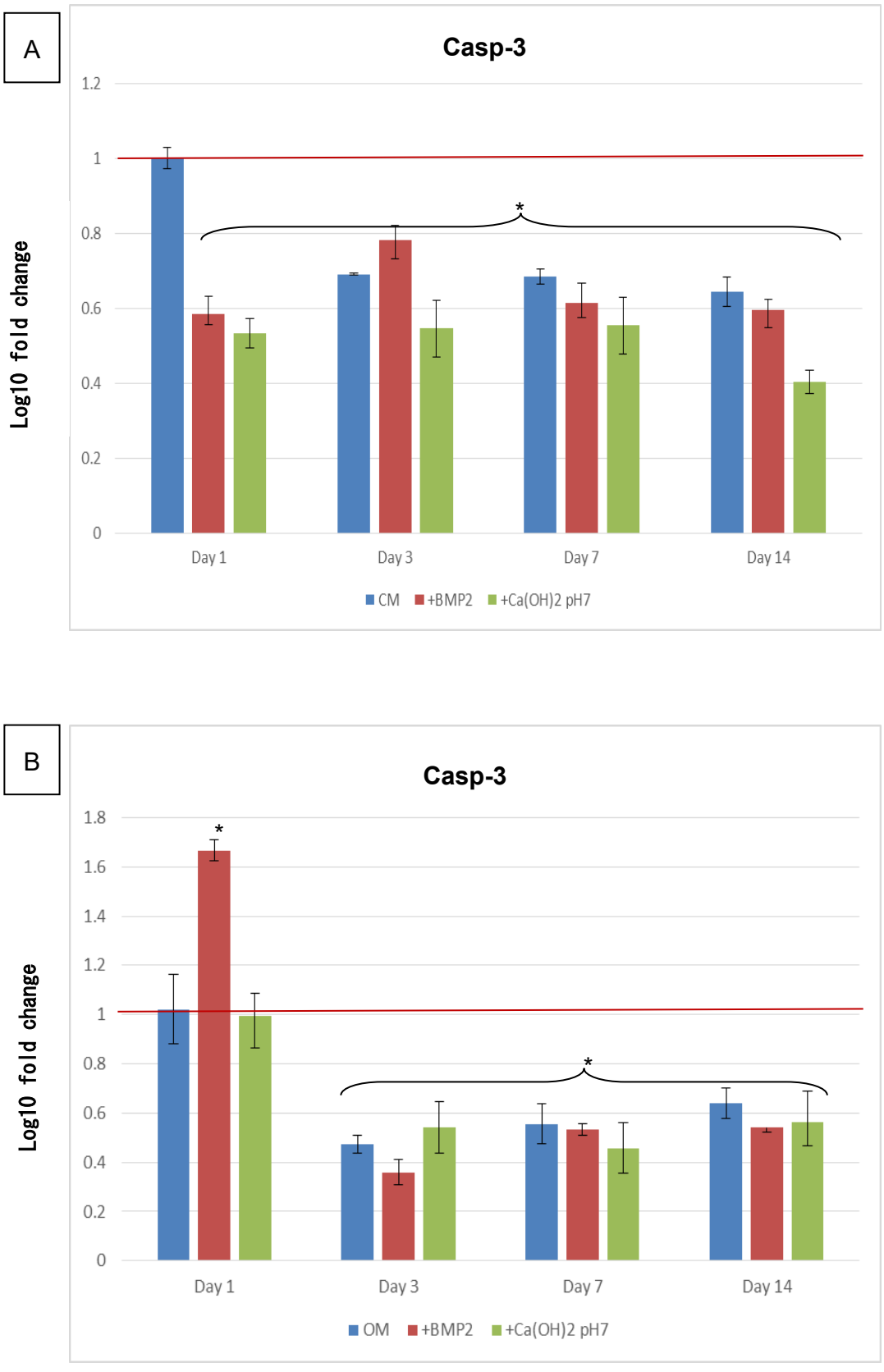


Figure 4- 5: Gene Expressions of CASPASE-3 of cells challenged with (A) culture medium (CM) or (B) osteogenic medium (OM) supplemented with either BMP2 or $Ca(OH)_2$ at pH7 and cultured for 1, 3, 7 and 14 days. * $p<0.05$. Error bars indicate standard error of the mean

4.3.4 qPCR Gene Expressions of Osteopontin and Bone Sialoprotein

qPCR was used to quantify level of expression of, bone related genes, osteopontin and Bone Sialoprotein after 14 days of cell culture (Figure 4.6). The culture medium supplemented group demonstrated no expression of osteopontin and bone sialoprotein at cell cultured with culture medium or culture medium supplemented with BMP2 and $Ca(OH)_2$-pH7. Osteogenic medium supplemented group demonstrated expression of both osteopontin and bone sialoprotein. However there were no significant differences ($P>0.01$) for expression of bone sialoprotein between cells treated with osteogenic medium and osteogenic medium supplemented with BMP2 and $Ca(OH)_2$-pH7. All osteogenic medium supplemented groups demonstrated a significant increase ($P<0.01$) in expression of osteopontin with significantly higher expression of osteopontin at cells treated with $Ca(OH)_2$-pH7 when compared with cells treated with osteogenic medium or BMP2.

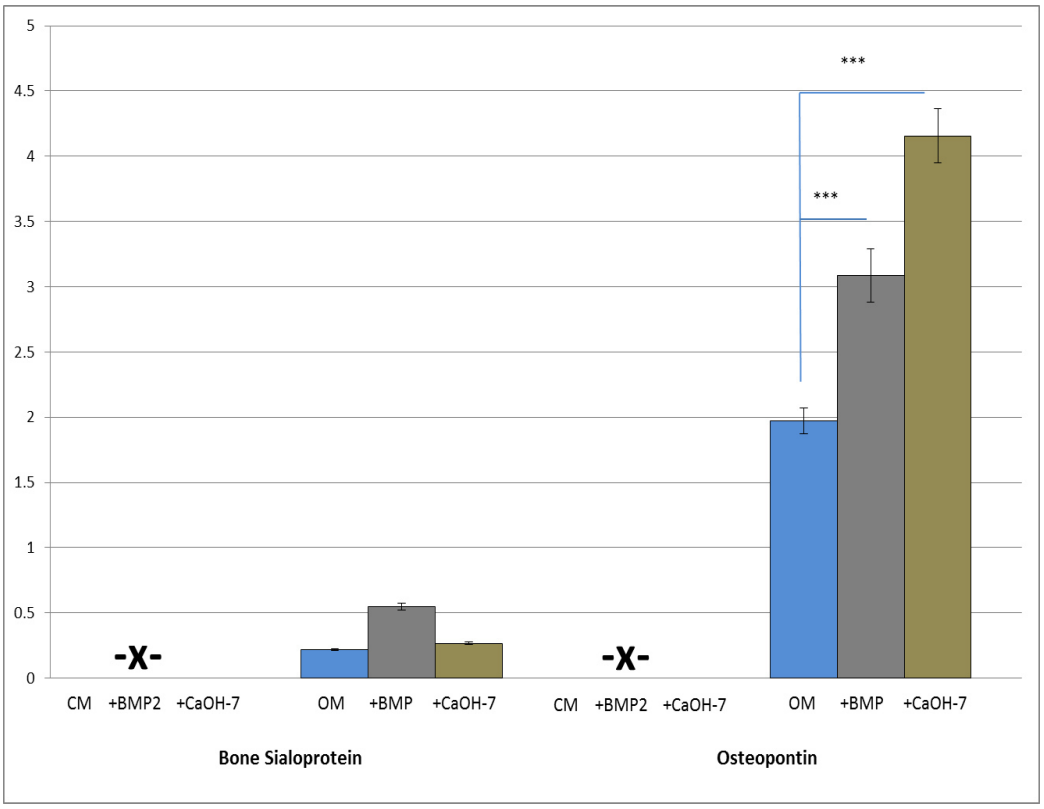


Figure 4- 6: qPCR Gene Expressions of osteopontin and Bone Sialoprotein from cells challenged with culture or osteogenic medium supplemented with either BMP2, Ca(OH)2 at pH7 and cultured for 14 days.CM :culture medium, OM: osteogenic medium, ***P<0.01. Error bars indicate standard error of the mean.

4.3.5 Immunocytochemical staining

4.3.5.1 PCNA and Caspase-3 Immunolocalisation

Within the culture medium supplemented group, PCNA (Figure 4.7) and Caspase-3 (Figure 4.9) were expressed in cells treated with culture medium and culture medium supplemented with BMP2, $Ca(OH)_2$ at pH7 and pH11 at day 1, 3 and 7. More cells were stained positive to PCNA were observed at day 3 in all treatment group. At day 14, PCNA and Caspase-3 were expressed in cells treated with culture medium and culture medium supplemented with BMP2 and $Ca(OH)_2$ at pH7 but not with cells treated with culture medium supplemented with $Ca(OH)_2$ at pH11 as no cells were available to stain. Negative controls where primary antibodies were omitted and control Isotype demonstrated only the dark blue nucleus with no non-specific staining (Figure 4.4 and 4.6).

Within the osteogenic medium supplemented group, PCNA (Figure 4.8) and Caspase-3 (Figure 4.10) were expressed at day 1, 3 and 7 in all cell groups. At day 14, PCNA and Caspase-3 were expressed in cells treated with osteogenic medium and osteogenic medium supplemented with BMP2 and $Ca(OH)_2$ at pH7 but not with cells treated with osteogenic medium supplemented with $Ca(OH)_2$ at pH11 as no cells were available to stain. Negative controls where primary antibodies were omitted and control Isotype demonstrated only the dark blue nucleus with no non-specific staining (Figure 4.5 and 4.7).

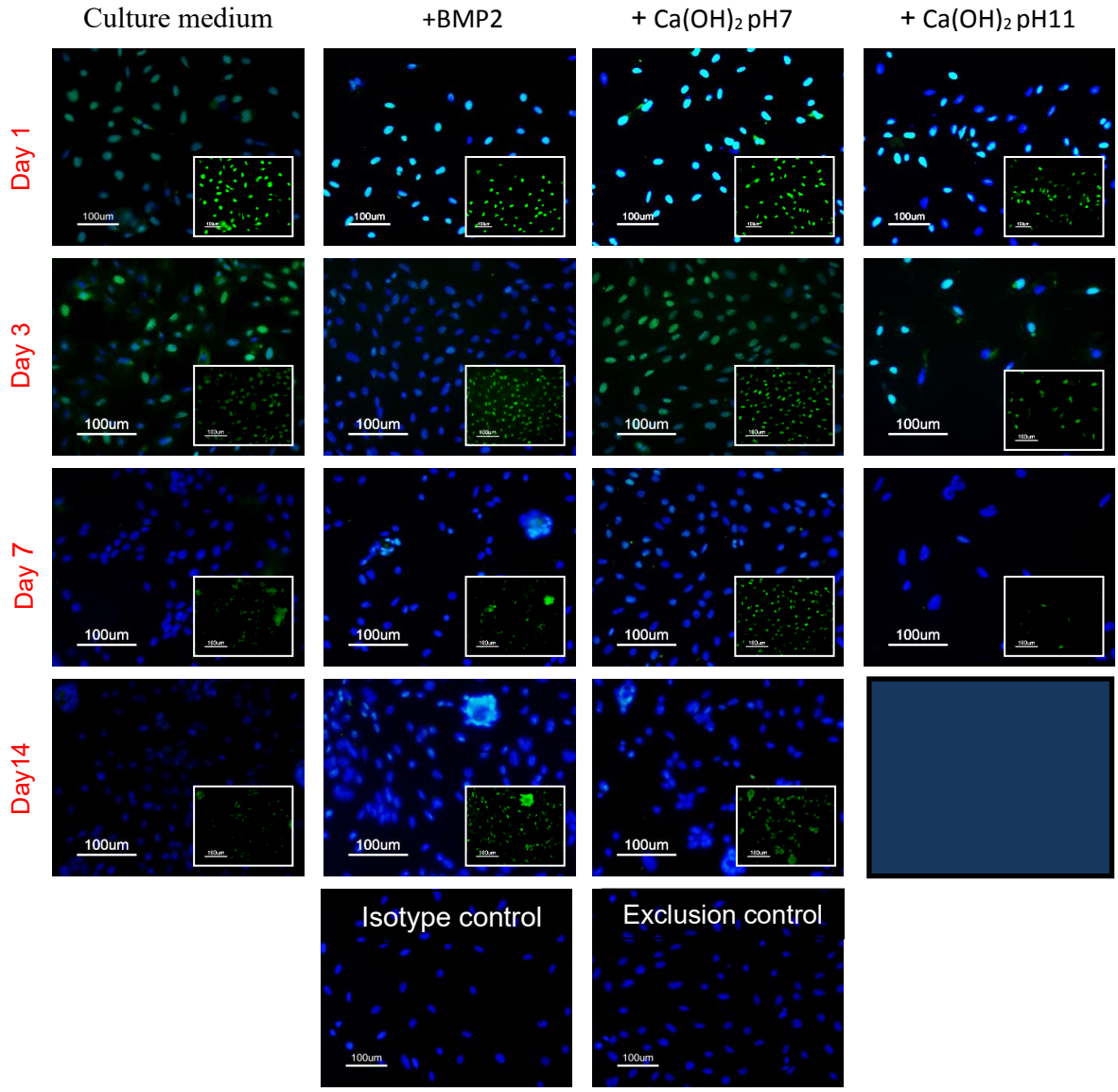


Figure 4- 7: PCNA immunolocalisation for cells challenged with culture medium supplemented with either BMP2, $Ca(OH)_2$ at pH7 or pH 11 and cultured for 1, 3, 7 and 14 days. PCNA was expressed at all cells groups. Isotype and exclusion antibody negative controls were only expressed the blue DAPI nuclear stain. Small boxes show cells with PCNA FITC stain without DAPI stain.

