The effect of Bisphosphonates on Human Mesenchymal Stem Cell Behaviour and its implication on Titanium Osseointegration

A thesis submitted

By

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Declaration

I, Nasser Raqe Alqhtani confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Dedication

I dedicate this thesis to my beloved parents, family and friends; without their patience, understanding, support and most of all love. The completion of this work would not have been possible. Many thanks.

Acknowledgments

I would like to express my sincere gratitude to the University College London, Eastman Dental Institute for giving me the opportunity to fulfil my dream of being a PhD student here. This project has been a journey of knowledge, faith and development and would have never been completed without the kindness of certain special people.

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Abstract

Introduction: Bisphosphonates (BPs) are chemical analogues related to pyrophosphate; they are well known inhibitors of osteoclast activity. During the last 40 years, BPs have been used in the clinic to treat various bone diseases characterized by excessive bone resorption, such as osteoporosis, malignant bone diseases, and hypercalcaemia of malignancy. Human mesenchymal stem cells (hMSCs) possess regenerative properties with the ability to differentiate into different cell types and are the first osteogenic cells to colonise an implant's surface. Titanium implants have been successfully used to replace missing teeth for over 30 years. The effectiveness of the implant depends on successful osseointegration; coating dental implants with different types of bone-inducing material has shown positive effects on osseointegration. The effects of BPs on the proliferation and osteogenic differentiation of hMSCs are still unclear.

Aims: The aim of this project is to investigate the effect of two types of commonly used BPs (alendronate (ALE) and pamidronate (PAM)) on hMSC proliferation and osteogenic differentiation, the extended effect of a single low-dose of BPs on hMSCs proliferative and osteogenic behaviour as well as the epigenetics changes, and the role of a low dose of BPs as an adjunct treatment to implant therapy.

Methods and material: The experiments were conducted in a two-dimensional in vitro model, which consisted of hMSCs suspended in growth medium in a sterile culture vessel. We investigated the effect of these drugs on cell proliferation, migration, DNA methylation. Osteogenic genes and differentiation markers were measured including calcium, collagen type I and alkaline phosphates activity (ALP).

Results: The data suggest that treating cells with a lower concentration (100 nM and 10 nM) of the drug induces significant stimulation of hMSCs osteogenic differentiation. Treating cells with a single low dose of drugs (100 nM and 10 nM) may permanently change the osteogenic behaviour of the hMSCs even after passaging the cells. A low dose of BPs significantly upregulated osteogenic markers, including calcium, collagen type I, and alkaline phosphatase. There was also a significant effect on proliferation and cell migration on the titanium surface. Finally, the data showed that there was a significant effect on epigenetic phenotype via DNA methylation.

Conclusion: These experiments indicate that a lower concentration of drugs may play an integral role in enhancing the proliferation and osteogenic differentiation of hMSCs. Furthermore, these findings suggest that BPs may more than compensate for the established positive effects of osteoclasts in bone density in osteoporotic patients. The enhancement of osteogenesis could also point to the use of low-dose BPs as an adjunct to implant placement in patients. Therefore, the administration of BPs may be pivotal in accelerating osseointegration and the bone healing process following implant placement.

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Glossary of Terms

ABBREVIATION	**FULL NAME**
$1,25\text{-}(OH)_2D_3$	1,25-dihydroxyvitamin D_3
ALE	Alendronate
ALP	Alkaline phosphatase
α MEM	Alpha Minimum Essential Medium
BPs	Bisphosphonates
BLCs	Bone lining cells
BMP	Bone morphogenetic protein
BMU	Basic multicellular unit
BSP2	Bone sialoprotein
^{o}C	Degree Celsius
Ca^{2+}	Calcium
Cbfa1	Core binding factor 1
cDNA	Complementary Deoxyribonucleic acid
Chr	Chromosome
Cl^{-}	Chloride
CO_2	Carbon dioxide
COL1A1	Collagen alpha-1
Ct	Threshold cycle in real time polymerase chain reaction
DDH_2O	Double-distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DMPs	Differentially methylated probes
DMRs	Differentially methylated regions

DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FPP	Farnesyl pyrophosphate
GM	Growth Medium
GTP	Guanosine 5'-triphosphate
HCL	Hydrochloride acid
HMG-CoA	hydroxy-3-methylglutaryl-coenzyme A (mevalonate pathway)
hMSCs	Human mesenchymal Stem cells
IGF	Insulin like growth factor
IL	Interleukin
K+	Potassium
MAPK	Mitogen-activated protein kinase
M	Molar
Ml	Milli litre
mM	Milli molar
M-CSF	Macrophage-stimulating growth factor
MMP	Matrix metalloproteinase
NaOH	Sodium hydroxide
NPP	Nitrophenylphosphate
NSAIDs	Non-steroidal anti-inflammatory drugs
nM	Nano molar
NF-κB	Nuclear factor kappa B
OC	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
OM	Osteogenic medium
PAM	Pamidronate

Pi	Phosphate (inorganic)
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
pH	Power of hydrogen
PPi	Pyrophosphate
P/S	Penicillin/Streptomycin
PTH	Parathyroid hormone
R_a	Roughness Average of a surfaces measured microscopic peaks and valleys
RANK	Receptor activator of nuclear factor κB
RANKL	Receptor activator of nuclear factor κB ligand
RUNX2	Runt-related transcription factor 2
SEM	Scanning Electron Microscopy
T	Trypsin
TCP	Tissue culture plastic
Ti	Titanium
TGF	Transforming growth factor
TNF	Tumour necrosis-related factor
µm	Micrometre
µM	Micro-molar
Wnt5a	wingless-type MMTV integration site family member 5A

1 Introduction

Human mesenchymal stem cells (hMSCs) have a significant capability to repair and regenerate bone tissue. Their osteogenic differentiation is controlled, and is greatly affected by local and systemic factors. However, bisphosphonates (BPs) are well-known inhibitors of osteoclast activity. *In vitro* studies have indicated that these agents may affect other cell lineages, including osteoblasts and fibroblasts. Furthermore, BPs may control osteoblast activity and bone formation via several osteogenic markers and genes. With regard to osteoblasts that originate from MSCs, it has been shown that an external trigger may affect cell behaviour and phenotype; this change is called an epigenetic phenomenon. These epigenetic changes modulate gene expression, which subsequently affects cell behaviour. However, the phenomenon of osseointegration is considered to be a complex biological event that consists of several cellular and molecular activities. Its success primarily depends on a high level of titanium biocompatibility. A variety of *in vitro* approaches have been used to enhance implant performance, using different coating materials.

1.1 Bisphosphonates

1.1.1 Discovery and structure

Historically, bisphosphonates (BPs) were synthesized for industrial purposes at the end of the eighteenth century in Germany by Baeyer and Hoffman. They were used to prevent scaling because they have the ability to inhibit calcium carbonate precipitation. In addition, BPs were mainly used in textiles and fertilizers. In 1960, Fleisch et al. conducted a study where calcification was induced by collagen and found that body fluid and plasma contained some inhibitors of calcium precipitation that were inactivated by

the phosphatase. A few years later, Fleisch and colleagues reported that inorganic pyrophosphate (PPi) prevents the calcification of collagen by binding to newly formed crystals of hydroxyapatite (Fleisch et al., 1966). BPs were then introduced to the medical field for treating bone diseases such as osteopetrosis, Paget's disease of the bone, and myositis ossificans progressiva (Bassett et al., 1969).

Chemically, BPs or diphosphonates are stable chemical analogues related to Pyrophosphate (PPi) that are characterized by the presence of a carbon atom on the backbone of the molecule. Structurally, this carbon atom provides the unique feature of making these compounds difficult to hydrolyse; at the same time, they also have a strong affinity for calcium ions. In addition, BPs are non-hydrolysable compounds due to the presence of two phosphate groups, which are bonded to the central carbon atom. This carbon atom has two additional bonds, which are not present in pyrophosphate (Figure 1-1). This bond may form, or the space could be occupied by hydrogen atoms or another group that will make it easier to synthesise more compounds with different chemical properties. This additive changes the BPs physicochemical, biological, therapeutic and toxicity features. Accordingly, these additives give each drug its own features and modes of action. BPs are chemically divided into either nitrogen-containing (N-BPs) or non-nitrogen containing BPs (NN-BPs). Thus, the presence of a nitrogen atom is an important factor for the drug's potency and determines its mode of action. Moreover, the nitrogen atom makes the BPs up to 100 times more potent compared to non-nitrogen BPs (Roelofs et al., 2006). ALE and PAM are examples of nitrogen-containing BPs; they have one primary nitrogen atom attached to their central carbon atom and are more effective because they are 10–100 times stronger than non-nitrogen containing BPs, such as etidronate (Watts, 1998). Moreover, ibandronate contains a tertiary nitrogen, it is even stronger than ALE and PAM (Bauss and Schimmer, 2006).

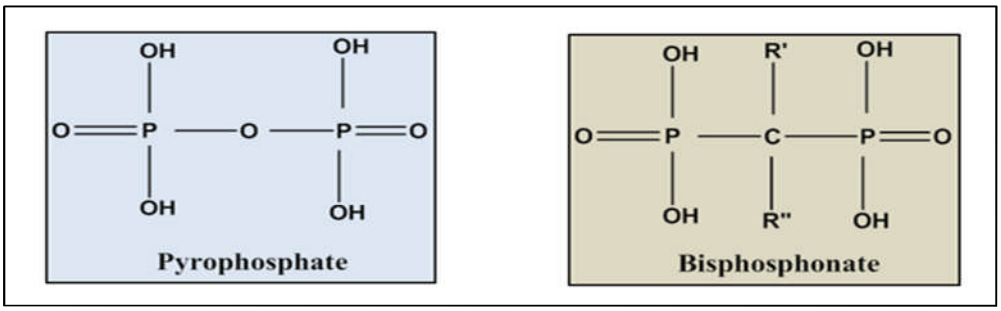


Figure 1.1: Pyrophosphate vs bisphosphonate

The chemical structure of bisphosphonate is considered a chemical analogue of inorganic pyrophosphate and is characterized by the presence of a carbon atom in the backbone of the molecule. This feature makes BPs more stable and resistant to thermal change and chemical degradation. The presence of R' and R'' side chains allow numerous substitutions that may lead to the formation of a large number of new compounds.

1.1.2 Mechanism of action

Regardless of whether they contain nitrogen, all of the BPs have the ability to inhibit bone resorption by interfering with osteoclast activity in a direct or indirect way. They also may affect adjacent cells, including osteoblasts, stem cells, fibroblasts, epithelial cells, and oral keratinocytes. Moreover, it has been reported that BPs have an effect on tumour cells (Biray Avci et al., 2015, Stefanovic et al., 2016). These drugs have several different mechanisms of action:

i. They inhibit bone resorption by inducing osteoclast apoptosis (Hughes et al., 1995).

ii. They prevent the development and recruitment of osteoclasts from hematopoietic precursors (Vitte et al., 1996).

iii. They stimulate the production of osteoclast inhibitory factors (Colucci et al., 1998).

iv. They inhibit the osteoclast enzyme farnesyl-diphosphate synthase (FPP) (Nuttall et al., 2012).

BPs have an affinity to bind with hydroxyapatite due to their carbon-substituted pyrophosphate structure, which gives them the unique feature of having selective action on the skeleton. Both types of BPs have a strong affinity to divalent metal ions, such as Ca^{2+}. These compounds also have a strong affinity for the Ca^{2+} of hydroxyapatite. N-BPs inhibit the osteoclast enzyme FPP in the HMG-CoA reductase pathway (Figure 1-2) (van Beek et al., 2003). Therefore, the FPP enzyme is a main pharmacologic target of N-BPs and clarifies the relationship between their structure and their potency (Dunford et al., 2008). Furthermore, the inhibition of FPP will prevent the prenylation of small GTPases, which play a crucial role in the production of isoprenoid lipids (Rogers et al., 2011). At the cellular level, BPs can affect osteoclasts directly by exerting a toxic effect on osteoclast retraction and cellular fragmentation. They also interfere with and alter the osteoclast's cytoskeleton. BPs indirectly inhibit osteoclast activity by modulating the osteoblast secretion of some intracellular signalling factors that stimulate the osteoclast. They mainly accumulate in the mineralized bone matrix and are released at sites with actively remodelling bone. Since they enter the body either orally or through an intravenous route, they are cleared from the circulation, taken up by osteoclasts, and transferred to sites of bone resorption.

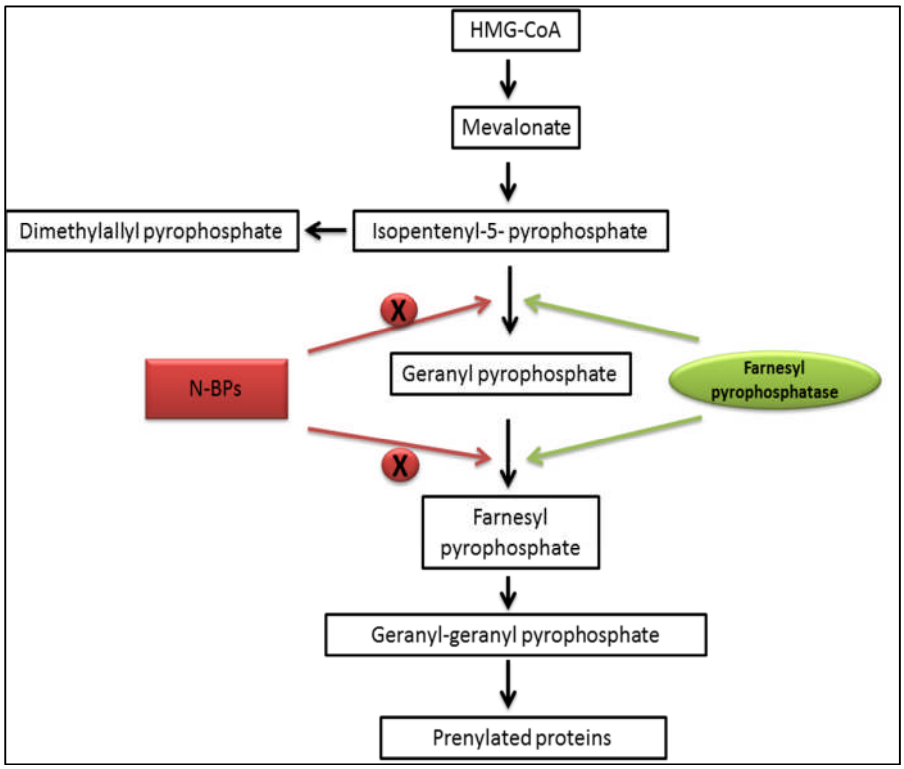


Figure 1.2: The HMG-CoA reductase pathway (mevalonate pathway)

The HMG-CoA reductase pathway is an important cellular metabolic process present in all eukaryotes and an important process for maintaining the cell membrane and the production of hormones and proteins, adapted with permission from (Rogers et al., 2011).

1.1.3 Clinical usage

Since their discovery, BPs have been used in the treatment of bone diseases characterised by excessive bone resorption, such as Paget's disease of the bone, hypercalcaemia of malignancy, osteogenesis imperfecta (OI), and all types of osteoporosis (Drake et al., 2008, Glorieux, 2007, Mundy and Yoneda, 1998, Ross et al., 2004, Russell, 2006, Russell, 2011). BPs have been extensively used in patients with Paget's disease of the bone as they selectively target the osteoclast (Dunstan et al., 2007). This disease is characterised by an increase in the number of osteoclasts, which subsequently leads to elevations in their activity and often produces bone fractures.

Hypercalcaemia of malignancy is a common condition that affects cancer patients, including those with breast and lung cancer and multiple myeloma (Stewart, 2005). The hypercalcaemia results from the excessive release of osteolytic factors, such as osteoclast activating factor (OAF) and parathyroid hormone-related protein (PTHrP). BPs are the treatment of choice in this disease; they may limit bone resorption by interfering with these factors (Ross et al., 2004). Breast cancer, for example, is treated mainly with intravenous (IV) PAM, zoledronic acid and ibandronate (Lipton et al., 2000, Rosen et al., 2004, Clemons et al., 2006, Body et al., 2003). All these drugs are prescribed to reduce bone pain and treat the skeletal complications associated with this type of cancer. In addition, the drug has been reported to have a direct effect on tumour cells by inducing cellular apoptosis. However, as previously mentioned, osteoporosis is a major health problem, and BPs are the drug of choice for this condition, including etidronate (Storm et al., 1990), ALE (Liberman et al., 1995) and risedronate (Reginster et al., 2000) .

In general, BPs have been extensively used in adults; recently, they have also been prescribed as a treatment option for children with OI. PAM is used intravenously at a dose of 9 mg/kg per year (Glorieux, 2007). Recently, ALE has also shown a promising result of limiting the number of fractures caused by OI (Cho et al., 2005). Clinically, due to their hydrophilic features, oral BPs have a low intestinal absorption rate of less than 1%, but they are highly retained in the bone. Most BPs are similar with a few differences, and they are capable of treating most bone resorption diseases. Since their approval for clinical use, at least 10 types of these drugs have been approved by the Food and Drug Administration (FDA) (Figure1-3). Oral BPs are absorbed mainly in the gastrointestinal tract (GIT), with small variations between the different types of BPs (Soares et al., 2016, Porras et al., 1999). Most of the drug is excreted unchanged by the kidney and is mainly taken up by bone; trace amounts are taken up by the kidney, liver and spleen. However,

when BPs are absorbed by the skeleton, they are taken in by osteoclasts and then can be released into the circulation once more to be taken up and embedded in the bone (Drake and Cremers, 2010).

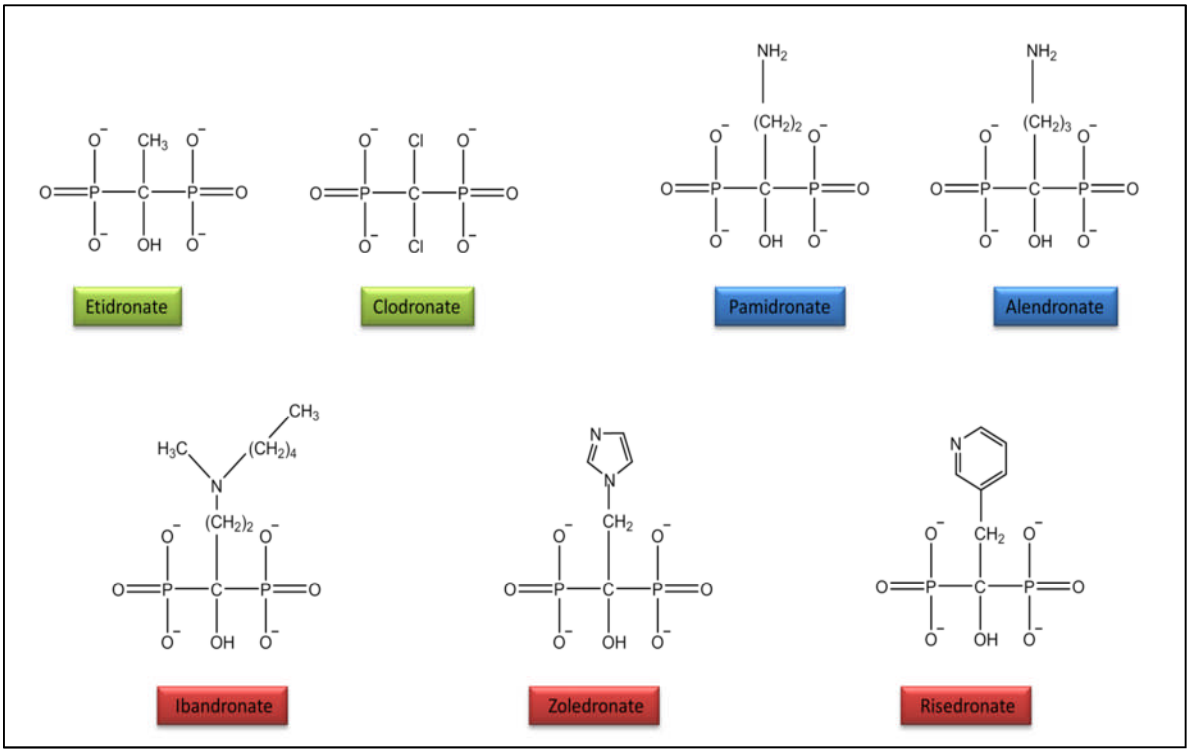


Figure 1.3: Chemical structure of BPs

Chemical structure of BPs that have been FDA-approved and are widely used in clinical practice. PAM and ALE contain only a basic primary nitrogen atom in their alkyl chain; this feature makes them 10 to 100 fold more potent than the simple BPs etidronate and clodronate. However, risedronate, zoledronate and ibandronate are considered to be the most potent BPs because of the presence of a nitrogen atom within a heterocyclic ring and also the presence of a tertiary nitrogen (Russell, 2006).

In general, BPs do not cause many side effects. The few that exist can be classified as early or late adverse effects, including upper GIT burning, musculoskeletal pain, hypocalcaemia and osteonecrosis of the jaw (Kennel and Drake, 2009). Osteonecrosis of the jaw (ischemic bone necrosis) is defined as exposed bone death that results from a decreased blood supply. Osteonecrosis may also occur as a result of infection, trauma,

medication (osteochemonecrosis), and radiotherapy (osteoradionecrosis). In the past, osteonecrosis of the jaw was mainly associated with radiation therapy, as recent evidence identified a correlation between BPs use and osteonecrosis of the jaw. Recently, osteochemonecrosis of the jaws that occurs as a result of taking BPs was given a new identification term known as bisphosphonate related/induced osteonecrosis of the jaws (BRONJ). Furthermore, BRONJ is considered a complication of taking this drug for osteoporosis, malignant bone disease and hypercalcaemia of malignancy (Marx, 2003). However, other studies have shown that additional agents can cause osteonecrosis' for example, two randomized clinical trials that investigated the effect of denosumab and the bisphosphonate, zoledronic acid implied that denosumab may be a new agent that can also induce BRONJ (Stopeck et al., 2010). Similarly, it has been reported that the incidence rates of denosumab- or zoledronic acid-related jaw osteonecrosis are 1.8% and 1.3% respectively; after four months of use, this prevalence increases to 2.2% (Scott and Muir, 2011). Bisphosphonates cause BRONJ by binding to the bone matrix and inactivating the osteoclasts via the ruffle border and the osteoblasts by inhibiting their proliferation, differentiation and mineralisation (Patntirapong et al., 2012, van Beek et al., 2003), while denosumab induced osteonecrosis of the jaw by binding to the osteoclast activator receptor and inhibiting the resorption of bone. The different pathology shown by these two necrotic mechanisms strongly suggests that ONJ is not just a specific BP, as originally represented by the term "BRONJ" but instead is more biologically complicated and would be better defined as chemo-osteonecrosis of the jaw (CONJ) (Anastasilakis et al., 2009). Recently, in 2014 an updated position paper from American Association of Oral and Maxillofacial Surgeons (AAOMS) recommended the changing of BRONJ to medication-related osteonecrosis of the jaw (MRONJ) (Ruggiero et al., 2014). This was

due to the fact that other drugs including denosumab had been implied to be a new agent that can induce ONJ.

1.1.4 Effect of bisphosphonate beyond osteoclasts

Beside their effects on osteoclasts, it has also been reported that BPs drugs may be involved and affect other cells, such as osteoblasts, osteocytes, fibroblasts, epithelial cells, keratinocytes and macrophages(Acil et al., 2012, Kim et al., 2011, McLeod et al., 2014). As stated previously, BPs are used to treat numerous bone diseases, including osteoporosis and bone metastases. The main target of BPs is the inhibition of osteoclast functions. Recently, it has been reported that osteoblasts may also be a possible target of BPs. In vitro studies have shown that BPs downregulate the receptor activator of nuclear factor kappa-B ligand (RANKL) and upregulate osteoprotegerins (OPGs) in osteoblasts. These findings clarify that the effects of BPs on osteoclasts may be mediated by osteoblasts (Maruotti et al., 2012, Viereck et al., 2002). Furthermore, other studies have reported that a high concentration of BPs may decrease the proliferation of osteoblasts (Acil et al., 2012). It has been hypothesised that BPs may enhance the bone mineral density via anabolic action on osteoblasts (Corrado et al., 2010). However, a high dose of BPs >10 μM will produce significant osteoblast apoptosis and decrease the cells' viability (Orriss et al., 2009, Huang et al., 2016, Patntirapong et al., 2012). Acil et al. conducted a study to investigate the cytotoxic effects of three types of BPs on osteoblasts: zoledronic acid (ZOL), PAM and ALE. They found that ZOL had the most negative effects on osteoblast proliferation compared to PAM and ALE. Moreover, it has been reported that a high dose of ZOL and PAM may limit the production of collagen (Acil et al., 2012). However, the angiogenetic effect of BPs on epithelial cells has been investigated with few conclusions (Walter et al., 2011). It has been reported that BPs may delay wound healing

by interfering with epithelial cells through the inhibition of type-I collagen (Ravosa et al., 2011).

Keratinocytes are highly specialised epithelial skin cells, and they represent 90% of epidermis cells that originate from keratinocyte stem cells. Their function is to form strong junctions with the nerves that supply the skin. These cells also play an important role in body immunity, as they are the first line of defence to prevent pathogens from entering the body. Reno et al. (2013) found in their study that treating human keratinocytes *in vitro* with a low dose of ZOL and neridronate stimulated the cells' proliferation (Reno et al., 2013). This effect was produced by decreasing the action of endogenous FPP levels, which work as an agonist of glucocorticoid receptors. Furthermore, it has been reported that a low dose of ZOL enhanced keratinocyte proliferation and produced an elevation of the keratinocyte-specific proliferation marker (K5) (Migliario et al., 2013). However, high doses of PAM and ZOL that are equivalent to the clinical dose had negative impacts on cell viability, migration and apoptosis of keratinocytes (Pabst et al., 2012, Kim et al., 2011).

However, fibroblasts are one type of specialised connective tissue cell. They are responsible for secreting extracellular matrix components, such as type I and/or type III collagen. These cells also participate in wound healing by migrating to the site of the wound and then proliferating to produce a collagenous matrix that will help isolate the affected tissue. The effect of BPs on fibroblast migration and proliferation have been investigated, and previous studies have shown that a dose that is close to the actual clinical regime inhibits fibroblast proliferation and leads to cell death (Scheper et al., 2009, Ravosa et al., 2011, De Colli et al., 2015, Tipton et al., 2011).

1.2 **Bone**

1.2.1 Bone structure

Bone can be defined as a mineralized connective tissue that makes up the major part of the body skeleton. It is a biological constructed material composed of cells and an extracellular matrix. This matrix is rich; composed of collagen fibres and non-collagenanous proteins. The cellular component consists predominantly of osteoblasts, osteoclasts and osteocytes. These cells together with the extracellular matrix form a scaffold to maintain the osteoarchitecture of the bone and subsequently equilibrizing the bone remodelling cycle (John P.Bilezikian and Martin, 2008, Boskey, 2013). Together with osteoclasts, the osteoblasts are dependent on each other for bone formation and resorption. The recruitment, proliferation and differentiation of both osteoblasts and osteoclasts are essential elements of the bone remodelling cycle.

Anatomically, bone mainly consists of three layers: the periosteum, the cortical and cancellous layers (Figure1-4). The periosteum is a fibrous connective tissue that covers all body bone surfaces except the articular surfaces where the bones are joined together. The periosteum is the outer layer that mainly consists of two components, the outer fibrous layer and the inner cellular layer that regulates cortical bone formation (Augustin et al., 2007). The outer layer is inelastic and contains less cells and more collagenous matrix. Furthermore, the superficial part of this layer is highly vascularized, which plays a vital role in providing a good blood supply to the bone and adjacent muscles. This layer also contains the nerves that travel with blood vessels (Dwek, 2010). The inner cellular layer houses more cells, such as multipotent mesenchymal stem cells, osteoprogenitor cells, osteoblasts, and fibroblasts, as well as micro-blood vessels and nerves (Allen and Ruggiero, 2014). This inner layer also serves as a transitional area between the inner layers of the bone and the overlying soft tissue. These layers together reveal that the

periosteum is a complex structure that contributes to bone growth and development and also participates in bone recovery and repair. The second bone layer is a cortical layer; its name refers to the cortex, which is dense and stiff. This layer gives the body's skeleton support, shape, protection and storage. The cortical layer represents 80% of the total bone mass of the skeleton (Clarke, 2008). Finally, the cancellous layer, which is also called the trabecular layer, represents the third layer, which is spongier and weaker compared to the cortical layer. This cancellous layer is highly vascularized and contains red bone marrow, where haematopoietic processes occur (Walter F. Boron, 2008).

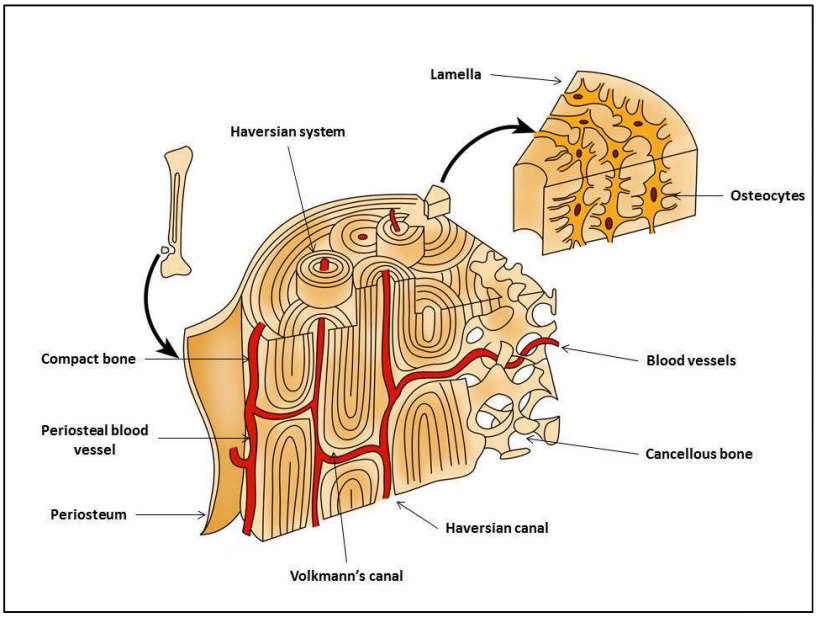


Figure 1.4: Micro-structural elements of the bone

The illustration shows a cross-sectional cut of the long bone (transverse and sagittal planes). The main micro-structural elements of the bone are shown from the surface to the bone's centre, including the periosteum, compact bone, and Volkmann's canals, the Haversian system and the spongey bone (cancellous bone), re drawn with permission from(Nanci, 2013).

1.2.2 Bone embryology and development

Human development begins when the fertilization process forms the zygote. Then, the zygote starts to produce identical cells, which give rise to the three main embryonic tissue layers: the ectoderm, mesoderm and endoderm. Each layer will participate in and produce specific organs in the human body systems, such as the nervous system and the musculoskeletal system. The human skeleton bones arise either from the ectoderm or the mesoderm. The mesoderm forms the axial skeleton, which specifically originates from the paraxial plate (the somatic part); the limb skeleton arises from the lateral plate of the mesoderm. The cranial neural crest portion of the ectoderm produces the facial and skull bones. These two origins participate in the bone ossification process, which mainly occurs by either intramembranous ossification or endochondral ossification. In intramembranous ossification, the bone forms directly within pre-existing mesenchymal tissue; this type of ossification occurs mainly in facial, skull and flat bones. The rest of the body skeleton is mostly formed by endochondral ossification. The intramembranous ossification process is primarily initiated by mesenchymal stem cells located within the embryonic soft fibrous connective tissue, which will later differentiate into specific bone-forming cells, such as osteoblasts. This process occurs at multiple sites within each skull and facial bone (figure1-5) (Nanci, 2013).

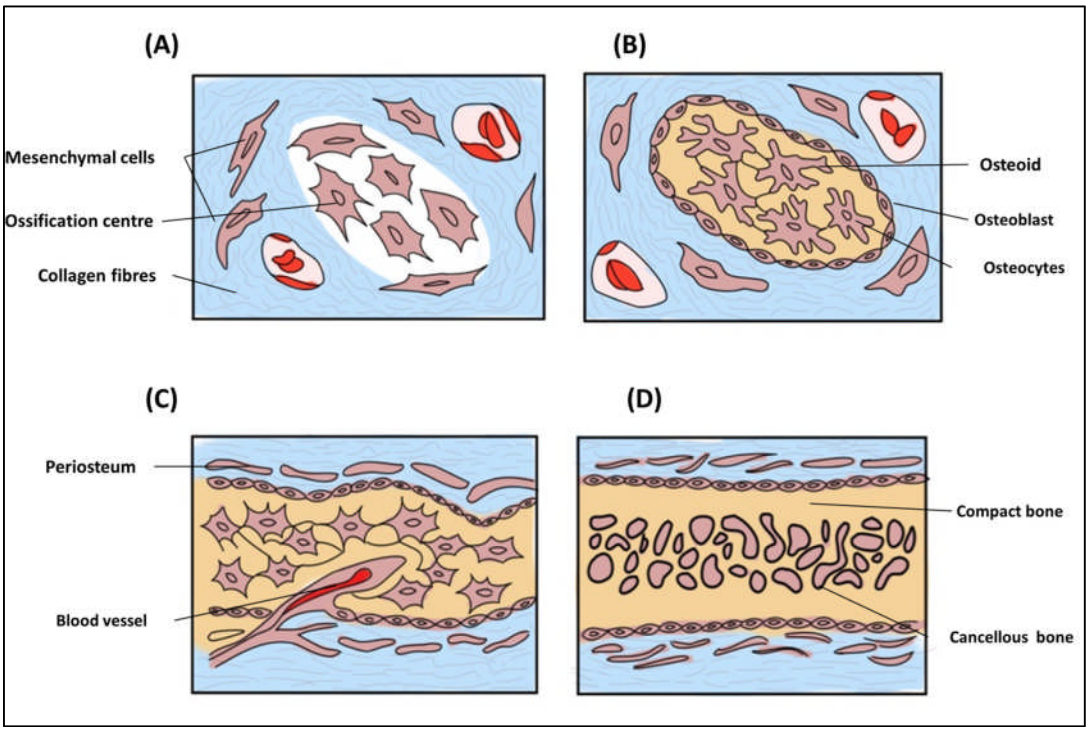


Figure 1.5: Intramembranous ossification

Intramembranous ossification processes consist mainly of four steps where bone forms directly within pre-existing mesenchymal tissue. The mesenchymal cells will later differentiate into osteoblasts where the bone will start to form, re drawn with permission from (Gerard J. Tortora, 2009).

However, the endochondral ossification process (bone forms within the hyaline cartilage that is generated from the mesenchyme) mainly occurs in long bone where cartilage is replaced with bone tissue. This process can be divided into primary and secondary ossification. The primary ossification process starts around the eighth intrauterine week of development in which the chondroblasts begin secreting cartilaginous matrix to form a hyaline cartilage model, and then the chondroblasts become trapped and differentiate into chondrocytes in the perichondrium to surround this model. The next stage is when the chondrocytes in the central model become enlarged and resorb some of the central part of this model, followed by matrix calcification, when the chondrocytes began to die. Then, the stem cells within the perichondrium start to differentiate into osteoblasts, forming a

compact bone collar around the calcified cartilage matrix. Finally, the blood capillaries from the periosteum invade the cartilage matrix and stimulate the remaining stem cells to form the primary ossification centre. The secondary ossification process takes the place around the bone epiphyses and involves replacing the calcified cartilage. These processes are completed around the twelfth intrauterine week (figure1-6) (Franz-Odendaal, 2011).