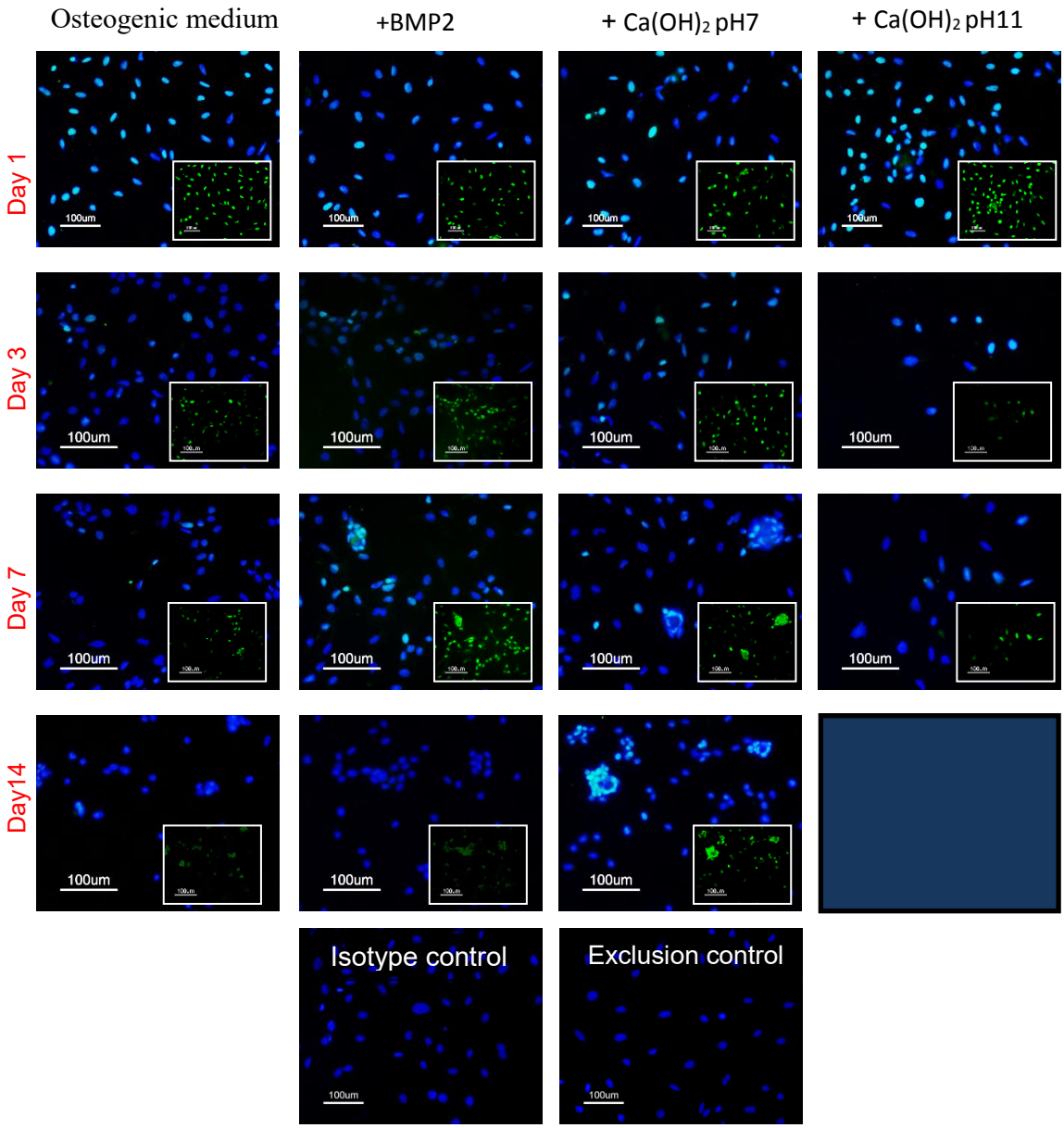


Figure 4- 8: PCNA immunolocalisation for cells challenged with osteogenic medium supplemented with either BMP2, $Ca(OH)_2$ at pH7 or pH 11 and cultured for 1, 3, 7 and 14 days. PCNA was expressed at all cells groups. Isotype and exclusion antibody negative controls were only expressed the blue DAPI nuclear stain. Small boxes show cells with PCNA FITC stain without DAPI stain.

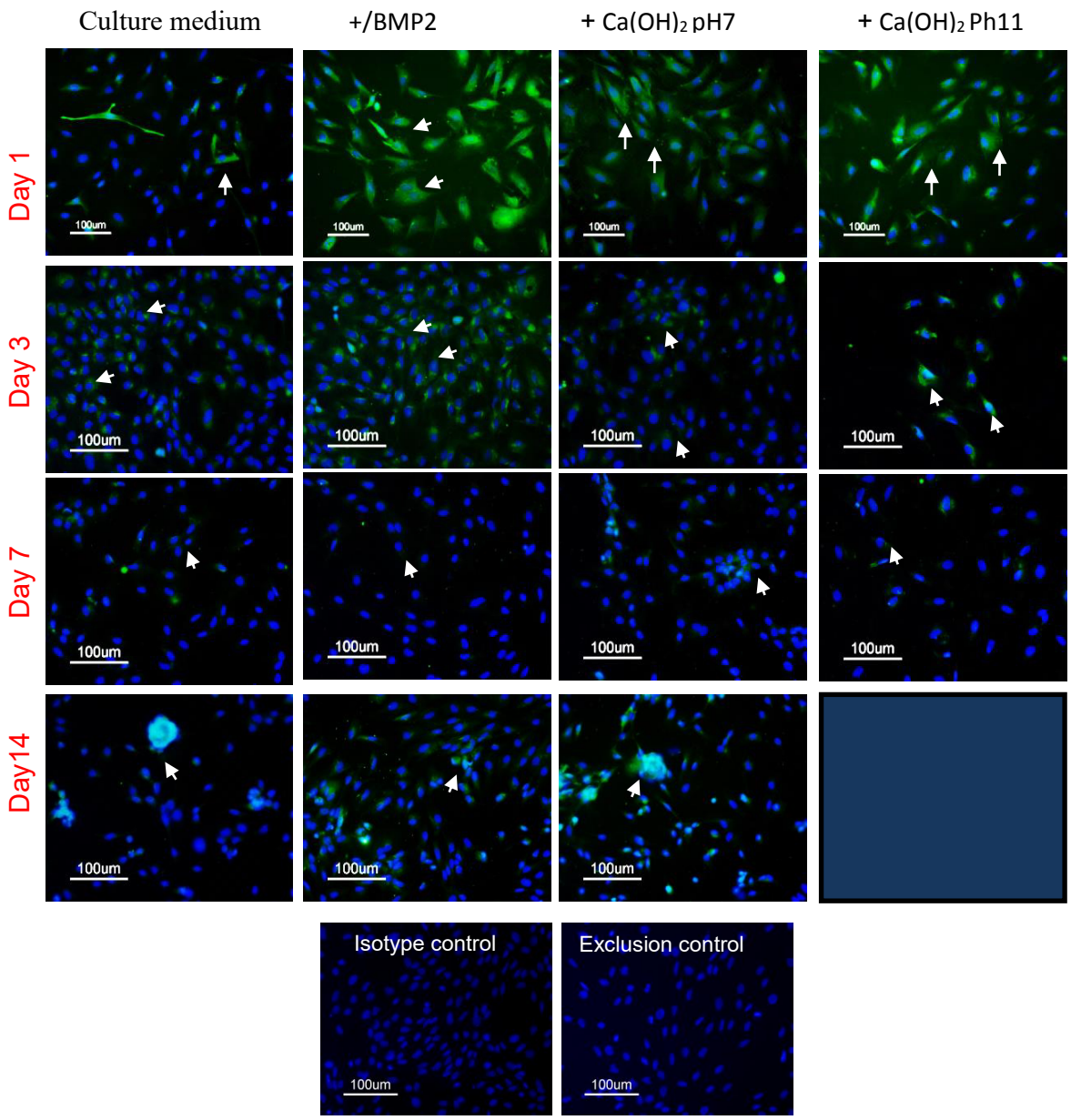


Figure 4-9: CASP-3 immunolocalisation for cells challenged with culture medium supplemented with either BMP2, $Ca(OH)_2$ at pH7 or pH 11 and cultured for 1, 3, 7 and 14 days. CASP-3 was expressed at all cells groups confirmed by green-FITC (arrow). Isotype and exclusion antibody negative controls were only expressed the blue DAPI nuclear stain.

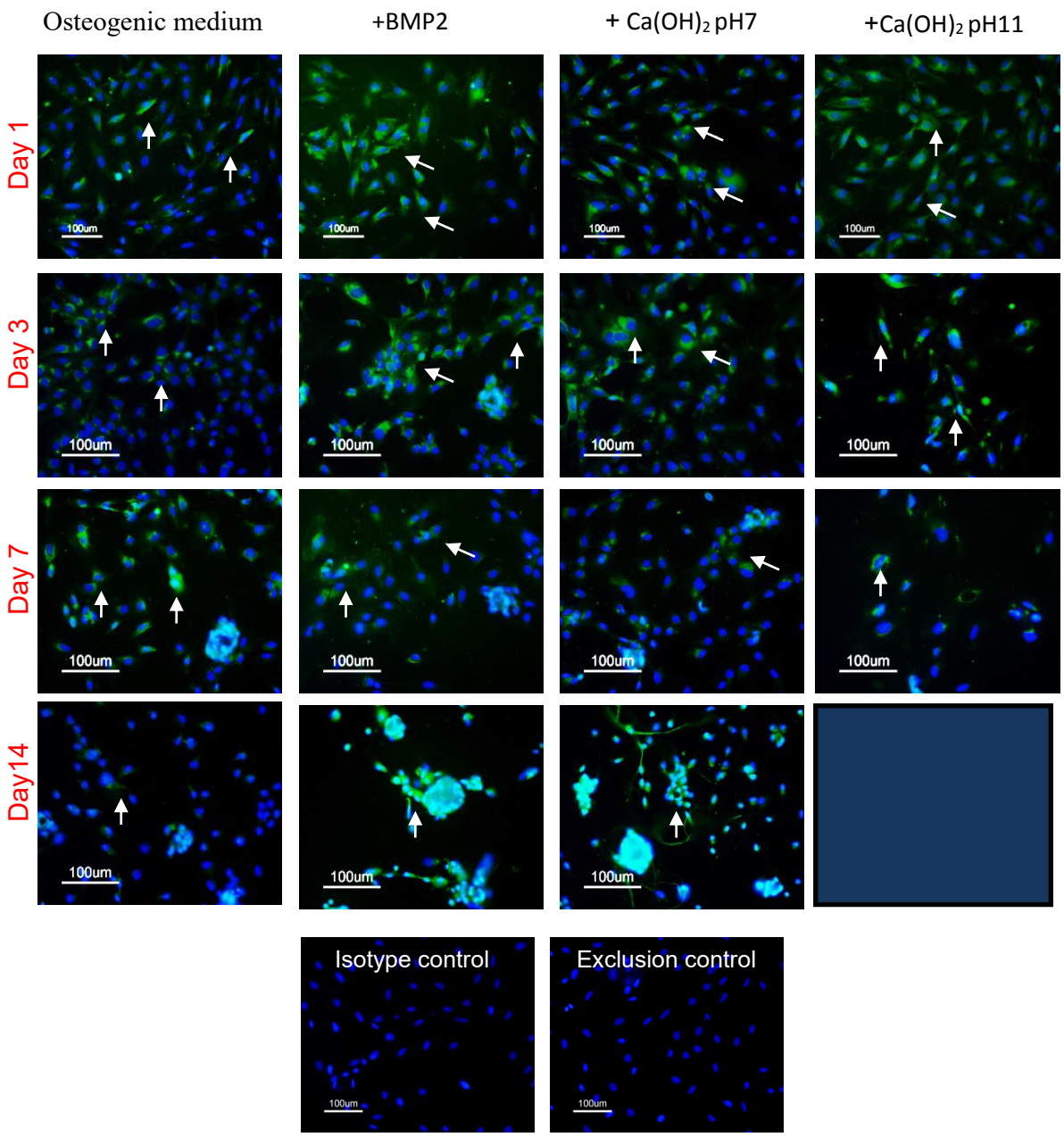


Figure 4-10: CASP-3 immunolocalisation for cells challenged with osteogenic medium supplemented with either BMP2, $Ca(OH)_2$ at pH7 or pH 11 and cultured for 1, 3, 7 and 14 days. CASP-3 was expressed at all cells groups confirmed by green-FITC (arrow). Isotype and exclusion antibody negative controls were only expressed the blue DAPI nuclear stain.

4.3.5.2 Osteopontin and Bone Sialoprotein Immunolocalisation

Within the culture medium supplemented group, BSP (Figure 4.11) and osteopontin (Figure 4.13) were not expressed at day 1, 3 and 7 in all cell groups. At day 14, BSP and osteopontin were expressed in cells treated with culture medium supplemented with BMP2 and $Ca(OH)_2$ at pH7 but not with cells treated with culture medium. BSP and osteopontin expressions were not observed in cells treated with $Ca(OH)_2$ at pH11 as no cells were available to stain. Negative controls where primary antibodies were omitted and control Isotype demonstrated only the dark blue nucleus with no non-specific staining (Figure 4.11 and 4.13).

Within the osteogenic medium supplemented group, BSP (Figure 4.12) and osteopontin (Figure 4.14) were not expressed at day 1, 3 and 7 in all cell groups. At day 14, BSP and osteopontin were expressed in cells treated with osteogenic medium and osteogenic medium supplemented with BMP2 and $Ca(OH)_2$ at pH7. BSP and osteopontin expressions were not observed in cells treated with $Ca(OH)_2$ at pH11 as no cells were available to stain. Negative controls where primary antibodies were omitted and control Isotype demonstrated only the dark blue nucleus with no non-specific staining (Figure 4.12 and 4.14).

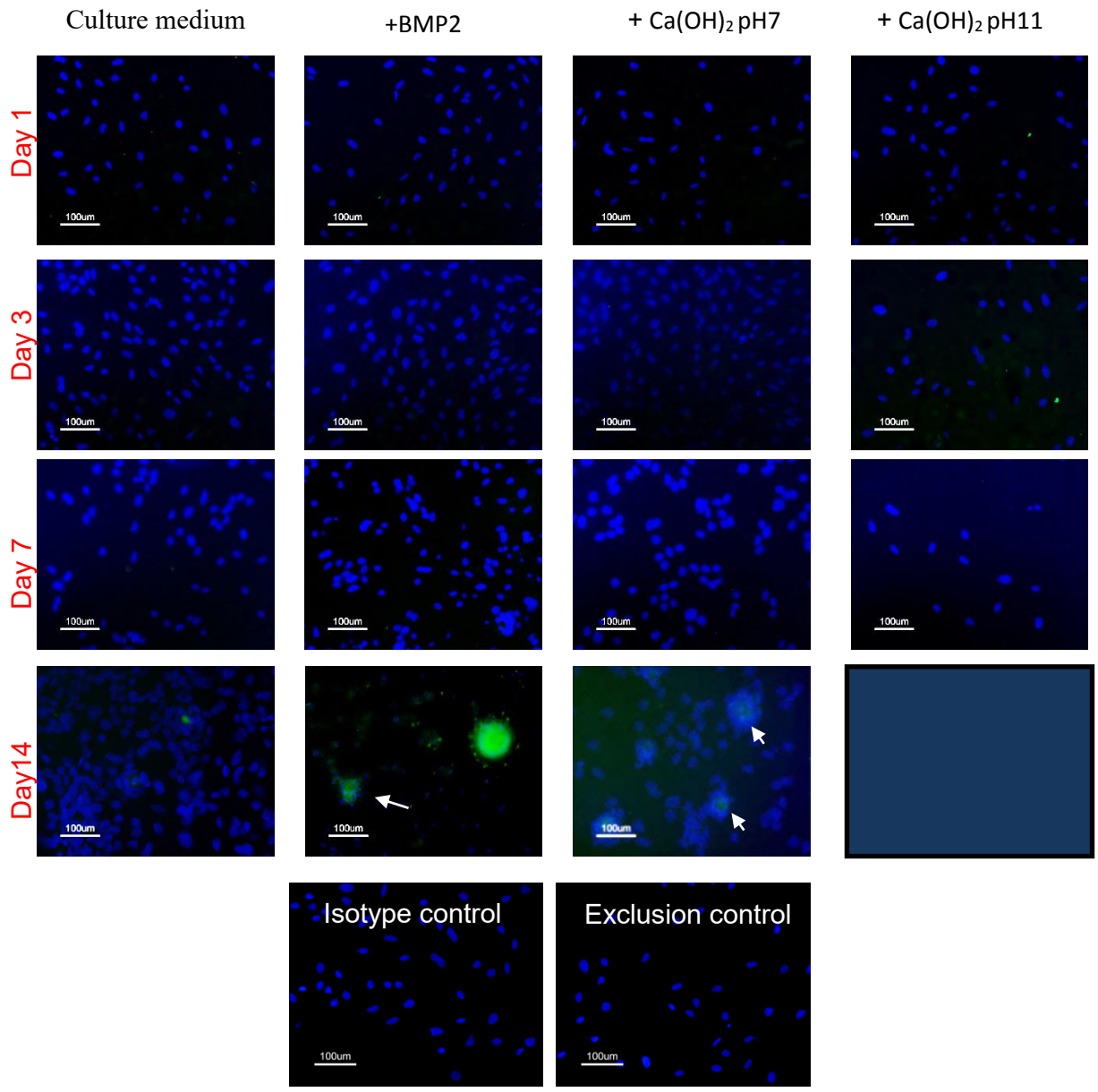


Figure 4- 11:BSP immunolocalisation for cells challenged with culture medium supplemented with either BMP2, $Ca(OH)_2$ at pH7 or pH 11 and cultured for 1, 3, 7 and 14 days. BSP was only expressed at day 14 at cells treated with culture medium supplemented with BMP2 and $Ca(OH)_2$ at pH7 as confirmed by green-FITC (arrow). Isotype and exclusion antibody negative controls were only expressed the blue DAPI nuclear stain.

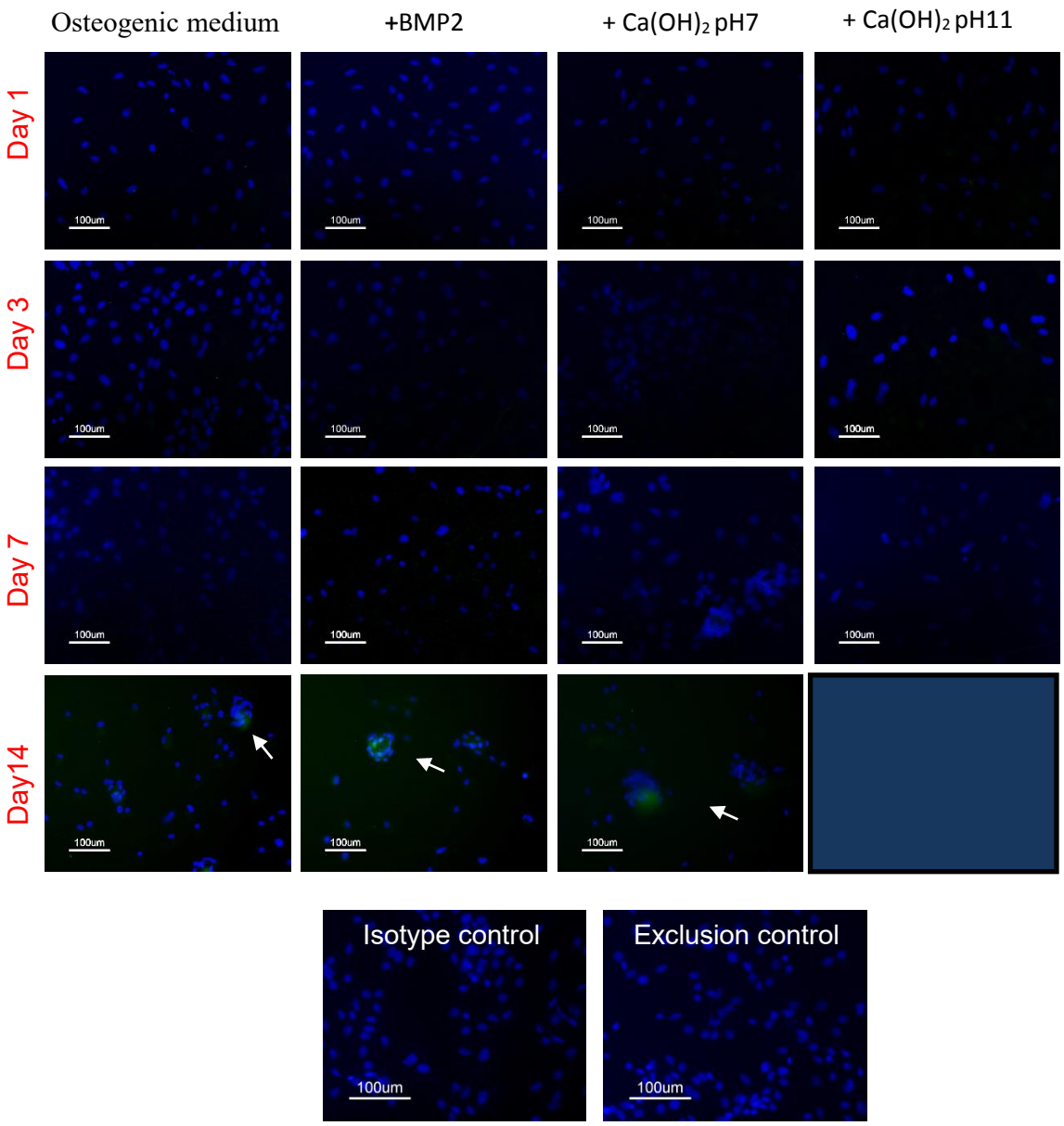


Figure 4- 12:BSP immunolocalisation for cells challenged with osteogenic medium supplemented with either BMP2, $Ca(OH)_2$ at pH7 or pH 11 and cultured for 1, 3, 7 and 14 days. BSP was only expressed at day 14 at cells treated with osteogenic medium and osteogenic medium supplemented with BMP2 and $Ca(OH)_2$ at pH7 as confirmed by green-FITC (arrow). Isotype and exclusion antibody negative controls were only expressed the blue DAPI nuclear stain.

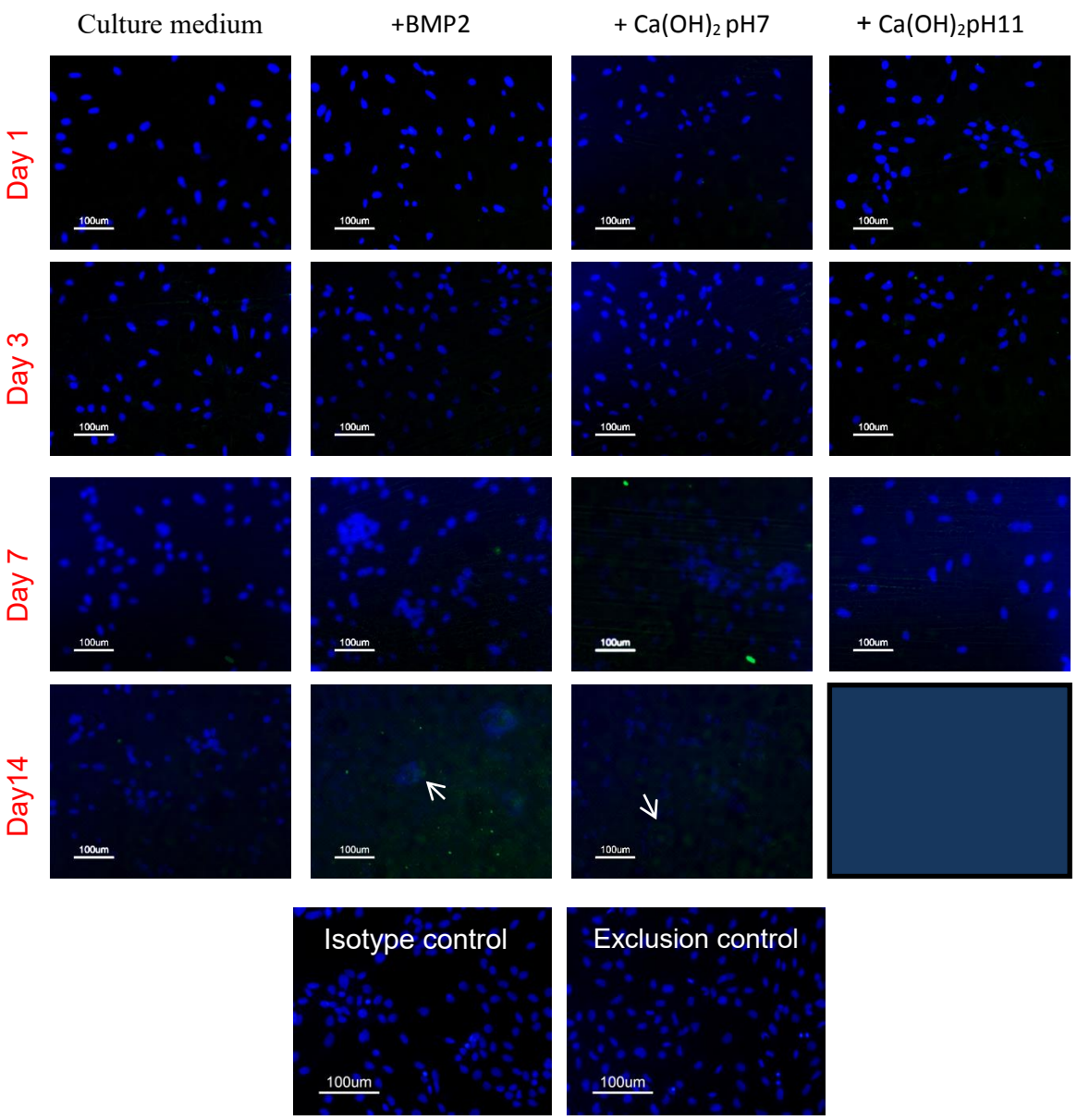


Figure 4- 13: Osteopontin immunolocalisation for cells challenged with culture medium supplemented with either BMP2, $Ca(OH)_2$ at pH7 or pH 11 and cultured for 1, 3, 7 and 14 days. OPN was only expressed at day 14 at cells treated with culture medium supplemented with BMP2 and $Ca(OH)_2$ at pH7 as confirmed by green-FITC (arrow). Isotype and exclusion antibody negative controls were only expressed the blue DAPI nuclear stain.

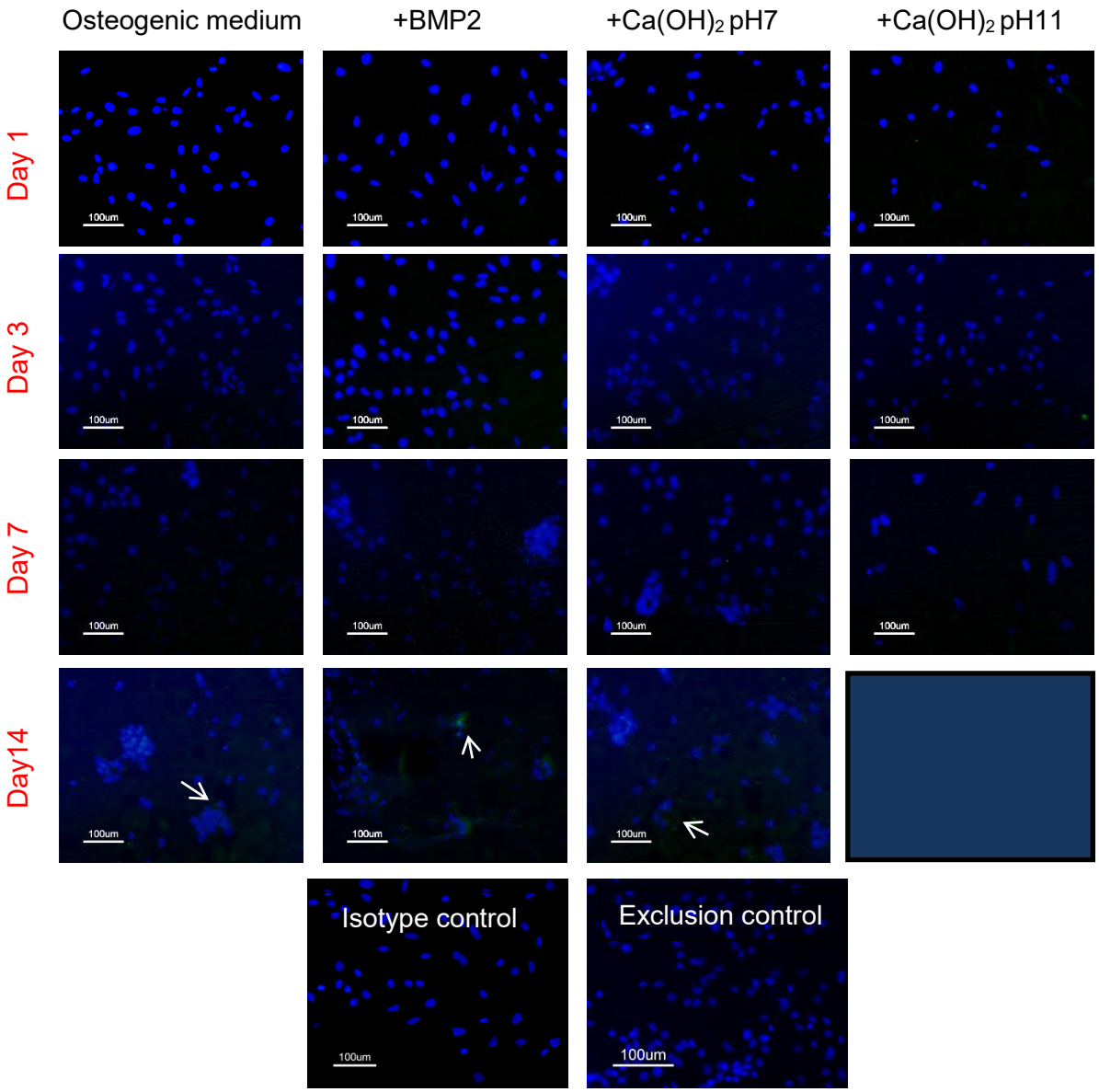


Figure 4- 14: Osteopontin immunolocalisation for cells challenged with osteogenic medium supplemented with either BMP2, $Ca(OH)_2$ at pH7 or pH 11 and cultured for 1, 3, 7 and 14 days. OPN was only expressed at day 14 at cells treated with osteogenic medium and osteogenic medium supplemented with BMP2 and $Ca(OH)_2$ at pH7 as confirmed by green-FITC (arrow). Isotype and exclusion antibody negative controls were only expressed the blue DAPI nuclear stain.

4.3.6 Alizarin Red Staining

Alizarin red staining was performed to investigate effects of calcium hydroxide on osteogenic differentiation. Cells were examined for Alizarin red staining after 14 days in culture (Figure 4.15). Within both culture medium and osteogenic medium supplemented group, cells cultured with $Ca(OH)_2$ at pH 7 and BMP2 were demonstrated multiple spherical nodules formation. Alizarin red staining was not detected in cells treated with culture medium or osteogenic medium supplemented with $Ca(OH)_2$ at pH11 as no cells were available to stain. Alizarin red staining was negative in cells treated with culture medium but not with osteogenic medium. Alizarin red staining demonstrated that cells challenged with $Ca(OH)_2$ at pH 7 demonstrated more nodule which was similar or higher than cells treated with osteogenic medium as well as BMP2.

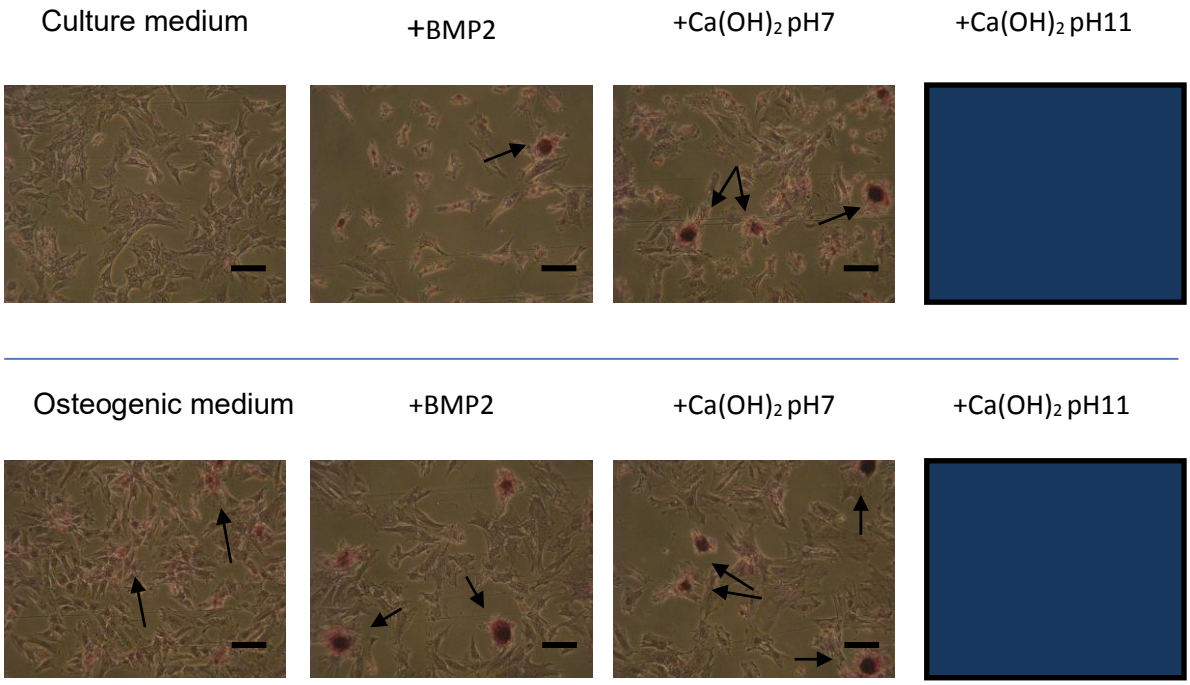


Figure 4- 15: Alizarin red staining for cells challenged with culture medium or osteogenic medium supplemented with either BMP2, $Ca(OH)_2$ at pH 7 or pH 11 at day 14. The red colour represents spherical nodules (arrow).