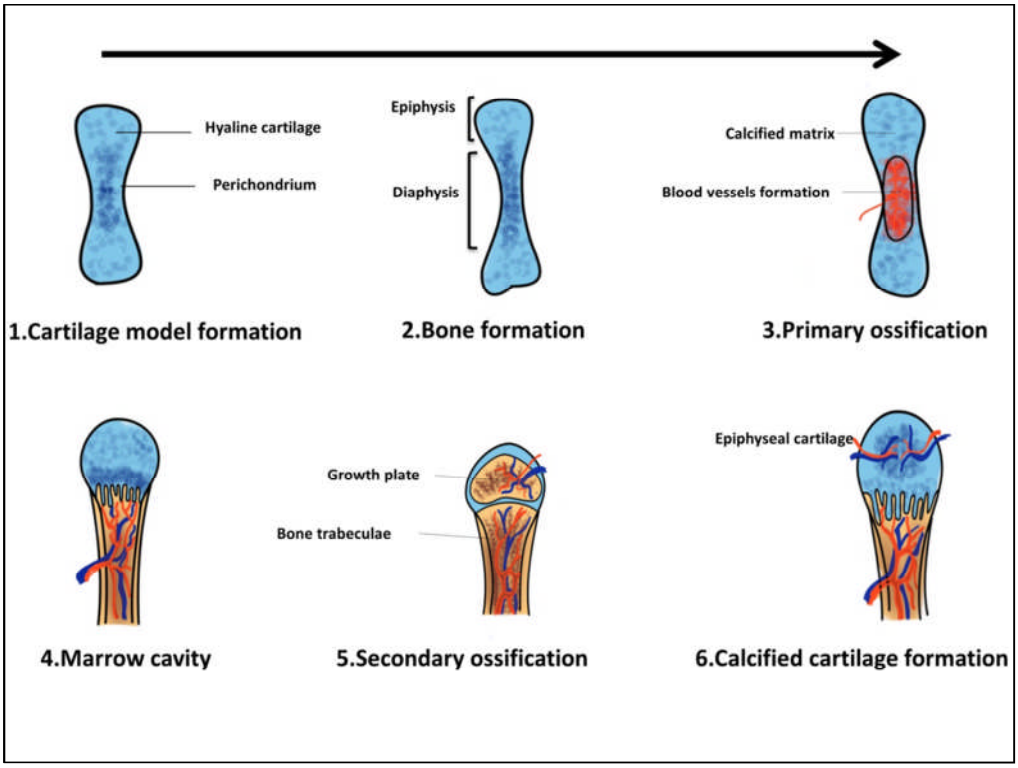


Figure 1.6: **The endochondral ossification phenomenon**

Endochondral ossification is divided into primary and secondary ossification. The primary ossification process starts when the chondroblasts begin secreting a cartilaginous matrix to form a hyaline cartilage model, followed by chondroblasts becoming trapped and differentiating into chondrocytes in the perichondrium to surround the cartilage. The chondrocytes in the central model become enlarged and resorb some of the central part of this model, followed by matrix calcification, when the chondrocytes began to die. Then, the stem cells within the perichondrium start to occupy this place and to differentiate into osteoblasts, forming a compact bone collar around the calcified cartilage matrix. The blood capillaries from the periosteum invade the cartilage matrix and stimulate the remaining stem cells to form the primary ossification centre. The secondary ossification process

takes place around the bone epiphyses by replacing the calcified cartilage, illustration was re drawn with permission from (Nanci, 2013).

1.2.3 Bone cells

There are two types of bone cells that maintain bone: osteoclasts and osteoblasts. The osteoblast family consists of osteoblasts that form bone; osteocytes are responsible for maintaining this bone as well as the lining cells that cover the bone's surface figure 1-7.

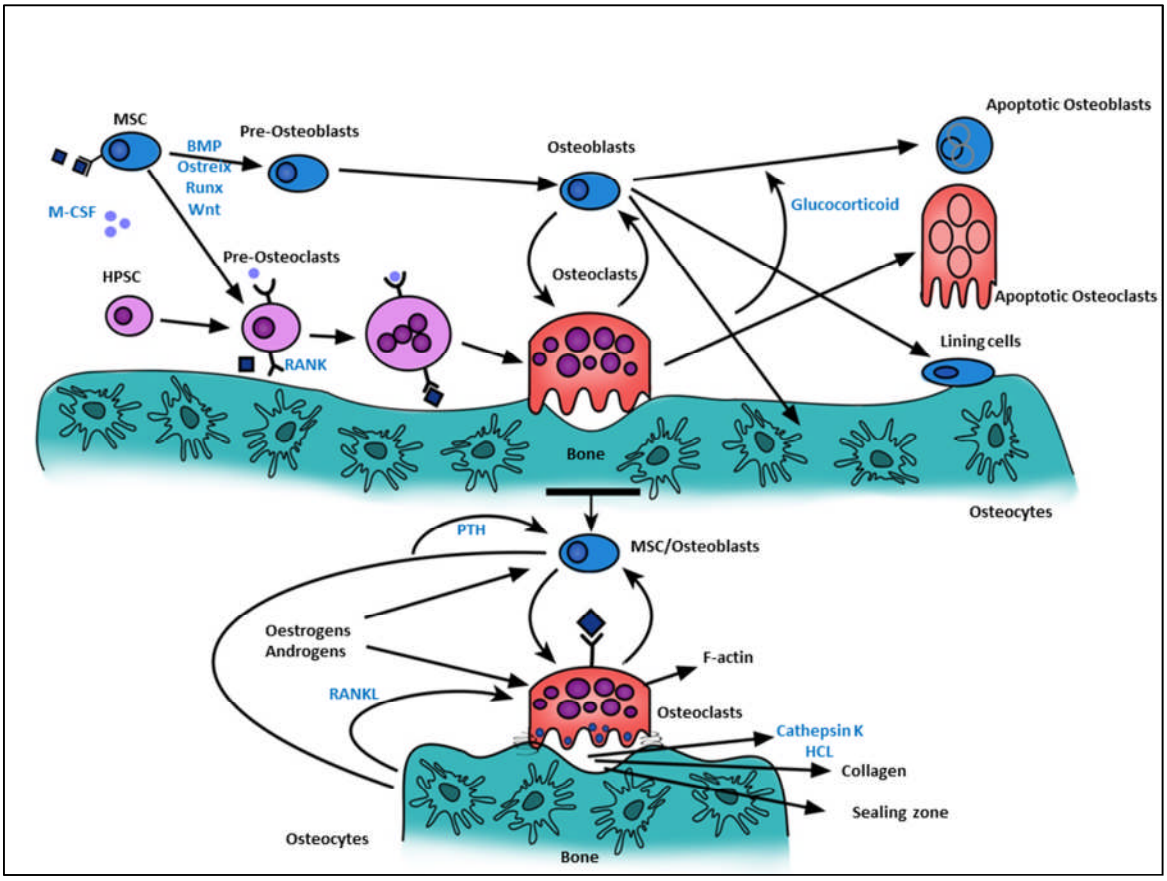


Figure 1.7: Origin of bone cells,

The osteoblasts originate from mesenchymal stem cells, Osteocytes are star-shaped cells derived from osteoblasts and located within the bone lacunae and bone lining cells that are located on the bone surface. The osteoclasts originate from haematopoietic stem cells. These cells are regulated by different systemic and local factors. Image adapted from (Hofbauer et al., 2014).

1.2.3.1 Osteoclasts

Osteoclasts are multinucleated cells that originate from haematopoietic stem cells, which mainly arise from the bone marrow. They are the major bone resorbing cells; this makes them unique and highly specialized cells. Osteoclasts are located on bone surfaces, in Haversian systems and occasionally on periosteal surfaces (Mundy, 1995). Microscopically, osteoclasts appear larger with abundant mitochondria compared to other bone cells. The inner surface of these cells also has a ruffled border to allow the cells to strongly attach to the bone's surface. These features are useful to identify and isolate these cells histologically. In addition, the presence of tartrate-resistant acid phosphatase is a useful marker of osteoclast identification. Osteoclasts are regulated by different signalling molecules, such as RANKL. This protein is produced by various body cells, including osteoblasts. RANKL play an important role in osteoclast activation by interacting with the RANK receptor, which is present on the osteoclast's surface. The interactions between RANKL and RANK are mainly involved in osteoclast differentiation and survival stages (Yasuda et al., 1999) and activate the osteoclast precursors to form mature osteoclasts. This interaction increases bone resorption. To balance this process, osteoblasts secrete an OPG protein that acts as a decoy for RANKL. Furthermore, osteoclast activities are regulated by various hormones and cytokines, such as parathyroid hormone (PTH), 1,25 dihydroxyvitamin D, transforming growth factor (TGF) and tumour necrosis factor (TNF) (Yasuda et al., 1999, Sims and Martin, 2014).

The sequence of resorptive activity in the osteoclast starts with the attachment of the osteoclast to the bone's surface, which creates a sealed acidic microenvironment that demineralizes the bone. This demineralization is followed by the degradation of the bone matrix due to the release of proteolytic enzymes, such as cathepsin B and acid phosphatase, and the production of hydrogen ions. Finally, the degraded matrix will be

transformed and released along the membrane opposite to the ruffled border(Mundy, 1995).

1.2.3.2 Osteoblasts

Osteoblasts are mononucleated cells that are the main bone-forming cells, and they originate from mesenchymal stem cells. Osteoblasts are mainly located on the bone's surface. They play an integral role in synthesizing the organic matrix of the bone. In contrast to osteoclasts, the osteoblasts are differentiated cells; they undergo mitotic division and display a high level of ALP activity. These cells differentiate into osteoblast progenitors and then into osteoblasts. Osteoblasts are responsible for the production and mineralization of the bone matrix. They also secrete collagen type I, the main component of the organic matrix, as well as several non-collagenous proteins. In addition, osteoblasts are responsible for the production of non-collagenous proteins, such as osteonectin (ON) osteopontin (OPN) and osteocalcin (OCN). Furthermore, osteoblasts secrete a variety of cytokines, several types of bone morphogenic proteins (BMPs) and transforming growth factor-β, platelet-derived growth factor (PDGF), insulin-like growth factors (IGF-I and IGF-II), ALP and fibroblastic growth factor (FGF) (de Oliveira et al., 2003). The secretion of these cytokines and growth factors regulates cellular activity, bone formation and bone metabolism.

Along with other growth factors, the bone morphogenic protein family plays an integral role in endochondral bone formation (Snyder et al., 2004). It has been reported that BMPs up-regulate the expression of ALP, collagen type 1 and OCN *in vitro* and stimulate cell differentiation (Yamaguchi et al., 2000). However, several genes have shown a regulatory effect on the osteoblast phenotype, such as runt-related transcription factor 2 (RUNX2), ostreix (OSX), ALP and Osteoprotegerin (OPG. Several studies have reported that the runx2 is the main regulator gene of the osteoblasts development (Komori, 2010, Paredes

et al., 2004). It has the ability to control and participate in different osteogenic signalling pathways as well as directly stimulating other osteogenic genes, such as OCN, RANKL, OPN and ALP. On the other hand, OSX is also one of the osteoblast-specific genes. It controls and regulates osteoblast genes, such as OCN, OPN and collagen type I (Nakashima et al., 2002, Zhou et al., 2010, Karsenty, 2008). Nakashima et al. found that removing this gene limits the ossification process of the osteoblasts (Nakashima et al., 2002). There are also some other genes that exert regulatory action, such as ALP and OPG; both are involved in cell growth, adhesion and differentiation.

Morphologically, osteoblasts have a cuboidal shape that allows these cells to function together. Microscopically, osteoblasts have prominent Golgi complexes, and the plasma membrane of osteoblasts is rich in ALP (Rosenberg et al., 2012, Florencio-Silva et al., 2015) .

1.2.3.3 Bone Lining Cells

Bone lining cells (BLCs) are flat shaped osteoblasts mainly found on the bone surface where the bone remodelling process is unlikely to occur (Miller et al., 1989). These cells have flat and ovoid nuclei, and their endoplasmic reticulums extended near the bone's surface. The bone cells (including bone lining cells) are a major part of the basic multicellular unit (BMU), an anatomical structure that is present during the bone remodelling cycle. The main function of these cells is still not completely clear. It has been reported that bone lining cells may interfere with direct communication between osteoclasts and the bone matrix, where bone resorption should not occur. Moreover, these cells exert their effects on osteoclast differentiation and the production of OPG and RANKL (Miller and Jee, 1987).

1.2.3.4 Osteocytes

Osteocytes are star-shaped cells derived from osteoblasts and located within the bone lacunae surrounded by mineralized bone matrix. Due to their long life, they represent 90–95% of the total bone cells (Bellido, 2014, Franz-Odendaal et al., 2006). This feature gives the osteocyte the ability to adapt the bone in response to mechanical forces. Osteocytes communicate with other bone cells via dendrites (Mundy, 1995). Recent reports have shown that the osteocyte may be involved in the bone remodelling and turnover processes. Following bone injury, osteocytes undergo apoptosis, which indirectly stimulates osteoblasts to form new bone (Cardoso et al., 2009). Osteocytes also have an indirect effect on BMPs and Wnts; both genes are responsible for bone formation and regulating the activity of osteoblasts. In addition, it has been reported that osteocytes can regulate bone resorption through pro- and anti-osteoclastogenic cytokines. Moreover, osteocytes secrete OPG, which is a decoy receptor for the RANKL that subsequently limits the action of RANK.

1.2.4 Jaw bones

The human jaws consist of an upper fixed jaw (maxilla) and a lower movable jaw (mandible). Both play an important role in biting, chewing and initial food digestion. The mandible is a horseshoe-shaped structure with a concavity that runs posteriorly. It serves as an anchor for the lower teeth and is the largest and strongest facial bone. The mandible consists of a horizontal arch (the body) and two vertical portions (rami) that are joined with the body at the mandible angle. Together with the rami, the body of the mandible provides attachment for the muscles of mastication. Histologically, the mandible has the same structure of flat bones, with a central spongy tissue surrounded by a compact tissue. On the other hand, the maxilla is the second largest facial bone and anchors the upper teeth. It is softer than the mandible and is also highly vascularized due to being mainly

composed of cancellous bone. Thus, the remodelling rate of the jawbones is higher than any other body bones (Dixon et al., 1997). However, the mandible and maxilla are covered with a thin layer of keratinized epithelium with numerous functions. This includes such as protection against external force as a mechanical barrier to harmful microorganism, minor salivary glands which together with major salivary glands that provide the principal source of lubrication in the oral cavity.

1.2.5 Osteoporosis

As humans grow, their normal bone mass is maintained by the balanced process of bone resorption and formation. Bone mass normally reaches its maximum peak during the third decade, and then a slow, age-related bone loss phenomenon begins. The definition of osteoporosis is age-related metabolic bone change characterised by excessive bone loss, low bone mass and deterioration of the microstructure of the bone, which subsequently will lead to weak and fragile bone (Papaioannou et al., 2008). Two types have been identified, and each one has its own unique features. Type I osteoporosis, which is also called postmenopausal osteoporosis, is mainly characterised by excessive cancellous bone loss and elevation of bone turnover. This type only affects postmenopausal women. Type II osteoporosis is called senile osteoporosis and is also characterised by an elevation of bone turnover that leads to hip bone fracture. This type usually affects women more than men. Osteoporosis is a complex disease and results from multiple risk factors, including age, gender, genetics, and environmental and hormonal influences. The major diagnostic parameters of osteoporosis are bone architecture, bone mineralisation and bone mechanical properties. Furthermore, bone regeneration slows down with ageing, and fractures manifest delayed healing, leading to morbidity and mortality in an elderly population. The most common treatment for these conditions is NBPs. These compounds as discussed before not only have a strong affinity for the Ca^{2+} of hydroxyapatite but also

affect the osteoclast enzyme farnesyl-diphosphate synthase (FPPS) in the HMG-CoA reductase pathway (Figure 1-2) (van Beek et al., 2003).

1.3 **Bone repair and implantology**

1.3.1 Bone remodeling

Bone remodelling is the process by which bone is turned over; it is the result of the activity of osteoblasts and osteoclasts. Remodelling is a balanced continuous process regulated by systemic and local hormonal factors that begins during the embryonic stage and continues throughout the life span. Indeed, remodelling is more rapid in childhood, where the rate of bone turnover is high to form new bone, and then decreases gradually as people age (Clarke, 2008). The bone remodelling process consists of five sequenced stages: activation, resorption, reversal, formation and finally, the resting stage. The first stage is initiated by the activation of osteoclasts when they resorb the old bone, which forms the resorption lacuna. Osteoblasts are then attracted to the resorption site. Subsequently, new bone will form and fill the resorption site. Furthermore, osteoblasts and osteoclasts are dependent on each other for bone formation and resorption. This process is affected by the recruitment of osteoblasts and osteoclasts. One important proposed finding is that osteoblasts regulate the differentiation and activation of osteoclasts via their interactions between the receptor antagonist of nuclear factor-b (RANK), the RANK ligand (RANKL) and the decoy receptor OPG (Karsenty, 2008). Any changes in the balance of bone formation or resorption will be presented as a clinical disease of the skeleton, such as osteoporosis.

Histologically, bone mainly consists of three layers: the periosteum, cortical bone and cancellous bone. The periosteum is the outer layer, and it consists of an outer fibrous layer and an inner cellular layer that regulate cortical bone formation.

However, the mandible, which is our interest in this study, is a horseshoe-shaped structure with a concavity that runs posteriorly. It serves to anchor the lower teeth and is the strongest facial bone. In contrast, the maxilla anchors the upper teeth and is softer than the mandible. The maxilla is highly vascularized and mainly composed of cancellous bone. Thus, the remodelling rate of the jawbone is higher than that of any other body bone. In addition, bone turnover by osteoclasts and osteoblasts also requires angiogenesis to maintain vascular integrity, which is an essential part of bone remodelling for the maintenance of bone mineral density and bone structure. Pathological situations resulting in the loss of bone's blood supply can lead to avascular necrosis (Seguin et al., 2008).

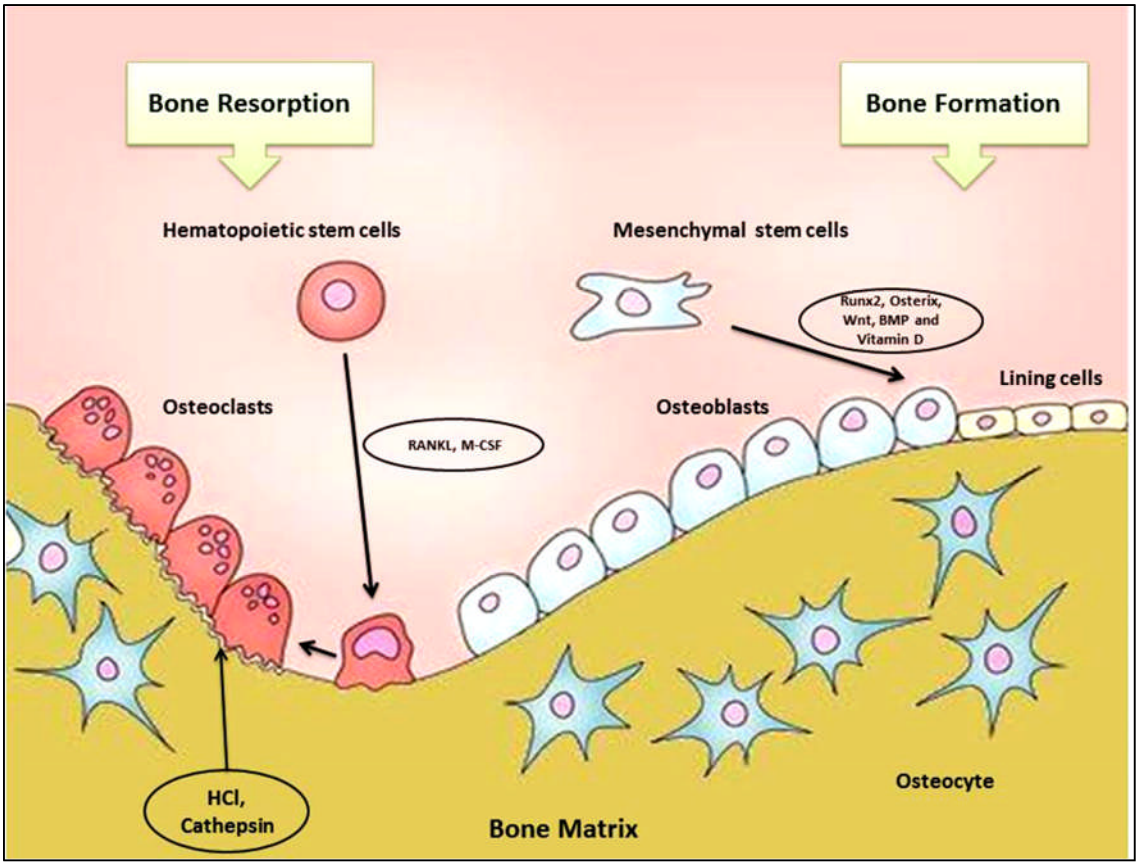


Figure 1.8: Bone remodelling cycle

This diagram shows the bone remodelling cycle. This cycle is initiated by the activation of osteoclasts followed by osteoblasts attracted to the resorption site. New bone will subsequently be formed, adapted with permission from (Imai et al., 2013)

1.3.2 Regulation of bone formation

As previously stated, bone is a complex structure that is regulated by different systemic and local factors. The systemic factors include PTH, calcitonin, 1,25 dihydroxyvitamin D, oestrogen, thyroid hormone, and growth hormone. PTH is produced by the parathyroid gland and is responsible for increasing the concentration of calcium levels in the blood. An elevation in PTH will indirectly stimulate osteoblast formation by increasing osteoclastic bone activity (Rosenberg et al., 2012). The real effect of PTH is believed to be associated with increasing the level of RANKL and inhibiting the expression of OPG (Florencio-Silva et al., 2015, de Oliveira et al., 2003). 1,25 dihydroxyvitamin D is the second most important hormone that plays an essential role in the bone remodelling process by stimulating osteoclast activity and mimicking the action of PTH with its indirect effect on osteoblasts to release RANKL and then to stimulate the osteoclasts. However, calcitonin is considered an antagonist of PTH because it lowers blood calcium levels. It also has an effect on osteoclasts' cell membranes, leading to cytoplasmic shrinkage and thus inhibiting bone resorption (Papaioannou et al., 2008). Growth hormone and thyroid hormone also participate in the bone remodelling process through their direct and/or indirect effects on osteoblast and osteoclast numbers and functions (Canalis, 2010, Jayesh and Dhinakarsamy, 2015).

Large groups of local factors are involved in the bone remodelling process, including cytokines, prostaglandin, BMPs and growth factors. The cytokines group includes the interleukin family released by the immune system, which act as intracellular mediators in the immune response. The interleukin family includes interleukin-1 (IL-1), interleukin-4 (IL-4) and interleukin-6 (IL-6), which regulate bone resorption by stimulating osteoclast or osteoblast proliferation. The production of IL-6 is correlated with 1,25 dihydroxyvitamin D and PTH (Yao et al., 2015). The growth factors include insulin-like

growth factors (IGFs), transforming growth factor β (TGF β), fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs); these factors are involved in bone formation and bone resorption.

1.3.3 Osseointegration

1.3.4 Definition and history

Osseointegration refers to the direct physical and mechanical contact between bone and its substrate without the formation of non-bone tissue between these contacts. In 1956, Branemark developed and defined this phenomenon as “a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant” (Figure 1-9). In his studies, Branemark showed that a titanium implant can permanently and completely fuse to the bone and cannot be separated without a fracture. He then suggested that this will offers a useful, long-term method for supporting dental prostheses (Branemark, 1983). Recently, the American Academy of Implant Dentistry (AAID) has defined this phenomenon as “the contact established without interception of non-bone tissue between normal remodelled and entailing sustained transfer and distribution of load from the implant to and within the bone tissue” (Jayesh and Dhinakarsamy, 2015).

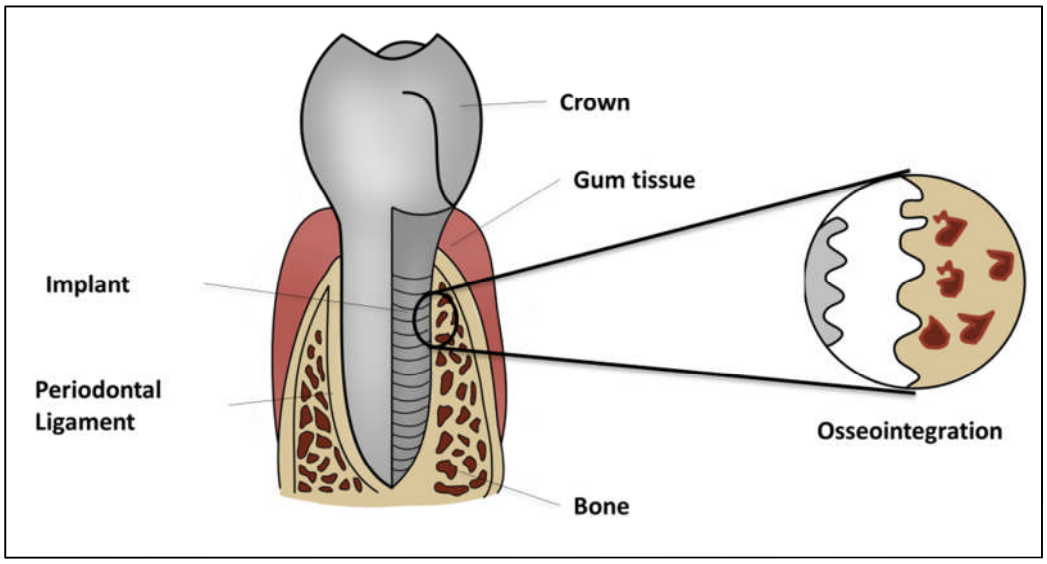


Figure 1.9: Osseointegration phenomenon

Osseointegration is a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant.

1.3.5 Biological principle of osseointegration

In order to produce a permanent and functional interaction between the implant and the bone, a good biological environment should be achieved. The outcome depends on different biological elements related to the surrounding tissue and implant topography as well as the loading process. The surrounding tissue, which includes both the bone and soft tissue, plays an integral role in accelerating osseointegration. There are three interlinked biological processes involved in osseointegration: the osteophyllic phase, the osteoconductive phase and the osteoadaptive phase (Davies, 1998, Berglundh et al., 2003). The osteophyllic phase (or osteogenesis) is the initial phase of the osseointegration process where the inflammatory response starts. Following implant placement into the marrow space, the blood will start to form a clot. Numerous cytokines and growth factors are then released, and each factor has a unique role and function in regulating cell proliferation and enhancing cellular adhesion to the implant surface. Stem cells and

osteoblasts begin to migrate, proliferate and differentiate. Subsequently, new bone will start to form. This phase usually lasts for four weeks. The second phase is osteoconductive, where the bone cells start to spread over the implant's surface, to mineralize and to produce a thin layer of bone called woven bone. This process normally takes up to three months from the time of implant placement. The final phase is the osteoadaptive phase, which normally begins four months following the implant's placement. During this stage, the implant begins to integrate into the surrounding bone, and the remodelling process creates more bone around the implant (Yao et al., 2015, Garg, 2004). However, an effective biological seal between the dental implant and the surrounding bone and soft tissue is considered an essential part of implant success. This seal inhibits the passage of microorganisms. If no seal is produced, these microorganisms may colonise the intra-area between the implant and bone; the formation of a permucosal zone could eventually lead to implant failure (Yeung, 2008).

1.3.6 Implant coating

Since the advent of titanium implants in dental practice, different types of coating materials have been considered as possible options for implant coating. These materials include bone growth factors (BGFs), calcium phosphate, carbon, fluoride, hydroxyapatite (HA) and BPs. Bone growth factors were once used as a coating material to enhance the bone density around the implant as well as for better healing outcomes (Al-Hezaimi et al., 2014, Thorey et al., 2011). Implants coated with BMPs have also shown better stability due to enhancing the bond strength of the implant's surface. Furthermore, it has been reported that BMP-2 coated implants may increase bone deposition around the implants. In contrast, IGFs and TGFs have been used to facilitate the bone healing process (Lynch et al., 1991). However, the main disadvantage of using bone stimulating factors is their

ability to be released over a period of time. Calcium phosphate and HA are some of the coating materials that have the potential to form a strong interstitial bond between the bone and the implant. This substance showed a considerable effect as osteoconductive material (Allegrini et al., 2006).

Calcium phosphate and HA-coated implants promote osteogenic differentiation, cell migration and adherence. The major concern of HA-coated implants is their long-term progress; the HA may undergo degradation and then resorption, which may lead to implant failure (van Oirschot et al., 2016, Klymov et al., 2016). Carbon has also been used as a coating option for the implant material, as it was found to be more stable and biocompatible than some other options. Furthermore, a carbon coating may improve osteoblast adhesion, proliferation and mineralisation (Poon et al., 2005, Harrasser et al., 2015). Despite these positive effects, there have been no long-term studies to support these data. It has been reported that fluoride-coated implants show a promising effect on osteoblast differentiation *in vitro*, but there was no impact on implant biocompatibility. In addition, fluoride-coated implants have shown a higher removal torque, which improves the implant's stability (Ellingsen et al., 2004).

BPs have been used as coating materials due to their selective inhibition of the osteolytic process by restricting the activity of osteoclasts. This action subsequently affects the remodelling process, and more bone will form. The impact of a local application of BPs on osseointegration was previously investigated by coating the implant with BPs and examining the effect on osteoclast function to accelerate wound healing and bone formation (Greiner et al., 2007). Moreover, it has been reported that the local application of BPs on an implant showed a significant increase in biomechanical strength of the fracture site (Greiner et al., 2008). However, these studies used a high-dose of BPs, which

may affect bone compaction and could impair the osseointegration process. In addition, in some studies, the local application of BPs did not improve the osseointegration and actually impaired wound healing. Furthermore, another *in vitro* study showed that using a soaking bone graft (allograft) with a high dose of ALE decreased biomechanical fixation and osseointegration (Jakobsen et al., 2007).

Table 1.1: Implant coating materials

Materials	Example	Studies	Outcome
Carbon coating	Currently not on the market; still being investigated	*In vitro*, *in vivo* and clinical studies	Improved biologic properties and histocompatibility, but studies are still under way
Bisphosphonates	Currently not on the market; still being investigated	No long-term studies available	No long-term studies available
Bone stimulating Factors	Currently not on the market; still being investigated	Pilot animal studies and clinical studies	Studies are still under way
Bioactive glasses and ceramics	Currently not on the market; still being investigated	Chemical, *in vivo* and *in vitro* studies	Studies are still under way
Fluoride coatings	OsseoSpeed	*In vitro* studies	Selective osteoblast differentiation results
Hydroxyapatite (HA)	Restore Implant system	*In vivo*, *in vitro* and retrieval studies	Most commonly used type of implant coating; other implant coating studies mainly use HA as a control
Titanium/titanium nitride	IonFusion	*In vitro*, *in vivo* and clinical studies	Titanium mechanical properties are considered in relation to the degree of osseointegration

***(Xuereb et al., 2015)**

1.3.7 Factors affecting osseointegration and implant outcome

Several factors have an effect on osseointegration success and failure. Factors that affect osseointegration success can be classified as implant-related factors, such as the implant's design, topography, material type, shape and coatings (Xuereb et al., 2015). The second factor is the surrounding bone anatomy and biology (Marco et al., 2005). The third factor is related to the mechanical stability and type of loading protocol used (Soballe, 1993). A fourth factor is the types of adjunctive materials that improve implant success, including the graft material, bone guided regeneration and drugs, such as simvastatin and BPs. On the other hand, factors that lead to osseointegration failure include implant mobility, micromotion, radiotherapy and drugs (cyclosporin, warfarin). Furthermore, some factors are patient-related, such as osteoporosis, diet, age and smoking (Mombelli and Cionca, 2006).

However, several implant materials have been used to replace missing teeth, knees and hip bones, including cobalt-chromium, zirconium, stainless steel and titanium. Titanium (Ti) is one of the best metals that is able to osseointegrate, thus increasing the lifespan of these implants. Ti is considered the best option to replace missing teeth, as 95% of dental implants are titanium alloys. Ti is number 22 on the periodic table and is one of the most abundant metallic elements on earth. Due it is biocompatibility, it is widely and extensively used in the medical field as a treatment option for replacing knees, hips and teeth. Chemically, Ti has many unique features, such as being nonmagnetic, its ability to tolerate high temperatures, being corrosion resistant and having a high melting point.

1.4 **Mesenchymal stem cells**

1.4.1 Definition and origin

Human mesenchymal stem cell (hMSCs) are undifferentiated cells known for their capacity to renew themselves and differentiate into different types of cells (Devine et al., 2001). Friedenstein and his colleagues; (1970) were the first research group to identify and define these cells as fibroblast precursors from the bone marrow (BM) (Friedenstein et al., 1970). The self-renewal feature of these cells is their ability to generate daughter cells that mimic their mother cell (Wright-Kanuth and Smith, 2001, Smith and Zsebo, 2001). These cells are believed to originate from the human bone marrow, which is also a source of haematopoietic stem cells (HSCs), and they represent only a small percentage of the total number of bone marrow cells (Campagnoli et al., 2001). HSCs mainly produce blood cells and lymphatic system precursors. In addition, several reports have shown that these cells can be present in different body tissues apart from the bone marrow, such as the adipose tissue(Zuk et al., 2002), placenta, umbilical cord (Wang et al., 2004), lung, amniotic fluid and primary teeth(Sunil et al., 2015). The primary goals of hMSCs are to maintain and repair the body tissues in which they exist. They have the ability to differentiate into different mesodermal origin cells, such as osteoblasts, fibroblasts and chondrocytes, adipocytes, myocytes and neurons. Compared to HCSs, hMSCs have a strong affinity to attach to tissue cultured on a plastic surface (Dominici et al., 2006, da Silva Meirelles et al., 2008). Earlier studies with hMSCs have verified that cells harvested from older donors have a short life span and diminished proliferative capacity compared to hMSCs harvested from younger donors (Stenderup et al., 2003). The *in vivo* identity of hMSCs is still controversial in the research field. They are present as a heterogeneous population of multipotent cells.

1.4.2 Biological Characteristics

The isolation of MSCs from different anatomical sites, like the periosteum, muscles, adipose tissue and skin, indicates that these cells are derived from a common embryonic ancestor. They are distributed across the entire body during the development stage, where they are responsible for mesoderm repair (da Silva Meirelles et al., 2008). hMSCs are preserved in body tissues in an undifferentiated form. They do not have any features typical of differentiated cells; instead, they have the characteristics of primary cells. Stem cells have some basic characteristics, such as self-renewal and the ability to differentiate into other cells. In addition, MSCs can be characterised according to their surface markers, morphology, differentiation capacity and immunomodulatory effects. There are different surface markers, which reflect the stem cells' behaviour and type. These markers indicate whether the MSCs are positive for CD105, CD73, and CD90 or negative for CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface markers (Dominici et al., 2006). These cells have unique morphological characteristics, which include a small cell body with a prominent and large nucleus that occupies most of the cytoplasm (Figure1-10). Intracellular components are present in small amounts, such as the Golgi apparatus, rough endoplasmic reticulum and mitochondria. Moreover, these cells have a fibroblastic morphology (Colter et al., 2000) and have demonstrated the ability to differentiate into different types of cells: for example, adipocytes, osteoblasts, chondrocytes and myogenic-like cells (Kolf et al., 2007). The time these cells remain in culture is crucial for them to differentiate into different cell lineages. For example, hMSCs lose their ability to differentiate into chondrocytes after the third passage, whereas their ability to differentiate into osteoblasts is reduced after the fifth passage. In addition, the differentiation ability of MSCs into cardiomyocytes also depends on the passage (Zhang et al., 2005). The immunomodulatory effects of stem cells include their ability to

interfere with immune system cells, including T-cells and dendritic cells. They are also able to generate a local immunosuppressive microenvironment by secreting some cytokines, including interleukin (IL), leukaemia inhibitory factor and stem cell factor (Haynesworth et al., 1996, Ryan et al., 2005).

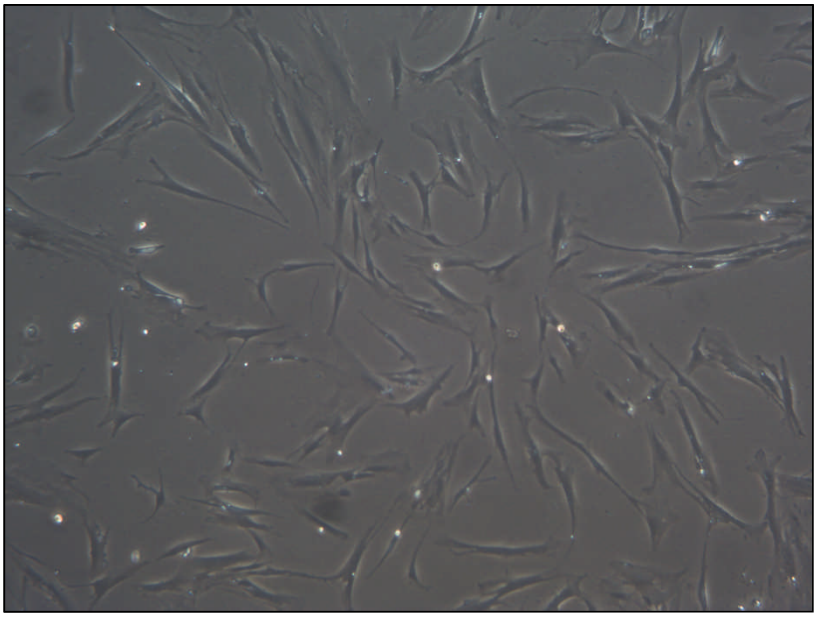


Figure 1.10: Human mesenchymal stem cells (hMSCs)

Human mesenchymal stem cells (hMSCs) are spindle shaped fibroblast-like cells. Original magnification × 100, phase-contrast light microscopy, the image was taken by the author.