4.4 Discussion

The previous chapter revealed that growth factors can be released from the *ex vivo* bone model following calcium hydroxide treatment; inducing cellular proliferation and differentiation. Calcium hydroxide demonstrated an ability to mediate dentinogenesis either through direct stimulation on the cells or by solubilisation of dentine extracellular matrix (Sangwan et al. 2013). By investigating the effect of calcium hydroxide on *in vitro* cell culture, this chapter interestingly demonstrated that continuous administration of calcium hydroxide at pH 11 is cytotoxic to the cells; however calcium hydroxide at pH 7 mediates proliferative and osteogenic capacity similar or higher than BMP2.

Calcium hydroxide dissociates into calcium and hydroxyl ions. Calcium has been shown to play critical roles during cellular proliferation, migration, differentiation, and mineralization of the cells (Torneck et al. 1983; Kulesz-Martin et al. 1984; Schröder 1985; Zayzafoon 2006). Alkaline conditioned medium, pH 7.8, has also been shown to enhance human dental pulp cells mineralization (Okabe et al. 2006). It has been suggested that cells of oral mucosa have a protective response and demonstrated resistance to the effects of highly alkaline drinking water pH 11.2 and pH 12 (Merne *et al.* 2001). Extracellular calcium is also important during bone remodelling as it induced chemotaxis of osteoblasts and monocytes at site of resorption (Sugimoto *et al.* 1993). Although presence of calcium shows beneficial effect to the cells, increase calcium concentration can have a reverse effect on the cells. Influx of calcium can initiate toxic cell death (Schanne *et al.* 1979). It could be suggested that the highly alkaline environment triggered by the hydroxyl group caused disruption of the integrity of the cell

membrane followed by influx of high concentration of calcium ions across the damaged cell membrane leading to toxic death of the cells cultured with calcium hydroxide at pH 11.

Cells appeared to be unable to tolerate the pH11 after 7 days which suggests that this pH was above the maximum that the cells can tolerate. Further culture of the cells in the alkaline medium was considered toxic environment for the cells that can not buffer or tolerate after 7 days. Calcium hydroxide at pH 12 is used in dentistry as an antimicrobial reagent providing a sterile environment at the superficial layer of dentine. However the results of this chapter demonstrated that alkaline pH is toxic and led to cell death as demonstrated by reduction in cell viability and cytological examination. It may be possible that calcium hydroxide at pH 11 can exert either apoptotic effect or necrotic effect. Caspase three is a protein that is activated in the apoptotic cell (Salvesen 2002; Ghavami *et al.* 2009) after apoptotic signalling events have occurred (Walters *et al.* 2009). Immunocytochemistry and PCR for casp-3 were performed to investigate the effect of calcium hydroxide on the cells. However, The alkaline environment appeared to reduce cell attachment, possibly due to cell death, and thus cells were difficult to find.

Addition of calcium hydroxide at pH 7 is similar to suplememting the cells with calcium only, since the effect of the hydroxyl group is eleminated during pH optimization. It has been reported that calcium has the ability to influence proliferation and differentiation of cells (Walker *et al.* 2006) and extracellular

calcium has also been shown to induce cellulaur differentiation without reducing cellular proliferation (Micallef *et al.* 2009). Result of this chapter demonstrated that cells cultured with calcium hydroxide at pH 7 demonstrated a similar effect to cells cultured with BMP2. BMP2 is also known to stimulate celluar proliferation and differentiation (Liu et al. 2009; Hakki et al. 2014). It is likely that both calcium and BMP2 were stimulting the proliferation and differentiation of the cells as demonstrated by the positive expression of PCNA, OPN and BSP and alizarin red staining.

Calcium hydroxide at pH 11 was applied continuously to the cells leading to a gradual decrease in cell number during the culture period. Calcium hydroxide at alkaline pH however was shown at previous chapter to facilitate cellular proliferation, migration and differentiation in *ex vivo* experiments through the release of growth factors. It could be that the concentraion used in this expirement was higher than cell's ability or the continuous addition of alkaline calcium hydroxide resulted in cell death found during cell culture with calcium hydroxide at pH 11. It has been reported that calcium hydroxide may be mitogenic at certain concentrations (Torneck et al. 1983) and the pH used in this experiment could be too high for cell survival.

PCNA, a proliferation marker, and capase-3, apoptotic marker, were expressed in all groups at gene as well as protein level. Peak of cellular proliferation has been demonstrated in several studies to be at day 3 (Einhorn 1998; Ai-Aql et al. 2008; Dimitriou et al. 2011). However PCNA gene expression was upregulated

at day 1, 3, 7 and 14 and this was also confirmed by immunolocalization. This could be because the cells cultured at same seeding density; rate of proliferation were similar among all groups except at alkaline calcium hydroxide where cells were decreased in number. Although culturing the cells at alkaline calcium hydroxide demonstrated a cytotoxic lethal effect, capase-3 was not only upregulated at cells cultured with alkaline calcium hydroxide but also expressed at the other group. This is likely a programmed cell death due to the nature of bone marrow stem cells were they constantly replaced due to continual bone turnover and remodelling during life-span (Parfitt 1984).

Immuolocalization demonstrated expression of osteopontin and bone sialoprotein at cells treated with calcium hydroxide at pH 7 and BMP2 at both culture and osteogenic medium groups. Expression of osteopontin and bone sialoprotein were also confirmed by qPCR analysis at osteogenic medium supplemented group but not at culture medium supplemented group. It could be that osteogenic medium was the cause of osteogenesis at gene and protein level not BMP2 or calcium hydroxide but osteogenesis was induced by culture medium supplemented by BMP2 and calcium hydroxide. These were confirmed by alizarin red staining as well as Immuolocalisation. Osteopontin and bone sialoprotein were analysed for gene levels at day 14 and it could be that their synthesis and gene expression were missed at day 14 at culture medium supplemented groups but osteogenic medium supplemented group have an additional factor for osteogenesis- induced by osteogenic medium. Alizarin red staining demonstrated multiple spherical nodules formed at cells treated with cells treated with osteogenic medium, BMP2 and calcium hydroxide at day 14

which represent the beginning of calcified deposition of the extracellular matrix. Results demonstrated that number of nodules was higher at cells treated with calcium hydroxide. Also osteopontin was upregulated at cells treated with calcium hydroxide. It has been reported that osteopontin have a high affinity to binds to multiple calcium ions (Chen *et al.* 1992; Kläning *et al.* 2014) inducing hydroxyapatite formation (Ito *et al.* 2004). Culturing of the cells for longer period will further investigate effect of calcium hydroxide on matrix calcification and mineralization.

Although both osteopontin and bone sialoprotein are bone related genes and they upregulated at cells treated with BMP2 and calcium hydroxide, osteopontin was highly expressed when compared with expression of bone sialoprotein. Osteopontin is a phosphorylated glycoprotein expressed by osteoblasts and involved in cell adhesion, migration, and survival (Sodek *et al.* 2000). It has been reported that osteopontin is first to be expressed during differentiation of osteoblast progenitors (Yamate et al. 1997) and prior to the expression of BSP (Liu *et al.* 1994; Lekic *et al.* 1996). The cells cultured at day 14 are still at stage of osteoblastic differentiation and are not fully mature osteoblast. This suggest that osteopontin was expressed more than bone sialoprotein. Furthermore, osteopontin was significantly upregulated in cells treated with calcium hydroxide more than cells treated with BMP2. This is likely indicate that calcium hydroxide is able to induce osteoblastic differentiation more than BMP2. It has been demonstrated that calcium ion induced mRNA expression of osteopontin (Rashid et al. 2003; Chang *et al.* 2008)

Calcium hydroxide is incorporated in multiple clinical materials and is used at different pH ranging from physiological to alkaline pH. Although calcium hydroxide is a gold standard treatment in endodontic therapies, it has been associated with some side effects related to its alkalinity, viscosity, and application time. The results of this chapter demonstrated that calcium hydroxide effect on the cell is different at different pH. While continuous supplementation of calcium hydroxide at pH 7 mediates proliferative and osteogenic capacity of the cells, calcium hydroxide at pH 11 is cytotoxic to the cells. Testing calcium hydroxide at different pH, concentration and application time will give better understanding of its effect on the cells and maximise its beneficial effect of mediating not only dentine regeneration but also bone regeneration.

Chapter 5.

Expression of bone morphogenic fusion protein in HEK cell lines and assessment of their biological activity

5.1 Introduction

Bone morphogenetic proteins (BMP) are important growth factors that have a wide range of developmental and post-natal functions. They have been shown to be important in skeletal system, for instance BMP2 is known to be osteoinductive agent; inducing commitment and differentiation of progenitor cells into osteoblasts (Wang *et al.* 1993; Ogasawara *et al.* 2004). It also plays a critical role as a chemoattractant agent, stimulating migration of progenitor cells and mature osteoblasts to the site of injury (Fiedler *et al.* 2002). BMP7, another bone morphogenetic protein, has been shown to stimulate proliferation, osteogenic differentiation and mineralization *in vitro* in human osteoporotic tissues inducing alkaline phosphatase activity and calcium production in growing osteoblasts (Pountos *et al.* 2010a). BMP7 has also been shown to stimulate osteogenesis, cementum regeneration, and new attachment formation at mandibular osseous defects (Giannobile et al. 1998) and in maxillary sinus floor augmentation procedures before placing implant (Bergh et al. 2000).

BMPs have been used in *in vitro*, animal models and human clinical trials to investigate and maximise their potential benefits in improving osteogenesis. BMPs, particularly BMP2 and BMP7, have been used clinically and

demonstrated a therapeutic effect for the treatment of bone diseases such as treating open tibia, non-union fractures and spine injuries (Friedlaender et al. 2001; Carragee et al. 2011). When compared with conventional fracture therapies such as bone grafts, BMP2 and BMP7 have been demonstrated to have effective and superior outcomes especially in healing of open tibial fractures and spinal fusion injuries (Garrison *et al.* 2007). In 2002, a large prospective study with 450 participants from France, Germany and the UK, patients received 1.5 mg/ml BMP2 for treating open tibia fractures. Results demonstrated enhanced bone repair and significant reduction of the risk of secondary intervention and infection (Govender *et al.* 2002b).

Although BMP2 and BMP7 have been approved for clinical use showing remarkable outcomes, there are still some concerns associated with their clinical treatments. A major obstacle for the therapeutic uses of BMP2 and BMP7 are the biological short half-life of the growth factor; about 30 min in case of BMP7 (Vukicevic *et al.* 1998) and ranging from 4-6 hours in case of BMP2 (Gruendler et al. 2001). Clinical use of BMP2 and BMP7 have been shown to be successful in some cases (McKay *et al.* 2007; White *et al.* 2007) and unsuccessful in other cases (Carragee et al. 2011), which could be that the concentration of BMP required by each tissue is different and tissues/ cells may respond differently to different BMPs. This could be due to the issue of the perfect time to add external BMP to the tissue and the present of local proteins that may interact with external BMP in an agonist or antagonist effect (Brazil *et al.* 2015). Noggin is one of the soluble BMP antagonist which binds to BMPs inhibiting BMPs from binding to their cell surface receptors and disabling the initiation of BMP signal

transduction in target cells (Groppe *et al.* 2002; Winkler *et al.* 2004). It has been demonstrated that BMP heterodimers are more resistant to inhibition by antagonists such as noggin (Zhu *et al.* 2006). Furthermore the high effective dose required which can reach into milligram and a series of potential side effects lead to the concerns in using BMP in the clinic. Some of the common side effects associated with BMP include inflammatory reactions, radiculitis, ectopic bone formation, osteolysis, malignancy (Carragee et al. 2011) and life-threatening events such as compression of the airway and/or neurological structures (Food and Drug Administration 2008). All of these limitations of BMP administration arose from the high dose of the protein required for bone formation, the poor stability and transient biological activity of the protein, insist the need of better way to enhance protein stability and reduce the concentration.

Gene therapy provides an alternative treatment strategy to deliver BMPs to the tissues. It has been shown that delivering BMP7 gene to periodontal bone defects in rats demonstrating bridging of the periodontal bone defects and periodontal tissue engineering through rapid osteogenesis and cementogenesis (Jin *et al.* 2003). Different studies have aimed to compare the effects of BMP2-7 heterodimer and BMP homodimers on bone regeneration. An *in vitro* studies demonstrated that a rhBMP2-7 heterodimer induced cell migration and cell differentiation with a significantly lower concentration and greater effect than the rhBMP2 and rhBMP7 homodimers (Zheng *et al.* 2010). In a bone defect model, a BMP2-7 heterodimer was also shown to induce new bone formation in a significantly higher quality and quantity of bone volume and bone implant contact greater than that of BMP2 and BMP7 homodimers in peri-implant bone defects

in minipig calvaria (Wang *et al.* 2012) and on minipig frontal skull (Sun *et al.* 2012). Both studies used collagen sponges with low-dose (30 ng/mL) BMP2-7 heterodimer, BMP2 or BMP7 homodimers and concluded that BMP could be applied at a low dose to reduce the cost and side effects of BMP (Sun et al. 2012; Wang et al. 2012). Growth factor therapy is required to be applied at an appropriate dose with a suitable carrier at the correct application time and with reduced costs and side effects of the therapy. Gene therapies are now being used as a promising technique that may provide a better way to enhance protein stability and reduce the cost and concentration of current clinical growth factor application. This chapter aims to transfect BMP2-2, BMP7-7 homodimers and BMP2-7 heterodimer into HEK cell lines followed by assessment of the efficacy of low dose of BMP protein on hBMSCs.

5.2 Materials and Methods

5.2.1 Preparation of Transformation and Transfection reagents

A 15.5 g of 2xYT microbial growth medium powder (Sigma Aldrich, UK) was dissolved in 500 ml of dH_2O and sterilized by autoclaving. After autoclaving, a 480 µL of ampicillin (100 mg/ml) was added to 480 ml medium at room temperature as ampicillin contained medium (2xYT-ampicillin) and 20 ml of the medium without ampicillin was used as ampicillin free medium. A LB-amp Broth/Agar was prepared by dissolving 17.5 g of LB-broth powder (Sigma Aldrich, UK) in 500 ml of dH_2O and sterilized by autoclaving. A 500 µL ampicillin (100 mg/ml) was added to the LB-broth solution at room temperature after autoclaving. The liquid agar was poured into bacterial plates, and left to set for 10 min prior storing at -4ºC. Full medium for cell culture was consisted of Dulbecco's modified Eagle's medium (DMEM), 10% foetal calf serum (FCS, Invitrogen, UK), and 1% antibiotics (100 units/ml penicillin G sodium, 0.1µg/ml streptomycin sulphate and 0.25µg/ml amphotericin; Invitrogen, Paisley, UK). Selective medium was made from the full medium in addition to 400ug/ml Zeocin™.

5.2.2 Transformation of competent cells

Three fusion proteins, BMP2 homodimer, BMP7 homodimer and BMP2-7 heterodimer, were previously constructed from a pS-L-Fc vector by Dr Xiao-Qing Wei, School of Dentistry, Cardiff University. Briefly, pS-L-Fc, a mammalian expression vector which was constructed to fuse two proteins, BMP2-2, BMP7-7

and BMP2-7, linked with a linker peptide (3xGGGGS), in-frame with the human IgG1 Fc region (Figure 5.1). A 50 µL aliquot of DH5 α competent cells (Invitrogen, Scotland) was mixed gently with 5ng plasmid. Cells were incubated on ice for 30 minutes then immediately transferred in a previously warmed water bath (42°C) for 20 seconds then replaced again on ice for 2 minutes. A 950 µL aliquot of room temperature ampicillin free medium was added to each tube. Cells were incubated on a shaking incubator (Innova 4300, New Brunswick scientific Co., Inc., USA) at 37°C at 225 rpm for 1 hour. After incubation, cells were centrifuged at 13.000 rpm for 15 seconds. Cell supernatants were discarded and the pellet was resuspended and plated out using a cell spreader into LB- agar plates containing (100 mg/ml) ampicillin. Plates were incubated overnight at 37°C to allow colony formation, and plates stored at -4ºC.

After colony formation, two single colonies were picked from each plate using a pipette tip and placed in a universal tube previously filled with 2ml of 2xYT-ampicillin. Cells were incubated on a shaking incubator at 37°C and 225 rpm for overnight. After incubation, 1 ml of the culture medium was used to extract DNA using a QIAprep miniprep kit for plasmid purification and digestion to select the best colony and the remaining medium was stored at -4ºC.

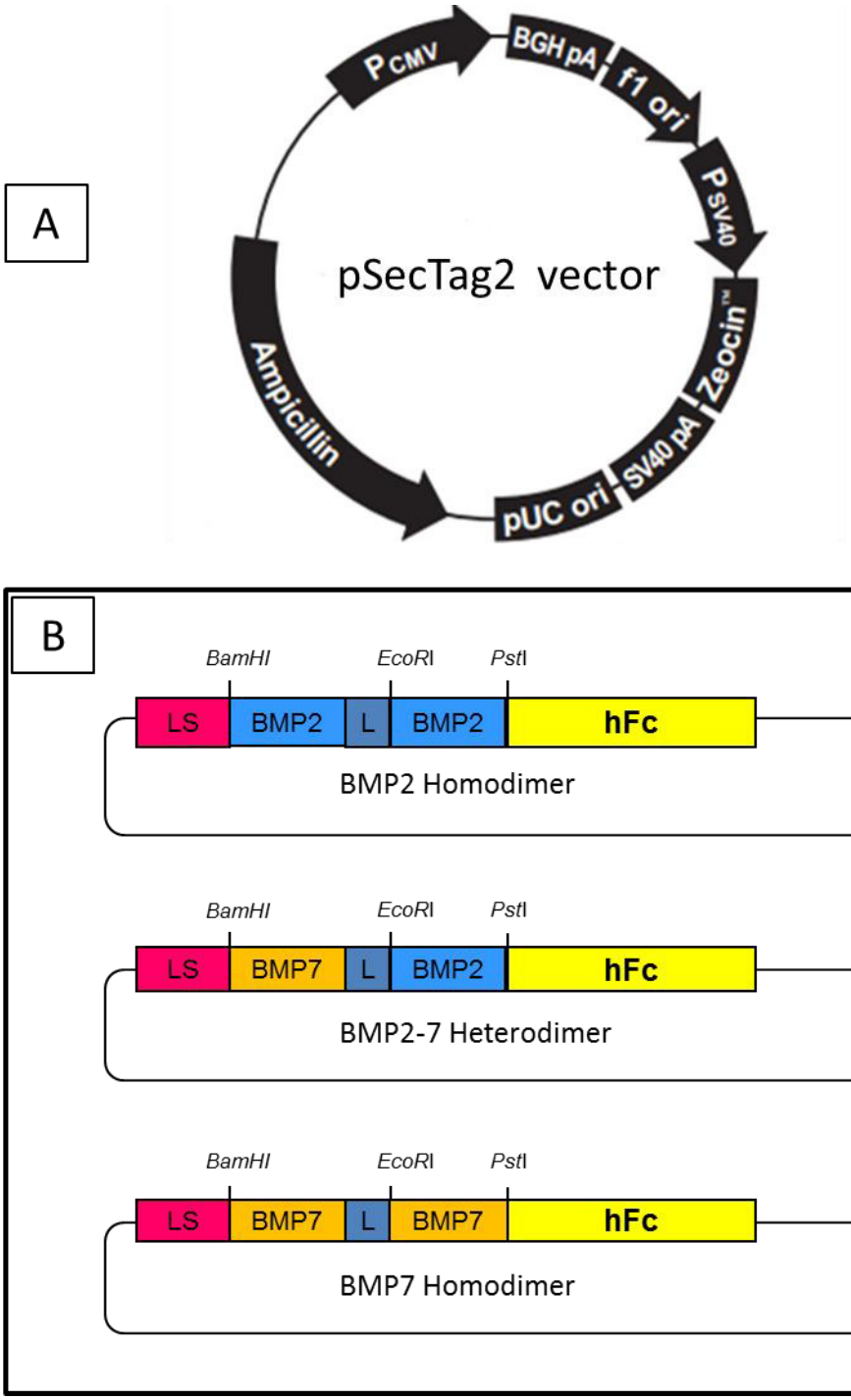


Figure 5- 1: A map of pSecTag2 vector (A) modified to construct BMP2-2, BMP2-7 and BMP7-7 fusion protein (B). Three fusion proteins BMP2-2, BMP7-7 and BMP2-7 were constructed from pSec-L-Fc vector. The vector carries s ampicillin and Zeocin™ resistance genes for antibiotic selection. It contains a human hinge Fc domain for intracellular stability, quantitative assay and purification.

5.2.2.1 Miniprep Plasmid Purification

A QIAprep spin miniprep kit (Qiagen, UK) was used for plasmid isolation and purification of bacterial cultures grown in 2xYT medium. 1ml of bacterial cell culture was centrifuged at 13.000 rpm for 3 min at room temperature. Pellets were resuspend in 250 µL Buffer P1 and transferred to a microcentrifuge tube prior to addition of 250 µL of Buffer P2. The solution was mixed thoroughly by inverting the tube 4–6 times until the solution became clear. 350 µL of Buffer N3 was added and the solution mixed immediately and thoroughly by inverting the tube 4–6 times. The solution was centrifuged for 10 min at 13,000 rpm. The supernatant was transferred to the QIAprep spin column prior centrifuging for 1 min and the flow-through was discarded. The QIAprep spin column was washed by adding 0.75 ml buffer PE and centrifuged for 1 min. The flow-through was discarded and the column was recentrifuged for 1 min to remove residual wash buffer. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube and 50 µL buffer EB was added to the center of the QIAprep spin column to elute DNA, left to stand for 1 min, and centrifuged for 1 min.

5.2.2.2 Plasmid Digestion

The purified DNA isolated by the miniprep was digested using three digestion enzymes. Single digests were performed using 2 µL miniprep DNA sample, 1 µL BamH1, 2 µL buffer E, 0.2 µL BSA and made up to a total volume of 20 µL with 15 µL dH_2O. Double digests were performed using 2 µL miniprep DNA sample, 1 µL EcoR1, 1 µL Pst1, 2 µL buffer H, 0.2 µL BSA and made up to a total volume of 20 µL with 14 µL dH_2O. Double digests were also performed using 2 µL

miniprep DNA sample, 1 µL BamH1, 1 µL EcoR1, 2 µL buffer E, 0.2 µL BSA and made up to a total volume of 20 µL with 14 µL dH_2O. All digestion enzymes and buffers were supplied from Promega (Promega, UK). All DNA/digests solutions were incubated in a previously warmed water bath (37 ºC) for 1 hour. After incubation, DNA/digests solutions were analysed using agarose gel electrophoresis (as in section 4.2.3.5). Accordingly, single and double inserts were identified.

5.2.2.3 Midiprep Plasmid Purification

After colony formation, 500 µL from a previously stored culture colony was incubated with 100 ml of 2xYT-ampicillin. Cells were incubated on shaking incubator at 37°C and 225 rpm overnight. After incubation, culture medium was centrifuged at 3800 rpm for 15 min at 4 ºC. DNA was extracted using a QIA plasmid plus midi kit (Qiagen, UK) for plasmid purification. Bacterial Pellets were resuspended in 4 ml Buffer P1 prior to adding 4 ml of Buffer P2. The solution was mixed thoroughly by inverting the tube 4–6 times until the solution became viscous then incubated at room temperature for 3 min. 4 ml of Buffer N3 was added and the solution mixed thoroughly by inverting the tube 4–6 times until the solution became clear. The lysate mix was transferred immediately into QIAfilter Cartridge, which was previously placed into a universal tube and incubated at room temperature for 10 min. During incubation, QIAGEN Plasmid Plus spin columns and tube extenders were placed into Vac-Man® laboratory vacuum manifold (Promega, UK). After incubation, the plunger was gently inserted into the QIAfilter Cartridge and the cell lysate was filtered into the tube. The QIAfilter Cartridge was removed and 2 ml Buffer BB was added to the cleared lysate in

the universal tube, and mixed by inverting 4–6 times. The lysate was then transferred into the tube extenders/QIAGEN Plasmid Plus spin column on the vacuum manifold. Vacuum was switched on until the liquid was drawn through all columns. The QIAprep spin column was washed by adding 700 µL buffer ETR and centrifuged for 1 min. The flow-through was discarded and the column was rewashed by adding 700 µL buffer PE and centrifuged for 1 min. The column was recentrifuged for 1 min to completely remove residual wash buffer. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube and 200 µL buffer EB was added to the center of the QIAprep spin column to elute DNA, let stand for 1 min, and centrifuged for 1 min. DNA samples were stored at -20ºC. To quantify the amount of DNA in each sample, a NanoVue (GE Healthcare, UK) was used. The NanoVue was standardised with 2 µL of Buffer EP as a blank before using 2 µL of DNA sample to quantify.

5.2.3 Determining Zeocin™ Sensitivity

Zeocin™, a selective antibiotic, was used to identify transfected cells. HEK- 293 cells (American Type Culture Collection, USA) were cultured in a 6-well plate in a seeding density of $5x10^4$ cells/cm^2. Cells were cultured for 24 hours in a full medium prior changing to medium containing varying concentrations of Zeocin™ (0, 100, 200, 400, 600, and 800µg/mL) to each well. Selective medium was replaced every 3–4 days and the percentage of surviving cells observed for 1 week. 400µg/mL was selected as the concentration leading to death of the majority of the cells.

5.2.4 HEK cells Culture and Transfection

HEK cells were cultured in full medium in a T75 flask until 80% confluent. Cells were then passaged using trypsin and counted before being transferred into 24-well plate. Cell density was adjusted to $1x10^5$ cells/cm^2 prior to seeding 1ml into each well of 24 well plate and cultured overnight. After 24hrs, 500 µL of medium was removed just before transfection. Transfection reagents were prepared according to the manufacture instruction, which included Lipofectamine 3000 and p300 reagent. Lipofectamine® 3000 Reagent 0.75 and 1.5 µL were diluted in two tubes of 25 µL serum free medium and mixed well. Master mix of DNA was prepared by diluting 2.5µg/µL DNA in a 50 µL serum free medium and 2 µL P3000™ Reagent and mixed well. Diluted DNA was added to each tube of Diluted Lipofectamine® 3000 Reagent in (1:1 ratio). DNA-lipid complex was incubated for 5 minutes at room temperature prior adding 50 µL of them to each well of HEK cell culture in 24-well plate. Transfected cells were incubated for 48 hours before passaging the cells into 100mm petridish for Zeocin selection (400ug/ml). Supernatant medium was collected from transfected cells and a control non transfected cell for hFc detection by ELISA.

5.2.5 hIgFc capture ELISA

A 96-well ELISA plate (Thermo Fisher Scientific , USA) was coated with 50µl of 0.5ug/ml Anti-Human IgG (Fc gamma-specific) biotin, diluted in 0.1M Na_2HCO_3/dH_2O and the plate incubated in 4°C for overnight. The plate was washed with ELISA wash buffer (0.05% tween 20, PBS) 3 times, prior adding 100µl of blocking buffer (10% FBS/PBS) and incubated at 37°C for 2 hours. The plate

was washed with ELISA wash buffer 3 times, prior adding 50µl of the sample or a series of double diluted standard (Human IgG) into each well followed by incubating the plate in 4°C for overnight. The starting point of the standard began from 20ng/ml and a double dilution was made to 0ng/ml. The plate was washed with ELISA wash buffer 5 times before adding 50 µl of 1/1000 diluted Biotin conjugated anti-human Fc specific antibody and incubated at 37°C for 1-2 hours. The plate was washed with ELISA wash buffer for 5-6 times prior adding 50 µl of 1/1000 diluted HRP Avidine in blocking buffer and incubated at 37°C for 1 hour. The plate was washed with ELISA wash buffer for 7 times before adding 50 µL of TMB and incubated at 37°C for colour development for 15 minutes. The reaction was stopped by adding 50 µL of stop buffer prior to reading in a plate reader at 450nm using SPECTRO star Omega plate reader v3 and MARS data analysis software v2.41 (BMG LABTECH). The colour intensity is proportional to the amount of Fc and hence BMP. The concentration of BMP-Fc captured in the samples was calculated by using standard curve and polynomial (order 4) equation to compare the absorbencies to standards of known concentrations.

5.2.6 Single Cell Colony Isolation and Culture Expansion

The plate was washed with 10 ml PBS twice after discarding medium, then 5 ml of PBS was added to cover the cells. The plate was tilted to expose the colony, and 10 µL PBS was added onto the colony and scraped it out with 200 µL tip. 12 single colonies were selected from each 100 petri dish plate. Selected colonies were visible as a white spot with clear edge and separated from other colonies. Each colony was mixed in 96-well plate with 40µl trypsin and incubated for 1-2 min to separate cells into single cell suspension. Cells were then transferred into

a 24-well plate and cultured overnight in 1ml of full medium. The medium was changed into selection medium (400µg/ml Zeocin) and cells cultured until confluent. Medium was collected and hIgFc level detected by ELISA. Cells with positive ELISA results were transferred into a 6-well plate for further expansion. After cells reached confluent, ELISA was made again for supernatants medium to identify colonies with positive hIgFc expression. Cells with a high positive hIgFc expression were sub cultured into a T75 flask for further expansion. Cells cultured in T75 flasks for 11 days without medium changes and 200 µL of supernatant medium were collected every other day. Collected supernatant medium were concentrated using a centrifugal concentrator Vivaspin 20 MWCO (Fisher Scientific, UK) and hIgFc concentrations detected by ELISA.

5.2.7 Assessment of functionality of BMP using human bone marrow stromal cells

Human bone marrow stem cells (hBMSCs), a kind gift from W Nishio Ayre (School of Dentistry, Cardiff University, UK), were seeded at 4000 cells/cm^2 and cultured with α-MEM supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen), 1% antibioticantimycotic and 1% L-ascorbic acid 2-phosphate (Sigma). Cells were incubated at 37^{o}C in 5% CO_2. Medium was changed every two days and mycoplasma testing was routinely conducted every passage. When cells reached approximately 80% confluence, they were passaged with accutase (Sigma), counted with a haemacytometer using Trypan Blue and reseeded at 4000 cells/cm^2. Cells were continually propagated and underwent multiple passages until sufficient cells were gained. Cells were seeded in 24-well plates and 48-well plates in triplicate at 4000cells/cm^2 and cultured with culture

medium or culture medium supplemented with 50ng/ml of either rhBMP2 (Invitrogen, UK) or BMP2-2 homodimer. Cells in 24-well plates were used for RT-PCR and 48-well plates for ALP and ARS assays. Cells were incubated at 37^{o}C in a 5% CO_2 atmosphere for 14 days during which medium was changed with fresh supplemented medium every third day and images were taken. Cells were washed with PBS and fixed with 4% paraformaldehyde at day 3, 7 and 14.