1.4.3 Osteogenic behaviour

MSCs that demonstrate osteogenic behaviour are one of the unique pluripotent characteristics of these cells. This finding has been confirmed by multiple reports where the isolated MSCs obtained from the bone marrow that were positive for some surface osteogenic markers differentiated into osteoblasts (Foudah et al., 2013, Kollmer et al., 2013). These markers include CD10, CD92, CD166, CD90 and CD105. There were also some osteogenic markers, such as ALP, RUNX2, OCN, OPN and Wnts5, which confirmed the osteogenic behaviour of the MSCs. These cells show pivotal and good

therapeutic potential in bone regeneration and growth, as well as the osseointegration of bone implants. Several reports have proposed that the hMSCs can be differentiated into osteoblasts following certain protocols, including the use of dexamethasone (Dex), ascorbic acid (Asc) and β-glycerophosphate (β-Gly) (Langenbach and Handschel, 2013a, Friedenstein et al., 1987) .

1.4.4 Epigenetics in the proliferation and osteogenic differentiation of hMSCs

Epigenetics is defined as the study and investigation of heritable phenotypic changes in cellular gene expression that are not involved in the genotype behaviour of both eukaryotic and prokaryotic cells (Deans and Maggert, 2015). These changes occur regularly and naturally, and can be predisposed by numerous physiological, physical and chemical factors. Moreover, epigenetic changes provide a framework for regulating the development and differentiation of hMSCs, along different lineages, such as osteoblasts, adipocytes and neurons (Vrtacnik et al., 2014). Molecularly, four epigenetic changes are primarily involved in various cellular activities: DNA methylation, histone modification, chromatin remodeling and non-coding micro RNA (Figures 1-11) (Egger et al., 2004, Han and Yoon, 2012). These changes are considered as crucial, as they allow organization of the transcription cellular program during embryonic development and tissue maintenance.

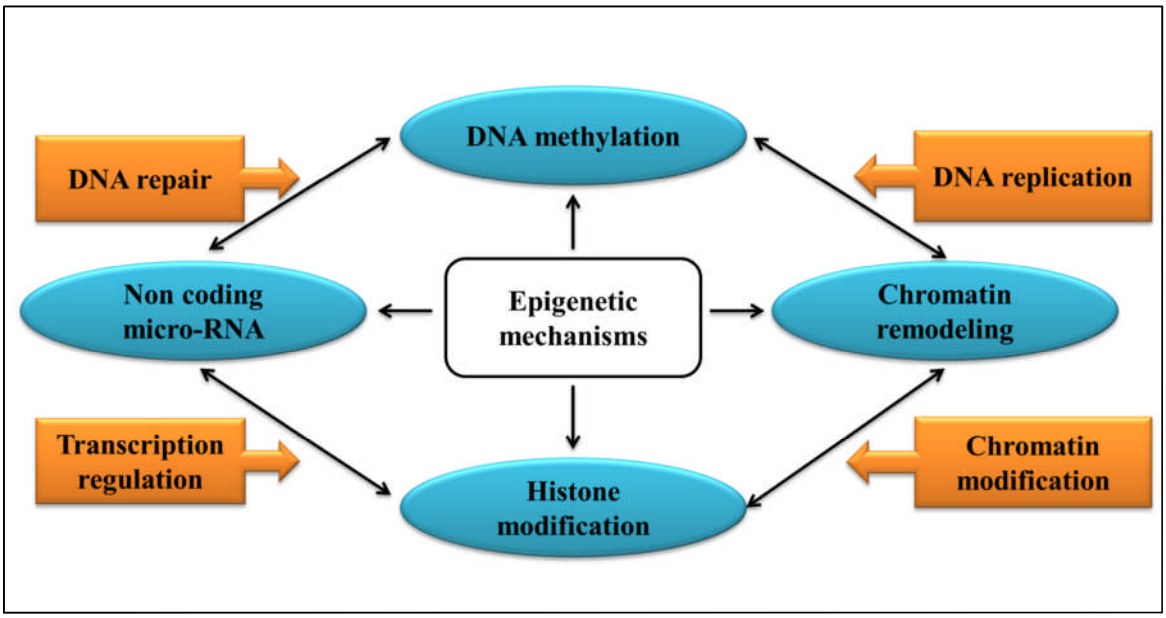


Figure 1.11 Epigenetics mechanisms

This diagram shows the different mechanisms that are involved in the phenotypic changes of human mesenchymal stem cells and their modifications. These mechanisms are involved in gene expression regulation as a response to external stimuli.

DNA methylation occurs via the addition of a methyl group to DNA; this will subsequently modify gene function and therefore cell behaviour. In addition, this phenomenon may starkly change cell gene expression, cell division and differentiation into specific tissues from as early as the embryonic stage (Smith and Meissner, 2013). However, DNA methylation is reversible, for example, by hydroxylation of the methyl groups (Wossidlo et al., 2011).

Cancer was the first of several diseases to be linked to epigenetic changes (Sharma et al., 2010); they are also associated with cardiovascular disease, diabetic mellitus and multiple sclerosis. It has therefore been proposed that epigenetic therapy may have potential in the treatment of some types of cancer, via the control of several DNA methylation inhibitors (Cortez and Jones, 2008).

As discussed earlier in this chapter, hMSCs have a promising outcome with regard to use in the treatment of different human diseases, including bone, cartilage and cardiac diseases (Teven et al., 2011). However, *in vitro* studies have shown a correlation between a lengthy culture period and morphological and functional changes of the cells (Galderisi and Giordano, 2014). These changes will subsequently affect the replicative behaviour of the cells, or replicative senescence, which can be manifested by limitation of cell proliferation and osteogenic differentiation. It has been reported that early passage of hMSCs, compared to late passage, appears to be regulated by epigenetic changes via control of cell aging (Wagner et al., 2008, Srinageshwar et al., 2016).

In the last decade, research has focused on improvement of the *in vitro* environment, to allow MSCs to differentiate to other lineages, by addressing gene modification and expression. It was recently discovered that epigenetics are involved in hMSC differentiation (Huang et al., 2015). In addition, some studies have shown a correlation between the mechanism of epigenetics and differentiation of MSCs via the control of some osteogenic genes (Tan et al., 2009). Moreover, DNA methylation exerts its regulatory effect on osteogenic differentiation to a greater extent than the other epigenetic markers. Dansranjavin et al. found that osteogenic differentiation of hMSCs was accompanied by downregulation of stem cell-related genes, such as Brachyury and LIN28, via hypermethylation of promoters of these genes (Dansranjavin et al., 2009). Furthermore, it has been reported that biological and mechanical stimuli can induce MSC osteogenesis through downregulation in the methylation of OPN promoters (Arnsdorf et al., 2010). This evidence shows that epigenetic changes play an important role in the majority of cellular molecular activities, as well as in cell aging and development. It has also been shown that the handling of the hMSCs epigenome may improve their osteogenic differentiation (Hsiao et al., 2010, Ozkul and Galderisi, 2016).

1.4.5 MSC and bone healing

MSCs have proved their effective role and involvement in bone turnover, haemostasis and differentiation into different types of cell lineages. Thus, the clinical use of cultured cells to form different body tissue has been proposed but still needs to be investigated. Pre-clinical studies have shown that stem cell therapy includes bone repair, cartilage repair, marrow regeneration, tendon repair, gene therapy and vascular repair (Bielby et al., 2007).

Both the *in vitro* and *in vivo* studies have proposed that a high density of bone marrow stem cells may enhance fracture repair and subsequently accelerate wound healing (Hernigou et al., 2005) . Hernigou et al. investigated the application of autologous bone marrow MSCs on tibial non-union fracture; they found that around 88% of tibial non-unions were treated successfully. Following bone trauma, the body responds by secreting local and systemic factors, including pro-inflammatory cytokines. Then the immune cells migrate to the injured site, and osteogenic progenitor cells start to proliferate and differentiate to form new bone. The hMSCs participate in one or more of these events. For example, in an inflammatory response, the hMSCs will modulate and limit the action of some immunosuppressive factors, including TGF β and prostaglandin E2. On the other hand, hMSCs start to produce some stem cell- derived factors and some cytokines, which will change the type of immune cells at the trauma site. Following the trauma, such as implant placement, the MSCs begin to differentiate into connective tissue cells, including chondrocytes and osteoblasts, to initiate the repair process and build up new bone.

hMSCs and osteoblast progenitors have been proposed to be one of the major cells in the osseointegration phenomenon (Tuan, 2011). These cells migrate and adhere to the titanium surface from day one following implant placement. They then start to create a non-collagenous scaffold that subsequently will regulate cell adhesion and differentiation.

This scaffold layer is mainly formed of calcified afibrillar containing mineralized osteoid with a 0.5-mm thick layer, rich in calcium and phosphorus (Meyer et al., 2004). The topography of the titanium surface indirectly suggests that it enhanced the hMSCs differentiation and osteogenic mineralisation (Jayaraman et al., 2004).

1.5 **Hypothesis**

A low dose of BPs potentially has a temporal effect on hMSC proliferation and differentiation.

1.6 **Aims and Objectives**

The aims and objectives of this study were as follows:

(i) To investigate the effect of BPs on the proliferation and osteogenic differentiation of hMSCs, by examining cellular proliferation, cellular mineralisation, alkaline phosphatase activity, cellular apoptosis and osteogenic gene expression.

(ii) To examine the temporal effect of a single low dose of BPs on the proliferative and osteogenic behaviour of hMSCs by investigating cellular proliferation and cellular mineralisation, and examine early and late osteogenic markers.

(iii) To investigate whether BPs exert a permanent phenotypical change on hMSCs by examining epigenetic changes using a DNA methylation marker.

(iv) To investigate whether low-dose BP treatment might induce proliferation, migration and osteogenic differentiation of hMSCs on Ti surfaces as a way of enhancing the rate and degree of osseointegration by examining cellular

proliferation, cellular attachment and retention, cellular migration, cellular mineralisation and morphological differences.

The experiments were conducted in a two-dimensional *in vitro* model, which consisted of cells suspended in growth medium in a sterile culture vessel. A summary of the experiments is presented as a flow chart in Figure 1.12, and shows the steps that were taken to address the study hypothesis.

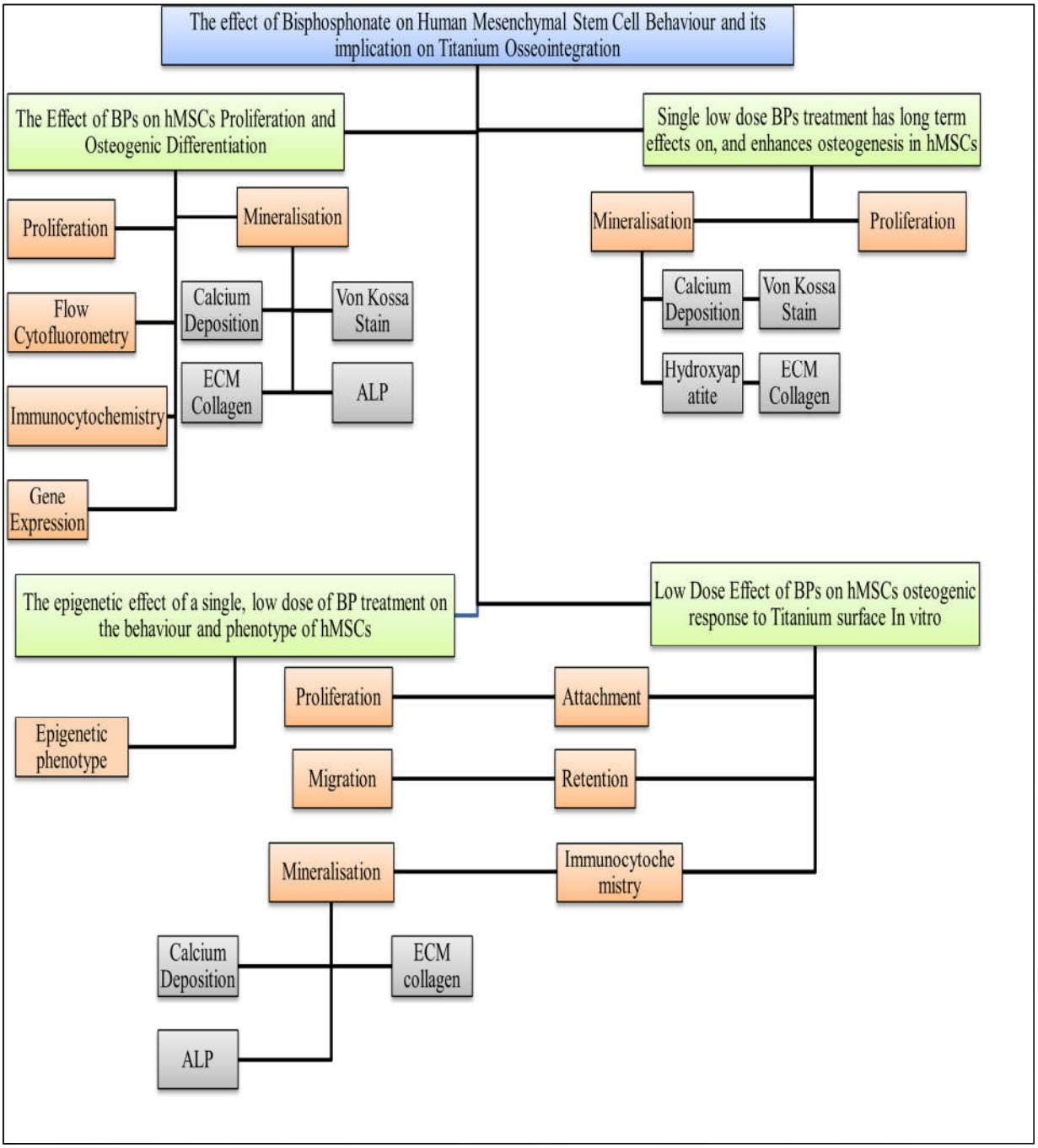


Figure 1.12: Outline of the experimental work

2 General materials and methods

2.1 Cell culture

In this project, we used primary human bone marrow-derived mesenchymal stem cells (hMSCs). These cells were purchased from the institute for Regenerative Medicine at the Texas A&M Health Science Centre College of Medicine (USA). The cells were undergoing the pre-characterisation process, which includes stem cell markers and osteogenic and chondrogenic differentiation. Moreover, these cells had the ability to adhere to plastic tissue culture vessels as one of their unique features. All cell culture and media preparation procedures were performed in a sterile laminar flow hood. The cell culture materials were sterile when acquired. Non-sterilised materials were autoclaved and oven dried at 60°C prior to use. The cell culture environment facilities are tabulated in Table 2-1. In addition, the general consumable, culture media and reagents are detailed in Tables 2-2 and 2-3, respectively. All cultured media and reagents were pre-warmed at 37°C for ~30 min.

Table 2.1: Cell culture environment

No.	Item	Manufacturer	Application
1	Laminar flow hood	NUAIRE Class II with UV-tube	Provide a clean work environment, was cleaned regularly with 70% ethanol, virkon and propanol wipes
2	Incubator	Thermo Scientific	Provide a 95% humidified, 5% CO_2 and 37°C environment for cells to grow
3	Water bath	Grant	Came with thermostat to warm up the culture media to 37°C, regularly cleaned
4	Inverted Microscope	Olympus CXK31	To regularly visualise the cells
5	Haemocytometer	Sigma	Standard 9 x (1x1 mm) grid with cover slip
6	Cryogenic storage	Thermo Scientific	Liquid nitrogen tank
7	Centrifuge	Sorvall	For large tissue culture samples
8	Microcentrifuge	Sanyo	For small tissue culture samples
9	Pipettes	Gilson	Calibrated pipette gun boy and 20, 200 and 1000 µl micropipettes
10	Autoclave	Systec	Pressurised steam sterilizer set at 126ºC for 20 min

Table 2.2: Cell culture consumables

No.	Item	Manufacturer	Application
1	Flasks	Helena Biosciences	Three sizes of polystyrene flasks were used: 25, 75 and 150 cm^2. Air permeable cap. The inner surface had a uniform flat growth surface.
2	Plates	Helena Biosciences	Multi-well polystyrene plates with removable lids. Two types were mainly used: 96-well and 24-well plates.
3	Tubes	Corning	Different polystyrene tubes were employed for different cell culture purposes; 50, 15 and 2 ml sizes were used.
4	Microcentrifuge tubes	Fisher Scientific	Autoclavable, different sizes 300 µl, 500 µl and 1 ml
5	Cryopreservation vials	Fisher Scientific	1.2 ml, self-standing, conical interior bottom
6	Pipette tips	Star lab	Disposable and sterilised, volume range: 10-1000 µl

Table 2.3: Cell culture media and reagents

No.	Item	Manufacturer	Application
1	Alpha minimal essential medium	Gibco	αMEM; L-Glutamine supplemented basal medium used for the hMSCs growth medium
2	Dulbecco's eagles medium	Gibco	DMEM; L-Glutamine supplemented low glucose basal medium used for hMSCs osteogenic media supplemented with osteogenic inductive elements.
3	Antibiotic	Sigma	A mixture of penicillin 10 units/ml and Streptomycin 10 mg/ml in dictation-free PBS. This was occasionally supplemented with the fungicide Amphotericin B at 0.2% of the culture medium.
4	Fetal bovine serum	Invitrogen	Nutrition sources in cell culture
5	Phosphate buffer saline	Lonza	Used for dilution and cell washing
6	Trypsin/EDTA	Life Technology	0.05% trypsin, 0.002% EDTA in phosphate buffered saline
7	Dexamethasone	Sigma	Water-soluble, anti-inflammatory glucocorticoid for osteogenic induction.
8	Ascorbic acid	Sigma	Collagen synthesis initiator in osteogenic media.
9	β-glycerophosphate	Sigma	Phosphate donor and Initiates osteogenic differentiation
10	Dimethylsulfoxide	Sigma	DMSO : used as a protectant during cold storage of living cells

2.1.1 MSCs isolation

The hMSCs isolation process were performed by the Tulane centre of Gene therapy, US. Briefly, bone morrow was aspirated from the iliac crest of three donors; two male and a female aged 22, 24 and 37 respectively. In order to obtain adherent MSCs; cells were separated using density centrifugation and then plated in tissue culture plastic (TCP). Cells were then grown using alpha minimum essential medium (α-MEM) supplemented with 16.5% foetal bovine serum (FBS) till cells reach 60-80% confluence. The cells were then harvested as passage 0 then re-expanded at passage 1 where the characterisation test was started. The characterisation of hMSCs was performed using flow cytometry to test hMSCs specific markers. The hMSC were positive to these surface markers CD147,

CD166, CD90, CD105, CD29, CD59 CD49c, CD73a and CD146. They were negative to these markers CD34, CD45, CD79a, CD11b, CD19 and CD14. The cells showed their ability to differentiate to fat, bone and cartilage (Prockop et al., 2001, Sekiya et al., 2004).

2.1.2 Mesenchymal stem cell culture

hMSCs from three donors were used in this project (N=3). The cells were cultured in 150 cm² tissue culture flasks (Helena Biosciences) in alpha minimum essential medium (α-MEM, Gibco BRL). This medium contained a 10% FBS (Invitrogen) and 1% of penicillin/streptomycin (P/S) (Sigma-Aldrich). The flasks were incubated at 37ºC at 5% CO_2 until they reached ~80% confluence. The medium was changed twice a week (every 3–4 days). When the cells became ~80% confluent (Figure 2-1), the GM was discarded and the cell monolayer was then washed twice with phosphate-buffered saline (PBS) to remove any excess culture medium and dead cells. The cell layer was then trypsinized using an appropriate volume of trypsin-EDTA (Invitrogen) in PBS. The flasks were then transferred to the incubator (Thermo Scientific, UK) and incubated at 37ºC for 5–10 minutes. To stop the action of trypsin, 10 ml of GM were added to each flask. The detached cell layer was then collected in 50-ml plastic tubes (Sarstaed) and subjected to centrifugation at 1000 rpm for 5 minutes at 37ºC. The supernatant was discarded, and the cells were re-suspended in a 10-ml culture medium. Next, the cells were counted in a Neubar chamber (haemocytometer) according to table 2.4. Briefly, the cells' numbers were estimated using a haemocytometer by adding 10 µl of the cell suspension to 10 µl of trypan blue. The total number of live, non-stained cells was counted. Then, the formula in Table 1 was used to calculate the total number of cells. The cells were then subcultured for further passaging or distributed for the desired experiment. This step was carried out by adding a known number to the culture flasks or plate. However, osteogenic media (OM) comprising Dulbecco's modified Eagle's medium (DMEM), low glucose pyruvate

(Gibco), 10% FBS (Invitrogen), and 1% penicillin/streptomycin (Sigma-Aldrich) further supplemented with 10 mM β-glycerol phosphate (Sigma-Aldrich), 50 μM ascorbic acid (Sigma-Aldrich) and dexamethasone (Dex) 10 nM (Sigma-Aldrich) was used to promote the osteogenic differentiation of hMSCs.

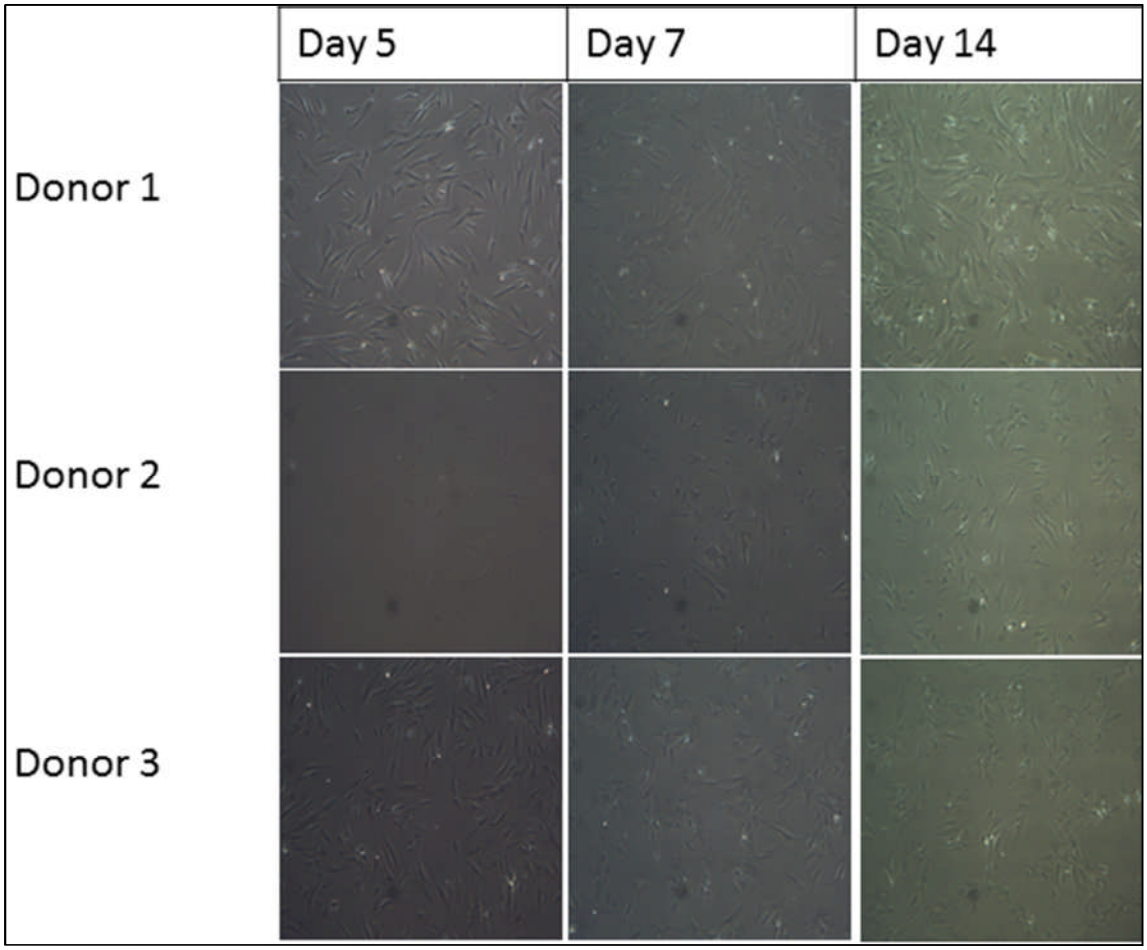


Figure 2.1: Human Mesenchymal Stem cells growth

A X10 objective view showing the growth of mesenchymal stem cells at different time points where the cells normally become confluent after 14 days in culture. These images show the fibroblastic features of these cells.

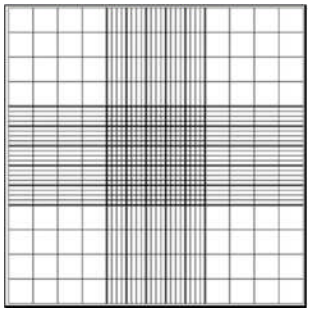
Variable	**Formula**	
Cell concentration	C (cells/ml)= A (cells) x Df x 10,000	
Total cell number	Cn (cells) = C x Vf	

Table 2.4: Cell counting method.

The formulas used to count the cultured and live cells in a 1:1 cell suspension with Trypan blue using a haemocytometer. In the formulas, C = cell concentration; A = average count from 4 x 1 mm^2 boxes of the haemocytometer; Df = dilution factor; and Cn = total cell number. The haemocytometer grid contains nine large squares each with a surface area of 1 mm^2. The depth of the chamber is 0.1 mm equivalent to 10 µl. The average cell count from four large corner squares was taken and multiplied by 10^4 to give the cell density in 1 ml of solution.

2.1.3 Cryopreservation

The cells were cryopreserved to be used in these experiments as required. Subconfluent cells were washed twice with PBS and then trypsinized using trypsin-EDTA. Centrifugation was carried out to form cell pellets, which were re-suspended at a density of 5–7.5 x 10^5 per ml in a freezing medium of (v/v) 10% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and 90% GM. An aliquot of 1 ml of the cell suspension was then transferred to marked cryovials. The cells were then frozen at -80°C overnight before being transferred to cryogenic liquid nitrogen tanks (Thermo Scientific).

2.2 Drug selection

The selected BP drugs reflected a specific route of administration and mechanism of action. PAM is an N-BP that is administered intravenously, whereas ALE is given orally. The tested BPs concentration ranges were based on the information stated in the literature

and also on preliminary data, which allowed us to exclude the cytotoxic dose. Both drugs were purchased from Sigma-Aldrich.

2.3 **Microscopy and imaging**

2.3.1 Light microscopy

An inverted light microscope Olympus CK31 (Olympus, Southall, Middlesex, UK) was used to regularly visualise cellular growth, confluence and morphology at different time points in the cell culture. Normal observation was also conducted with a magnification of X4 and X10.

2.3.2 Fluorescent microscopy

The fluorescent microscope is useful equipment based on its light source. This tool is used to study specimens, which can be made to fluoresce, and is equipped with excitation and emission filters. The target sample can be viewed by natural fluorescence or labelled with fluorescing markers, which will turn into visible images. Samples were labelled with an appropriate marker. The Leica–DMIRB fluorescence microscope equipped with a COOLSNAP Monochrome Camera was used for this project.

2.3.3 Image analysis

The images were analysed using ImageJ (National Institutes of Health (NIH)) and Image Pro Plus software, version 4.5 (Media Cybernetics, Marlow, Buckinghamshire, UK).

2.4 **Proliferation assays**

Proliferation was measured using an Alamar blue assay. Alamar blue is a dark blue water soluble and non-toxic solution of dye called resazuirin. The proliferation of these cells at different time intervals was assessed using the Alamar blue assay (AbD Serotec). A 10% dye solution was added to each well, and cells were incubated for 4 hours at 37ºC and 5% CO_2. Next, 100-µl aliquots were transferred into black, 96-well plates (Fisher Scientific). The fluorescence intensity (Excitation: ʎ= 530 nm, emission: ʎ= 590 nm) was measured using a plate reader (BioTeK FLX800). The total cell numbers were calculated via interpolation through the use of a known standard curve (Figure 2.2).

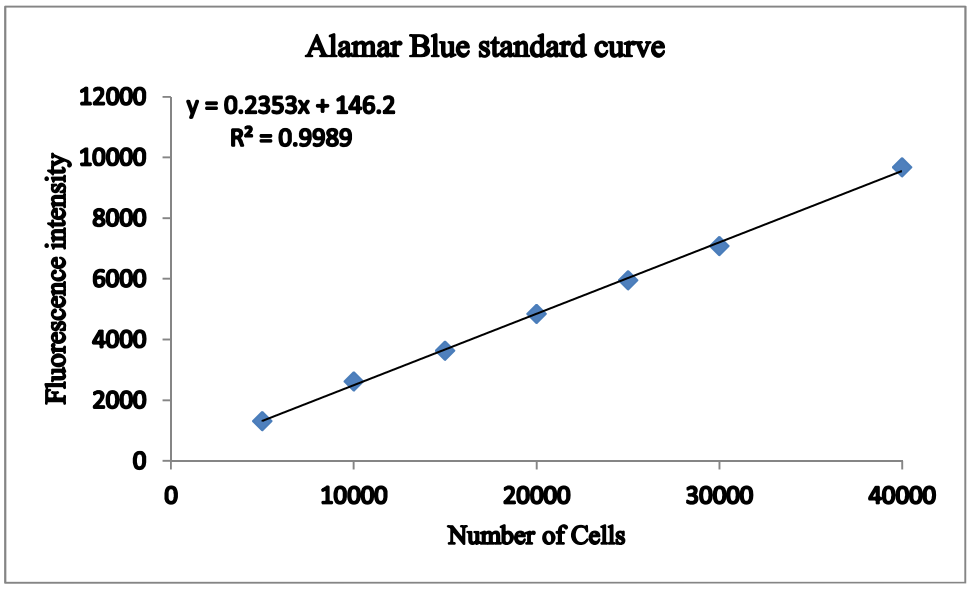


Figure 2.2: A standard curve of the Alamar Blue assay,

The figure shows a line graph of seven points with a mean fluorescence intensity plotted against cell numbers. The sample was grown in 96-well plates by seeding a known number of cells from the cells suspension.

2.5 **Mineralisation assays**

2.5.1 Calcium deposition assay

The colorimetric quantichrom kit (BioAssay Systems) was used according to the manufacturer's instructions. This method was employed to detect calcium ions in a sample solution. Following the application of a working solution, a blue colour will form when it comes in contact with calcium ions; its intensity will depend on the degree of concentration. By the end of the experiment, the cell monolayers had been washed twice with PBS then lysed with 500 µl 1M HCl for ≤ 1 h on the orbital shaker. After that, a 5 µl lysate was transferred to a clear-bottomed 96 well plate. To this, 200 µl of freshly prepared working reagent was added. Absorbances were read at 562 nm using a plate reader (Tecan, M200).

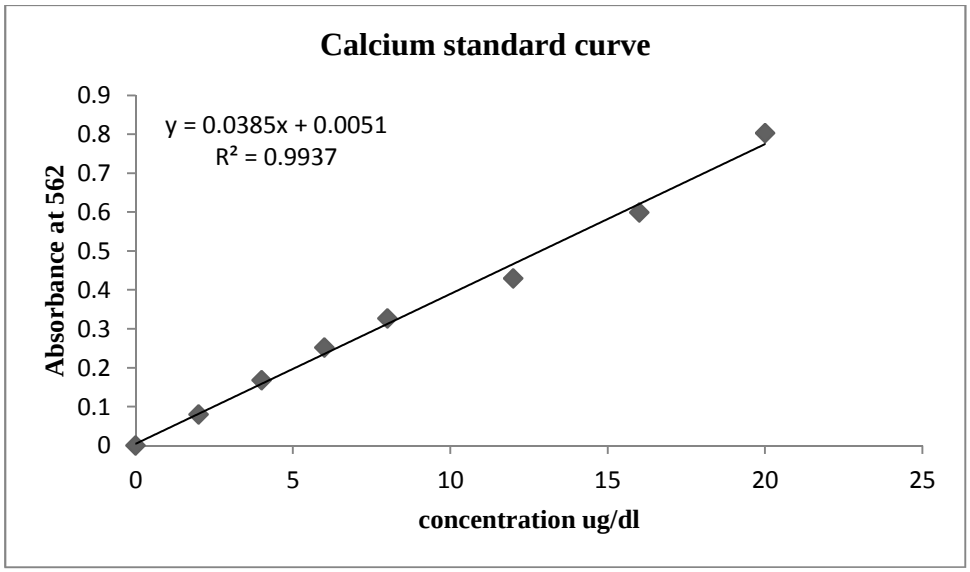


Figure 2.3: Standard curve of calcium assay.

The figure shows a line graph of eight points of calcium salts formed in a 96-well plate. Each point represents the mean of three replicates (n=3).

2.5.2 Von Kossa stain

This section spotlights one of the calcium stain methods developed by Von Kossa to semi-quantify the mineralisation produced by cells, such as osteoblasts. The main principle of this method is to stain calcium ions in the presence of light and also to replace them with silver ions. By the end of the experiment, the cell monolayers had been gently washed twice with PBS. The sample was then fixed in 5% glutaraldehyde for 30 min followed by several washes with distilled water. Using Von Kossa (Abcam), the sample was incubated with Silver Nitrate Solution (5%) for 60 minutes with exposure to ultraviolet light or 100 watt incandescent (BONMAY, OEM, China). The sample was then washed with distilled water and incubated with sodium thiosulphate solution (5%) for 2–3 minutes. After that, the cells were incubated with nuclear fast Red Solution for 5 minutes followed by several washes and dehydrated very quickly in three changes of fresh Absolute Alcohol. Finally, the samples were then examined using an Olympus BX50 optical microscope (Olympus, Southall, Middlesex, UK) along with a CoolsnapPRO-cf camera (Media Cybernetics, Marlow, Buckinghamshire, UK). Images were processed to semi-quantify the calcium crystals using Image-Pro Plus software v4.5 (Media Cybernetics, Marlow, Buckinghamshire, UK).

2.5.3 Alkaline phosphatase assay (ALP)

ALP activity was assessed using the colorimetric SensoLyte® pNPP Alkaline Phosphatase Assay Kit (Cambridge Bioscience) according to the manufacturer's instructions. Briefly, hMSCs 1×10^4 cells/well were seeded into 24-well plates and grown for 24 h at 37ºC and at 5% CO_2. The medium was changed to OM supplemented with different concentrations of the drugs (ALE and PAM). By the end of the experiment, the cells had been washed twice in PBS, lysed with 500 µl 0.1% triton X-100, scraped into microcentrifuge tubes and incubated at 4ºC for 10 minutes. The cell suspension was

centrifuged at 2500Xg for 10 minutes at 4ºC. To assess the enzyme activity, all standards and cell lysates were incubated with p-Nitrophenyl Phosphate (pNPP) substrate solution at 37°C for 30 min. and all of the reaction mixtures were transferred to a 96-well microplate and absorbances read at 405 nm using a plate reader (Tecan, M200).

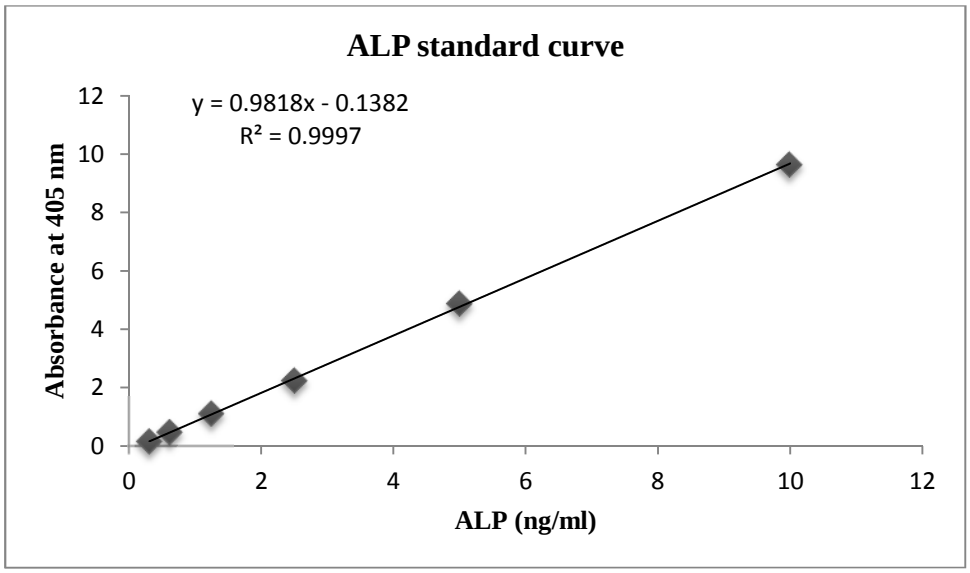


Figure 2.4: Standard curve for the alkaline phosphatase quantification assay

A line graph with 6 points of the standard curve formed on a 96-wells plate. Each point represents the mean of three replicates (n=3).