5.2.7.1 Alkaline phosphatase staining

Fixed cells were washed with washing buffer made from 0.05% Tween 20 in PBS. Cells were incubated with substrate solution made by dissolving one BCIP/NBT tablet (SigmaFast^{TM} BCIP-NBT; Sigma Aldrich) in 10 ml distilled water. Plates were placed at room temperature in the dark for 5-10 min. Cells were washed with washing buffer then replaced with PBS. Cells were evaluated under light microscope and images captured using Canon P1234 camera (Canon UK Ltd., UK).

5.2.7.2 Alizarin red staining

Fixed cells were washed with distilled water then incubated with Alizarin Red S staining solution. Alizarin Red S staining solution was prepared by dissolving 1g Alizarin Red S (sigma, UK) in 50ml distilled water and pH adjusted to 4.1 - 4.3 with 0.1% NH_4OH. Plates were placed in the dark at room temperature for 45 min. Cells were washed four times with distilled water and then replaced with PBS. Cells were evaluated under a light microscope and images captured using Canon P1234 camera (Canon UK Ltd., UK).

5.3 Results

5.3.1 DH5α Transformation

DH5α competent cells were used for cells transformation with BMP2-2, BMP2-7 and BMP7-7. After transformation, cells were plated out onto LB- agar plates containing (100 mg/ml) ampicillin which allowed selection of only transformed cells. Examination of the plates demonstrated that plates number 1 (BMP2-2), 2 (BMP2-7) and 3 (BMP7-7) demonstrated colonies formation, when compared with the control plate number 4. Colonies formation at transformed plates demonstrated that successful transformation was achieved (Figure 5.2).

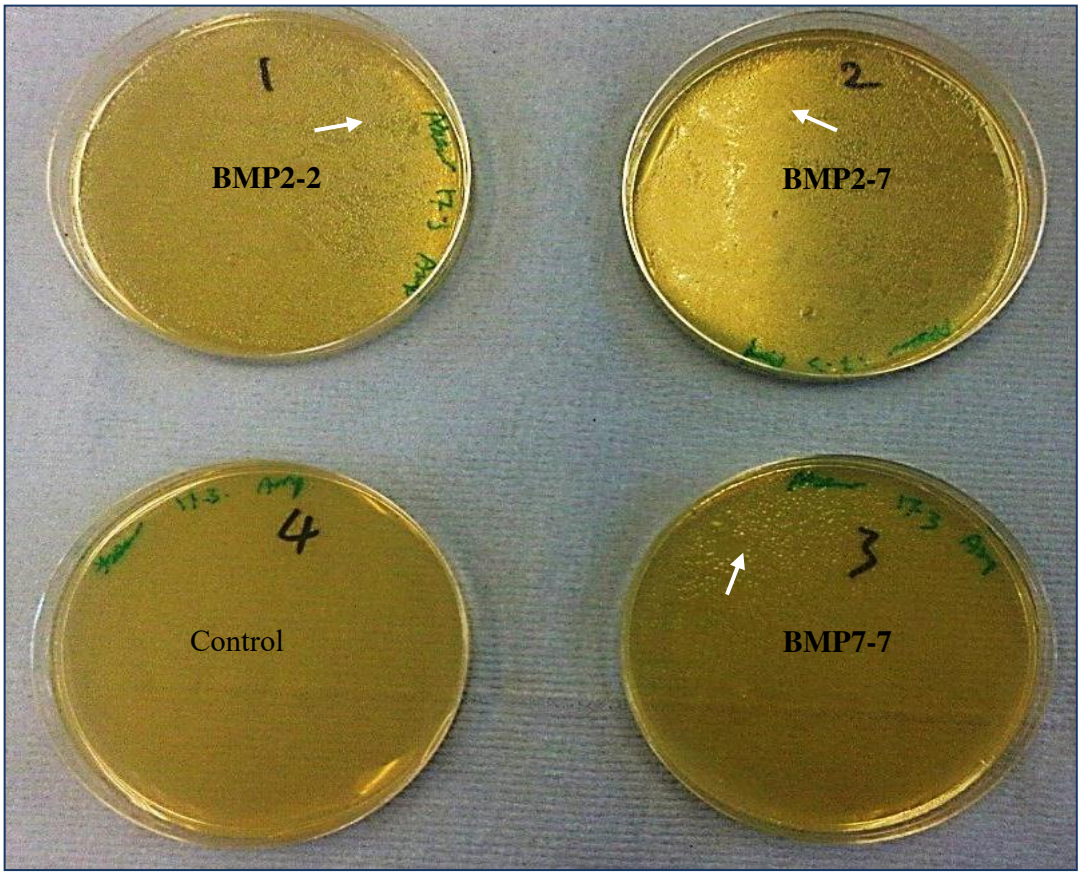
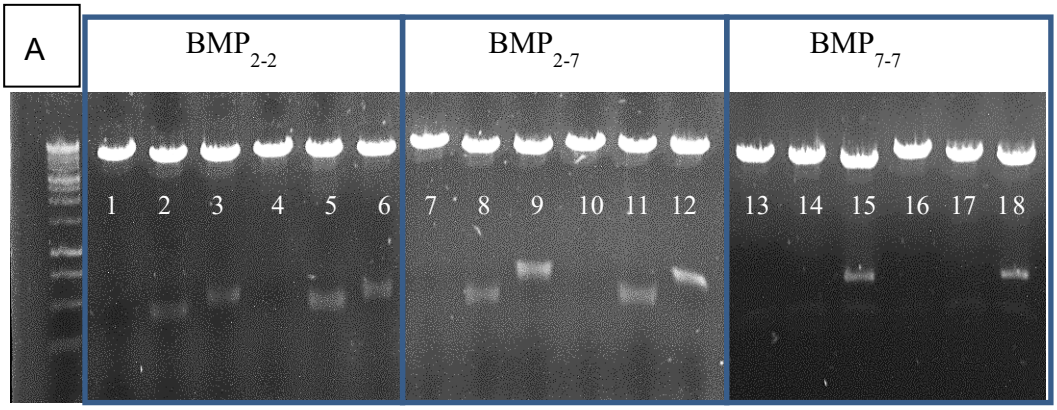


Figure 5- 2: DH5α competent cells transformed with BMP2-2, BMP2-7, BMP7-7 and control. Plates number 1 (BMP2-2), 2 (BMP2-7) and 3 (BMP7-7) demonstrated colonies formation (arrow). No colony formation was seen at the control plate.

5.3.2 Plasmid Digestion

Agarose gel electrophoresis was used for further transformation analysis. Two colonies were used from each transformed sample and digested with three restriction enzymes. The number of bands appeared in each lane was corresponded to the restriction enzyme used. Lane number 1, 4, 7, 10, 13 and 16 demonstrated single inserts because one digestion was made by BamH1. All other lanes demonstrated double inserts because a double digestion was made by either ECOR1/Pst1 or BamH1/ECOR1. As predicted, all lanes demonstrated a ~5Kb band corresponding to the pSec vector. Other bands were related to BMP2= 350bp and BMP7= 400bp (Figure 5.3).

A



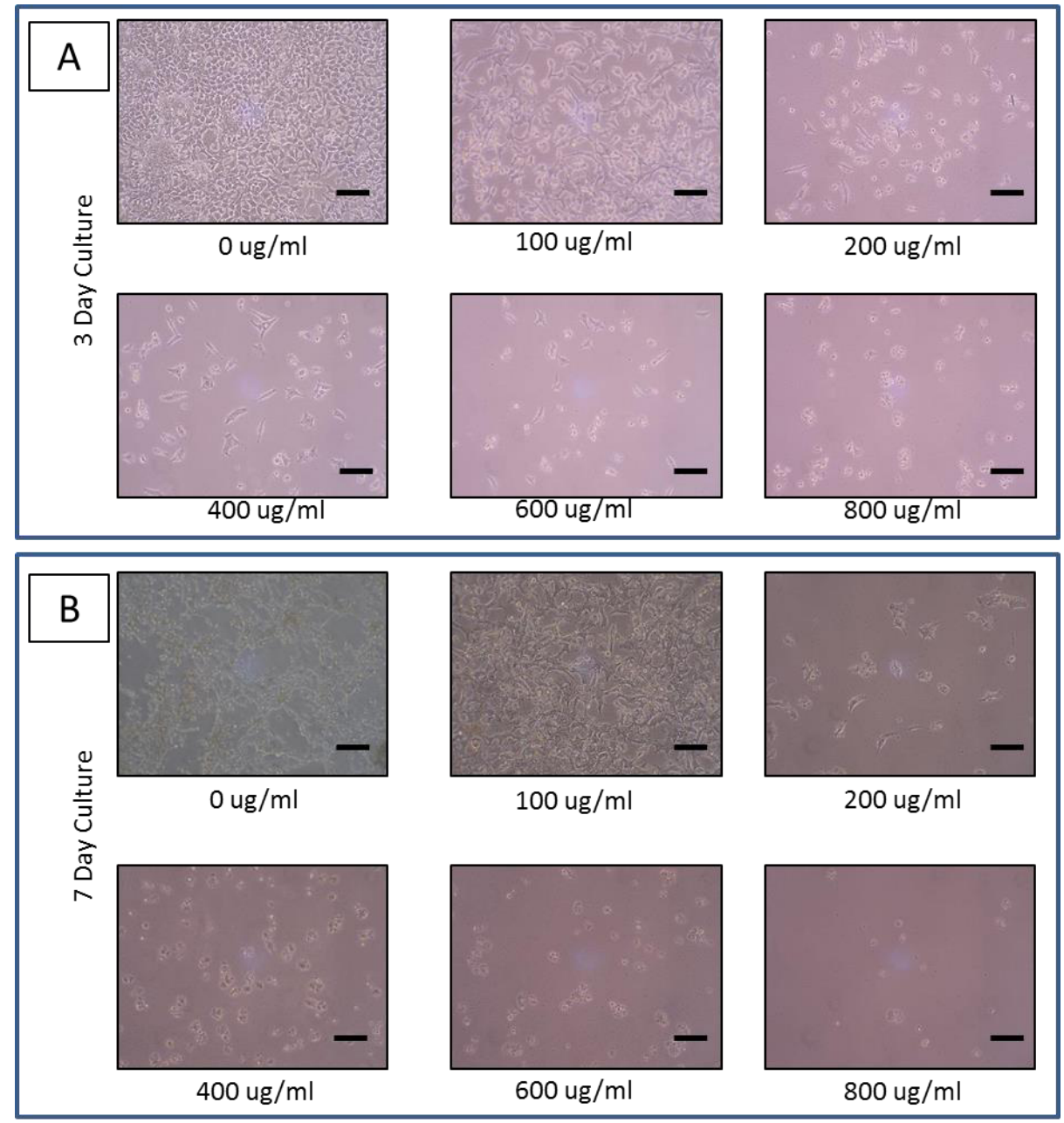
B

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Sample	BMP2-2, colony 1			BMP2-2, colony 2			BMP2-7, colony 1			BMP2-7, colony 2			BMP7-7, colony 1			BMP7-7, colony 2		
Restriction Enzymes	BamH1	ECOR1, Pst1	BamH1, ECOR1	BamH1	ECOR1, Pst1	BamH1, ECOR1	BamH1	ECOR1, Pst1	BamH1, ECOR1	BamH1	ECOR1, Pst1	BamH1, ECOR1	BamH1	ECOR1, Pst1	BamH1, ECOR1	BamH1	ECOR1, Pst1	BamH1, ECOR1

Figure 5- 3: single and double insert after cell transformation were detected by agarose gel electrophoresis (A). Numbers represent sample and restriction enzymes as seen in details in the table (B). Bands are shown corresponding to approximately BMP2= 350bp and BMP7= 400bp. All lanes contain a ~5Kb band corresponding to the pSec vector. Ladder= 1000 Kg. Restriction enzymes: BamH1, ECOR1 and Pst1, BamH1 and ECOR1.

5.3.3 Determining Zeocin™ Sensitivity

Cells were cultured with different Zeocin concentrations and monitored for 7 days to identify the concentration that can be used during culture for antibiotic selection. Cells were cultured with medium supplemented with Zeocin at concentrations of (0, 100, 200, 400, 600, and 800µg/mL). At day 7, most of the cells were viable at cells cultured with 100 and 200µg/mL Zeocin when compared with the control (0µg/mL). However, all cells died when cultured with 600µg/mL, and 800µg/mL. The majority of the cells died at cells cultured with 400µg/mL. Thus the 400µg/mL concentration was used for antibiotic selection (Figure 5.4).



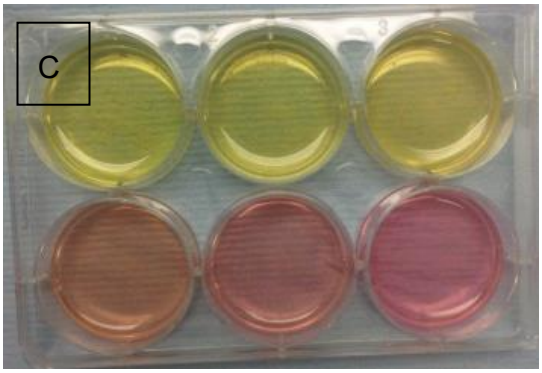


Figure 5- 4: Determining Zeocin™ Sensitivity. Cell cultured with different Zeocin concentration and cultured for 3 days (A) and 7 days (B). Plate (C) demonstrated colour change of the medium with live cells (yellow) and dead cells (pink). Scale bar, 100µm

5.3.4 hIgFc capture ELISA

ELISA was used to determine the efficacy of cell transfection by detecting the concentration of hFc and hence BMP from the supernatant medium after lipofectamine transfection. hFc was detected in supernatant medium collected from cells transfected with BMP2-2, BMP2-7 and BMP7-7 at both 0.75ug and 1.5ug DNA concentration. No hFc was detected in control transfections as well as HEK cells demonstrating that successful transfection was achieved (Figure 5.5).

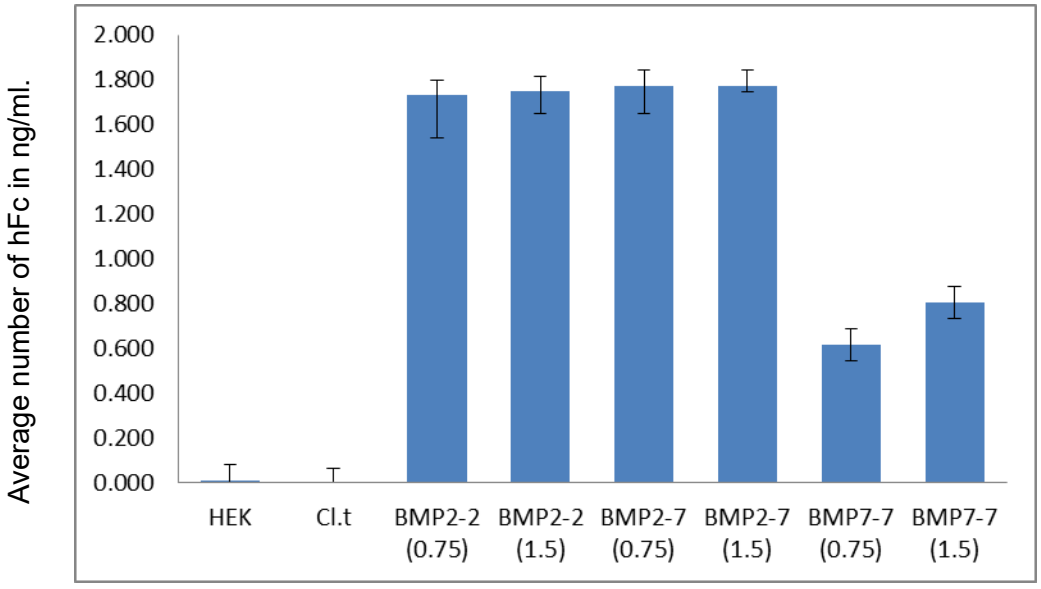


Figure 5- 5: ELISA for hFc to identify efficacy of HEK cells transfection. Comparison of hFc detected at supernatant medium collected from cells transfected with BMP2-2, BMP2-7 and BMP7-7 at 0.75 and 1.5ug DNA concentration. Error bars indicate standard error of the mean

5.3.5 Colony formation

After cell transfection, cells were passaged and cultured with medium supplemented with zeocin for antibiotic selection. Zeocin is lethal to un-transfected cells (Figure 5.6). Light microscope images demonstrated colonies formation after transfection and antibiotic selection. Plates with transfected cells demonstrated survival and proliferation with multiple colonies formed throughout the plate. However, no cells were available at control plates during the entire culture period (Figure 5.6).

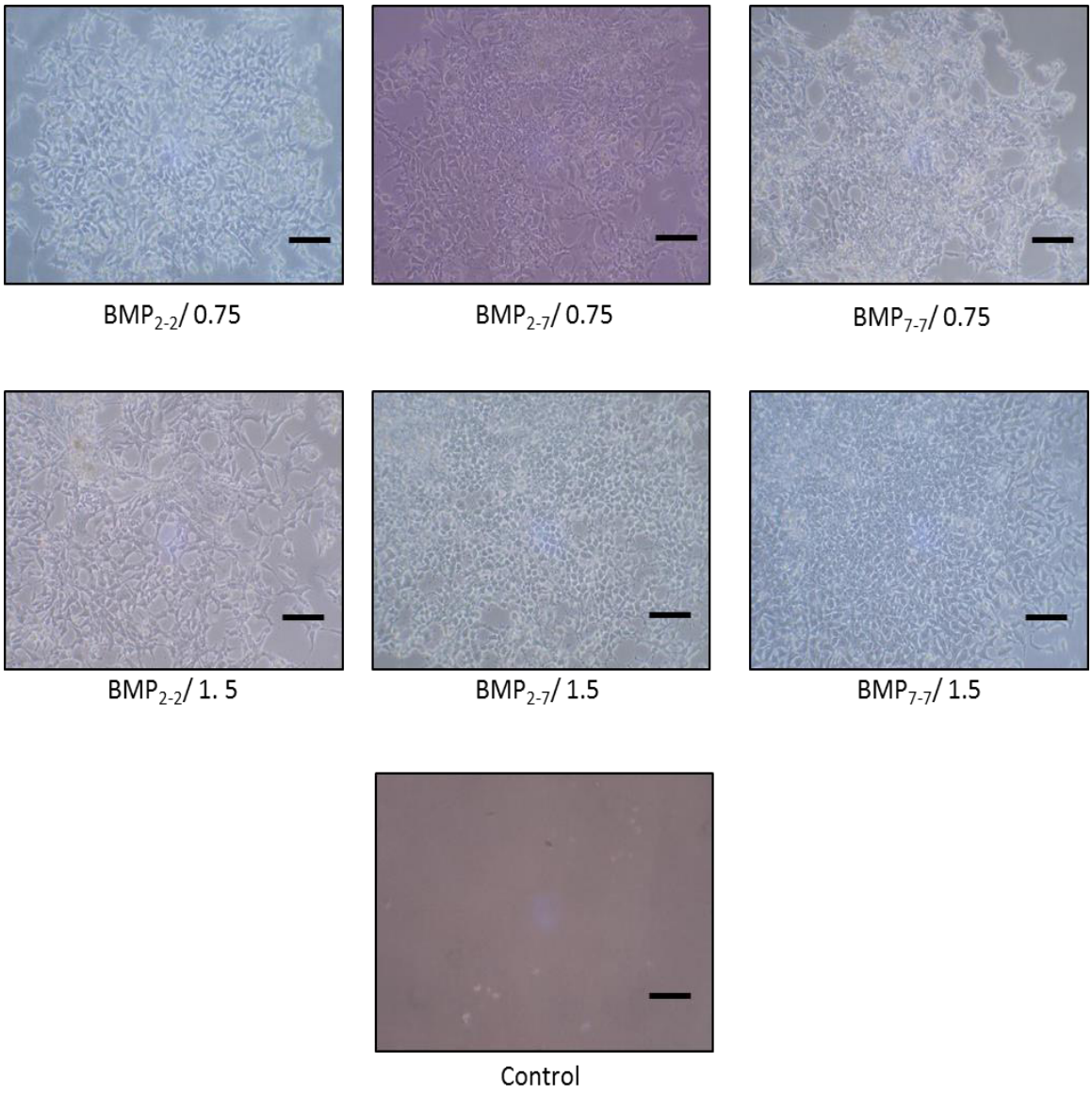


Figure 5- 6: Example of colony formed after transfection and antibiotic selection. Colonies formed at all transfected plates but not at the control plate. Scale bar, 100µm

5.3.6 Assessment of functionality of BMP2-2 on hBMCs

5.3.6.1 Cellular morphology

hBMCs cells were cultured for 14 days in culture medium or medium supplemented with either BMP2-2 or rhBMP2 to investigate the effect of BMP2-2 on hBMCs. During the culture period, cells of all groups were observed to increase in number and demonstrated a fibroblast like morphology with the characteristic long, spindle, bipolar shape. Greater numbers of cells were observed at day 7, when compared with day 3. After 14 days in culture, there were marked variations in the proliferative capacity between the groups. Cells cultured in BMP2-2 demonstrated high proliferative capacity greater than cells cultured with BMP2 or control culture medium (Figure 5.7).

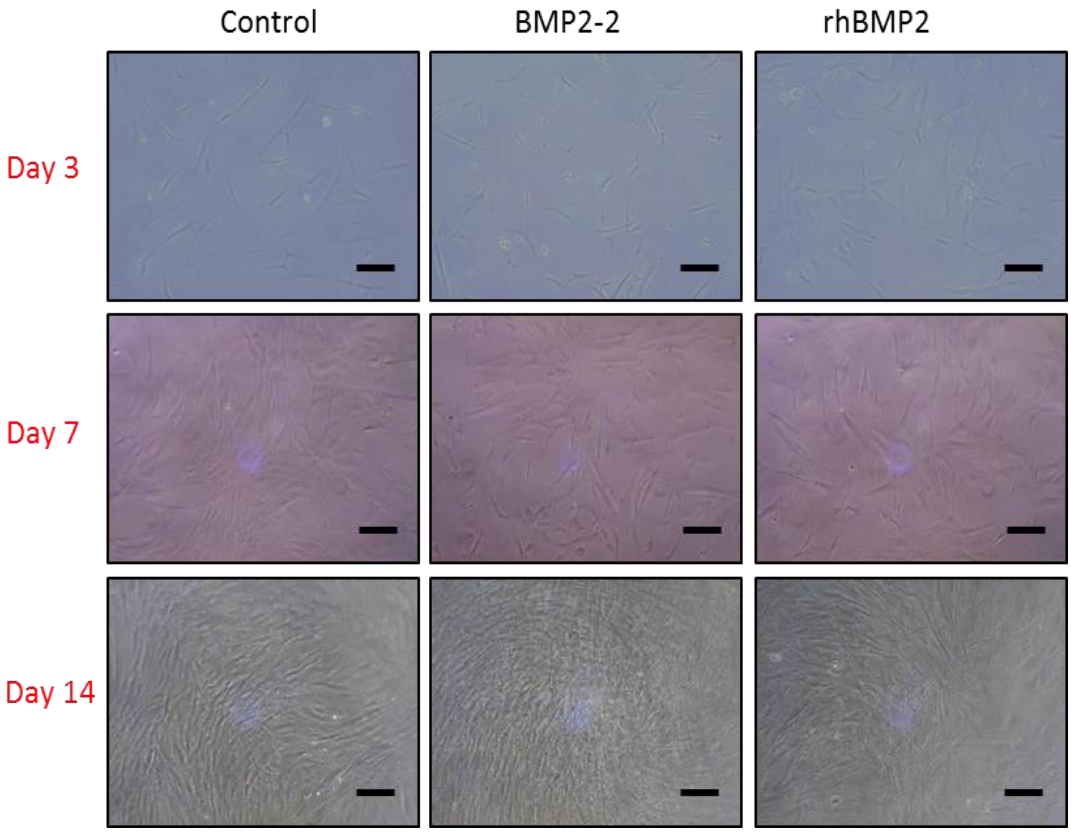


Figure 5- 7: hBMCs cells cultured in culture medium or medium supplemented with either BMP2-2 or rhBMP2 for 14 days. Scale bar, 100µm

5.3.6.2 Alkaline phosphatase staining

Alkaline phosphatase staining was used to investigate the effects of BMP2-2 on the cells. After 3 days in culture, cells of all groups were demonstrated negative staining to alkaline phosphatase. When compared with cell cultured at control culture medium or BMP2, cells exhibiting positive staining for the alkaline phosphatase could be observed at day 7 in cells cultured with BMP2-2. Cells appeared with the characteristic alkaline phosphatase blue –violet colour. When compared with cells at day 7, cells cultured with BMP2-2 demonstrated high staining to alkaline phosphatase demonstrating multiple cells stained dark blue-violet at day 14. Control culture and cells cultured with rhBMP2 demonstrated no staining to alkaline phosphatase at day 14 (Figure 5.8).

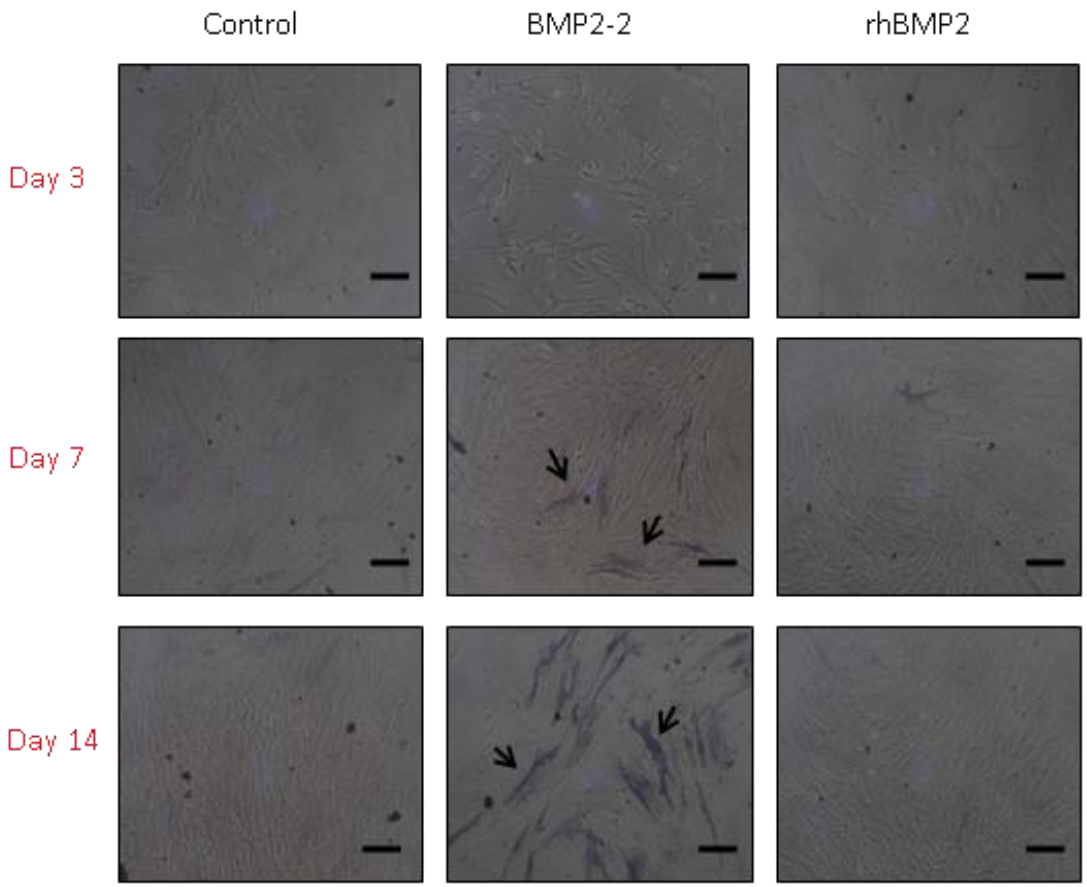


Figure 5- 8: Alkaline phosphatase staining for hBMCs cells cultured in culture medium or medium supplemented with either BMP2-2 or rhBMP2 for 14 days. Cells cultured with BMP2-2 demonstrated positive staining to alkaline phosphatase at day 7 and 14 (arrow). Scale bar, 100µm

5.3.6.3 Alizarin red staining

Alizarin red staining was used to investigate effects of BMP2-2 on osteogenic differentiation. Cells at all groups demonstrated similar negative staining to alizarin red staining at day 3 and 7. However, at day 14 cells cultured with BMP2-2 stained positively with alizarin red staining demonstrating multiple cells with red colour. Control culture and cells cultured with rhBMP2 demonstrated no alizarin red staining at day 14 (Figure 5.9).

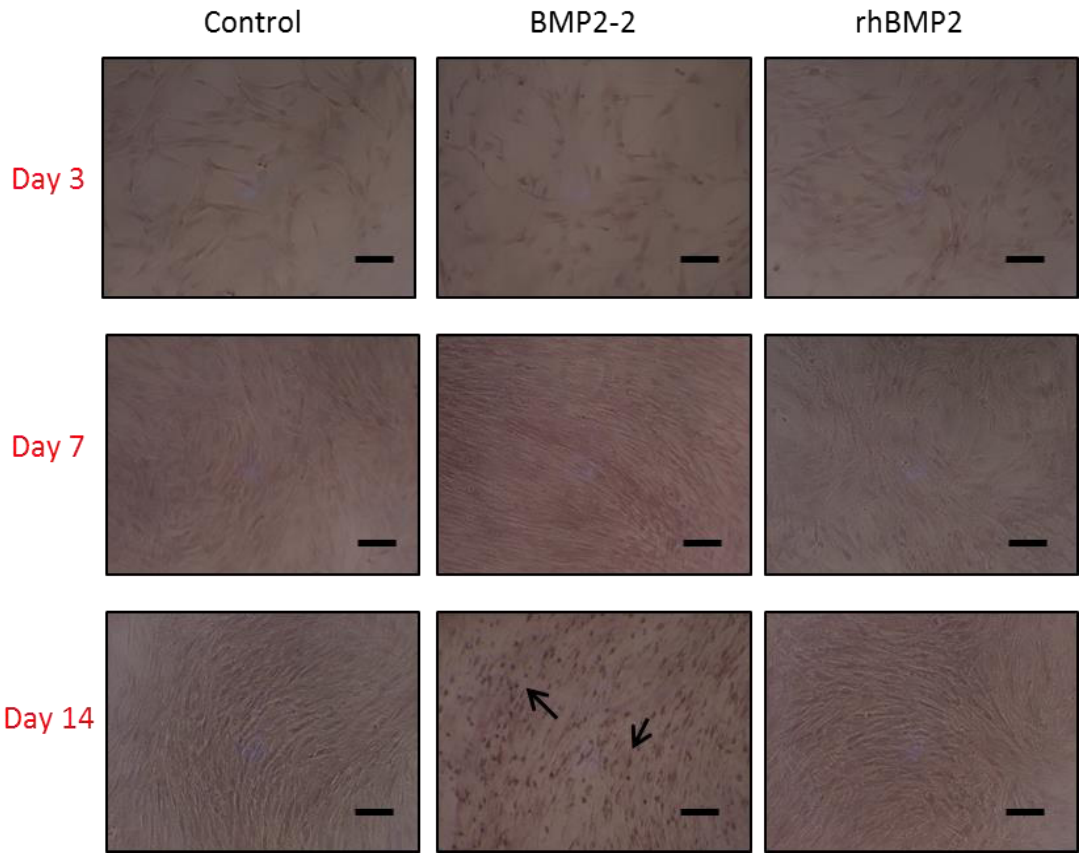


Figure 5- 9: Alizarin red staining for hBMCs cells cultured in culture medium or medium supplemented with either BMP2-2 or rhBMP2 for 14 days. Cells cultured with BMP2-2 demonstrated positive staining to alizarin red staining at day 14 (arrow). Scale bar, 100µm

5.4 Discussion

The results presented in this chapter demonstrate that BMP2-2 and BMP7-7 homodimers and BMP2-7 hetrodimer were successfully transformed into DH5α competent cells and successfully transfected into HEK cells. BMB2-2 homodimer expressed by HEK cells was successfully demonstrated osteogenic potential. The osteogenic ability of BMB2-2 homodimer was greater than commercial recombinant human bone morphogenetic protein-2 and at a nanogram dose.