2.6 Collagen deposition quantification

The extracellular matrix collagen was analysed using the Sirocel collagen assay kit according to manufacturer's instructions (Biocolor). Briefly, cell monolayers were homogenised with ice cold 0.5 M acetic acid supplemented with 100 µg/ml porcine pepsin. Samples were concentrated overnight with 200 µl isolation and concentration reagent at 4°C. Concentrated samples were centrifuged at 12,000 revolutions per minute (rpm) for 10 minutes for pellet collagen, while the albumin-rich supernatant was discarded. Collagen pellets were stained with Sirius Red in picric acid for 30 minutes, and then centrifuged at 12,000 rpm for 10 minutes. The pellets were washed in an acid-salt

solution to remove any excess unbound dye. A bound stain was eluted in 250 µl alkali solution. Absorbances were measured at 555 nm using a plate reader (Tecan, M200).

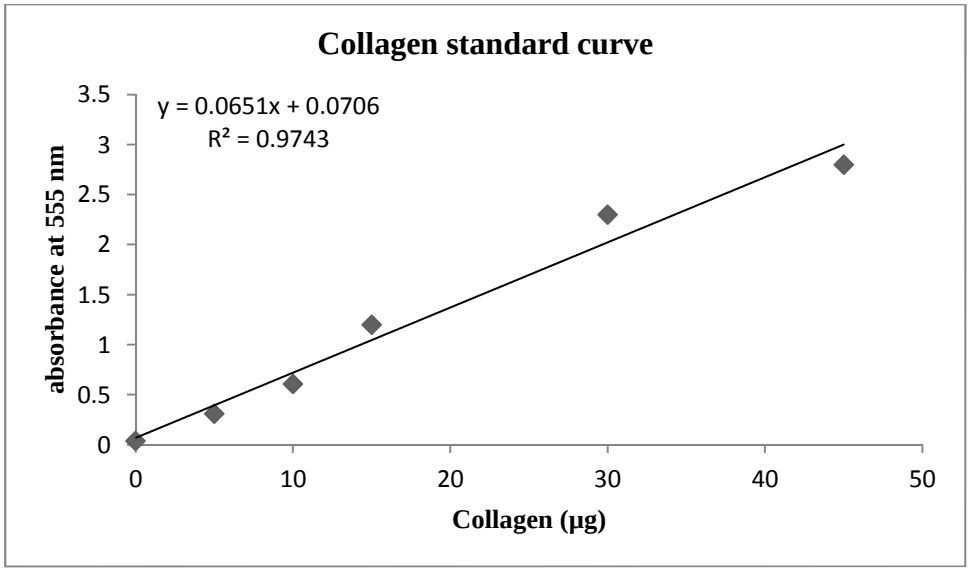


Figure 2.5: Collagen standard curve

6-point standard curve produced from a stock (1 mg/ml^{-1}). Each point represents mean of three replicates (n=3).

2.7 Immunocytochemistry

Immunocytochemistry is a useful method to visualize the target proteins or peptides in cells. This approach uses different biomolecules that will bind the required protein. This biomolecule is called an antibody, which will link to the fluorescent dye, which will give a signal (fluorescence) that is later detected by a microscope.

Table 2.5: Fluorescent markers used for immunocytochemistry

No.	Item	Manufacturer	Application
1	Type 1 collagen antibody	Sigma	A primary antibody to localize type 1 collagen; at 1:1000 dilutions.
2	Anti-vinculin antibody	Abcam	A primary antibody to localize the hMSCs adhesion protein (vinculin); at 1:200 dilution
3	Goat anti-mouse IgG	Life technologies	Secondary antibody of the vinculin

4	Actin antibody	Life technologies	A phalloidin-iFlour 488 was used at 1:1000 dilution to
5	4′, 6-diamidino-2-phenylindole (DAPI)	Sigma	Used for nuclear visualisation; at 1:300 dilution
6	Hydroxyapatite	Lonza	Used for visualisation HA crystal formation.

2.8 **Statistical analysis**

Statistical analysis between pairs of means was carried out using the student t test and multiple comparisons were performed by one-way analysis of variance (ANOVA) in GraphPad Prism software (v5.04) and Microsoft Excel 2010 followed by Bonferroni post-test; the representative data is presented as mean ± standard deviation (SD), with $p<0.05$ considered to be statistically significant.

Results

3 The Effect of BPs on hMSCs Proliferation and Osteogenic Differentiation

3.1 Introduction

The hMSCs *in vitro* can be induced to undergo osteogenic differentiation into osteoblasts, which will be molecularly, morphologically, and functionally similar to osteoblasts (Dominici et al., 2006). The commitment of these cells to differentiate into osteoblasts is regulated by a variety of signalling molecules. These signalling molecules including Wnt family, TGF-β family, transcriptional factors, and BMPs family play an integral role in the osteogenic differentiation process (Augello and De Bari, 2010). The bone formation is an important process for body development, whereas the remodelling process is a programmed cycle which is continuous and runs throughout life. This cycle starts with hMSCs differentiation into osteoprogenitor cells which will differentiate into pre-osteoblasts; later, these cells became mature osteoblasts (Augello and De Bari, 2010, Bielby et al., 2007, Birmingham et al., 2012, Devine et al., 2001, Florencio-Silva et al., 2015). The hMSCs osteogenesis is considered important for maintaining bone remodelling and bone formation (Birmingham et al., 2012). However, the proliferation of hMSCs is an important factor in developing different cell types, which makes them an interesting target for medical applications to repair damaged tissue (Horst et al., 2012). They are recruited very early at the injured and damaged site and participate in the healing process (Rennert et al., 2012). Several studies extensively investigated the effect of the BPs on osteoclasts, as these cells were the main targets of BPs approved for clinical usage. However, besides their effect on the osteoclasts, it has been reported that

BPs affect other cells such as osteoblasts, keratinocytes, and fibroblasts. The present evidence indicates that high doses of BPs inhibit proliferation of these cells (Acil et al., 2012). A few reports have also shown that BPs affect the osteoblast osteogenesis process (Corrado et al., 2010). Moreover, BPs may modulate the osteoblasts' activity and bone formation; however, the biological mechanisms of how BPs affect several osteogenic markers, genes, and proliferation remain unknown. On the other hand, high doses of BPs have been found to induce cellular apoptosis on different cell types such as macrophage (Moreau et al., 2007).

The aim of this chapter is to clarify the effect of descending doses of BPs on hMSCs proliferation and osteogenic differentiation *in vitro*. This analysis was conducted by evaluating the hMSCs' proliferation and differentiation. The early and late osteogenic markers included ALP, type I collagen deposition, and calcium deposition. In addition, the osteogenic mineralisation of hMSCs was measured by quantifying calcium formed at different points in time: 7, 14, and 21 days. The ECM collagen deposition was also measured, as well as the ALP level, over the same period. The osteogenic genes, including transcriptional factors, extracellular matrices, and the osteogenic regulators were studied. These genes are as follows:

- Runt-related transcription factor 2 (RUNX2): This gene is one of the osteogenic master genes and is mainly responsible for initial differentiation of osteoblasts.
- Transforming growth factor beta (TGF-β1): This gene controls the growth, proliferation, differentiation, and apoptosis of osteoblasts.
- ALP: This gene is also one of the early indicators for cell differentiation.
- Osteocalcin (OC) or bone gamma-carboxyglutamic acid-containing protein (BGLAP): This gene is mainly a bone matrix associated gene.

- Collagen alph-1 (COL1A1): One of the extracellular matrix component regulators.
- Bone sialoprotein (BSP2): One of the bone matrix component proteins.
- Wnt5a: This gene is regulating the BMP expression as well as hMSCs' proliferation.
- Osteopontin (OPN): One of the bone matrix component proteins.

3.2 **Materials and methods**

3.2.1 Cell culture

In this chapter, we investigated the effect of two types of commonly used BPs (ALE and PAM) on hMSCs' proliferation and osteogenic differentiation. hMSCs were plated at a density of 1×10^4 cells/well in 1.9-cm^2 wells containing MSC GM or OM with 10% FBS and 100U/ml each of penicillin/streptomycin and incubated at 37ºC in humidified 5% CO_2. The cell culture was conducted as described in section (2.1.2).

3.2.2 Assessment of Cell Proliferation

The effects of BPs (ALE and PAM) on hMSCs' proliferation were measured by seeding $1x10^4$ cells per well in a 24 well plate. The cells' numbers were serially measured at different points of time: days 1, 3, 7, and 14 using alamar blue assay as described in section (2.5).

3.2.3 Assessment of Cells Mineralisation

The hMSCs' osteogenic differentiation was assessed by seeding 10,000 cells /well in a 24 well plate in OM. Cell cultures were maintained by changing the media every 2-3 days. The osteogenic markers were evaluated as described in the next sections.

3.2.3.1 Von Kossa Stain

The Von Kossa stain method was used as a semi-quantitative approach to assess hMSCs' mineralisation at 21 days post seeding. The methods for this approach are described in section (2.9.3).

3.2.3.2 Quantification of Calcium Deposition

The total calcium formed by cells was examined at 7, 14, and 21 day post seeding with a colorimetric quantichrom kit (BioAssay Systems) according to the manufacturer's instructions, as described in section (2.5.1).

3.2.3.3 Quantification of ECM Collagen

The total amounts of ECM formed by hMSCs were measured at 7, 14 and 21 day post seeding using the Sirocel collagen assay kit according to manufacturer's instructions (Biocolor) as described in section (2.6). The total amount was measured from three replicates as it was difficult to measure form a single well due to the small quantities produced by small cells number.

3.2.3.4 Type 1 collagen staining

In order to visualise collagen type I under a florescence microscope, a specific antibody for collagen was used. The process was initiated by washing the monolayer with PBS and then fixing it in ice-cooled methanol fixation for 15 minutes at -20° C. The permeability of the monolayer with cooled acetone for 1 minute at -20° C. The antibodies were blocked using 10% goat serum in PBS for 30 minutes. The primary antibody was applied for another 30 minutes, followed by a 3x wash with PBS at least 5 min each. The samples were incubated overnight. The next day, samples were incubated with a secondary antibody at room temperature for 30 minutes followed by three washes with PBS. To detect the nuclei, a DAPI stain was applied to the samples for 5 minutes. After that, the sample was left to dry and then evaluated under a florescence microscope.

3.2.3.5 Alkaline Phosphatase Activity

The ALP activity was assessed using the colorimetric SensoLyte® pNPP Alkaline Phosphatase Assay Kit (Cambridge Bioscience) according to the manufacturer's instructions as described in section (2.9.3). The ALP activity was measured at 7, 14, and 21 day post seeding.

3.2.4 Apoptosis and necrosis assay

Apoptosis is defined as programmed cell death due to a healthy trigger. In contrast, necrosis is sudden cell death caused by external stimuli, such as trauma, inflammation, toxicity and drugs. In order to identify whether the drug may affect the cell apoptosis or necrosis processes, the FITC Annexin V staining kit was used according to the manufacturer's instructions (BD Biosciences, UK). With this kit, we can identify the degree of cells in apoptosis at an earlier stage. Also, this kit is used to quantitatively determine the percentage of cells within the selected population that are actively undergoing apoptosis or necrosis. In this experiment, cells were cultured with drugs in GM or OM for 24 hours. Cells were then washed twice with cold PBS, and the cells were resuspended in a 1X Binding Buffer (concentration of 1 x 10^6 cells/ml. 100 µl of this solution was then transferred to a 5 ml culture tube. Thus, 5 µl of FITC Annexin V and 5 µl PI was added. Cells were then gently vortexed and were incubated for 15 min at RT (25°C) in the dark. Finally, a 400 µl of 1X Binding Buffer was added to each tube; the sample was then analysed by flow cytometry (BD FACScan™ System, BD Biosciences, UK).

3.2.5 Gene expression

In order to analyse the gene expression changes a real time-polymerase chain reaction (RT-PCR) was used. A double delta Ct method ($2^{-\Delta\Delta Ct}$) was employed for this analysis.

This approach determines the threshold cycle (Ct), which represents the cycle number at which the reaction number inters log exponential phase of amplification. This method consists of three main steps: RNA extraction, reverse transcriptase, and Taqman probe based RT-PCR assay of relative level of gene expression. A full description of each steps is provided in the following section.

3.2.5.1 RNA extraction

At the end of each experiment, the total amount of RNA was extracted using an RNeasy Mini kit from Qiagen according to the manufacturer's instructions. This kit contains a silica membrane column to which the RNA adheres. The adherent cells were washed three times with PBS and homogenized with an RLT buffer (for 24 well plates, we used 350 µl). The total RNA was precipitated by mixing the same amount with 70% ethanol in distilled water. The mixing volume was transferred to a silica membrane column, which captured the total RNA, which would be later undergoing a series of washes of different buffers. Finally, RNA was eluted from the membrane using a 50 µl of RNase-free water per column. To evaluate the integrity and quantity of the extracted RNA, a spectrophotometer ((Tecan, M200) with Tecan NanoDrop at an excitation level of 260 nm and an emissions level of 280 nm was used.

3.2.5.2 Reverse transcriptase

A high-capacity reverse transcription kit purchased from Applied Biosystems was used to reverse the transcribed RNA to cDNA according to the manufacturer instructions. In general, the cDNA was prepared so that 10 ng of the RNA was used in every reaction in subsequent PCR. The required amount of cDNA was calculated for the PCR in order to achieve the correct amount of RT master mix. Table (3.1) outlines the amount of RT master mix components needed for a 20 µl reaction. Sample were prepared in 0.2 ml

PCR tubes, placed in a thermocycler machine (100™PTC, MJ Research, Inc, US), and the cycle underwent the following conditions: incubated at 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and 4°C for overnight. The samples were then collected and stored in -20 °C until further usage.

Component	**Volume/Reaction (µl)**
10✕ RT Buffer	2
10✕ RT Random Primers	2
25✕ dNTP Mix (100 mM)	0.8
MultiScribe™ Reverse Transcriptase	1
Nuclease-free H_2O	4.2
Total	10 µl/reaction

Table 3.1: Components of the RT master mix and their volumes used in this study according to the manufacturer's instructions.

3.2.5.3 Real-time quantitative polymerase chain reaction (qRT-PCR)

The Applied Biosystems 7300 real-time PCR system and related sequence detection software was used to perform the real time quantitative polymerase chain reaction (qRT-PCR). Each sample was examined in triplicate for expression of the target genes and endogenous control (GAPDH). Each reaction was 25 µl table (2.6), which had been optimised previously. A 25µl reaction with 5 µl of cDNA per reaction which was equal to 100ng of RNA was performed. The samples were then run for a 1h 45 min cycle to

obtain the Ct value. The Ct value of each gene was normalised to the endogenous control (GAPDH) to establish the delta Ct value. The relative fold of the expressed genes were calculated for each replicate. The Taqman probes used are tabulated in table 3.3.

Component	**Volume (µl)**
TagMan® Universal PCR master Mix	12.5
TagMan® probes	1.25
CDNA	5
Nuclease free water	6.25
Total	25 µl /reaction

Table 3.2: Reagents used to prepare the qRT-PCR master mix according to the manufacturer's instructions.

Gene	**Probe ID**
Runx-2	Hs00231692_m1
OPN	Hs00959010_m1
Wnt5a	Hs00998537_m1
ALP	Hs01029144_m1
COL1a1	Hs00164004_m1
TGF-B1	Hs00998133_m1
OC	Hs01587814_g1
BSP2	Hs00913377_m1
GAPDH	Hs03929097_g1

Table 3.3: TagMan probe catalogue number for q-PCR analysis

3.2.6 Image Analysis

Light microscopy was used to visualise the cells stained with Von Kossa stain, then the image was processed using the Image Pro Plus software. For the HA samples, we used a Leica–DMIRB fluorescence microscope equipped with a COOLSNAP Monochrome Camera. Images were collected and processed with the image J Imaging System.

3.3 **Results**

3.3.1 Proliferation

The dose responses over different time points on hMSCs were studied. The data show treating cells with ALE (100nM and 10nM) significantly stimulated cells' proliferation on days 7 and 14 compared to the cells treated with GM only. A similar effect was observed with the group treated with PAM (10nM), where the drug significantly stimulated proliferation at days 7 and 14. A cytotoxic effect was observed with cells treated with 100µM from both drugs, as shown in figure (3.1). However, from day 3, growth of cells that had been treated with 100µM became slow, and proliferation was significantly decreased ($p<0.05$), as shown in figure (3.1 & 3.2). Furthermore, under the microscope observation the cells became round and detached, and cells death was observed.

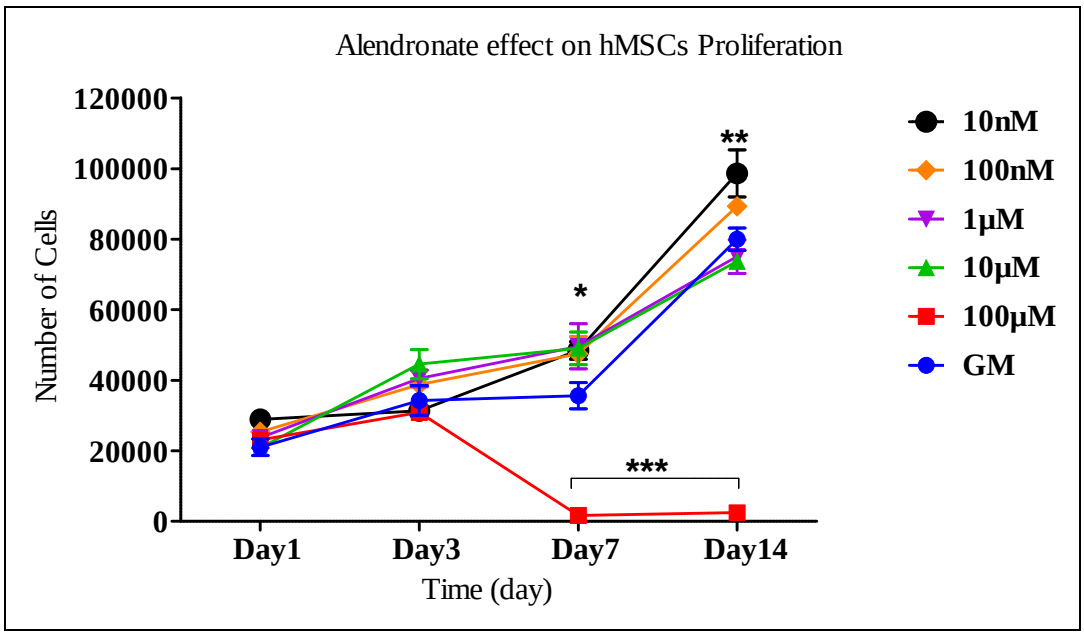


Figure 3.1: The effect of different concentrations of ALE on hMSCs' proliferation.

The effect of different concentrations of ALE on hMSCs' proliferation at different time points using Alamar blue assay. The data showed that at days 1 and 3, cells were proliferated at similar rates on all concentrations. Then, at 100 µM there was a cytotoxic effect on cells proliferation on days 7 and 14. On days 7 and 14, there was a significant stimulation of hMSc proliferation at 10nM. Each line represents Mean±SD, $p<0.05$ treated group vs. control (GM).

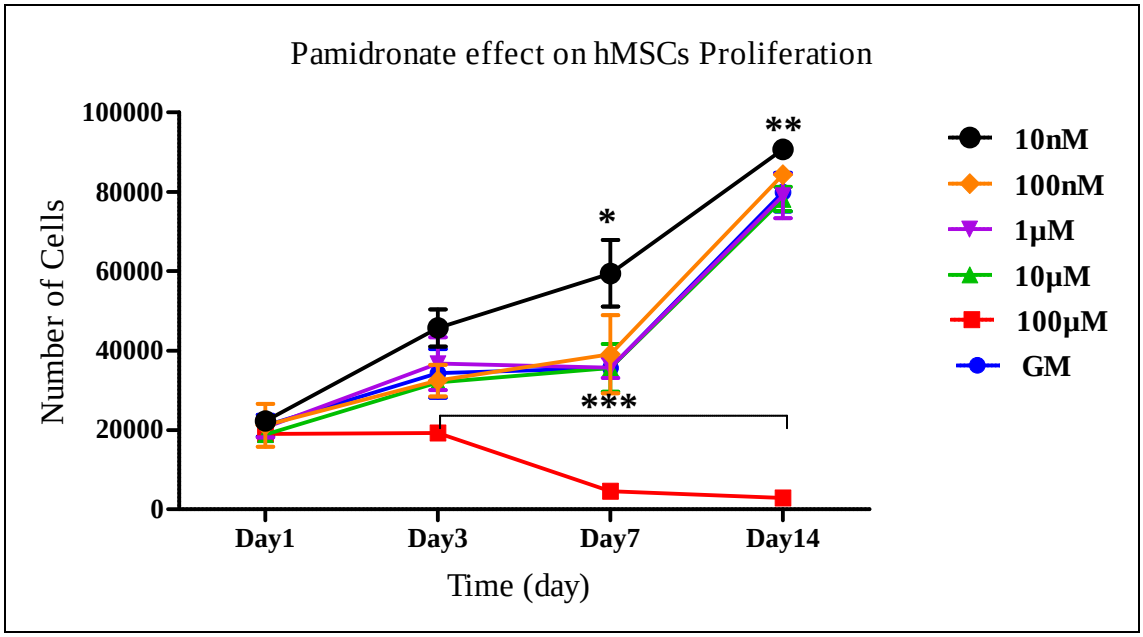


Figure 3.2: The effect of different concentrations of PAM on hMSCs proliferation.

The effect of different concentrations of PAM on hMSCs' proliferation at different time points was studied using Alamar blue assay. Results showed that at 100 µM, there was a cytotoxic effect on cells' proliferation on days 3, 7, and 14. A small dose of PAM (10 nM) shows a significant stimulation of hMSCs' proliferation on days 3, 7, and 14. Each line represents Mean±SD, $p<0.05$ treated group vs. control (GM).

3.3.2 Mineralisation

3.3.2.1 Quantification of Calcium Deposition

The effect of ALE and PAM on calcium deposition was evaluated on day 7, 14 and 21 post seeding. The results of this experiment are illustrated in figure (3.3). The data shows that the low dose of ALE (10nM) at days 14 and 21 has significantly stimulated the cells to deposit more calcium compared to the group that had been treated with OM ($p<0.05$). Cells that been treated with 10µM and 1µM show less deposited calcium

compared to the control group treated with OM only at day 21. Similarly, the group treated with PAM 10nM also shows stimulatory effects on calcium deposition produced by cells, whereas the effects of 10µM and 1µM are significantly less than the group treated with OM only ($p<0.05$), as shown in figure (3.4).

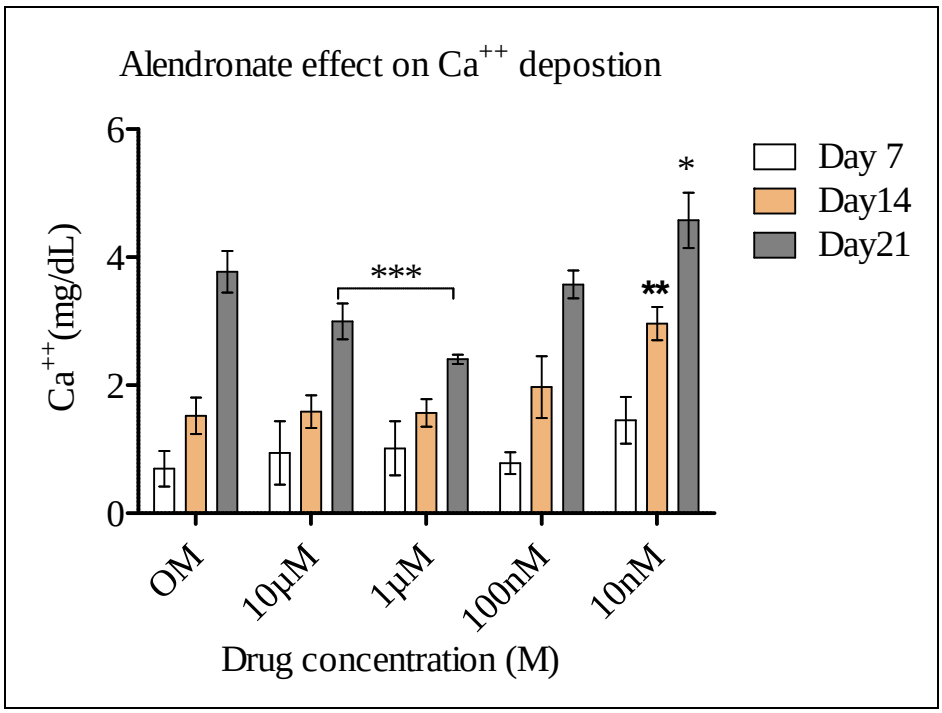


Figure 3.3 Quantification of total calcium deposition following ALE treatment,

Bar chart showing the effect of different doses of ALE on the total amount of deposited calcium produced by osteogenic differentiated hMSCs in OM at days 7, 14, and 21. The total amount of deposited calcium was significantly higher for the group treated with 10nM at days 14 and 21. However, cells that had been treated with 10µM and 1µM showed less deposited calcium compared to the control group treated with OM. Each bar represents Mean±SD. $p<0.05$ treated group vs. control (OM).

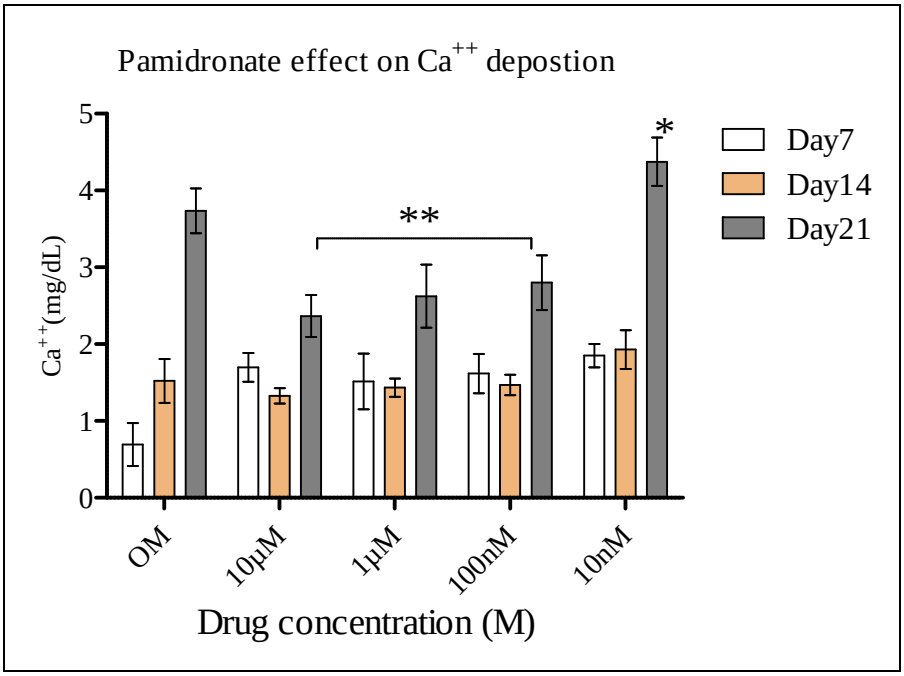


Figure 3.4 Quantification of total calcium deposition following PAM treatment,

Bar chart showing the effect of different doses of PAM on the total amount of deposited calcium produced by osteogenic differentiated hMSCs in OM at day 21. The total amount of deposited calcium was significantly higher for the group treated with 10nM at days 14 and 21. However, cells that had been treated with 10µM, 1µM and 100nM showed less deposited calcium compared to the control group treated with OM. Each bar represents Mean±SD, $p<0.05$ treated group vs. control (OM).

3.3.2.2 Von Kossa stain

A von Kossa stain was used to semi-quantify and visualise the mineralised nodules. The images showed a significant effect on mineralised nodule formation when the sample treated with ALE and PAM (10nM) was Von Kossa positive and showed more mineralised nodules in comparison with the control group treated with OM only. The effects of PAM (100nM) were much higher than the effects of ALE; the cells produced more calcium as shown in figures (3.7 and 3.8). The mineralised nodules were semi-

quantified using Image Pro Plus software, version 4.5 (Media Cybernetics, Marlow, Buckinghamshire, UK) to calculate the percentage of the black area per 100µm^2, and the results correlated with visual inspection as shown in figures (3.5 to 3.8).

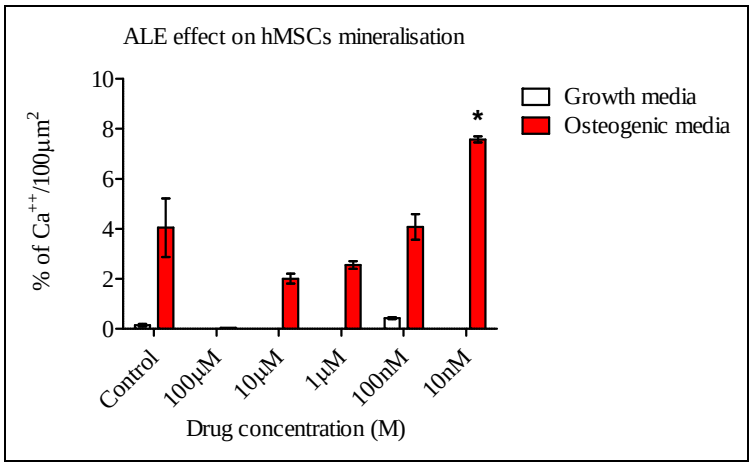


Figure 3.5 Semi-quantitative assessment of cell mineralisation with Von Kossa Stain following ALE treatment

Bar graph showing that the group treated with ALE (10nM) had more mineralised nodules produced by hMSCs after 21 days in culture than the group treated with OM media only. Each bar represents Mean±SD, *=p<0.05, treated group vs. control (OM).

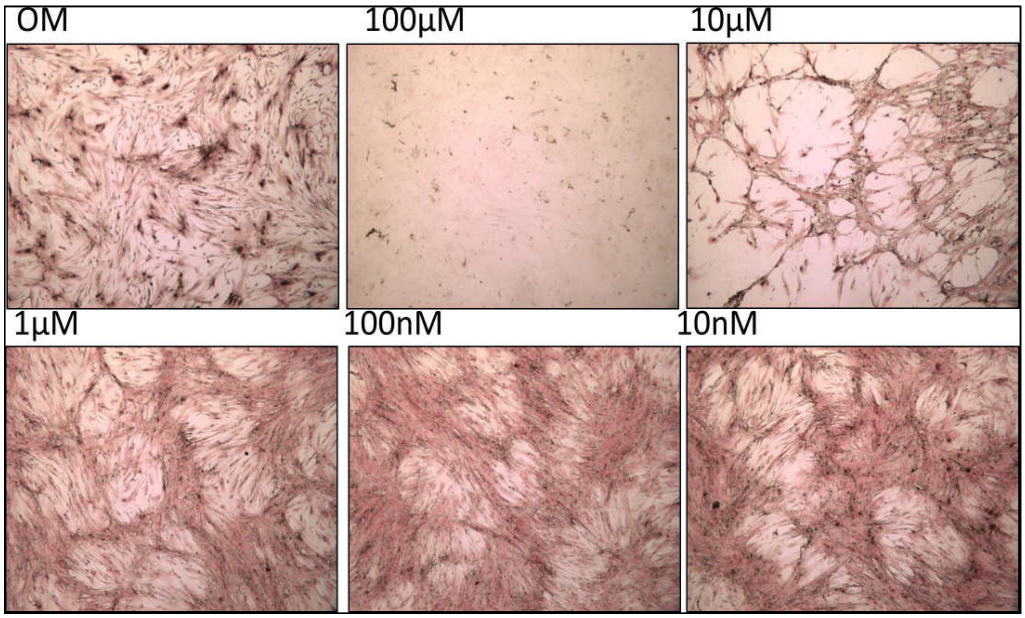


Figure 3.6 Von Kossa stain for calcium mineralisation following ALE treatment at day 21

Light microscopy images showing that the group treated with ALE 10nM had more mineralised nodules (black area) produced by hMSCs after 21 days in culture than the group treated with OM media only. Images were taken using X 40 objective.

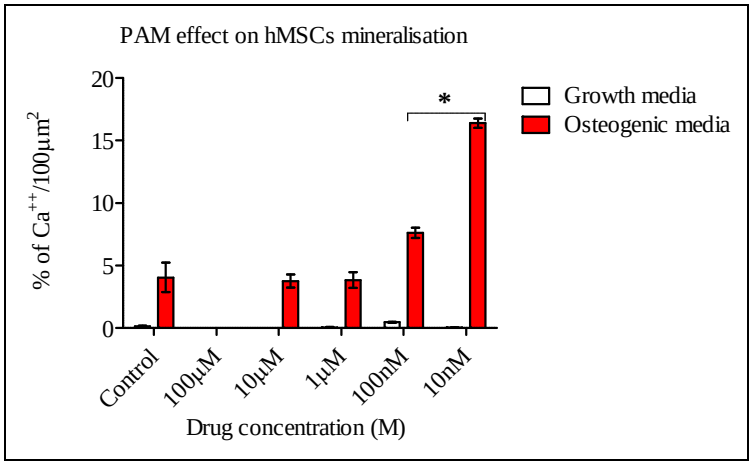


Figure 3.7 Semi-quantitative assessment of cell mineralisation with Von Kossa Stain following PAM treatment

Bar graph showing that the group treated with PAM (100nM and 10nM) had more mineralised nodules produced by hMSCs after 21 days in culture than the group treated with OM media only. Each bar represents Mean±SD, *=p<0.05, treated group vs. control (OM).

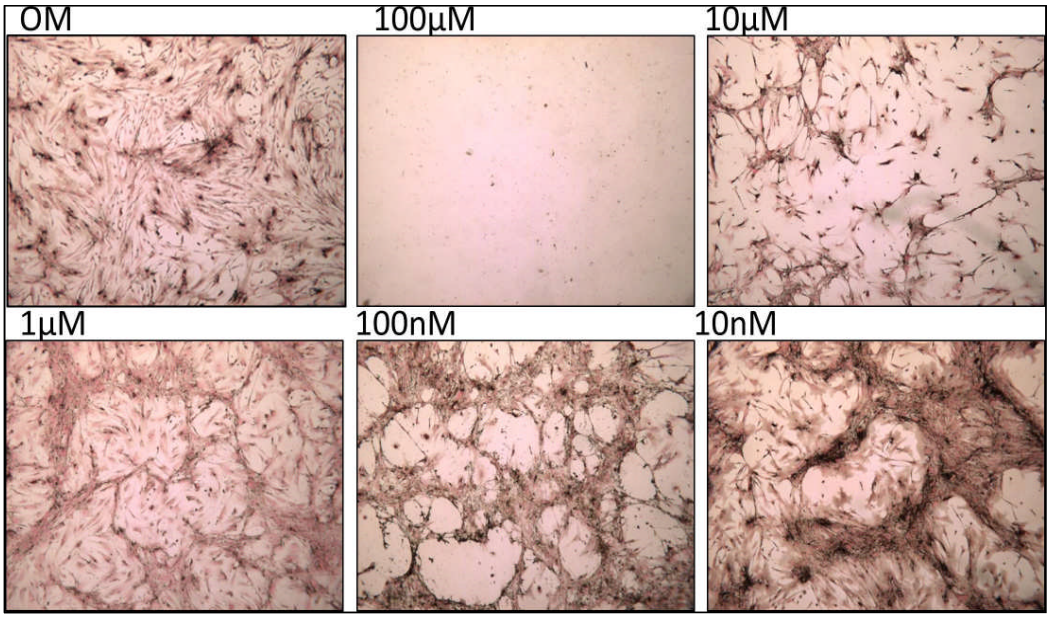


Figure 3.8 Von Kossa stain for calcium mineralisation following PAM treatment at day 21

Light microscopy images showing that the group treated with PAM (100nM and 10nM) had more mineralised nodules (black area) produced by hMSCs after 21 days in culture than the group treated with OM media only. Images were taken using X 40 objective.

3.3.2.3 Alkaline Phosphatase Activity

The effect of ALE and PAM on the ALP activity was measured on days 7 and 14 post seeding. The results show that the group treated with ALE (100nM and 10nM) at day 7 was significantly stimulated by the ALP activity compared to the control groups treated with OM only, as shown in figure (3.9). Similarly, the group treated with PAM (1µM, 100nM, and 10nM) at day 7 was significantly stimulated by the ALP activity compared to the control groups treated with OM only, as shown in figure (3.10). There was no significant effect at day 14 for any groups or either drug.

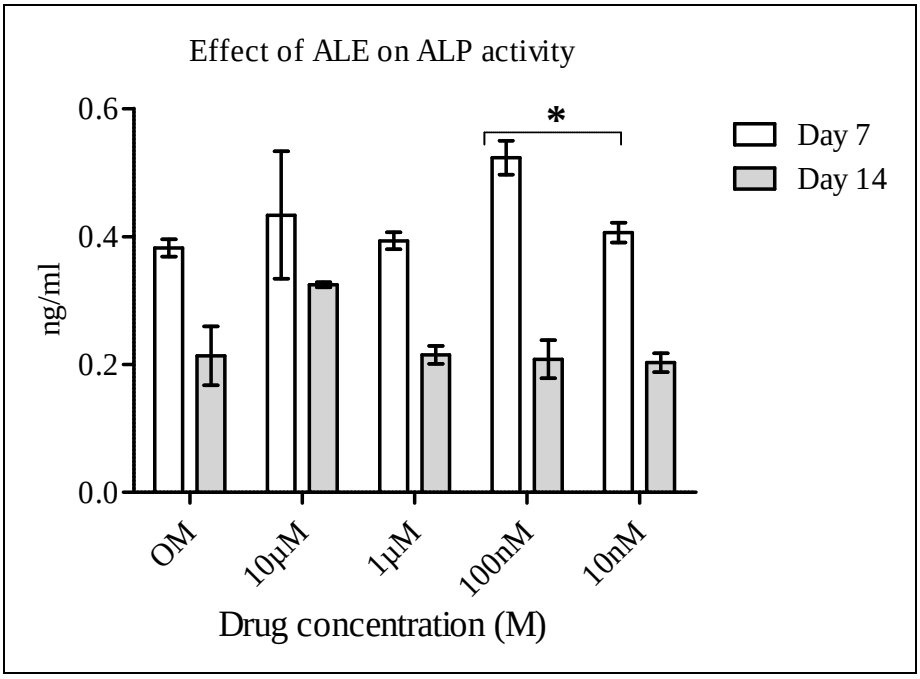


Figure 3.9 : The effect of ALE on ALP activity

ALP activity after seven days' incubation was significantly increased in all cells treated with the 100nM and 10nM of ALE when compared to the control group treated with OM only. There was no significant effect at day 14. Each column represents the mean±SD. *=p<0.05, treated group vs. control (OM).

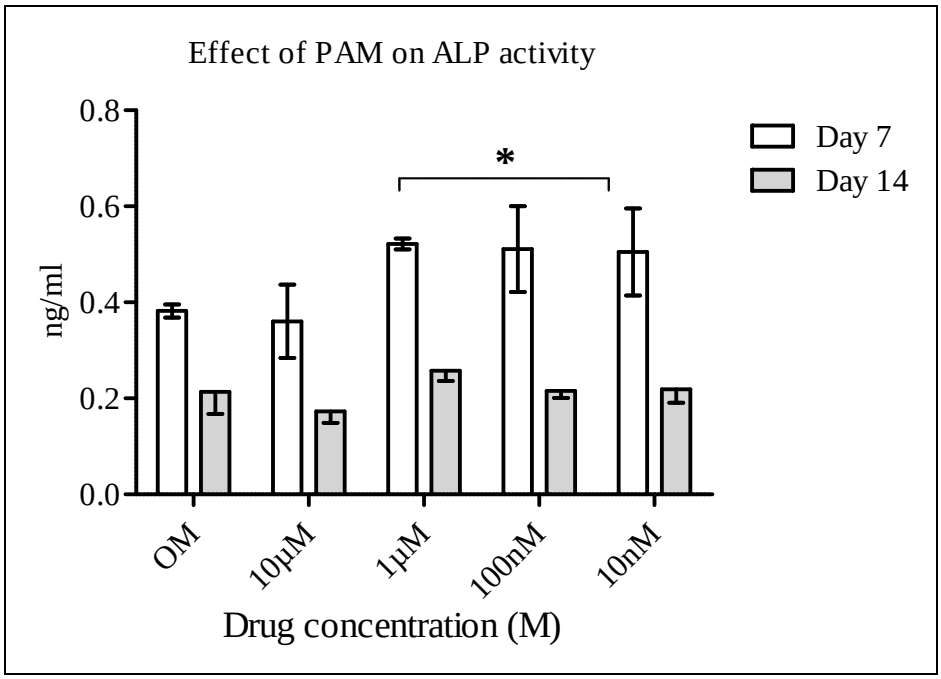


Figure 3.10: The effect of PAM on ALP activity

ALP activity after seven days' incubation was significantly increased in all cells treated with the 1µM, 100nM and 10nM of PAM when compared to the control group treated with OM only. There was no significant effect at day 14. Each column represents the mean±SD. *=p<0.05, treated group vs. control (OM).

3.3.3 Quantification of ECM Collagen Deposition

The effects of dose response over different time points 7, 14, and 21days on type I collagen deposition were analysed. The results of this experiment are illustrated in figure (3.11). The data shows that the low dose of ALE (1µM and 100nM) significantly stimulated the cells to deposit more collagen compared to the group that been treated with OM only at day 21. Cells that been treated with 10µM showed less deposited collagen compared to the control group treated with OM only at day 21, as shown in figure (3.12). The group treated with PAM 10nM also show significant stimulatory effects on collagen deposition produced by cells, whereas the effect of 10µM and 1µM is significantly less

than the group treated with OM only at day 21. However, there was no significant effect for any groups treated on days 7 and 14 with either drug.

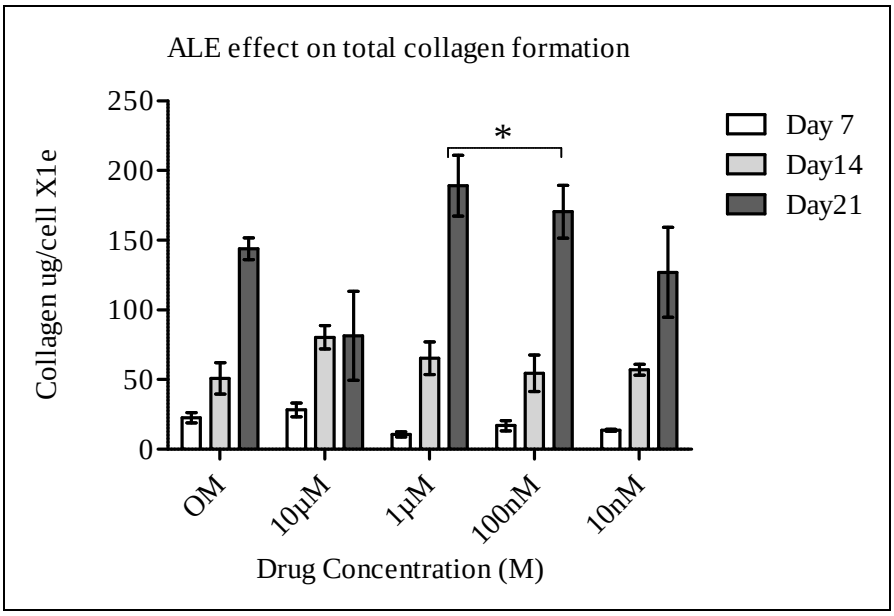


Figure 3.11: Quantification of ECM collagen following ALE treatment

Bar chart shows that at day 7, 14, and 21, extracellular collagen formation was analysed. The results showed that ALE (10µM and 100nM) had significantly stimulated hMSC to produce collagen following treatment with drugs for 21 days when compared to cells that had been treated with OM media only. In addition, there was no significant change after 7 and 14 days. Each column represents the mean±SD. *=p<0.05, treated group vs. control (OM).

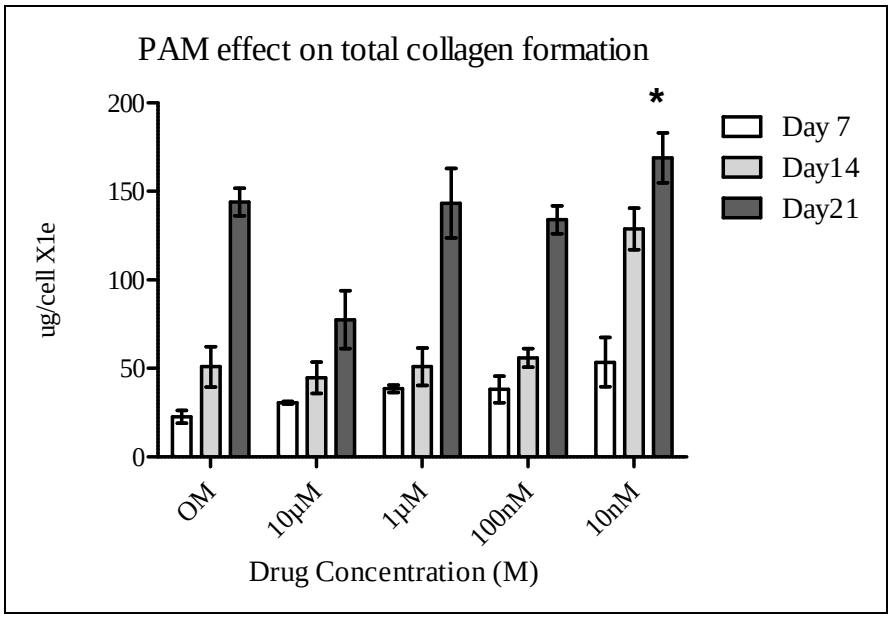
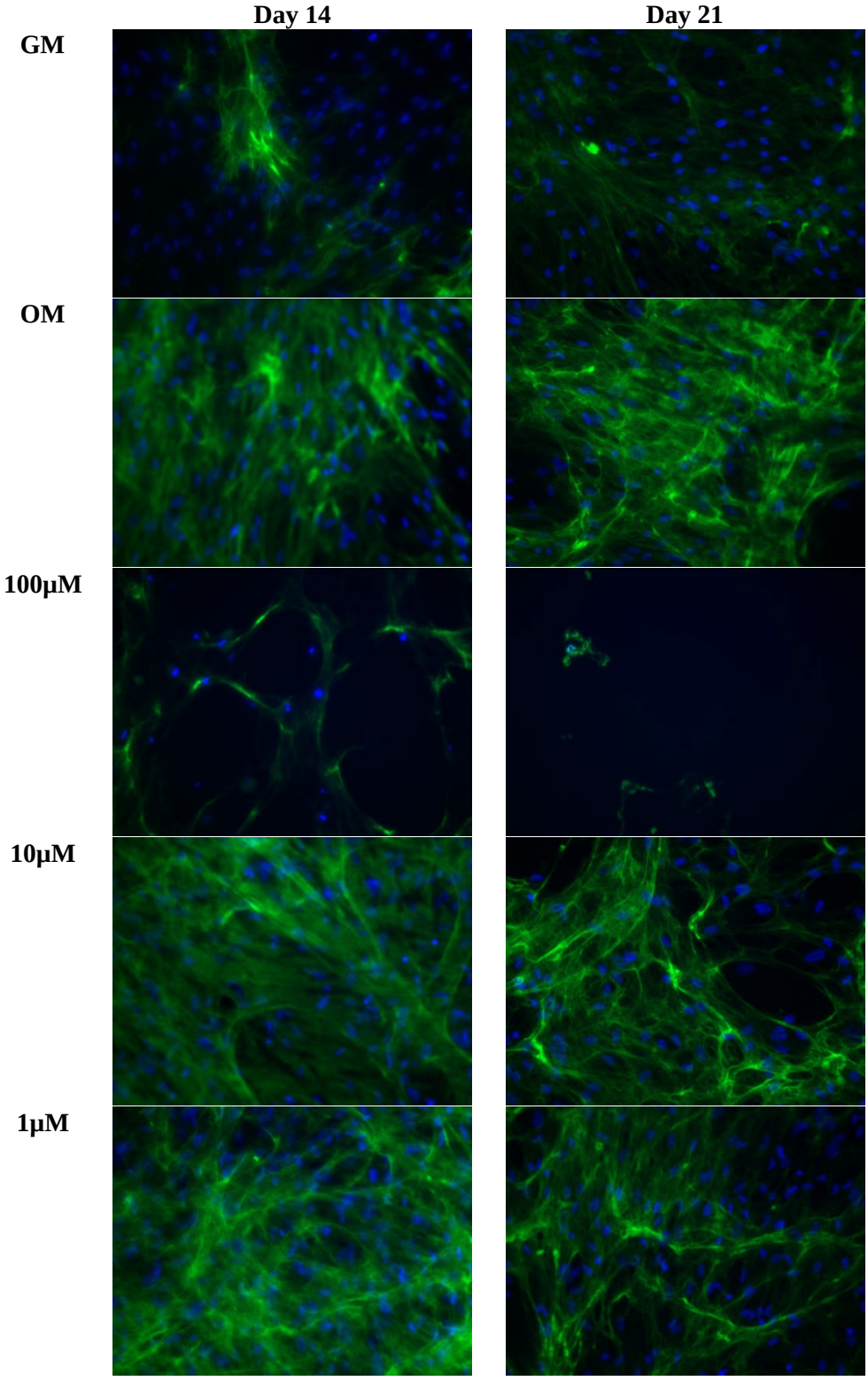


Figure 3.12: Quantification of ECM collagen following PAM treatment

Bar chart shows that at days 7, 14, and 21, extracellular collagen formation was analysed. The results showed that PAM (10nM) had significantly stimulated hMSC to produce collagen following treatment with drugs for 21 days when compared to cells that had been treated with OM media only. In addition, there was no significant change after 7 and 14 days. Each column represents the mean±SD. *=p<0.05, treated group vs. control (OM).

3.3.4 Immunocytochemistry

The collagen type I antibody were labelled and visualised under florescence microscope at days 14 and 21 post seeding. The images show that the collagen appeared more abundant in cells treated with ALE and PAM (1µM, 100 nM, and 10 nM) on days 14 and 21 compared to the cells treated with OM and GM only, as shown in figure (3.13 and 3.14). These images were correlated with the quantification of ECM collagen as stated in the previous section.


Day 14
Day 21
GM
OM
100µM
10µM
1µM

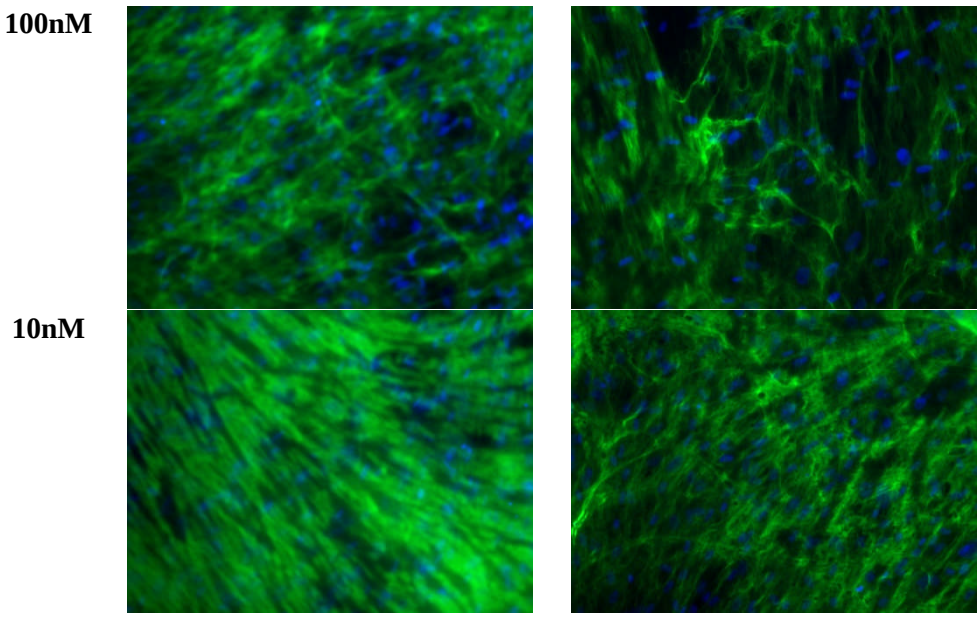
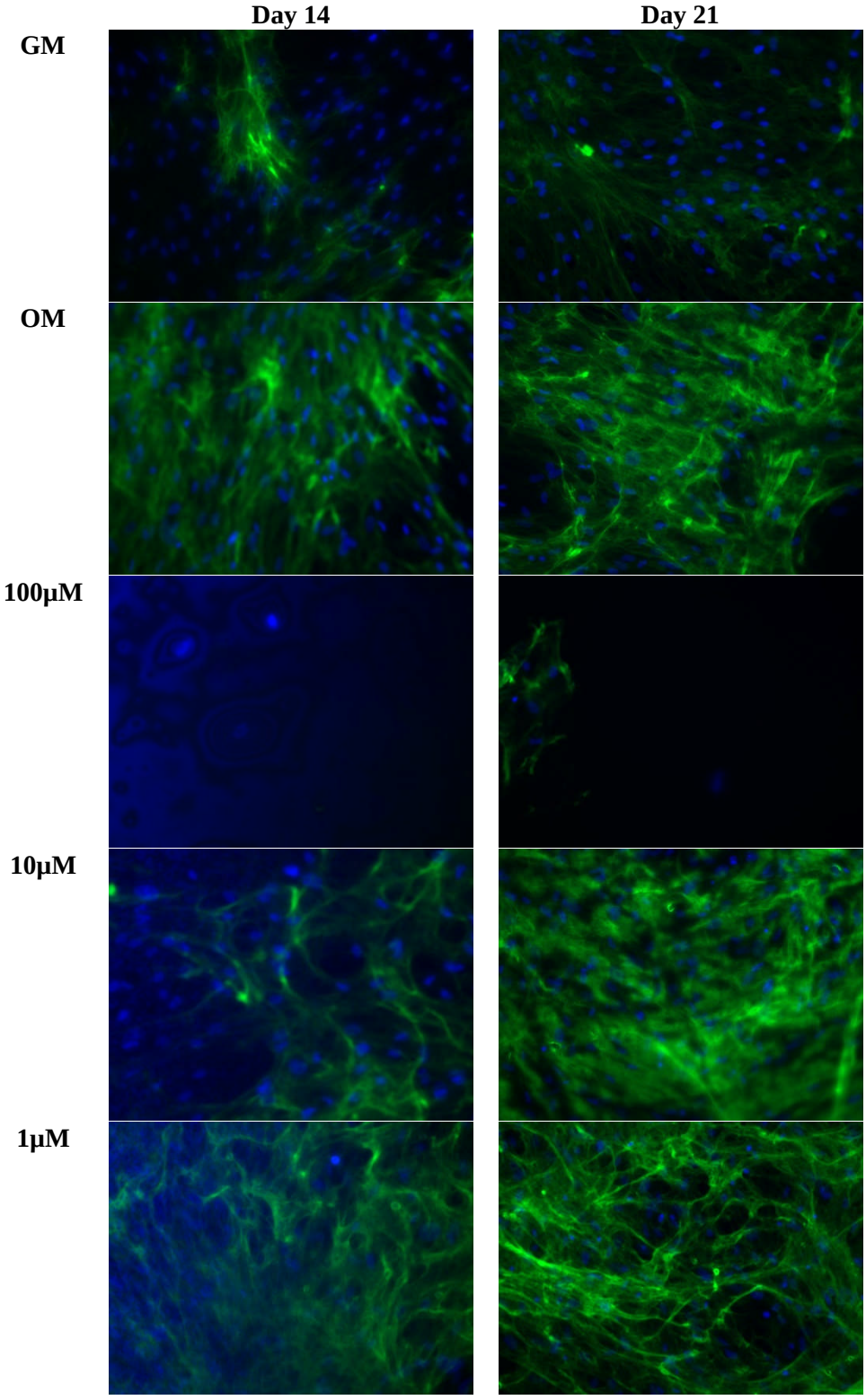


Figure 3.13: Florescent microscopy images showing the effect of low doses of ALE on collagen type I

The images show that the collagen expression appeared more abundant in cells treated with the ALE 1µM, 100 nM, and 10 nM on days 14 and 21 compared to the cells treated with OM and GM only. Images were taken using X 40 objective. Scale bar=50 µ M.


Day 14
Day 21
GM
OM
100µM
10µM
1µM

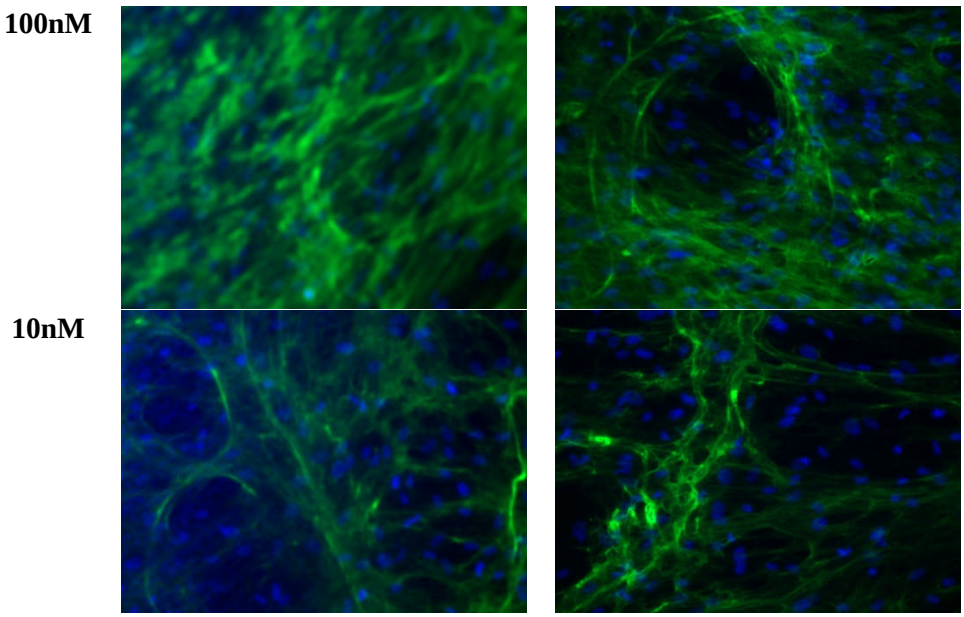


Figure 3.14: Florescent microscopy images showing the effect of low dose of PAM on collagen type I

The images showed that the collagen expression appeared more abundant in cells treated with the PAM 1µM, 100 nM, and 10 nM compared to the cells treated with OM and GM only. Images were taken using X 40 objective. Scale bar=50 µM.

3.3.5 Apoptosis and necrosis

In order to investigate the early effects of the high dose of ALE and PAM on hMSCs' apoptosis and necrosis the cells were labelled with annexin-V-FTIC. A high dose ALE and PAM (100µM and 10µM) show a significant effect on hMSCs' apoptosis compare to the control group that been treated with GM only after 24 hours of incubation. The data shows that the percentage of apoptotic cells was higher for the group treated with the 100µM compared to the group treated with 10µM for both drugs as shown in figure (3.15 and 3.16).

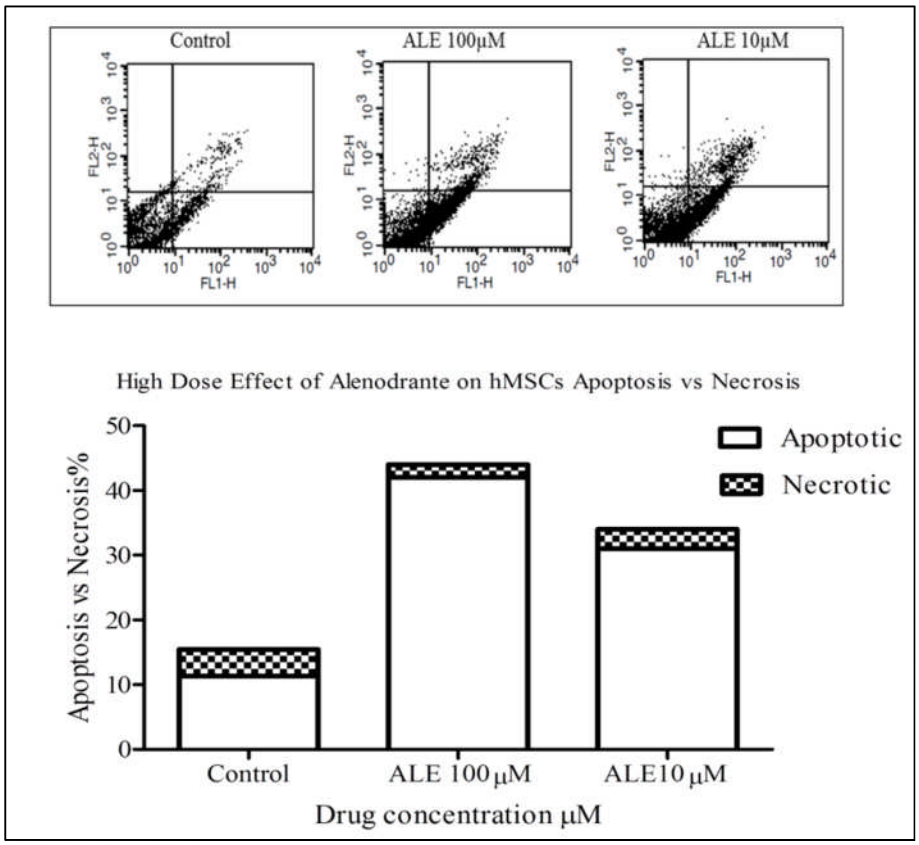


Figure 3.15: Cytological effect of ALE on hMSCs

Bar graph shows a cytological effect of high dose of ALE (100µM and 10µM) on hMSCs after 24 hours of incubation using annexin-V-FTIC stain. The two doses of ALE were exhibiting a significant apoptotic effect compared to the group treated with GM only. In addition, the rate of apoptotic cells was significantly high compared to the necrotic rate. Furthermore, the data presented on the top image lower right quadrant shows the percentage of early apoptotic cells.

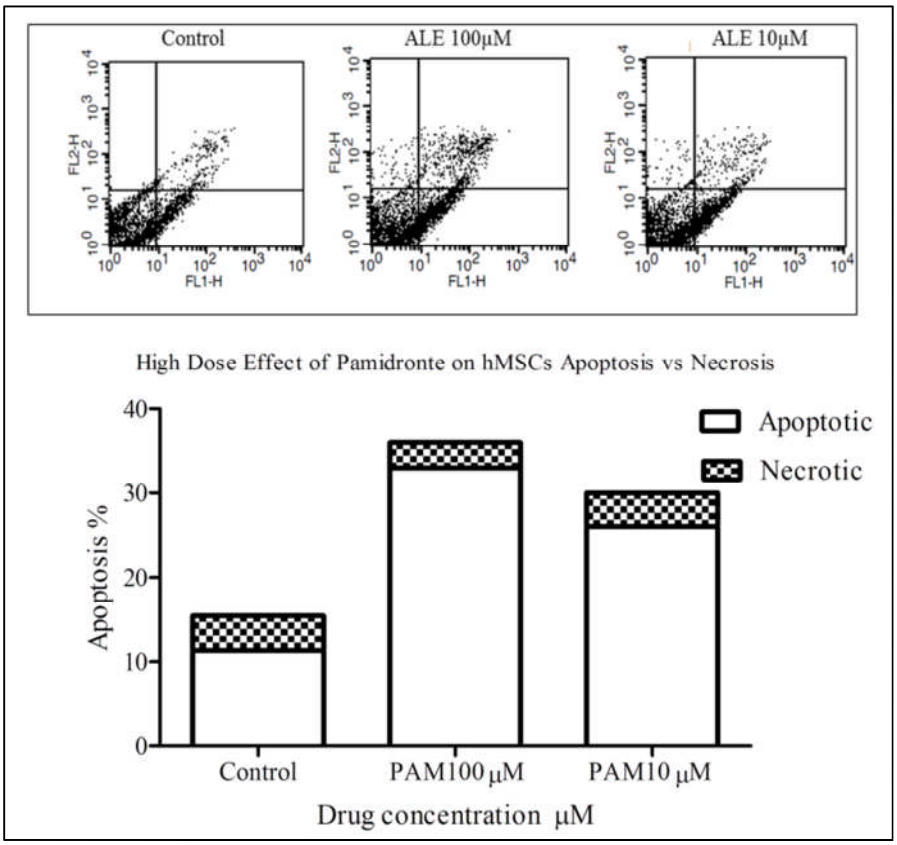


Figure 3.16: Cytological effect of PAM on hMSCs

Bar graph shows a cytological effect of high dose of PAM (100µM and 10µM) on hMSCs after 24 hours of incubation using annexin-V-FTIC stain. The two doses of ALE were exhibiting a significant apoptotic effect compared to the group treated with GM only. In addition, the rate of apoptotic cells was significantly high compared to the necrotic rate. Furthermore, the data presented on the top image lower right quadrant shows the percentage of early apoptotic cells.

3.3.6 Gene Expression

3.3.6.1 RunX2

The effect of ALE and PAM on temporal change of Runx2 which is one of the osteogenic transcriptional master genes was analysed using two doses (100nM and 10nM) at different time points: days 1, 3, 7, and 14 post seeding in OM. The real time PCR method was used to conduct this analysis. The results shown a significant upregulation for the group treated with ALE (100nM and 10nM) on day 3 compared to the control group treated with OM only. The gene was less expressed for the group treated with PAM (10nM) on day 3. There was no significant change for all groups on days 1, 7, and 14, as shown in figure (3.17).

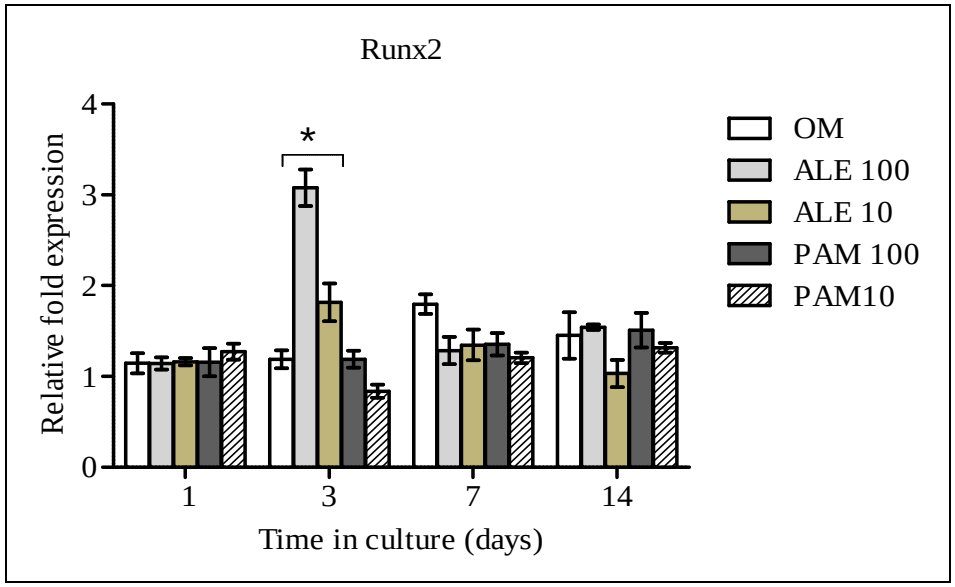


Figure 3.17: Effect of ALE and PAM on Runx2 expression,

Cells were seeded at 30,000 cells/well and the level of gene expression was examined at 1, 3, 7, and 14 days post seeding in OM. The bar chart shows a significant upregulation for the group treated with ALE (100nM and 10nM) on day 3 compared to the control group treated with OM only on day 1. The gene was less expressed for the group treated with PAM (10nM) on day 3. The rest of the groups, Runx2 was expressed at the baseline at days 1, 7, and 14; *=p<0.05, treated group vs. control (OM) day 1.

3.3.6.2 Osteopontin (OPN)

The effects of ALE and PAM on temporal change of the OPN gene, which is one of the bone matrix regulators genes, was analysed using two doses (100nM and 10nM) at different time points: day 1, 3, 7, and 14 days post seeding in OM. The results show a significant upregulation for all groups treated with ALE (100nM) and PAM (10nM) on day 14, compared to the control group treated with OM only. The gene was less expressed for the rest of the groups on days 3 and 7, as shown in figure (3-18).

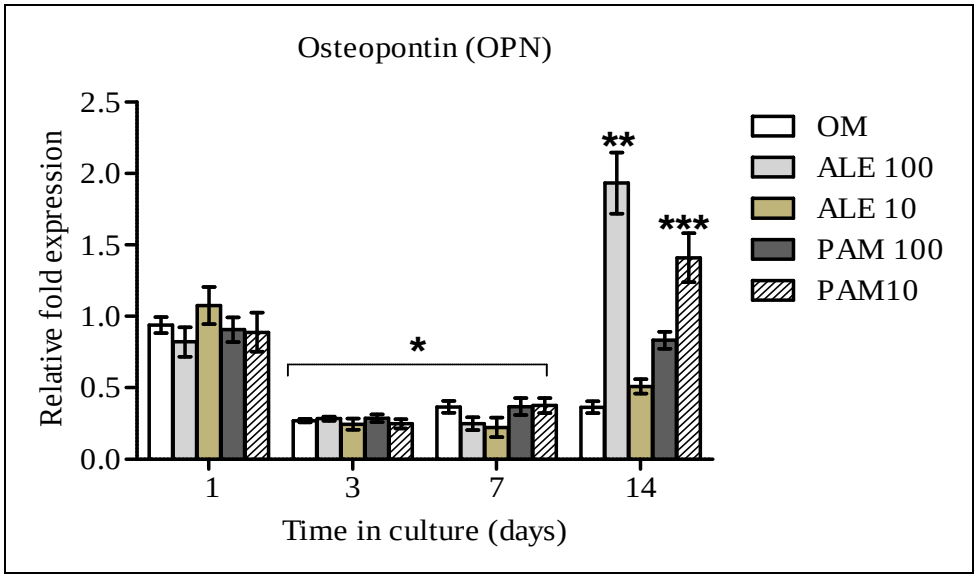


Figure 3.18: Effects of ALE and PAM on OPN expression,

Cells were seeded at 30,000 cells/well, and the level of gene expression was examined at 1, 3, 7, and 14 days post seeding in OM. The bar chart shows a significant upregulation for the group treated with ALE (100nM) and PAM (10nM) on day 14 compared to the control group treated with OM only. The gene was less expressed for all groups on day 3 and all groups on day 7. Each bar represents Mean±SD,*, **and *** =p<0.05 treated group vs. control (OM) day 1.

3.3.6.3 Wnt5a

The effects of ALE and PAM on Wnt5a, which is one of the BMP regulators, was analysed using two doses (100nM and 10nM) at different time points: days 1, 3, 7, and 14 post seeding in OM. The results show a significant upregulation for the group treated with ALE (10nM) and PAM (100nM) on day 1 compared to the control group treated with OM only. Also, there was a significant upregulation for the group treated with ALE (100nM) and PAM (100nM and 10nM) on day 14. The gene was less expressed for the group treated with ALE (100nM) on day 1. There was no significant change on days 3 and 7 as shown in figure (3.19).

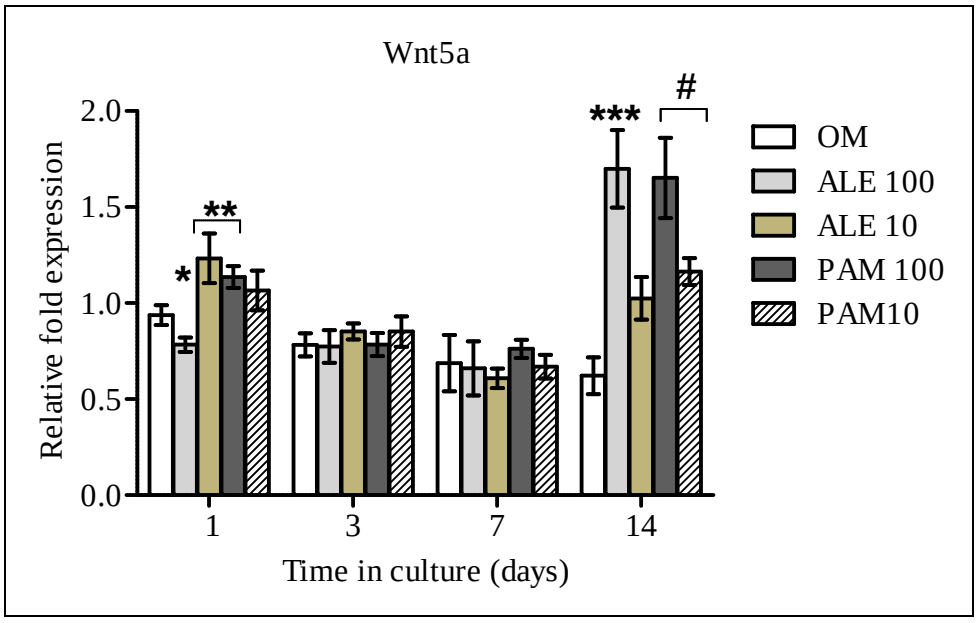


Figure 3.19: Effect of ALE and PAM on Wnt5a expression,

Cells were seeded at 30,000 cells/well, and the level of gene expression was examined at 1, 3, 7, and 14 days post seeding in OM. The bar chart shows significant upregulation for the group treated with ALE (10nM) and PAM (100nM) on day 1 compared to the control group treated with OM only. Also, there was a significant upregulation for the group treated with ALE (100nM) and PAM (100nM and 10nM) on day 14. Each bar represents Mean±SD,*.,*** and # p<0.05 treated group vs. control (OM) day 1.**

3.3.6.4 Alkaline Phosphatase (ALP)

The effect of ALE and PAM on ALP gene was analysed using two doses (100nM and 10nM) at different time points: days 1, 3, 7, and 14 post seeding in OM. The results show a significant upregulation for the group treated with ALE (100nM and 10nM) on day 3 compared to the control group treated with OM only. The gene was less expressed for the rest of the groups on day 3 and all groups on day 7. Furthermore, the ALP was significantly downregulated for all treated groups on day 14, as shown in figure (3.20).