Although E.coli is commonly used for protein expression due to its rapid, ease of use and relatively low cost (Portolano *et al.* 2014), production of high yield of recombinant protein is challenging due to low expression and poor protein stability (Baneyx 1999). Mammalian cells such as Chinese hamster ovary (CHO) and HEK cells demonstrate superior advantages in production of recombinant protein more than E.coli, particularly HEK cells have better advantages over other expression vehicles due to their ease of culture and high transfection efficiency (Huh *et al.* 2007; Nettleship *et al.* 2015). Transfections were made using Lipofectamine® 3000 reagent protocol. In summary the cells were transfected by DNA-lipid complex and cultured for 48h. Results from ELISA demonstrated that BMP2-7 heterodimer, BMP2-2 and BMP7-7 homodimer were detected at supernatant in all test groups but not at the control cells which confirm transfection. Transfections were also confirmed by culturing transfected

cells with Zeocin™ antibiotic. Colonies were formed in all test groups but not at the control plate. The pSecTag2 vectors carry Zeocin™ resistance gene which acts as a selective antibiotic. Cells that have this gene were able to survive at the medium but cells that do not have Zeocin™ resistance gene were destroyed at Zeocin™ containing medium thus no cells were available at the control plate. Furthermore it was mandatory to determine the appropriate concentration when using Zeocin™ as using it at a high dose (e.g. 700ug/ml) were lethal to the cells. Thus using Zeocin™ at 400ug/ml was capable to do the selection for transfected cells that carry Zeocin™ resistance gene.

Cells cultured for 11 days, during which supernatant medium were collected and BMP concentrations were quantified by ELISA and concentrated using centrifugal concentrator Vivaspin. However there were no high expression of BMP7-7 homodimers and BMP2-7 heterodimers enough to conduct the experiment of testing their biological functions on the cells. Low protein expression is a common issue across protein expressions from mammalian cells. There are several techniques which can be used in the future to increase expression of the protein from mammalian cells. It has been reported that lowering culture temperature to 30–33°C resulted in higher expression of a variety of recombinant proteins using CHO cells (Kaufmann *et al.* 1999; Yoon *et al.* 2003) and HEK cells (Lin *et al.* 2015). These studies suggest that the enhancement of protein expression after culturing the cells at low temperature is mainly because hypothermia increases cellular productivity of recombinant proteins while reducing the growth rate of the cells, through inducing cell growth suppression due to cold-induced cell cycle arrest (Kaufmann et al. 1999; Yoon et

al. 2003; Lin et al. 2015). Recent studies demonstated a novel and accessible protocols to express and purify milligram quantities of protein through performing transient transfections of suspension grown HEK cells at shaking incubator (Portolano et al. 2014) or using 10-layer cell factories at standard incubator (Aydin *et al.* 2012). Furthermore it has been suggested to culture transfected cells under the antibiotic selection pressure to maximize the chances of higher percentage of pure population of cells containing the gene of interest (Novo *et al.* 2012). A possible way to increase expression of the protein also is by selecting hundred colonies after colonies formation and select the highly positive colonies as the best expression cells after protein screening (Strukov and Belmont 2008).

Cells cultured in medium supplemented with BMP2-2 demonstrated ALP staining more than cells cultured in control medium or medium supplemented with rhBMP2. ALP is expressed by osteoblasts and osteoblasts precursor and it has been reported that ALP is up-regulated during differentiation (Walsh *et al.* 2000). rhBMP2 has been shown to regulate and induce ALP gene expression in cultured osteoplastic cells (Takuwa *et al.* 1991). Cells cultured in medium supplemented with BMP2-2 homodimer expressed ALP at day 14 more than at day 7. ALP expression has been found to be continuously increased over the time course (Rawadi *et al.* 2003) demonstrating peak expression at two weeks (Sun *et al.* 2012). Although both proteins were used in the same concentration, results demonstrated that BMP2-2 demonstrated superior effect over rhBMP2. rhBMP2 has been used clinically but in a very high dose, a minimum of 1.5mg/ml, and associated with serious post-operative complications (Carragee

et al. 2011). The concentration used in this experiment was 50ng/ml which was below the ED50 of the rhBMP2. This suggests that BMP2-2 has a better mineralization effect at a low concentration and can reduce the clinical complication demonstrated form rhBMP2 treatment. BMP2-2 was produced by HEK cells; however rhBMP2 was produced by E-coli. Since BMP2-2 produced by mammalian cells this could reduce the complications created by using protein produced from bacterial or viral origin. In this study, CHO cells were failed to express BMP fusion proteins when compared with HEK cells. HEK cells are originated from human cells and they could be better than CHO cells which are originated from hamster. These promising results can allow the use of BMP2-2 clinically in a safe, effective and low concentration.

BMP2-7 heterodimer, BMP2-2 and BMP7-7 homodimer were purified and expressed in the supernatant mammalian HEK cells. The vector used in this investigation was constructed by inserting a (3XGGGGS) linker to link the two dimers together. When compared with conventional disulphide linked dimers available commercially (Zheng et al. 2010; Sun et al. 2012; Wang et al. 2012), this linker has been shown to give greater protein solubility, flexibility, stability and function because it improves fusion protein expression and folding as well as protein half-life (Chen *et al.* 2013). Alizarin red and alkaline phosphatase staining results demonstrated an enhanced activity of (3XGGGGS) linked BMP2-2 homodimers and revealed regeneration potential greater than disulphide linked rhBMP2. These promising results are encouraging to further investigate and compare the biological activity of BMP2-7 heterodimer, BMP2-2 and BMP7-7 homodimer. It has been reported that using BMP 2-7 heterodimers

in relatively low dosage induced new bone formation in a significantly higher efficacy more than BMP2-2 and BMP7-7 homodimers (Zhao et al. 2005; Wang et al. 2012). Thus further investigation is required to assess the osteoinductive potentials of each protein and their effect on migration, proliferation and differentiation of osteoblasts cells.

BMP2-7 heterodimer, BMP2-2 and BMP7-7 homodimer have been used in peri-implant bone defects in minipig calvaria (Wang et al. 2012) and on minipig frontal skull (Sun et al. 2012). BMP fusion proteins could be used in the future at *ex vivo* fractured rat mandible model described in this thesis to gain better understanding of the roles of BMP2-7 heterodimer, BMP2-2 and BMP7-7 homodimer in bone repair. Agarose beads could be soaked in BMP fusion proteins then applied to the fracture site. Another application to investigate the biological activity of these fusion proteins would be asses its effects on dental implants osseointegration, dental and periodontal tissue regeneration. It has been demonstrated that BMP heterodimers are more resistant to inhibition by antagonists such as noggin (Zhu et al. 2006). Another avenue of investigation could be treating fractured rat mandible model topically with BMP2-7 heterodimer, BMP2-2 or BMP7-7 homodimer followed by trowel type culture in the presence of various BMP antagonists. This would allow a greater understanding of the osteoinductive abilities of theses fusion protein and hence its resistance to antagonists.

Chapter 6.

General discussion and Future Direction

Bone has been reported to contain numerous growth factors which are proposed to be released from the matrix during injury and play an essential role during bone repair. The thesis set out mainly to investigate the hypothesis that calcium hydroxide can release endogenous growth factors from mandibular bone. Moreover, the use of combinations of growth factors, such as the combinations found in the bone matrix, may be more effective in promoting bone healing compared with single growth factor therapy such as BMP2, which has found clinical application, but requires high doses to be effective. The thesis successfully identifies and compares the effect of bone matrix derived growth factor combinations and recombinant BMP2 on cellular behaviour in mediating bone repair processes using *ex vivo* fractured mandible slices model. The model was demonstrated an effective way to investigate cellular effect of growth factor during early bone repair process.

By comparing the effect of calcium hydroxide and BMP2 on *ex vivo* fractured mandible slices, both treatments demonstrated maintenance of tissue architecture throughout the section. Fractured mandible slices treated with calcium hydroxide however exhibited a significant increase in cell numbers and immunopositivity to bone markers when compared with BMP2 treated slices. Calcium hydroxide treated slices were demonstrated a significant increase in

release of VEGF, TGF-β1 and BMP2 from treated surfaces. The release of these cocktail of growth factors from bone surfaces by calcium hydroxide can effectively stimulates cellular reparative processes during bone regeneration and consequently will definitely avoid issues regard single growth factor therapy.

Calcium hydroxide at pH 11 was applied continuously to the cells while applied topically for 5 min on the fractured mandible slices followed by washing with PBS. Thus the alkaline medium caused a decrease in cell number at *in vitro* but increased in cell number at *ex vivo* model. The alkaline pH of calcium hydroxide was lethal to the cells at in vitro but was beneficial to the cells at *ex vivo*. Calcium hydroxide at pH 11 facilitates growth factors released from bone matrix stimulating migration and proliferation of the cells. This suggests that calcium hydroxide at pH 11 could be not lethal to the cells if applied to the cells for less time. Studies support that calcium hydroxide at pH 11 plays dual effects on cells as well as on the bone matrix. It could be that single cell culture at *in vitro* can not withstand the alkaline pH like cells surrounded by bone matrix at *ex vivo* fractured mandible slices which provide support to the cells to tolerate the alkaline pH.

Growth factors are essential during wound healing and tissue regeneration (Raja et al. 2009). During repair process, every growth factor has precise role, at specific concentration and at definite exposure time. Application of different growth factors at same time may work in agonist or antagonist way. Supplementation or overexpression of VEGF has been shown to inhibit BMP2

expression and osteogenesis in rat mesenchymal stem cells and ectopic bone formation in *in vivo* (Schonmeyr et al. 2010). BMP2 and TGF-β1 have been shown to have opposite effects on osteoblast differentiation and mauration in which TGF-β1 inhibited the ability of BMP2 to induce cell mineralisation (Spinella-Jaegle et al. 2001). However, scaffolds with dual release growth factor (VEGF & BMP2) have shown to enhance bone regenerative capability in a critical sized femur defect in which VEGF was released more after 7-days in culture up to 28 days then an increased in BMP2 compared to VEGF was observed (Kanczler et al. 2010). Thus delivering combination of different growth factors should be applied in an optimal ratios and precise delivery system. Results of this thesis have been demonstrated that using calcium hydroxide can provide an effective way to utilise endogenous growth factors. Avenue for future study is to investigate if calcium hydroxide can stimulate bone regeneration greater than scaffolds with dual release growth factor. Clinical application of calcium hydroxide on bone will be easier and avoid the obstacles of constructing a scaffold with multiple growth factor release which will need a long time to be approved for clinical use.

Several experimental and clinical trials have supported the safety and efficacy of using exogenous growth factors and some growth factors have been licensed for clinical use particularly BMP2 and BMP7 (Axelrad and Einhorn 2009). Current clinical BMPs therapies use a single BMPs treatment to treat bone injuries. However, multiple BMPs are expressed during bone development and fracture healing. The high dosages of BMP2 and BMP7 at current clinical therapies are due to the short half-life of the protein. Using Fc domain and (3XGGGGS) linked

protein were successfully stimulated bone osteogenesis greater than commercially available recombinant BMP2. It has been demonstrated that Fc domain and (3XGGGGS) linker provide greater protein solubility, flexibility, stability and function as it improves fusion protein expression and folding as well as protein half-life (Chen et al. 2013). Clinical use of BMP2 has been associated with several complications such as hematoma, swallowing and breathing difficulties (Shields *et al.* 2006; Smucker *et al.* 2006) and epidural heterotopic ossification (Joseph and Rampersaud 2007). These significant side effects are either caused by the high dose which can reach to milligram, the delivery mechanism or off-label use of the growth factors. However using calcium hydroxide to release endogenous matrix-bound growth factors can provide alternative safe and effective way for bone repair and regeneration. Calcium hydroxide has been used for ages to facilitate formation of reparative dentine and demonstrates ability to release TGF-β1 from the dentine (Tomson et al. 2007) and from bone slabs (Smith et al. 2011). Further Investigation of applying calcium hydroxide at pH 11 on *in vitro* cells culture for 5 min and topical application of calcium hydroxide at pH 7 on *ex vivo* fractured mandible slices is advisable. Looking at that will give better understanding on the effect of calcium hydroxide at different pH on the cells.

Bone repair is mainly carried out by synthesis and deposition of bone that carried out by bone cells, particularly osteoblast cells, which are post mitotic cells and have a short half-life (Almeida and O'Brien 2013). For bone formation to continue uninterrupted throughout the healing process, osteoblast cells need to be constantly replaced to supply injury site with new cells originating from

osteoprogenitor cells (Park *et al.* 2012). Results demonstrated that periodontal ligament stem cells can provide a good source for multipotent stem cells that have osteogenic potential and able to differentiate into osteoblast cells during fracture repair. Another venue of future investigation would be looking to marker of PDLSCs such as periostin and α-smooth muscle actin (αSMA) that proved to be a useful marker for identification of stem cells in the periodontium (Horiuchi *et al.* 1999; Fujii *et al.* 2008; Fujii et al. 2010). PDLSCs can be harvested easily from impacted or extracted teeth and used for investigating effect of calcium hydroxide and BMP2 and BMP7 homodimers and BMP2-7 heterodimer. PDLSCs can also be isolated and transplanted with a scaffold into fractured bone to investigate chemically derived growth factors on enhancing bone repair.

The novel findings gathered from the studies of this thesis indicate that *ex vivo* mandible model can be fractured and cultured with trowel type culture for long time without gross damage to the adjacent tissue. *Ex vivo* mandible model and Trowel type cultures can be considered a suitable and reproducible way to investigate effect of novel material. *Ex vivo* fractured mandible model can be stimulated by exogenous growth factors (BMP2) and chemically induced endogenous growth factors. Interestingly, calcium hydroxide is found to induce release of TGF-β1, BMP2 and VEGF from the bone matrix which influences cellular reparative processes. Although the studies were carried out on mandibular bone, the clinical end point of these results can be extended beyond fractured mandibular bone. For instance calcium hydroxide can be used to facilitate osteointegration through inducing endogenous growth factor release from the bone before implant, plates and screw placement. It can be used after

tumour resection to improve healing after bone reconstruction. Furthermore, calcium hydroxide can be apply on bone surface before placing scaffold and bone graft to accelerate bone regeneration through the release of endogenous growth factors. All of these suggestions will accelerate bone reparative processes and improve bone repair clinical outcomes.

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