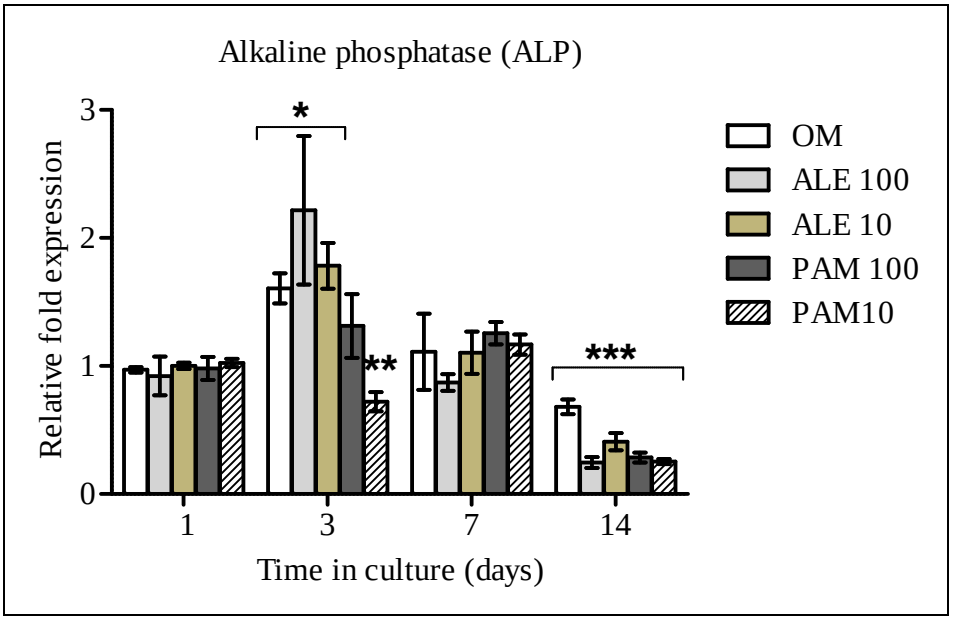


Figure 3.20: Effect of ALE and PAM on ALP expression,

Cells were seeded at 30,000 cells/well and the level of gene expression was examined at 1, 3, 7, and 14 days post seeding in OM. The bar chart shows a significant upregulation for the group treated with ALE (100nM and 10nM) on day 3 compared to the control group treated with OM only. The gene was significantly downregulated for all group treated with ALE and PAM on day 14. Each bar represents Mean±SD; * = p<0.05, treated group vs. control (OM) day 1.

3.3.6.5 Collagen Type 1 Alpha (COL1a1)

The effect of ALE and PAM on temporal change of COL1a1gene was analysed using two doses (100nM and 10nM) at different time points: day 1, 3, 7 and 14 post seeding in OM. The results show a significant upregulation for the group treated with ALE 100nM on day 3 compared to the control group treated with OM only. The gene was less expressed for the group treated with PAM (100nM and 10nM) on day 3 and all groups on day 7. There was a significant downregulation of all groups on day 14, as shown in figure (3.21).

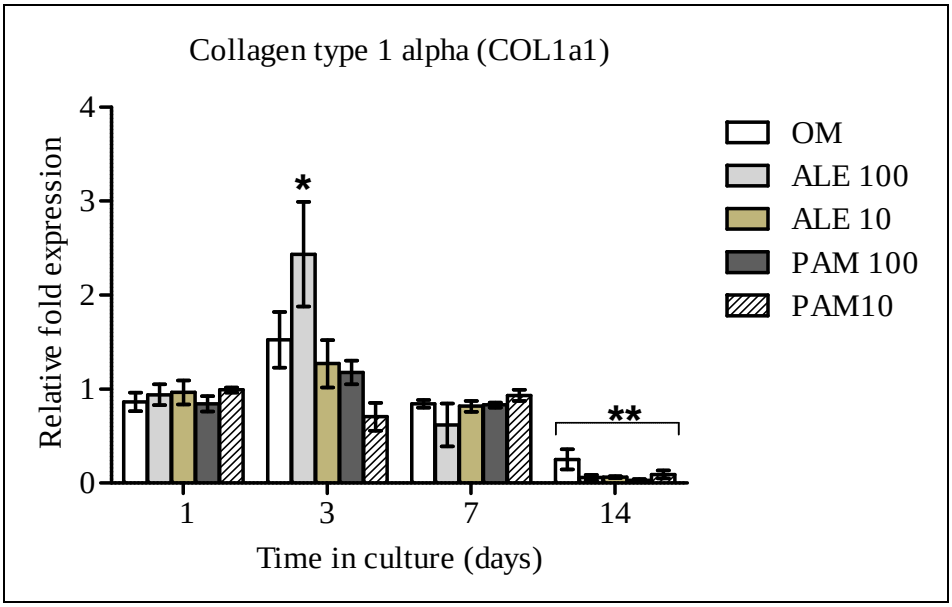


Figure 3.21: Effect of ALE and PAM on COL1a1 expression,

Cells were seeded at 30,000 cells/well, and the level of gene expression was examined at 1, 3, 7, and 14 days post seeding in OM. The bar chart shows a significant upregulation for the group treated with ALE 100nM on day 3 compared to the control group treated with OM only. The gene was less expressed for the rest of the groups on day 3 and all groups on day 7. There was a significant downregulation for all groups on day 14. Each bar represents Mean±SD, $p<0.05$, treated group vs. control (OM) day 1. *=$p<0.05$, treated group vs. control (OM) day 1; **=$p<0.05$ ALE (100nM) vs. control (OM) day 14.

3.3.6.6 Transforming Growth Factor Beta 1 (TGF-ß1)

The effect of ALE and PAM on temporal change of TGF-ß1 was analysed using two doses (100nM and 10nM) at different time points: days 1, 3, 7, and 14 post seeding in OM. The results show that the TGF-ß1 was expressed at the baseline, and there was no significant effect from both drugs at all-time points as shown in figure (3.22).

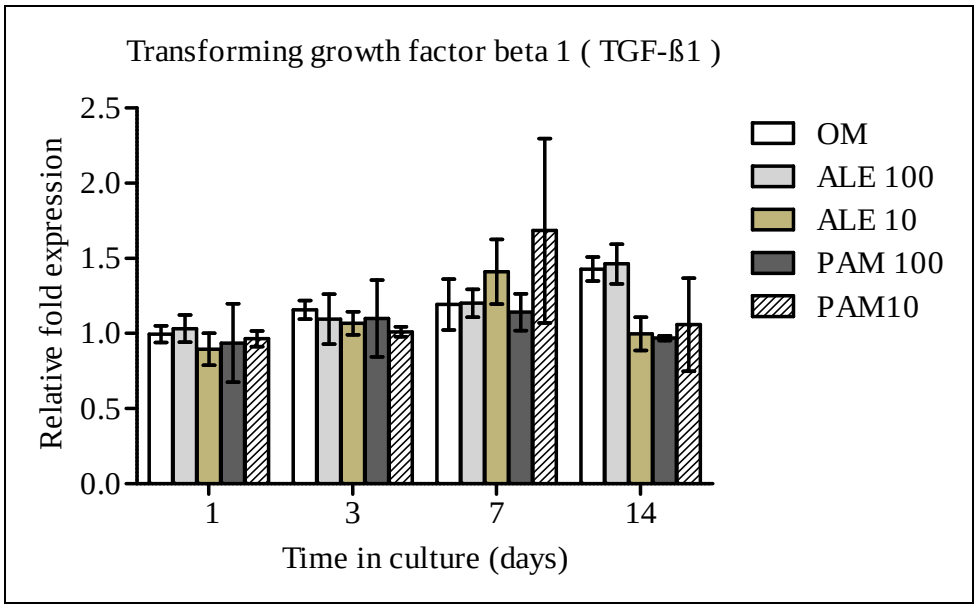


Figure 3.22: Effect of ALE and PAM on TGF-ß1 expression,

Cells were seeded at 30,000 cells/well and the level of gene expression was examined at 1, 3, 7, and 14 days post seeding in OM. The bar chart shows TGF-ß1 was expressed at the baseline for all groups treated by both drugs at days 1, 3, 7, and 14.

3.3.6.7 Osteocalcin (OC)

The effect of ALE and PAM on temporal change of OC, which is a metabolic regulator of pro-osteoblasts, was analysed using two doses (100nM and 10nM) at different time points: days 1, 3, 7, and 14 post seeding in OM. The results show a significant upregulation for the group treated with ALE and PAM (100nM and 10nM) on days 3, 7, and 14 compared to the control group treated with OM only on day 1. The gene was upregulated for the groups treated with ALE (100nM) on day 14 compared to the group treated with OM only, as shown in figure (3.23).

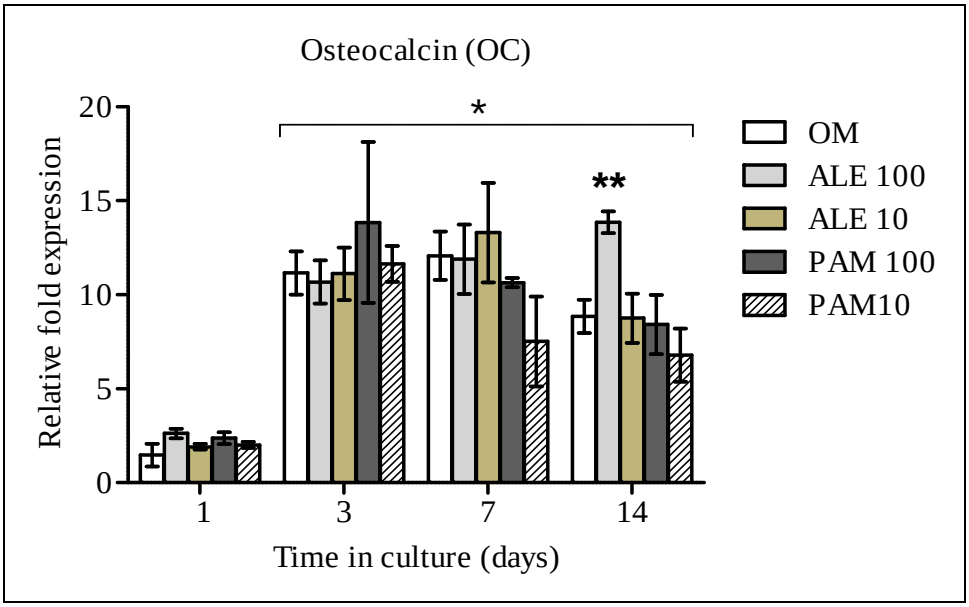


Figure 3.23: Effect of ALE and PAM on OC expression,

Cells were seeded at 30,000 cells/well, and the level of gene expression was examined at 1, 3, 7, and 14 days post seeding in OM. The bar chart shows a significant upregulation for the group treated with ALE and PAM (100nM and 10nM) on days 3, 7, and 14 compared to the control group treated with OM only on day 1. The gene was upregulated for the group treated with ALE (100nM) on day 14. Each bar represents Mean±SD, *=p<0.05, treated group vs. control (OM) day 1; **=p<0.05 ALE (100nM) vs. control (OM) day 14.

3.3.6.8 Bone Sialoprotein Type 2 (BSP2)

The effect of ALE and PAM on temporal change of BSP2, which is one of the bone extracellular matrix regulators, was analysed using two doses (100nM and 10nM) at different time points: days 1, 3, 7, and 14 post seeding in OM. The results showed a significant upregulation for the group treated with ALE (10nM) and PAM (100nM) on day 7 compared to the control group treated with OM only on day 1. The gene was significantly downregulated for all groups on day 14, as shown in figure (3.24).

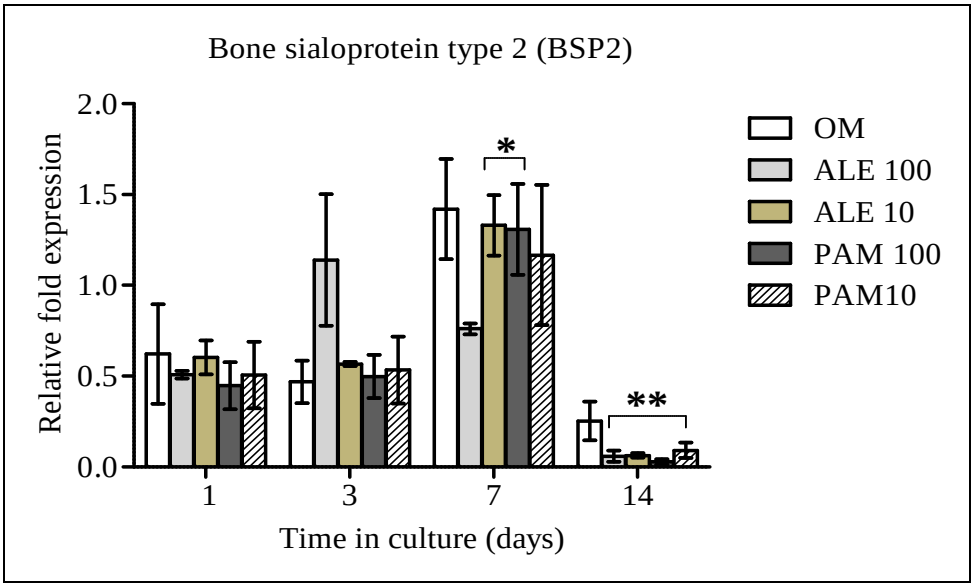


Figure 3.24: Effect of ALE and PAM on BSP2 expression

Cells were seeded at 30,000 cells/well, and the level of gene expression was examined at 1, 3, 7, and 14 days post seeding in OM. The bar chart shows a significant upregulation for the group treated with ALE (10nM) and PAM (100nM) on day 7 compared to the control group treated with OM only on day 1. The gene was downregulated for all groups on day 14. Each bar represents Mean±SD; $p<0.05$ treated group vs. control (OM) day 1.

3.4 **Discussion**

This chapter investigates the effects of two types of commonly used BPs (ALE and PAM) on hMSCs' proliferation and osteogenic differentiation. Over the lifetime, the hMSCs' self-renewing which is also known as proliferation is considered an important feature for these cells to develop and maintain other types of tissue, such as connective tissue and adipose tissue (Kobolak et al., 2015). These cells are believed to be sensitive to any external trigger, whether it is chemical, physical, or biological. (Sundelacruz and Kaplan, 2009). However, recent studies suggest that some types of BPs may affect osteoblast fibroblasts, keratinocytes, and hMSCs' proliferation and differentiation, whether by a positive or negative effect. To further investigate that effect, we chose to analyse the effects of ALE and PAM on the hMSCs, as these cells are some of the major cells involved in tissue regeneration (Bielby et al., 2007). Our data suggest that treating cells *in vitro* with ALE and PAM at different time points have two types of effects on cells' proliferation. The small dose of ALE and PAM (100nM), which is 1,000 times less than actual clinical doses, stimulates the cells' proliferation from day 7 post seeding. However, we found that the high dose that mimics the actual clinical dose inhibits the cells' proliferation from day 3 post seeding. This effect may occur via activation of some signalling pathway that is involved in hMSCs' proliferation, such as Wnt5's signalling pathway (Reya and Clevers, 2005, Guan et al., 2014).

The restriction of the proliferation rate of the groups treated with high doses was mainly due to the apoptotic effect. ALE and PAM have similar chemical structures; the only difference is the R2 chain that bonds to the central carbon atom. This similarity gives a clarification of why these two groups have a similar apoptotic effect.

The effects of ALE and PAM on the hMSCs' osteogenic differentiation and mineralisation *in vitro* was determined by analysing the early and late osteogenic markers, including calcium deposition, collagen formation, and ALP level. The results of this analysis show that the osteogenesis of hMSCs is a dynamic process, which makes them sensitive to external triggers, including chemical factors. In this experiment, we have found that the low doses of both ALE and PAM significantly stimulate the early and late osteogenic markers, including ALP, calcium, and collage type I. ALP is believed to be an early osteogenic and differentiation marker, where the hMSCs start to differentiate into osteoblasts. A few reports have found that BPs may have a direct effect on osteoblasts cell lines MG-63 and enhance the mineralisation rate (Xiong et al., 2009). In our study, we go up further to the main origin of OB: the hMSCs. This effect indicates that these drugs may act centrally acting on the osteogenic process, which will commit the cells to produce more bone. Moreover, in this chapter we observed that at day 7 post seeding, there were elevations of the level of ALP, confirming that the ALE and PAM may have a regulatory effect on this enzyme. Further, as to the early effects, both drugs showed a stimulatory effect on calcium deposition and collagen formation. This gives an additional indication about how the drug may involve both intracellular and ECM osteogenic activity. These findings were consistent with Von Kossa staining, which has provided a qualitative indication of the effect of both drugs on hMSC osteogenesis towards a mineralising osteoblasts phenotype and effective in stimulating ECM mineralisation following drugs treatment.

The osteogenic genes that mainly regulate the hMSCs' osteogenesis were evaluated. These genes including Runx2, Wnt5a, OPN, BSP2, ALP, Colla1a1, OC, and TGFb1 were examined at different time points. Runx2 is considered a master transcriptional factor that

regulates the osteoblasts' phenotypes and regulates other genes involved in the osteogenesis process, such as OC (Paredes et al., 2004, Lian et al., 2003). The Wnt5a gene was investigated, as it is considered a significant regulator of BMP that participates in osteoblasts' differentiation (Nemoto et al., 2012, Wall et al., 2009). BSP2 and OPN have potential roles in calcium deposition and HA formation, as well as promoting osteoblast mineralisation (Zhou et al., 1995, Hunter and Goldberg, 1993). ALP is the enzyme responsible for early mineralisation of the osteoblasts and is involved in osteoblast metabolism (Orimo, 2010). Collagen is considered an important protein that regulates the extra-cellular matrix and plays an integral role in making a connection between the surface integrins and other ECM components (Ferreira et al., 2012). Furthermore, the COL 1A1 has also participated in cell adhesion, proliferation, and osteogenic differentiation of osteoblasts (Roehlecke et al., 2001, Schlie-Wolter et al., 2013). OC is considered one of the osteoblasts' specific genes and is involved in osteoblasts' differentiation (Hauschka et al., 1989). TGFb1 is a multi-functional gene that plays an integral role in osteoblast formation and apoptosis (Chen et al., 2012).

The analysed genes have shown differences in expression between doses and time intervals, where most of the differences occurred at early stages. Both drugs show a similar effect on gene expression. In this chapter, we have found there was an upregulation of Runx2 for the group treated with ALE (100nM and 10nM). This finding indicates that the drug has an early chronological effect on hMSCs' differentiation. The Colla1a1 was upregulated for the group treated with ALE (100nM) initially; but it was down regulated at day 14. This finding may also reflect and correlate with a significant deposition of ECM collagen, as stated before; however, the collagen has shown that it responds to external force or stimuli (Jagodzinski et al., 2004). It may show that the

chemical stimuli counted as an additional trigger that affects this gene. To further analyse the effect of BP on hMSCs' osteogenesis, the OC gene was analysed. The finding suggests there was a temporal effect and upregulation at days 3, 7, and 14 compared to the day 1 control. TGFb1 was not observed to be affected by both drugs at any time points with the exception at day 7, which shows there was an effect, but it was not significant.

In further investigation, the ALP gene was analysed, as it has a role in early mineralisation. It was upregulated for the group treated with ALE (100nM and 10nM) at day 3, which gives a clear indication of its potential association with chemical triggers to stimulate cells' differentiation. However, this finding was correlated with enhancement of ALP activity, as stated earlier in this chapter. The OPN was upregulated on day 14, which gives an indication that the ALE and PAM have late effects on calcium deposition. On the other hand, BSP2 was upregulated at day 7 for the group treated with ALE 10 nM. Moreover, an analysis of COL 1A1 and OC showed that these genes are affected by drug treatment. OC was strongly and continuously affected by ALE and PAM from day 3 until day 14. These effects support previous findings where the drug stimulated cells' mineralisation and they give a strong indication that the drug directly regulates hMSCs via controlling the OC gene. The COL 1A1 was significantly upregulated at day 3 for the group treated with ALE 100nM only. This leads us to highlight that it is important that these genes may respond to the external triggers, whether mechanical, physical, or chemical.

Our finding suggests that at the cellular level, compared to PAM, the ALE had a strong influence in osteogenic genes' expression, cells' proliferation and osteogenic differentiation. However, the exact mechanism of this effect on the anabolic activity of

hMSCs is still not fully understood. From our findings, we believe that the effect is mainly related to direct controlling osteogenic genes, which will subsequently affect the differentiation process.

3.5 **Significant findings**

The results of the analysis conducted in this chapter suggest that there is an influence by the low doses of ALE and PAM on hMSCs' proliferation and osteogenic differentiation. This influence causes the hMSCs to enhance their proliferation rates and deposit more calcium and collagen in the extracellular calcified matrix.

The data showed that hMSCs are morphologically and behaviourally sensitive to chemical stimuli. This was supported by genetic findings, which indicate that the drugs commit the hMSCs towards an osteogenic lineage.

3.6 **Summary of the results**

These experiments show that lower concentrations of ALE and PAM may play an integral role in enhancing hMSCs' proliferation and osteogenic differentiation. In addition, the low doses of ALE and PAM have multiple effects on different hMSCs' activities. These effects are summarised in the below points:

- Low doses of ALE and PAM stimulate and enhance hMSCs' proliferation from day 7 post seeding.
- Low doses of ALE and PAM stimulate the early and late osteogenic markers, including calcium, collagen, and ALP.

- Genetic expression of key osteoblast phenotype and physiology is stimulated by BPs. Low doses of ALE and PAM promote the expression of osteoblast genes, including Runx2, OPN, BSP2, OC, Wnt5a, ALP, and COL1A1.
- ALE is more effective than PAM in hMSCs' activities.

Finally, BPs may have direct stimuli on osteoblasts' mineralisation. These findings suggest that BPs may more than compensate for the established positive effects of osteoclasts on bone density in osteoporosis patients.

4 Single low dose BPs treatment has long term effects on, and enhances osteogenesis in hMSCs

4.1 Introduction

Since the hMSCs were discovered by Friedenstein and his colleagues, they have generally played a crucial role in tissue regeneration and have been involved in regenerative medicine (Friedenstein et al., 1970, Mo et al., 2016, Yamada et al., 2015). They also play an integral role in bone regeneration and bone healing , showing a promising effect when transplanted to bone defects (Knight and Hankenson, 2013). hMSCs seem to be sensitive to both chemical and physical external stimuli (Chen et al., 2016). It has been reported that the growth of hMSCs on a soft hydrogel scaffold changes the osteogenic behaviour, as it is believed that memories of a mechanical change are influenced by past stiff environments (Yang et al., 2014). In addition, the substrate elasticity has effects on the stem cells' differentiation behaviour whereby the degree of substrate elasticity correlates with the stiffness of corresponding tissue, including bone, muscles and nerves (Engler et al., 2006). Furthermore, at the microenvironment level, external physical factors control stem cell function by interfering with the cellular signalling pathways (Sun et al., 2012). Gilberto et al. found that stem cells originating from muscle show that they memorise the last mechanical stimuli, which may stay even after the in vivo application (Gilbert et al., 2010). The behaviour of hMSCs is also thought to be influenced and affected by the surface topography of the substrate, even at the nanoscale level (Lavenus et al., 2015). . Some drugs, such as retinoids, have an effect on hMSCs differentiation and behaviour (Gudas and Wagner, 2011). This action and effect takes place mainly via controlling some stem cells' transcriptional genes, such as cellular retinoic acid binding protein 2

genes (CRABP2). Furthermore, it has been reported that immunosuppressant drugs have a direct effect on the human central nervous system-derived stem cells' differentiation and proliferation in vitro (Sontag et al., 2013).

From the previous chapter, we have found that a low dose of BPs promotes osteogenic differentiation and proliferation of hMSCs in vitro (Alqhtani et al., 2014). The low dose of BPs such as PAM and ALE upregulate the early and late osteogenic markers such as ALP, calcium deposition, hydroxyapatite (HA) and extracellular collagen deposition.

The aim of this chapter is to investigate the extended effect of a single low dose of BPs on hMSCs' proliferative and osteogenic behaviour by evaluating the ECM mineralisation parameters and to assess the ability of the hMSCs to synthesise osteoblastic markers. This analysis was conducted by evaluating the hMSCs' proliferation; the early and late osteogenic markers included ALP, type I collagen deposition, calcium deposition and HA.

4.2 Materials and methods

4.2.1 Cell culture

In this chapter, the effects of a single low dose of ALE and PAM (100nM and 10nM) on the behaviour of hMSCs were investigated. The hMSCs were plated at a density of 5×10^5 cells/75cm^2 flasks containing MSC GM and cultured according to the protocols described in section (2.1.1). After 24 hours the medium was changed with GM containing BPs at both 100nM and 10nM, and the hMSCs were incubated for 24 more hours. The cells were then washed and trypsinized and sub-cultured again without further exposure to BPs.

4.2.2 Proliferation

Proliferation were measured by seeding 5000 cells/well in a 24 well plate. The cells' numbers were serially measured at different points of time, days 1, 3, and 7 using alamar blue assay as described in section (2.4).

4.2.3 Assessment of Cells Mineralisation

The hMSCs' osteogenic differentiation was assessed by seeding 10,000 cells /well in a 24 well plate in OM. Cell cultures were maintained by changing the media every 2-3 days. The osteogenic markers were evaluated as described in the next sections.

4.2.3.1 Alkaline Phosphatase Activity

The ALP activity was assessed at day 7 post seeding using the colorimetric SensoLyte® pNPP Alkaline Phosphatase Assay Kit (Cambridge Bioscience) according to the manufacturer's instructions as described in section (2.5.3).

4.2.3.2 Quantification of Calcium Deposition

The total calcium formed by cells was examined on day 21 post seeding with a colorimetric quantichrom kit (BioAssay Systems) according to the manufacturer's instructions, as described in section (2.5.1).

4.2.3.3 Von Kossa Stain

The Von Kossa stain method was used as a semi-quantitative approach to assess hMSCs' mineralisation in 21 days post seeding. The methods for this approach are described in section (2.5.2).

4.2.3.4 Hydroxyapatite (HA)

HA is an essential inorganic component and represents ~70% of the total mass of the bone. To measure the total amounts of HA formed by hMSCs; OsteoImage mineralisation assay (Lonza) was used according to the manufacturer's instructions. By the end of the experiment, the culture media was removed, and the cells were then washed twice in PBS then fixed in 4% paraformaldehyde for 15 mins. Fixative agent was discarded followed by two washes with diluted wash buffer. Following this, the cells were incubated with staining reagent in the dark for 30 min at room temperature. The cells were then washed several times with a diluted wash buffer. After that, an appropriate amount of wash buffer was added to the wells for microscopy viewing and plate reader analysis. The amounts of the HA minerals were quantified using a plate reader (BioTeK FLX800); fluorescence: (Excitation λ= 492 nm, emission λ= 520 nm). Visual examination was carried out using a Leica–DMIRB fluorescence microscope equipped with COOLSNAP Monochrome Camera. Images were collected and processed with the image J Imaging System.

4.2.4 Quantification of ECM Collagen

The total amounts of ECM formed by hMSCs were measured on 21 days post seeding using the Sirocel collagen assay kit according to manufacturer's instructions (Biocolor) as described in section (2.6). The total amount was measured from three replicate as it was difficult to measure form a single well due to the small quantities produced by small cells number.

4.3 **Results**

4.3.1 Proliferation

The proliferation of 5000 cells/24 wells of hMSCs in GM was examined on 1, 3 and 7 days. ALE (100nM and 10nM) stimulated significant cell proliferation in all cells on Day 3 and Day 7. Also, cells that had been treated with 10nM PAM on Day 3 and Day 7 showed significant proliferation when compared to control cells that were treated with GM only. Cells treated with 100nM PAM promoted cell proliferation, but this increase was not statistically significant. Both drugs have shown a similar effect on cell proliferation at Day 1 as shown in figure (4.1).

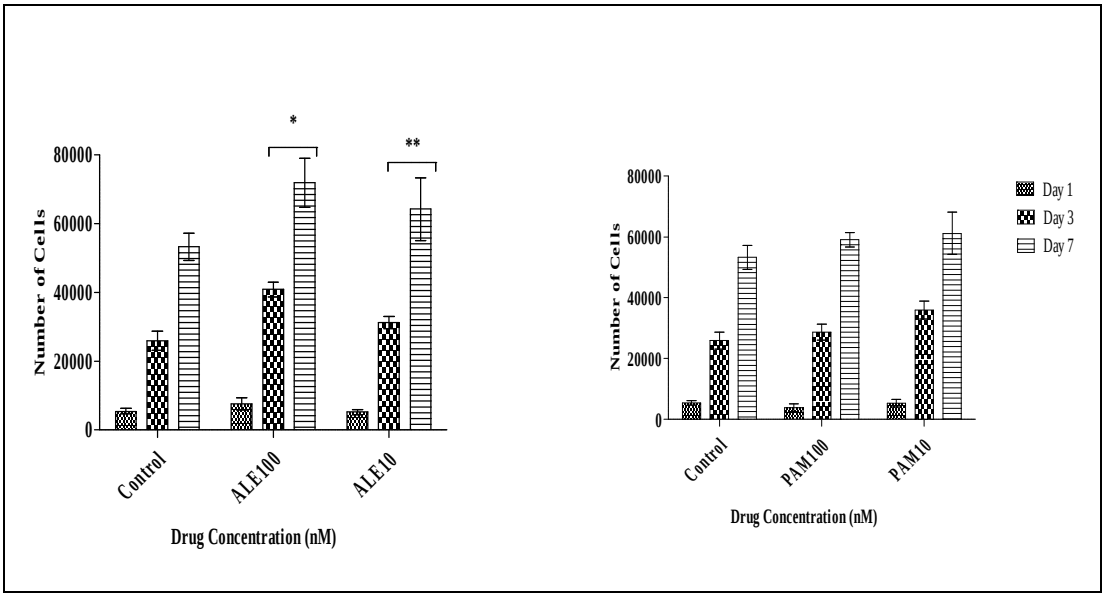


Figure 4.1: The extended effect of ALE and PAM on cell proliferation

A total of 5000 cells/24wells were seeded. The proliferation was examined on days 1,3 and 7. The effect of the ALE (100nM and 10nM) significantly stimulated cells proliferation at Day 3 and Day 7. Cells treated with 10nM PAM promoted cell proliferation, but this increase was not statistically significant. Data represent mean±SD. *&=p<0.05, treated group vs. control.**

4.3.2 Alkaline phosphates activity

ALP enzymes are one the most useful early osteogenic markers. The level of ALP activity was studied after seven days of incubation in OM. The data showed that the groups treated with a low dose of ALE and PAM (100nM and 10nM) significantly stimulated the ALP activity when compared to the control group that was treated with OM only as shown in figure (4.2).

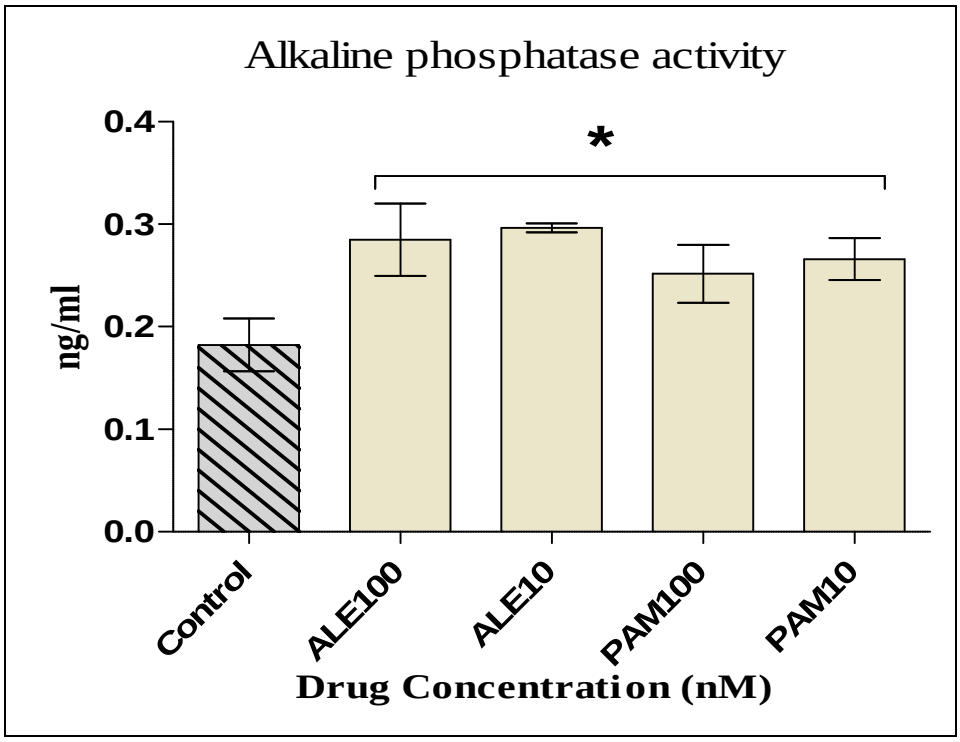


Figure 4.2: The extended effect on ALP activity.

ALP activity after seven days incubation was significantly increased in all cells treated with the lower dose of BP drugs when compared to the control group treated with osteogenic media only. Each column represents the mean±SD. *=p<0.05, treated group vs. control (OM)

4.3.3 Calcium deposition assay

Markers that were linked to early and late stage osteogenesis were analysed. These markers included calcium deposition, collagen type I and ALP. The data suggests that treating cells with a single low dose of ALE (100nM & 10nM) changes the osteogenic behaviour of the hMSCs even after passaging the cells but it was not statistically significant. Data showed that PAM drug significantly stimulated calcium deposition after three weeks when compared to cells that had been treated with OM only as shown in figure (4.3).

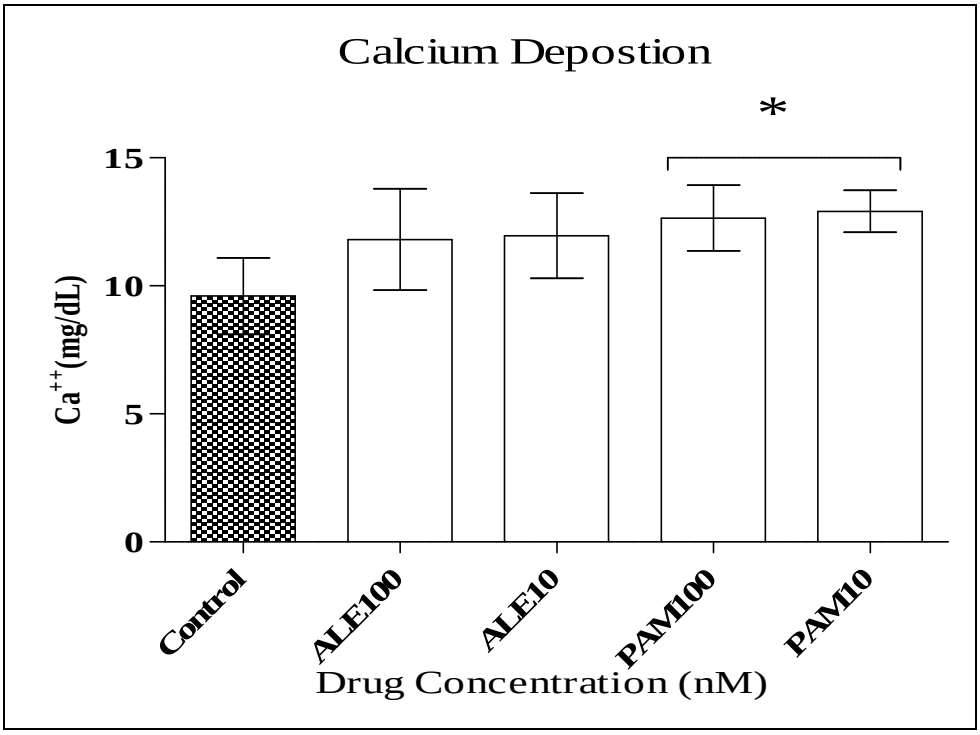


Figure 4.3: The extended effect of PAM and ALE on calcium depostion.

Cells were divided into five groups, with each group treated with different ALE and PAM concentrations (100 nM and 10 nM). (A): At Day 21, calcium deposition was analysed. The results showed that PAM group significantly stimulated hMSC mineralisation following drug treatment for 21 days when compared to cells that had been treated with OM only. Each column represents the mean±SD. *=p<0.05, treated group vs. control (OM).

4.3.4 Von Kossa stain

A von Kossa stain was used to semi-quantify and visualise the mineralized nodules (figure 4.4). The images showed an extended significant effect on mineralized nodule formation when the sample treated with ALE and PAM (100nM&10nM) were von Kossa positive in comparison with the control groups, which were treated with OM only. The mineralized nodules were semi-quantified using Image Pro Plus software, version 4.5 (Media Cybernetics, Marlow, Buckinghamshire, UK) to calculate the percentage of black area per 100µm^2, and the results correlated with visual inspection.

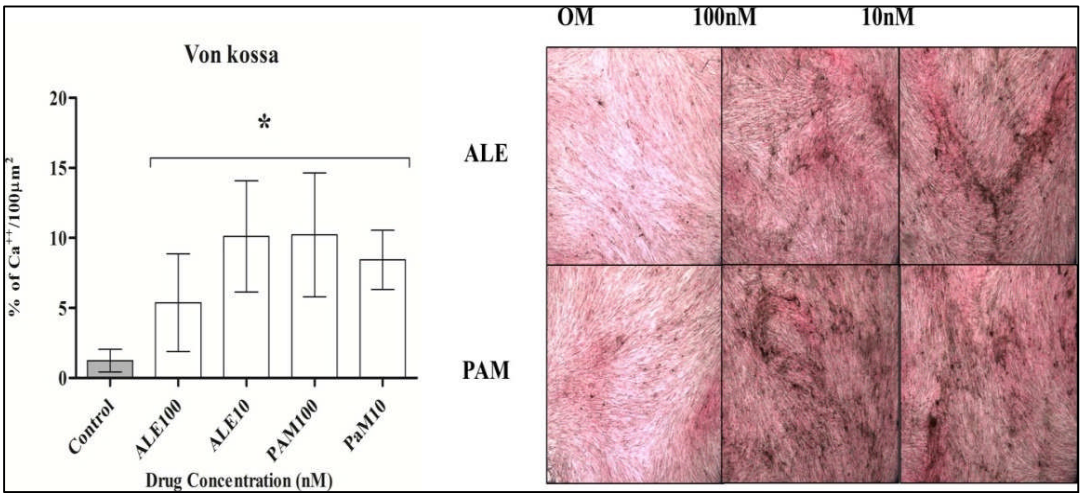


Figure 4.4: Von Kossa stain of mineralized nodule formation

Light microscopy images showing that there were more mineralized nodules (black area) produced by hMSCs after 21 days in culture than the group treated with OM media only. Images were taken using X 40 objective. *=p<0.05, treated group vs. control (OM).

4.3.5 Collagen deposition

Extracellular matrix collagen was assayed with a Sircol collagen assay kit from Biocolor following the manufacturer's instructions. The results showed that both ALE and PAM significantly stimulate hMSCs to produce more collagen at 21 days following a single low dose of drugs (100nM &10nM) than the other group that was treated with OM only as shown in figure (4.5).

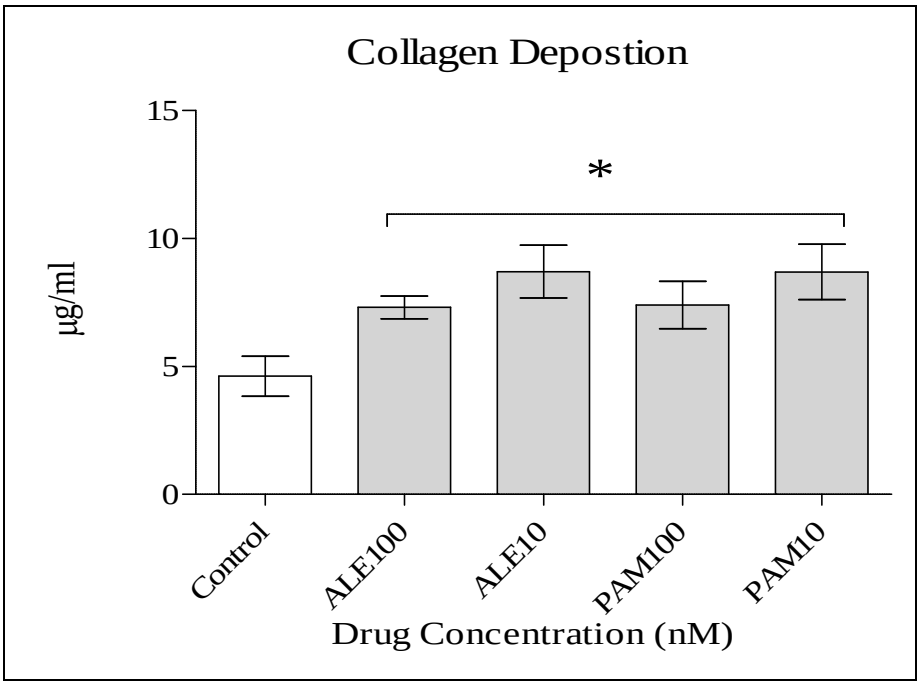


Figure 4.5: The extended effect on the production of Collagen deposition after 21 days.

At day 14, extracellular collagen formation was analysed. The results showed that PAM significantly stimulated hMSC to produce collagen following treatment with drugs for 21 days when compared to cells that had been treated with osteogenic media only. In addition, ALE significantly stimulated hMSC to produce collagen following treatment when compared to the control. Each column represents the mean±SD. *=p<0.05, treated group vs. control (OM).

4.3.6 Hydroxyapatite

A Leica DMIRB fluorescence microscope was used to visually examine HA nodule formation. Results showed that after 21 days of culture in OM following the single dose treatment with ALE and PAM there was a clear difference in the appearance and formation of the hydroxyapatite nodules when compared to the control group that had been treated with OM only. The hydroxyapatite depositions were semi-quantified using Image Pro Plus software, version 4.5 to calculate the percentage of fluorescent area per 100μm^2, and the results correlated with visual inspection as shown in figure (4.6).

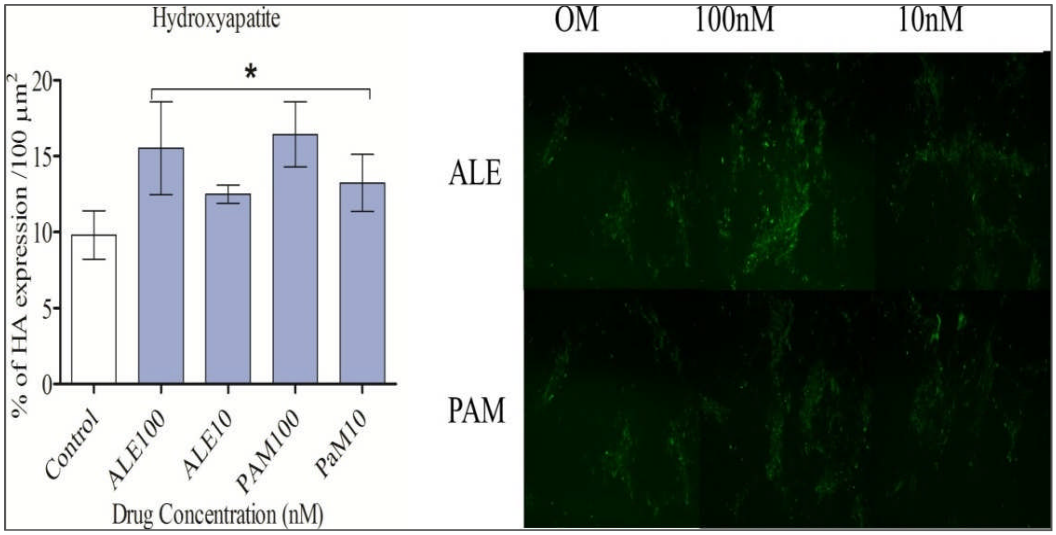


Figure 4.6: The extended effect on the production of hydroxyapatite by hMSCs after 21 days.

ALE and PAM (100nM and 10nM) significantly stimulated the production of HA. Florescent microscopy images showed more deposited hydroxyapatite was produced by hMSCs than the group treated with OM media only. Images were taken using X 40 objective. *=p<0.05, treated group vs. control (OM).

4.4 **Discussion**

The stem cells, with their unique features such as self-renewal and the capability to differentiate to other cell lineages, has become a promising target for regenerative medicine, cell therapy and drug development (Avior et al., 2016, Krampera et al., 2006). This part of project aimed to investigate the extended effect of PAM and ALE on hMSCs proliferation and osteogenic differentiation by studying the early and late osteogenic markers. Several studies have reported that stem cells, in conjunction with a biomaterial scaffold, were used to engineer different tissues and then to repair tissue defects (Willerth and Sakiyama-Elbert, 2008, Hasan et al., 2016, Zippel et al., 2010, Ghasemi-Mobarakeh et al., 2015, Battiston et al., 2014). These materials include collagen, alginate, agarose, polymethyl methacrylate (PMMA) and fibrin (Horst et al., 2012). The scaffold will provide a good environment for stem cells to proliferate and differentiate and will subsequently form the damaged tissue or organ (Sundelacruz and Kaplan, 2009). Accordingly, it is still a challenge that cell response varies based on the substrate type, which may affect the cell behaviour and growth. However, several signalling molecules were involved in stem cell differentiation, including BMP, FGF, TGF-β and Wnt signalling family (Augello and De Bari, 2010). In this chapter we have shown that BPs, as external triggers, could be an additional factor involved in stem cell development and differentiation.

Proliferation of hMSCs is crucial for their successful use in clinical practice. As previously reported, the low dose of ALE and PAM enhanced and accelerated hMSCs proliferation and differentiation to other cell lineages (Alqhtani et al., 2014, Abtahi et al., 2012, Kim et al., 2011, Maruotti et al., 2012, McLeod et al., 2014, Pabst et al., 2012). In this study we have found that ALE and PAM have an extended effect on and accelerated hMSC proliferation at Day 3 and Day 7 on a plastic tissue culture substrate. ALE

promotes cells proliferation more than PAM, which may relate to their chemical structure. It is still unclear whether this difference may involve cell signalling pathways such as MAPK pathways. These pathways are believed to have a crucial role on mammalian cell growth and development, including stem cells (Zhang and Liu, 2002, Geest and Coffer, 2009). However, hMSCs are one of the most sensitive cells to both mechanical and chemical external stimuli (Wu et al., 2013). Despite this sensitivity, our data supports these findings with even more permanent effects.

The ability of hMSCs to differentiate to mature osteoblasts is a crucial factor for bone formation (Prockop, 1997). This process was extensively studied in vitro by adding osteogenic factors including ascorbic acid, dexamethasone and β-glycerol phosphate into the culture medium of hMSCs (Langenbach and Handschel, 2013b). However, these factors together are not likely to be present within the hMSCs' natural environment in vivo. On the other hand, bone cells and bone tissue extracts have been used as osteogenic regulators in vitro of hMSCs without the involvement of osteogenic factors (Birmingham et al., 2012, Tsai et al., 2012, Wang et al., 2007, Ball et al., 2004, Wang et al., 2013). This regulatory effect could be a result of intracellular signalling and the biochemical factors involved in hMSC differentiation in vivo. In the present study, we have found that the BPs have an extended effect on the early and late osteogenic markers.

ALP is one of the early osteogenesis markers involved in the calcification of the bone matrix, and it was assessed at an early point (Masrour Roudsari and Mahjoub, 2012). Both drugs were found to have a stimulatory effect on ALP activity at Day 7 (figure 4-6). This finding suggests that the drug has an early impact on osteogenic differentiation and the production of extracellular minerals.

Both ALE and PAM promote a significantly higher level of calcium deposition using qualitative and quantitative approaches (figure 4-2). The semi-quantitative approach of

detecting and visualising the calcium deposition was accomplished through the use of the von Kossa stain, which correlates with the quantitative methods. The von Kossa stain was used to confirm the presence of physiological mineralization nodules. Using this approach to detect the mineralized nodules might not be sufficient to confirm the presence of calcium deposition. Furthermore, the bone mineral level when using a HA parameter was significantly increased, since HA represents up to 70% of the total mass of bone and gives the bone its rigidity (Clarke, 2008). The clinical application of HA is mainly in bone graft substitutes or in implant coating to promote bone formation around the implant and to restore the missing bone, as was previously reported (Ong and Chan, 2000, Fillingham and Jacobs, 2016). In the implant world this approach has some disadvantages, such as fragility and brittleness, which may lead to implant failure (Allegrini et al., 2006). In this chapter we showed that BPs stimulate the osteoblast to produce more HA minerals, which may work as an adjunct method of enhancing the contact and accelerating bone healing. Interestingly, the osteogenic differentiation process takes up to four weeks, and we have found in our study that the drugs may even shorten this time period to three weeks. This finding was in close agreement with previous reports that demonstrated that external mechanical and chemical stimuli upregulate and accelerate the osteogenic differentiation process (Chen et al., 2016, Ge et al., 2009).

Extracellular collagen is one of the major insoluble proteins in the extracellular matrix of bone (Lodish H, 2000). Previous studies demonstrated that the deposition of collagen in the extracellular matrix is considered as an essential factor in the bone microenvironment that participates in the haematopoiesis process (Klamer and Voermans, 2014, Klein, 1995). Thus, the deposition of collagen helps in facilitating bone healing and the bone remodelling cycle as the haematopoiesis is involved in this process. We found that both drugs have a stimulatory effect on extracellular collagen type I, which may play an

integral role in bone turnover, especially with bone related problems. Therefore, treating hMSCs with a low dose of BPs may be involved in the maintenance and growth of bone. Furthermore, from these findings we wanted to assess the ability of the extended effect of this drug in controlling and regulating the initiation of hMSCs' osteogenic behaviour on wound healing, as these cells are considered the first cells recruited to sites of bone trauma and around implant surface (Davies, 2003).

Several medical and nonmedical problems may impair the bone formation and remodelling, such as diabetes mellitus, osteoporosis, smoking and alcohol abuse (Guo and Dipietro, 2010). In addition, the bone healing process is altered with age, where there may be less bone formation, a slower rate of cell differentiation and slower bone remodelling (Kasper et al., 2009). In addition, the hMSC proliferation is decreased, resulting in a decreased ability to divide with elderly people (Tan et al., 2015). Both of the in vitro and in vivo studies have proposed that a high density of bone marrow stem cells may enhance fracture repair and subsequently accelerate wound healing (Hernigou et al., 2005). Hernigou et al. investigated the application of autologous bone marrow MSCs on a tibial non-union fracture; they found that around 88% of tibial non-unions were treated successfully. All these factors increased the risk of complications and affected the bone quality. Thus, the extended effects of BPs on hMSCs have afforded promise in the treatment of these conditions. Moreover, hMSCs in combination with drugs may be used as a potential approach to repair bone defects and in healing wounds. Also, this finding presents a promising systematic approach to accelerating osseointegration and increasing bone-implant contact without coating implants.

The hMSCs have the ability to generate new multiple cell lineages throughout their life which will give a specific tissue. This study has shown that the BPs may direct the stem cell differentiation behaviour towards one lineage. As differentiation is a complex process

that is mediated by different internal and external factors, it is still challenging to determine at what stage or which factor is still involved in this effect. However, the permanent nature of the changes suggests a possible epigenetic mechanism operating which we will be investigating in the next chapter.

4.4.1 Summary of the results

The outcome of this chapter has shown that the BPs enhanced both early and late osteogenic markers. Also, there was an extended effect on hMSCs' proliferation behaviour. They indicate these drugs have a strong effect on cells in depositing calcified matrix and producing more collagen. These findings may present a promising approach for using these drugs as an adjunct to implant therapy. The findings also indicate that these cells are highly sensitive to external chemical, mechanical, and electrical stimuli. In addition, the impact of hMSCs' ability to change their behaviour even after passaging has implications regarding stem cell therapy. Moreover, understanding hMSC regulation is a key factor for their clinical usage and application in tissue engineering.

5 The epigenetic effect of a single, low dose of BP treatment on the phenotype of hMSCs

5.1 Introduction

It has been shown that a low dose of BPs has an observable effect on the proliferation and osteogenic differentiation of hMSCs (Alqhtani et al., 2014). It is believed that this temporal effect is related to the upregulation of some of the osteogenic markers that control different aspects of osteoblastic growth and differentiation. One area of interest that has been highlighted is whether the effect may extend to affecting the epigenetic phenotype of hMSCs via DNA methylation. As has been previously stated, epigenetics relates to heritable, long-standing changes at the gene expression level without alterations in the underlying DNA sequence. It is considered to be the key link between the genotype and phenotype, in addition to controlling the biological aspect of cells (Im and Shin, 2015, Barros and Offenbacher, 2009). DNA methylation is a well characterised epigenetic mechanism that is mainly associated with inactivation of the X chromosome, the imprinting of specific genes and some cell-specific type gene expression (Han and Yoon, 2012, Fernandez-Tajes et al., 2014). It has previously been reported that any alteration in the degree of DNA methylation will affect the behaviour of cells such as hMSCs (Turinetto et al., 2016). This effect may guide the cells with regard to committing their differentiation to a specific cell lineage. Furthermore, at the gene level, these changes also direct and control gene expression, via gene promoters (Guilak et al., 2009). It has been reported that the stem cell can memorise external stimuli, such as mechanical and physical stimuli, following extended exposure (Yang et al., 2014). It is believed that epigenetics changes play a crucial role in cellular senescence and aging (Pollina and

Brunet, 2011). DNA methylation primarily occurs at the CpG cytosine sites, where 70–80 % of this process occurs (Jabbari and Bernardi, 2004). It has generally been reported that certain chemicals, such as cytosine analogues 5-aza-cytotidine and 5-aza-2-deoxycytidine, as well as non-nucleotide cytosine methylation inhibitors, control DNA methylation (Zheng et al., 2008).

The hypothesis of this chapter is that a low dose of BPs would exert control over hMSC phenotype via epigenetic changes. This control would primarily result from modulation of gene expression via DNA methylation.

5.2 **Methods and materials**

5.2.1 Cell culture

The effects of a single, low dose of two BPs (ALE and PAM) on the epigenetic phenotype of hMSCs were investigated. The hMSCs were plated at a density of 5×10^5 cells/75cm^2 in flasks containing MSC GM, and were cultured according to the protocols described in section 2.1.1. After 24 hours, the medium was changed to use GM containing BPs at both 100nM and 10nM, and the hMSCs were incubated for a further 24 hours. The cells were then washed, the medium was changed and the cells were grown for a further 2 weeks, during which time the medium was changed twice a week. On reaching this stage, the cells were then ready for DNA extraction.

5.3 **Epigenetic and DNA methylation**

Epigenetics is the study of a phenotypic trait that is caused by internal or external triggers, which affect cellular genes and cell behaviour. These triggers influence different processes, such as DNA methylation. The DNA methylation approach is the most commonly used, and it consists of three main steps: DNA extraction, Bisulphite conversion and Meth450k microarray processing. A full description of each step is provided in the following section.

5.3.1 DNA extraction

All DNA was extracted using a QIAamp® DNA Mini kit from Qiagen, according to the manufacturer's instructions (Qiagen, UK). This kit contains a silica membrane column to which the DNA adheres. The adherent cells were washed with PBS and then discarded, after which 0.1–.25% trypsin EDTA was then added. The cells were then collected from the GM and centrifuged for 5 min at 300xg. The supernatant was removed, and the cell

pellet was re-suspended in PBS until it reached the appropriate volume. This step was followed by the application of proteinase K, after which 200 µl AL buffer was added, the sample was mixed for 15s and was then incubated at 56°C for 10 min. An appropriate amount of ethanol (96–100%) was added, and the sample was then mixed. This mixture was then transferred to the QIAamp silica membrane column, followed by the addition of different buffers to AW1 and AW1 with centrifugation in between. Finally, the DNA was eluted with AE buffer or water. The integrity and quantity of the extracted DNA was evaluated using spectrophotometry with the Tecan Nano Drop at an excitation of 260 nm and an emission of 280 nm.

5.3.2 Bisulphite conversion

***Performed by UCL Genomics centre, UCL.**

The process was begun by conversion of 500ng of genomic DNA using a DNA Methylation™ Kit (Zymo Research, USA), according to the manufacturer's instructions. In brief, 5 µl M-Dilution buffer was added to each DNA sample. The samples were then incubated at 37°C for 15 minutes, after which 100 µl of the conversion reagent was added to each sample. The samples were then left in the dark at 50°C for 12 hours and then at 0-4°C for 10 minutes. A total of 400 µl M-binding buffer was added to each sample and mixed well, and the samples were then centrifuged at 3,000 x g for 5 minutes. A total of 400 µl M-wash buffer was added to each sample, the samples were then centrifuged at 3,000 x g for 5 minutes, and 200 µl of M-desulphonation buffer was added to each sample. The samples were then incubated at room temperature for 15–20 minutes, after which they were centrifuged at 3,000 x g for 5 minutes. These steps were followed by the addition of 500 µl M-wash buffer, followed by centrifugation at 3,000 x g for 5 minutes, another wash and then centrifugation at 3,000 x g for 10 minutes. Finally, the DNA was

eluted by adding 15 µl M elution buffer to each sample and then centrifuging each sample for 3 minutes. The DNA was then ready for the Meth450k microarray processing step.

5.3.3 Meth450k microarray processing

***Performed by UCL Genomics centre, UCL.**

The processing was conducted according to the Infinium HD Assay protocol (15019519_B, 2015; Illumina Inc., San Diego, USA). In brief, 500ng high quality bisulphite converted DNA was whole-genome amplified overnight at 37°C for 20–24 hours, using a deep well plate. The DNA was then fragmented at 37ºC for 1 hour, and placed in a hybridisation oven for 15 mins. This step was followed by DNA precipitation and then resuspension in hybridisation buffer. The samples were hybridised on to bead chips using a liquid-handling robot (Freedom Evo, Tecan Ltd, Switzerland) and incubated at 48°C for 16–24 hours. The fragmented and amplified DNA samples annealed to locus-specific 50-mers (covalently linked to one of over 500,000 bead types) during hybridisation. This step was followed by washing of the un-hybridised and non-specifically hybridised DNA. The bead chips were then prepared for staining and extension. Single-base extension (SBE) of the oligos on the bead chips, using the captured DNA as a template, incorporated detectable labels on the bead chips and determined the methylation level of the CpG sites of interest. The process of SBE and staining was carried out using the liquid-handling robot. The staining procedure itself involved signal amplification using multi-layer immunohistochemical staining. Finally, the bead chips were scanned using an iScan scanner with autoloader (Illumina Inc, San Diego, USA).

5.3.4 DNA methylation analysis

The DNA methylation data processing was run through different steps, including quality control, background correction, data normalisation, filtration and analysis, using R software version 3.3.2 and Bioconductor Illumina Human Methylation450 packages, as described in (Hansen and Fortin, 2016) and shown in Figures (2-9) and (2-10). The data were fully inspected for complete DNA bisulphide conversion, and the average beta values of each CpG residue were calculated. Data were normalised using the functional normalisation function (Fortin et al., 2014) within the Minfi Bioconductor package (Aryee et al., 2014). Cross-reactive probes were also filtered out by matching to the list published by Chen et al. 2013 (Chen et al., 2013). The normalised and filtered data was analysed for differential CpG probes using Limma (Ritchie et al., 2015). M-values were used in the regression. To avoid infinite M-values, beta values of exactly 0 or 1 where shifted to the value of 1×10^{-4} using the shiftBeta function in Harman (Oytam et al., 2016). These adjusted beta values were logit transformed to M-values. For the limma differential methylated probe analysis, a simple linear model of $y \sim 0 + x$ was used. The detection of differentially methylated ranges (DMRs) was also undertaken using DMRcate (Peters et al., 2015). Gene ontology and KEGG pathway analysis was undertaken using the 'gometh' function in the missMethyl Bioconductor package(Phipson et al., 2015) (Phipson et al., 2015).

The Human Methylation 450 Bead Chip contains over 485,000 probes. Therefore, to detect the significant hypermethylated and hypomethylated genes-associated probes, the limma decide tests function was performed to classify a series of related t-tests as up, down or not significant. The default was Benjamini–Hochberg-false discovery rate adjusted p values ($p<0.05$).

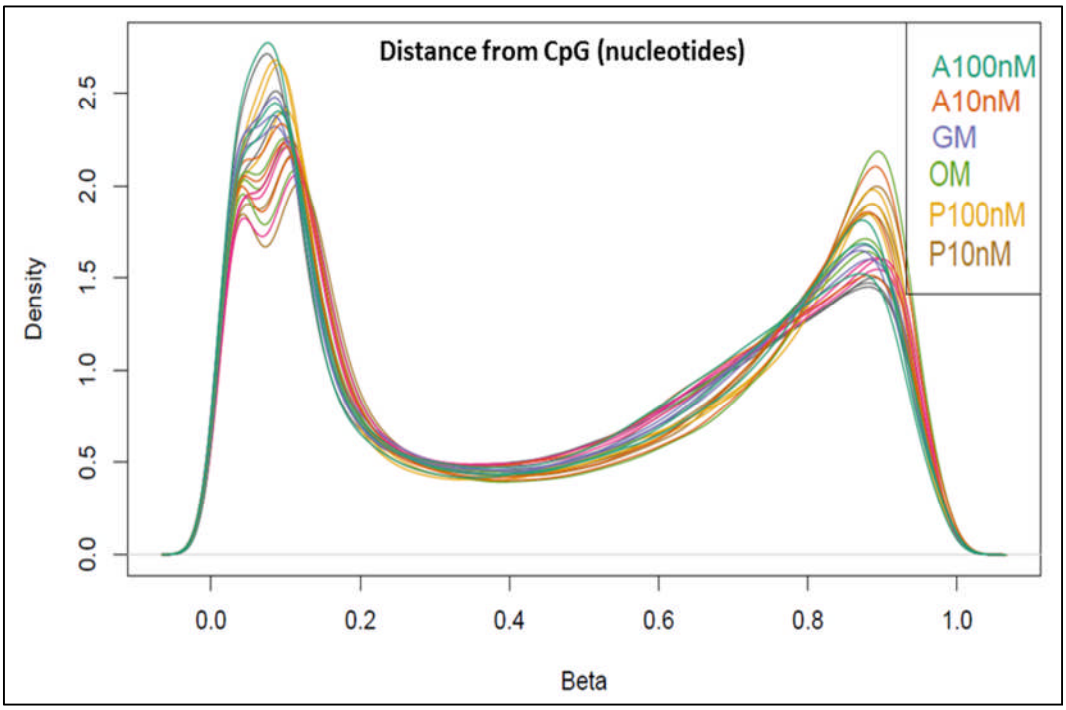


Figure 5.1: Beta values distribution of 450K data

A density plot. The graph shows the beta values distribution of 450K data for each sample, which were considered as part of a quality control check using Type I and Type II probes.

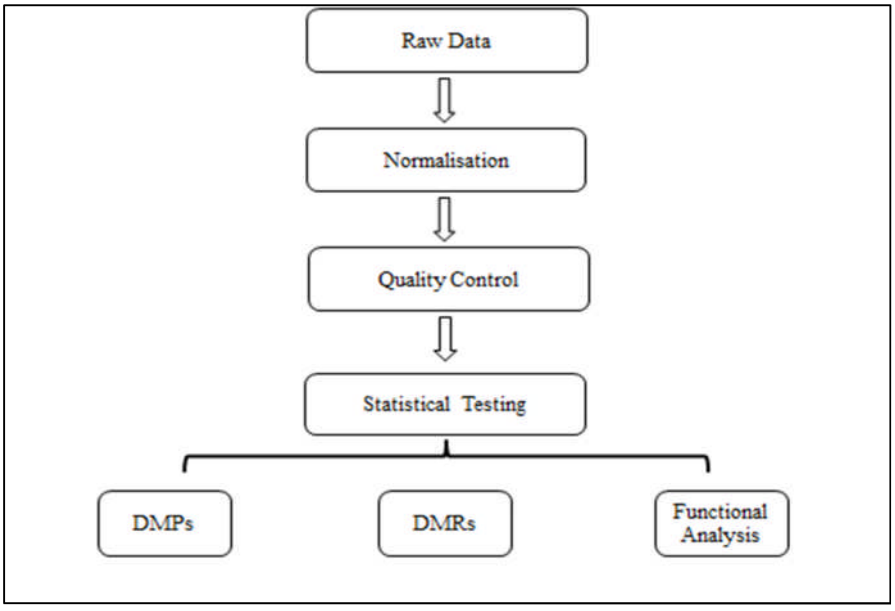


Figure 5.2: Illustrated diagram of DNA methylation analysis process

The DNA methylation data analysis process using R software version 3.3.2 and Bioconductor Illumina Human Methylation450 packages

5.4 **Results**

5.4.1 Overview of the DNA methylation analysis

DNA was extracted from treated cells, bisulfite-treated and the DNA methylation state inspected using Illumina HumanMethylation450 beadchip (450K) arrays. The 450K data was normalised and then further preprocessed to remove array probes not meeting quality criteria. In initial analysis, we discarded a total of 47,177 array probes which failed in any sample (detection p-value > 0.01), 17,541 probes with a SNP and 32,324 cross-reactive probes as identified by (Chen et al., 2013). The data showed that 395,865 gene probes were detected. Each contrast was evaluated as follows -1 indicated lower methylation, 1 signified significantly higher methylation and 0 indicated failure to refute the null hypothesis 0 (not significant). Overall, 3,450 probes were differentially hypomethylated (1,048) or hypermethylated (3,402) in the group treated with 100nM PAM. In the group treated with 10nM PAM, 6,250 probes were differentially either hypomethylated (1,517) or hypermethylated (4,733). This significant finding was observed when using a relaxed multiple testing threshold where $p<0.05$, as shown in Table 5-1. In contrast, no significant differentially methylated probes were detected when $p <0.05$ in the group treated with ALE, at both 100 nM and 10 nM doses. However, when the p value was relaxed at =0.1, a few probes were significantly methylated - 23 for the 100nM ALE group and one for the 10nM ALE group, as shown in Table 5-2. The results will be discussed in detail in the following sections.

Table 5.1. Significant CpG probe summaries of p=0.05 where -1 signifies lower methylation, 1 indicates significantly higher methylation and 0 signifies failure to refute the null hypothesis 0 (not significant).

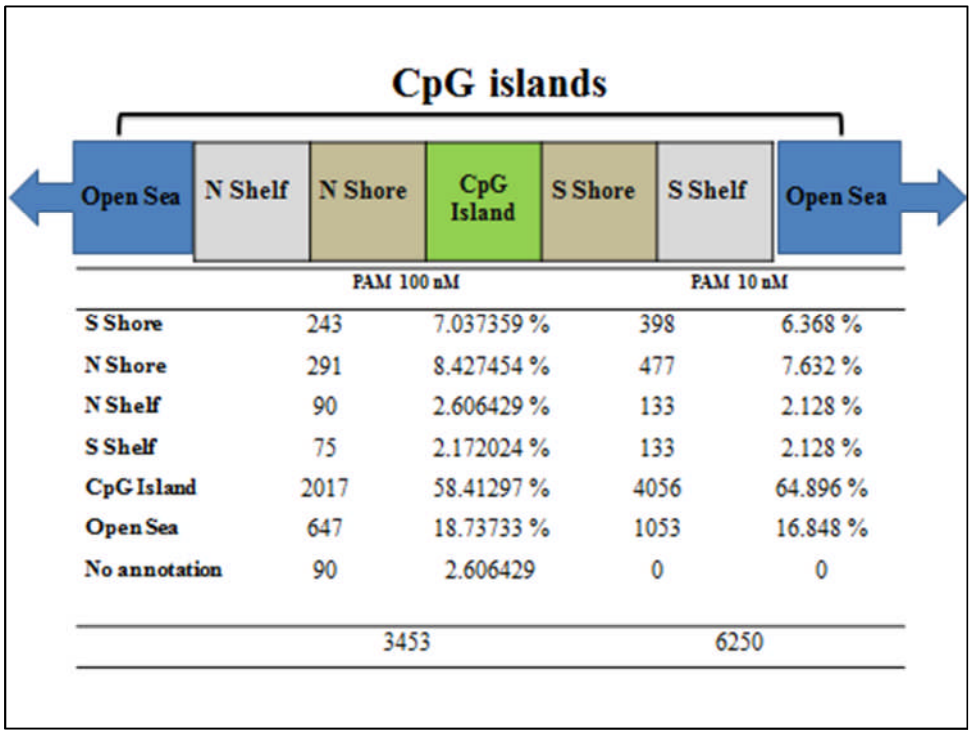
	ALE 10-GM	ALE 100-GM	PAM10 – GM	PAM 100 – GM	OM – GM	ALE 100 - ALE 10	PAM100 - PAM10
-1	0	0	1517	1048	2	0	0
0	395,865	395,865	389,616	392,413	395,862	395,865	395,865
1	0	0	4,732	2,404	1	0	0

Table 5.2. Significant probe summaries of p=0.10 where -1 signifies lower methylation, 1 indicates significantly higher methylation and 0 signifies failure to refute the null hypothesis 0 (not significant).

	ALE 10-GM	ALE 100-GM	PAM10 – GM	PAM 100 – GM	OM - GM	ALE 100 - ALE 10	PAM100 - PAM10
-1	1	19	3283	3843	2	0	0
0	395,864	395,842	382,527	385,851	395,860	395,865	395,865
1	0	4	10055	6171	3	0	0

5.4.2 Genomic and functional distribution of differentially methylated CpG locations

Following the drug treatment, the distribution methylated probes of CpG were analysed according to genome island, shores, shelves and open sea. The data showed that, for the group treated with 100 nM and 10 nM PAM, the CpG island primarily had the highest percentage of the significantly methylated probes, with values of 58.41 and 64.9, respectively. In contrast, the N shelf was associated with the lowest percentage of methylated probes, as shown in Figure 5-1.



	PAM 100 nM		PAM 10 nM	
S Shore	243	7.037359 %	398	6.368 %
N Shore	291	8.427454 %	477	7.632 %
N Shelf	90	2.606429 %	133	2.128 %
S Shelf	75	2.172024 %	133	2.128 %
CpG Island	2017	58.41297 %	4056	64.896 %
Open Sea	647	18.73733 %	1053	16.848 %
No annotation	90	2.606429	0	0
	3453		6250	

Figure 5.3: Distribution of methylated probes in CpG island regions.

CpG island had the highest percentage of the significantly methylated probes, with values of 58.41 and 64.9 for 100nM and 10nM PAM, respectively.

We also investigated the pattern of DNA methylation across the genome according to the functional distribution in locations within the gene transcript structure. Each transcript was divided into functional regions, beginning from TSS1500 (1,500 base pairs within the transcription start site), TSS200 (200 base pairs within the transcription start site), 5′ UTR (5 prime untranslated region), the first exon, 3′ UTR (3 prime untranslated region) and the gene body. The data revealed that the least DNA methylation occurred in the 3'UTR region at both doses of PAM. The most methylation occurred in the body and the TSS200 region. The overall pattern was the same in the first exon,TSS1500 and 5'UTR for both doses of PAM as shown in Figure 5-2.

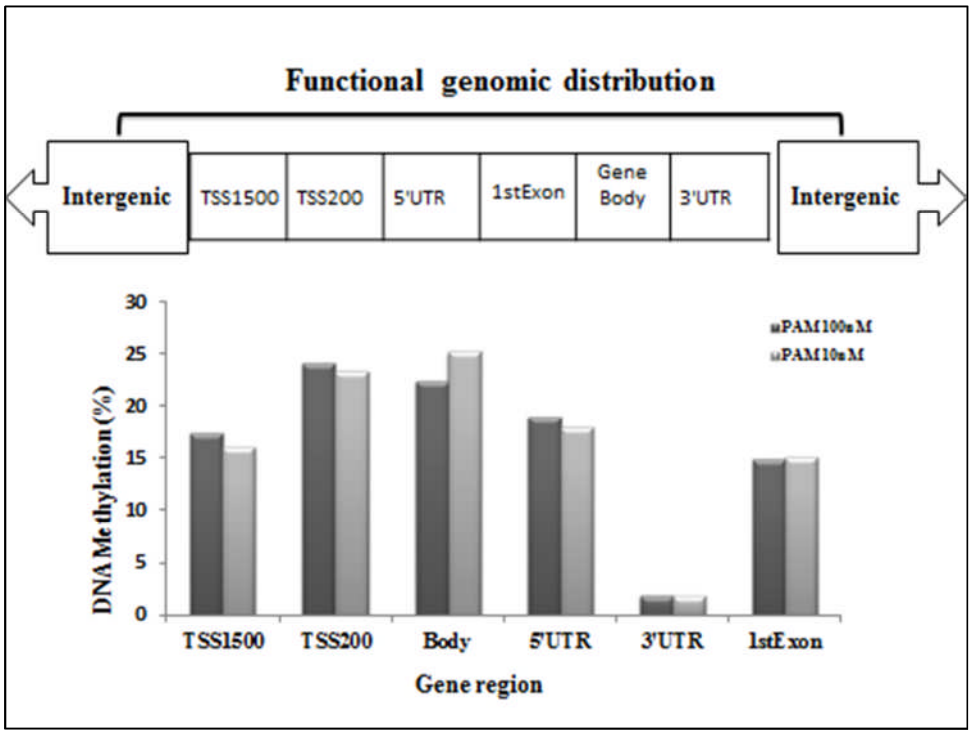


Figure 5.4: DNA methylation pattern.

The bar chart shows the percentage of the most affected DNA regions, whereby the 3'UTR was the least affected and the TSS200 and gene body were the most affected at both PAM doses.

5.4.3 Chromosomal distribution of differentially methylated probes

Across all of the chromosomes, the data showed an accumulation of methylated probes in certain chromosomes. The majority of the methylated probes were located in chromosomes 6, 12, 17 and 19 following an application of 100nM PAM. Similarly, for the group treated with 10nM PAM, the most affected chromosomes included chromosomes 1, 6, 11 and 19, whereby most of the methylated probes were present following application of the 10nM PAM dose, as shown in Figure 5-5.

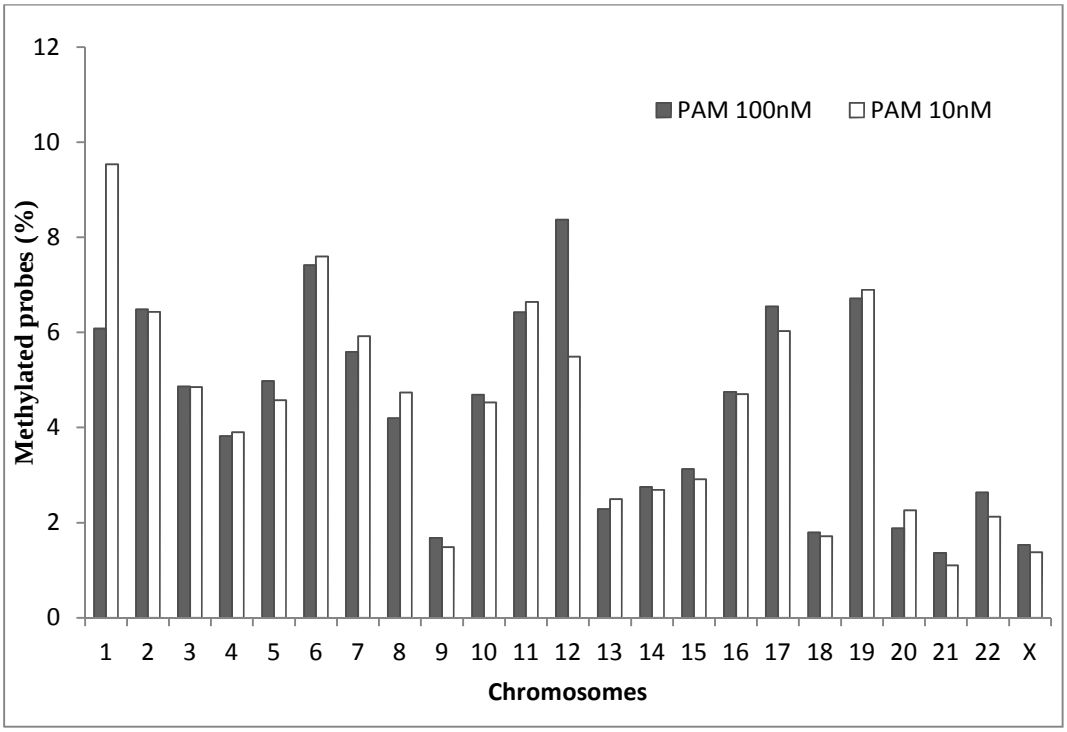


Figure 5.5: Chromosomal distribution of methylated probes.

For the group treated with 100nM PAM, notice the difference in the most affected chromosomes, including 6, 12, 17 and 19, where most of the methylated probes are present. For the group treated with 10nM PAM, the most affected chromosomes included chromosomes 1, 6, 11 and 19, where most of the methylated probes were present.

5.4.4 Differentially methylated probes (DMPs)

As mentioned previously, the total number of DMPs observed for the group treated with 100 nM PAM was 3,450 probes and for the group treated with 10nM PAM was 6,250 probes, as compared to the control group. Figure 5-7 and 5-8 indicates the heat map of 100 most significant differentially methylated probes of PAM 100nM and PAM 10nM. Furthermore, a summary of the top 25 most significantly DMPs is given in the appendix Tables 8-3 and 8-4. The effect reflects the most affected probes, with their genes as well as the island locations. The table columns show the probe ID, island location, gene name, gene location, P values and adjusted P values. The genomic features of DNA methylation were based on for DMRs mainly based on CpGs island. These features were categorised

as gene body, 5′UTR, 3′UTR, TSS200 and TSS1500. For example, the most significant probe for the group treated with 100nM PAM was cg22867714, which is a promoter of the Von willebrand factor gene. For the group treated with 10nM PAM, cg20733436 was the most significant probe, and is responsible for the TMED7-TICAM2 gene.

However, the MDS projection (Figure 5.6) implies PAM induces a larger shift in the methylome than other treatments. Further, with the PAM replicates clustering much closer, it is easier to detect small differences in methylation using a differential test and two of the ALE beadchip arrays have higher number of failed probes than the other beadchip arrays. A relaxation of the multiplicity-correction p-value cutoff to p=0.10 yields some ALE DMPs, however, this is marginal compared to the number of DMPs in PAM comparisons and similar to the OM comparison (Table 5-2). Removal of the two ALE samples with higher probe failure rates did not have a large impact on the number of significant probes. In this instance, with the multiplicity-correction p-value cutoff to p=0.10, there were 4 DMPs for the ALE 100 nM with GM comparison, but none for the ALE 10 nM with GM, nor ALE 100 nM with ALE 10 nM comparison. To further investigate the lack of DMPs for the ALE comparisons, we considered the differences in medians for each group. A single outlier sample in a group size of three, will increase the within-group variance, making it harder to find significant probes, but it will not shift the median. Therefore we computed median beta value differences between the ALE group and GM group and likewise, PAM with GM and OM with GM. We examined the median beta differences across groups for the subset of subsets significant in either of the PAM with GM comparisons.

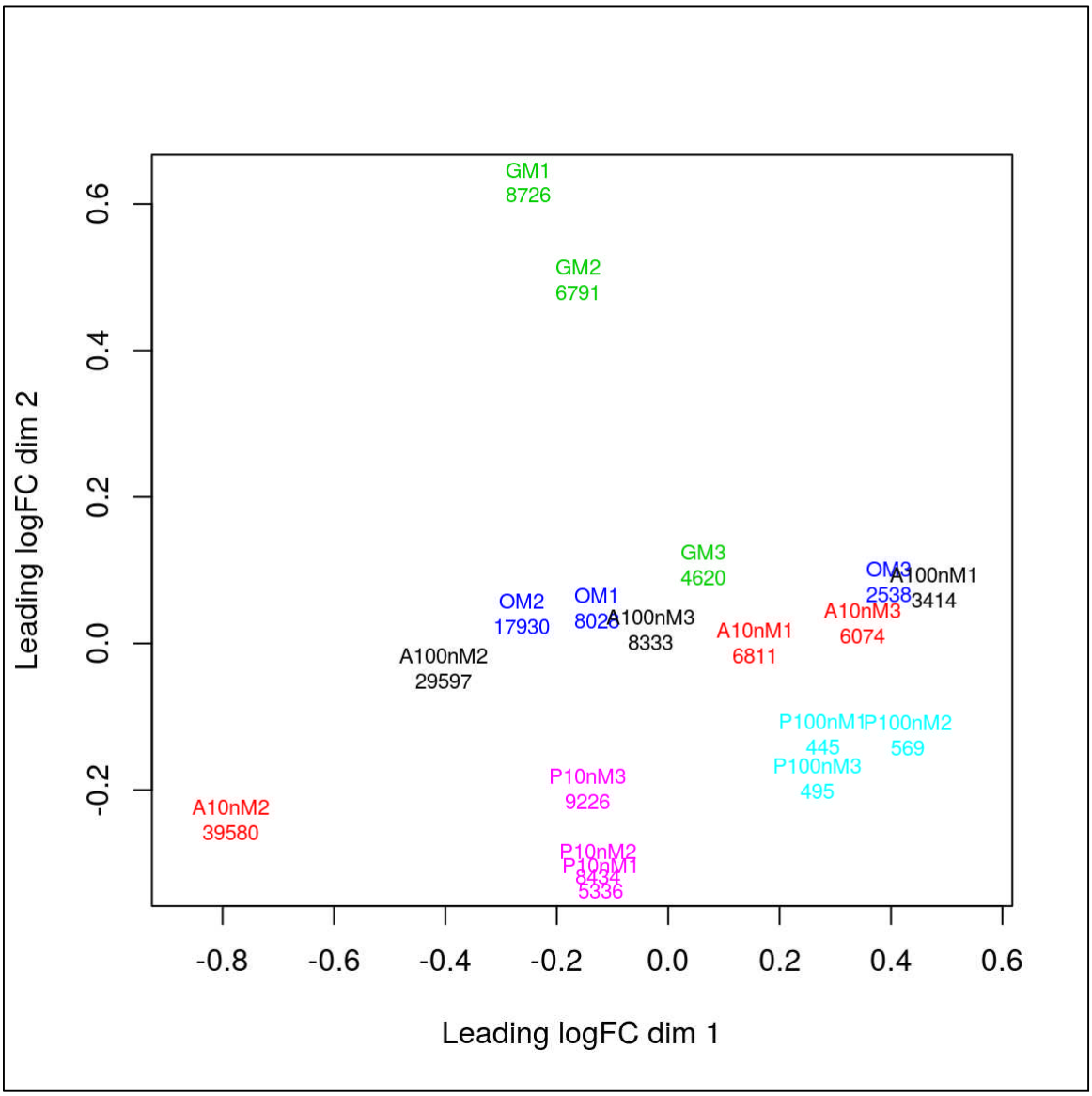


Figure 5.6: Multidimensional scaling (MDS) plot

Classical multidimensional scaling (MDS) plot of the 100,000 most variable probes. Experimental group is denoted by colour and the number under the sample name denotes the sum of probes for that beadchip array failing the detection threshold.

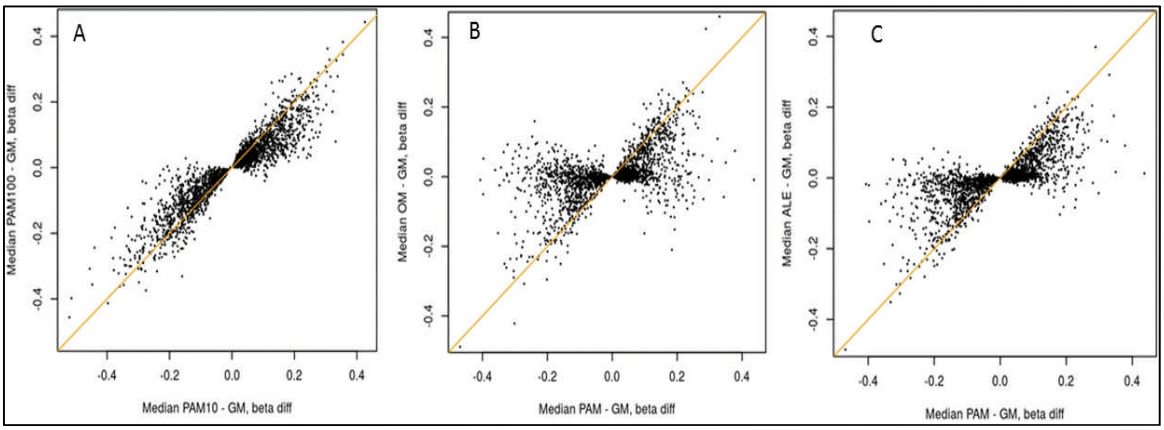


Figure 5.7 : Median beta differences

Comparing median beta differences across groups. The groups for comparison are a) PAM 10 nM and PAM 100 nM, b) collective PAM (10 nM and 100 nM) with OM and c) collective PAM with ALE. Plotted probes are constrained to the 4,733 DMPs significant in either of the 10 nM or 100 nM PAM comparisons with GM.

Figure 5.7 (A) shows that, relative to GM, the median differences in beta were mostly similar between the PAM 100 nM and PAM 10 nM groups across the 4,733 PAM DMPs (extensive tracking on the orange line). Figure 5.7 (B and C)show that some ALE and OM probes have shifted betas compared with GM, but many probes do not. Collectively, our global and DMP analyses suggest there is less modulation of the methylome by treatment with ALE and OM and what changes do occur aren't being detected as significantly different due to the larger within-group variance in the ALE and OM groups. This within-group variance seems partially technical in nature, with one beadchip array per group having a higher number of failed probes. We note that most of the DMPs had small effect sizes. Of the PAM 10 or 100 nM DMPs, only 112 (2.4%) and 91 (2.5%), respectively, had mean beta difference of greater than 0.25.

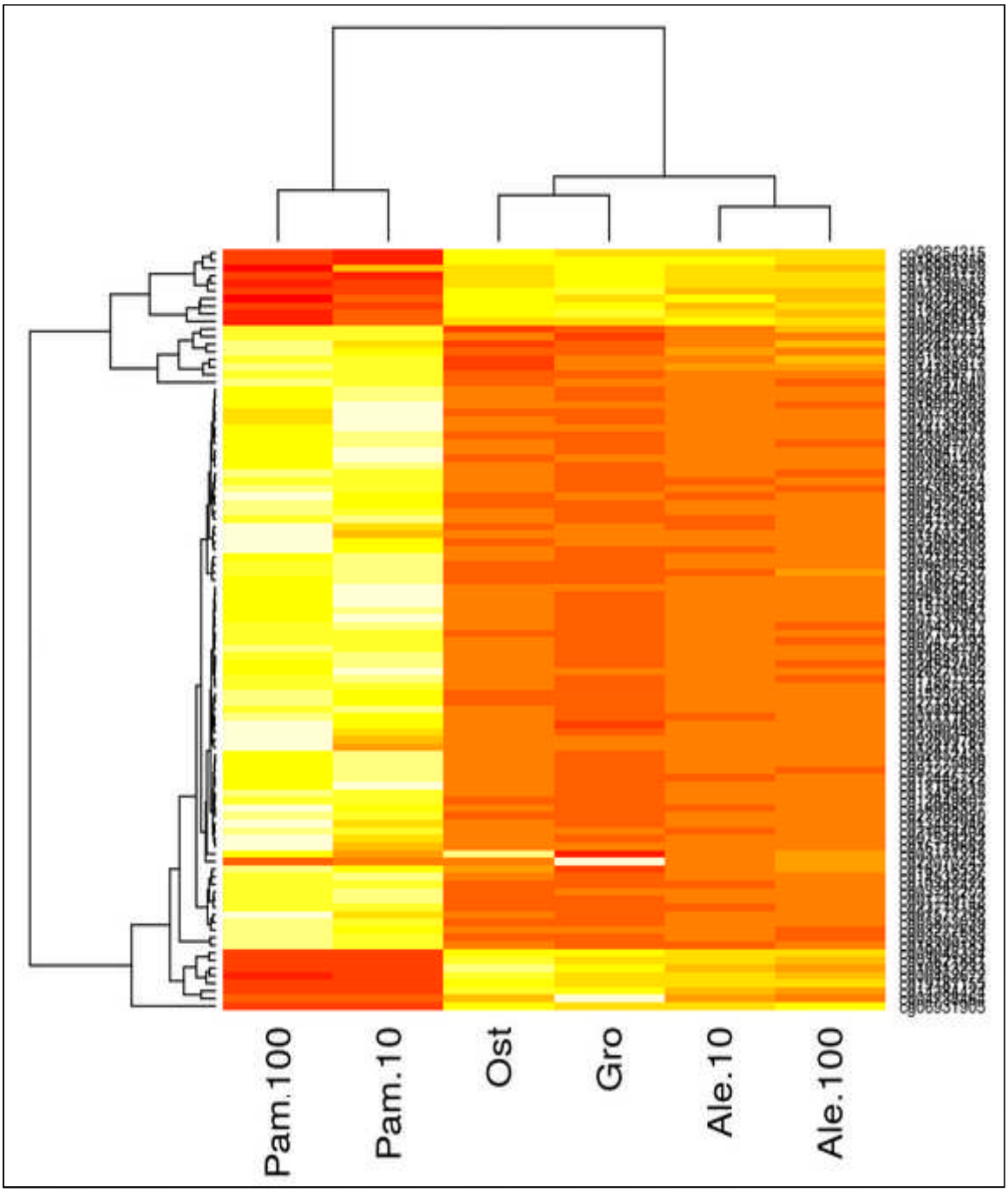


Figure 5.8: Heat map of 100 most differentially methylated PAM 100 nM probes

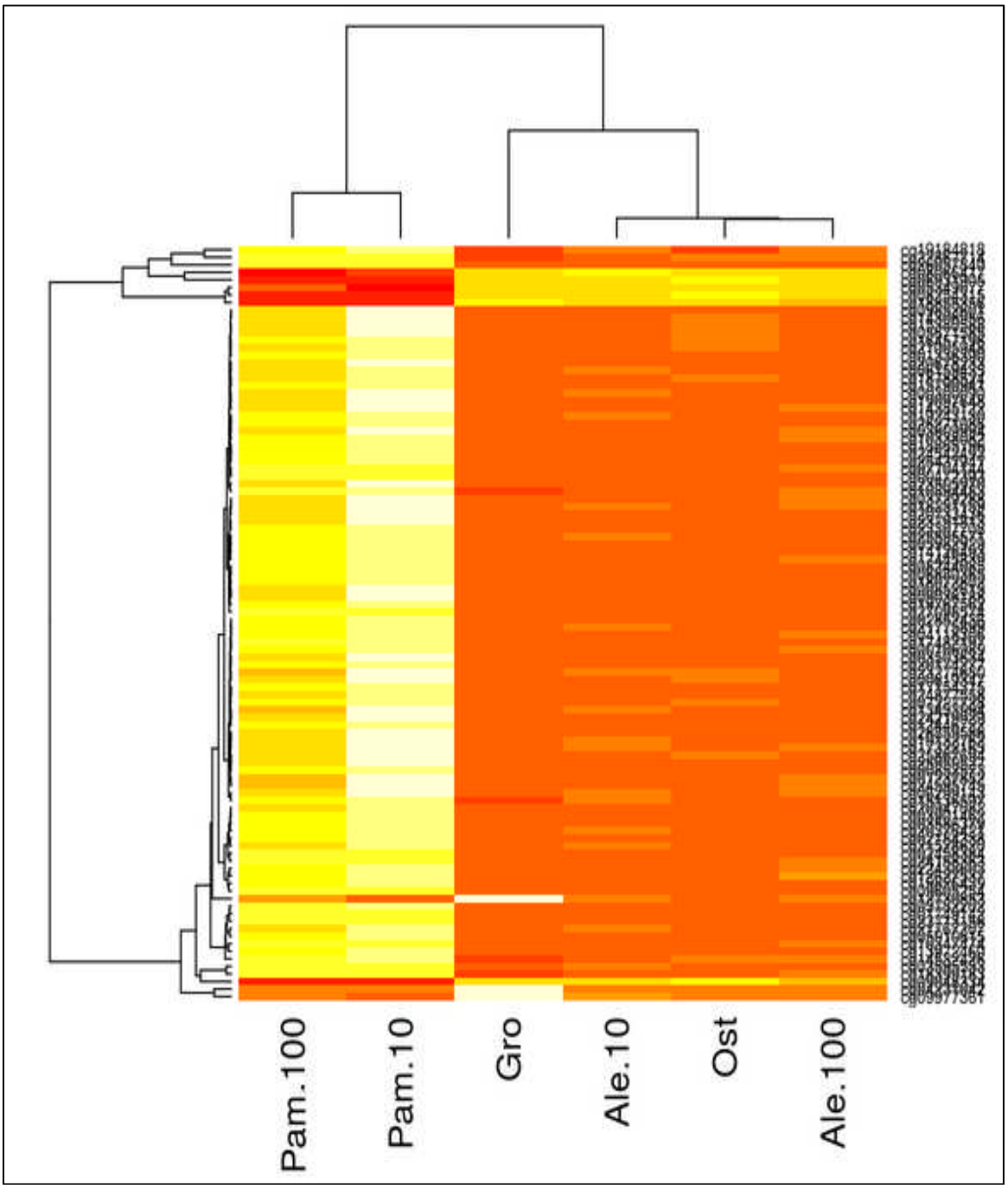


Figure 5:9: Heat map of 100 most differentially methylated PAM 10 nM probes

5.4.5 Differential methylation regions

A DMRcate package (developed by CSIRO, Australia) was used to analyse and explore the most DMRs, as well as differential tests for CpG sites. This was carried out by setting up the DMRcate to require at least three CpGs within a DMR to report it. This analysis was carried out on probes that passed a relaxed adjusted p value test, followed by the Benjamini-Hochberg test. The detection of differentially methylated regions (DMRs) was also undertaken using DMRcate. Considering that neighbouring CpG sites have a high correlation in their methylation state (Guo et al., 2017), the detection of DMRs identifies regions likely to represent robust methylation changes of biological importance. We found 386 DMRs for the PAM 100nM and 515 DMRs for the PAM 10 mM comparisons. Matching across the two sets of DMRs showed 210 regions had overlapping genome coordinates . The appendix tables 8.3 and 8.4 summarise the top 25 DMRs, including the gene name and transcription type. In order to identify the most detectable CpGs, the average percentage change of DMR values was calculated, βs, after normalisation by best and worst performing samples.

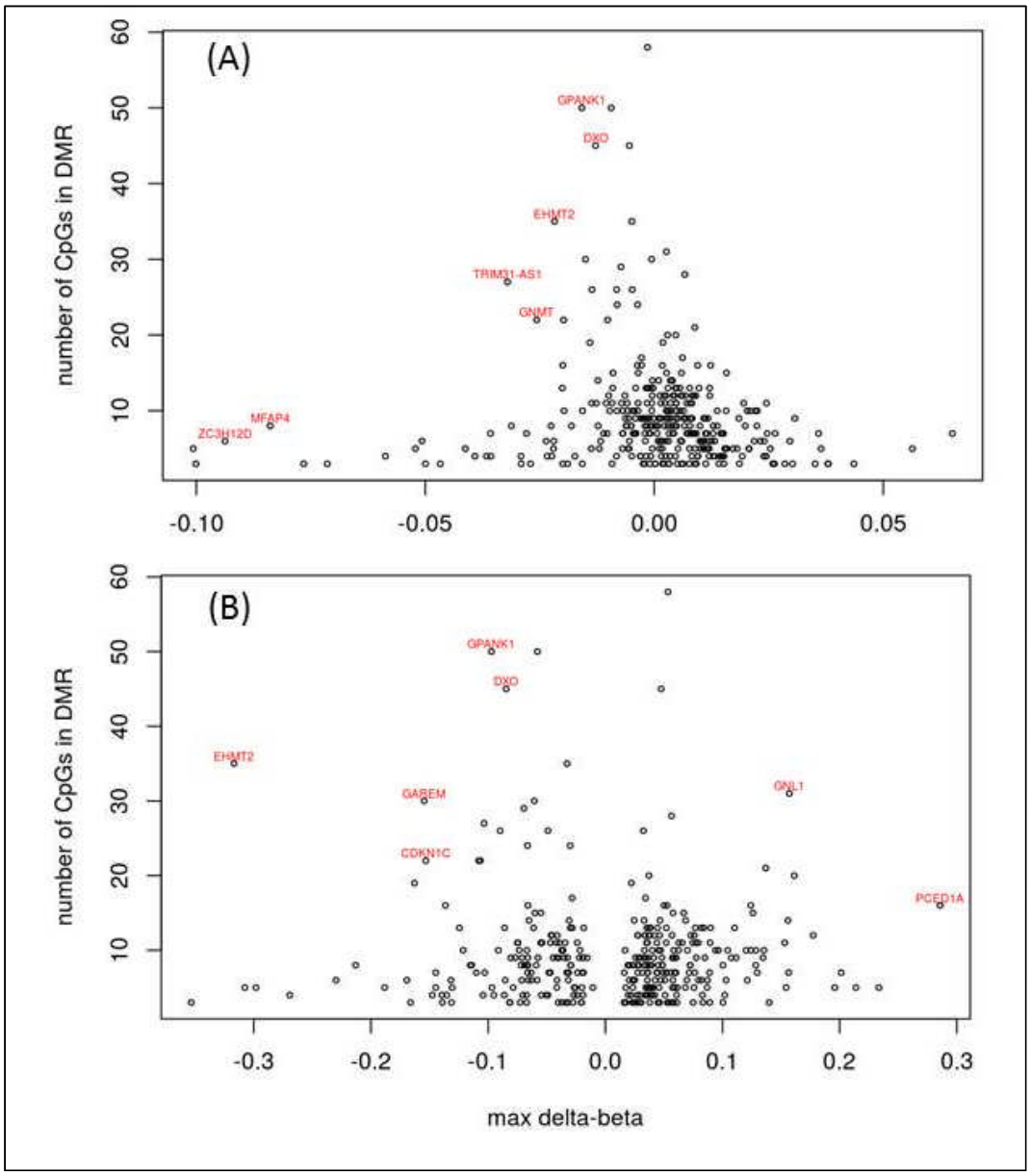


Figure 5.10: Maximal change in methylation of Δ beta values

DMR characteristics – maximal change in methylation (max Δ beta) by the contiguous length (number of CpGs). Those DMRs above the 95th quantile for DMR absolute delta-beta by number of CpGs have proximal gene names given in red. Only the DMRs for the a) PAM 10 nM and b) 100 nM contrasts which have common genomic region overlaps of at least 1 bp are plotted.

5.4.6 Gene ontology enrichment

To undertake gene ontology (GO) enrichment, we used Gometh as it controls for bias due to the variable number of CpG array features per gene. DMPs with an adjusted p-value of <=0.01 were analysed for enrichment with Gometh and the top 50 GO terms recorded. The same exercise was also performed with only those DMPs which overlap any of the DMRs. For all three analyses, no p-values were significant after false discovery rate (FDR) correction. However, common themes across the analyses are GO terms associated with insulin signalling, development, inflammation/immune processes and responses to cellular damage (such as UV or gamma radiation).

5.5 Discussion

In this chapter, we showed that the low dose of BPs was associated with epigenetics changes in hMSCs via DNA methylation mechanisms. The dependency of the effect of DNA methylation on BP dose and type was expected. It was shown that both the 100 nM and 10nM PAM doses had an effect on hMSC phenotype and behaviour. This effect can be explained as being due to a cellular interaction with external stimuli, such as chemical stimuli. In addition, our data suggest that the low dose of PAM may have acted as an external trigger that may have committed the cells to differentiate to a specific lineage, as well as increasing their proliferation rate and accelerating osteogenic differentiation. In this chapter we showed that the low doses of 100nM and 10nM PAM significantly affected a wide range of gene probes. Each gene has a unique role in cellular development, including proliferation and differentiation. The role and involvement of a wide range of these genes in the life, growth and development of cells, particularly stem cells, remains to be elucidated.

DNA methylation, as one type of epigenetic modification, can be affected by a variety of factors, such as age, gender, medical condition and environmental factors. In this project, we assessed single DMPs as well as DMRs following single dose application of BPs. However, we primarily focused on DMRs, as these provide strong evidence compared to individual DMPs, and this evidence is statistically powerful (Lister et al., 2009, Jaffe et al., 2012). With regard to the DMPs, we investigated the relationship between the most affected probes in relation to chromosome type and genomic distribution. Interestingly, our data showed that both chromosomes 11 and 19 were associated with the most methylated probes in both groups treated with 100nM and 10nM PAM doses. This is because chromosome 11 is one the most gene-rich chromosomes, and chromosome 19 has the highest gene density (Grimwood et al., 2004). This also explains the increase in cell proliferation rate following BP treatment. Furthermore, these observations highlighted the differences between groups treated with BPs in terms of dose, most affected probes and chromosomal distribution. The genomic distribution of the most affected probes was mainly located in the gene body and the TSS1500 region. This may have been due to an increase in transcriptional activity, as well as gene expression (Maunakea et al., 2010). Furthermore, our data showed that the significant DMPs were mainly found in the CpG island for both the 100nM and 10nM PAM doses; these findings suggest that methylation in the CpG island regions is more susceptible to external factors, such as BPs. However, ALE failed to produce significant probes. This could have been due to a small number of replicates or because it is mostly biological in nature. In addition, although ALE may have an effect on epigenetic changes, this effect may involve different mechanisms, such as histone modifications and chromatin remodeling.

The current findings have some limitations, such as the fact that the drug effects were studied in an *in vitro* environment, and other factors may be involved in these mechanisms in the *in vivo* environment. Therefore, the *in vitro* approach is considered as a useful measure for early screening of potential targets and also provides an early step in testing drug effects and possible implications. However, in an attempt to overcome this limitation, we examined at least three methylation sites in CpG for each gene regulatory region. Furthermore, epigenetic changes may be influenced by the source of hMSCs, whereby the cells may have already undergone initial alterations in DNA methylation, but to the best of our knowledge no clear evidence of that has previously been shown.

5.5.1 Significant findings

The data show that low, single doses of BPs have an effect on hMSC genotype and behaviour. Methylation of most of the genes probes where each gene has a role in cell development and commitment was observed. These findings may provide an explanation for increased hMSC proliferation, as well as osteogenic differentiation. Most of the DMPs were located in chromosomes 11 and 19, and most of the gene probes involved were responsible for early hMSC development and growth.

5.5.2 Summary of the results

The epigenetic effect of a single low dose of PAM via DNA methylation explains, and provides evidence of, why this drug increases hMSC proliferation and osteogenic differentiation via the control of several gene probes. It also appears that the drug may have direct control over stem cell memory. In addition, our findings showed that there was a correlation between differential methylation in hMSCs and low-dose BP treatment. These findings provide evidence of the potential functional importance of methylation in hMSC phenotype. Finally, together, these finding suggest that an understanding of the

phenotypic changes that occur during the life cycle of hMSCs could provide a clear vision with regard to the success of using these cells in the medical field and in understanding the underlying mechanisms of these external triggers.

6 Low Dose Effect of BPs on hMSCs osteogenic response to Titanium surface In vitro

6.1 Introduction

In recent decades, titanium (Ti) implants have been successfully used to replace missing teeth. This success is mainly due to the good biocompatibility of Ti and the phenomenon of osseointegration. The surface properties of the implant play an integral role for implant osseointegration (Le et al., 2007, Colombo et al., 2012). Also, the topography and chemistry of the surface properties can control the quantity and quality of adhered cells to the implant (J.I.Rosales-Leala, 2010, Logan and Brett, 2013). It has been reported that optimal surface roughness can lead to effective osseointegration (Halldin et al., 2014). This effect was due to increased osteoblast proliferation, differentiation and matrix protein production, e.g. collagen type I.

The coating of metal dental implants with different bone inducing materials can induce positive effects. These materials include, bone stimulating factors, BPs, fluoride, HA and calcium phosphate (CaP), and titanium/titanium nitride (Logan et al., 2014, Xuereb et al., 2015). CaP which is mainly composed of HA has been shown to induce osteogenic differentiation and osteoblasts growth on implant surfaces (Yang, 2001). It has been reported that coating implants with growth factors such as BMP stimulates bone formation and improves implant success (Wikesjo et al., 2008). HA also accelerates new bone formation by activation of osteoblast proliferation and differentiation (Kweon et al., 2014, Lee et al., 2014, Yang et al., 2010). However, the long-term survival of HA-coated dental implants is still a controversial clinical issue. Plasma spraying is a commonly used technique to coat implants, but this technique has the disadvantage of requiring

substantially thick coating layers and furthermore, controlling the final coating composition is difficult. However, titanium implants are widely used in the clinic because of their strength, low stiffness and light weight.

We have shown in chapter 3 that ALE and PAM promote osteogenic differentiation of hMSCs *in vitro* (Alqhtani et al., 2014). These findings suggest that BPs may have a direct stimulus on osteoblast mineralisation. It has also been reported that coating implants with bisphosphonate-eluting fibrinogen may improve implant osseointegration, but there are risks in developing chronic osteomyelitis (Abtahi et al., 2012).

The aim of this chapter was to investigate whether low doses of BP enhanced proliferation and osteogenic differentiation of hMSCs on Ti surfaces. We investigated the effect of these drugs on cell proliferation, migration, adhesion and attachment. Osteogenic differentiation markers were measured including calcium, collagen type I and ALP.

6.2 **Experimental protocols**

6.2.1 Sample preparation

Polished titanium discs 15 mm in diameter were used and had been designed to fit into the wells of 24-well tissue culture plates (Helena Biosciences). In order to measure the surface hydrophobicity of smooth titanium, a contact angel measurement was performed using an optical contact angle meter (KSV Instruments Ltd., CAM200). A droplet (2 µl) of distilled deionized water (DDH_2O) was placed on the substrate surface, and the image of the drop was recorded and analysed using KSV CAM 200 instrument and software. The surface roughness and topography were analysed using profilometry (Scantion, Proscan 1000). An area of 6.25 mm^2 was examined (n=6); the Proscan provided software allowing us to analyse the surface roughness by obtaining the Ra value. These values

were visually confirmed and analysed using an FEI XL30 FEG Scanning Electron Microscope (FEI, Eindhoven, Netherlands) (Figure 6.1).

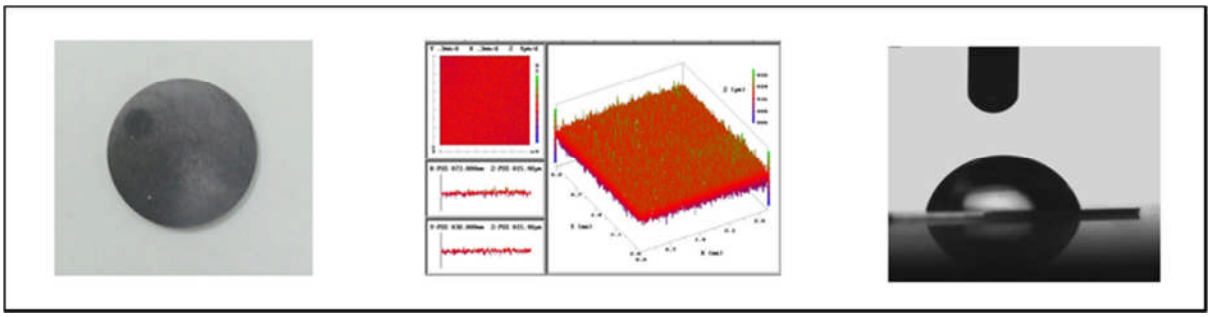


Figure 6.1: Titanium disc preparation

15-mm diameter polished titanium discs were used and were designed to fit into the wells of 24-well tissue culture plates. The surface roughness and topography were analysed using profilometry where the Ra value=0.63 µm and the contact angle were performed with a mean of 69.1 ± 3.7° (n=9).

To prepare the Ti discs for cell culture experiments, the discs underwent multiple steps, as shown in (Figure6.2). The discs were sonicated for 5 minutes in isopropanol on both sides in an ultrasonic cleaning tank (RS Components Ltd.UK) followed by immersion in DDH_2O for 15 minutes then allowed to air dry. The discs were then transferred to a fume hood and immersed in 0.1-N nitric acid inside the hood for 10 minutes followed by DDH_2O for 2 minutes. Finally, in order to sterilise the discs, they were placed in an ultra-violet (UV) machine for 20 mins on each side (BONMAY, OEM, China).

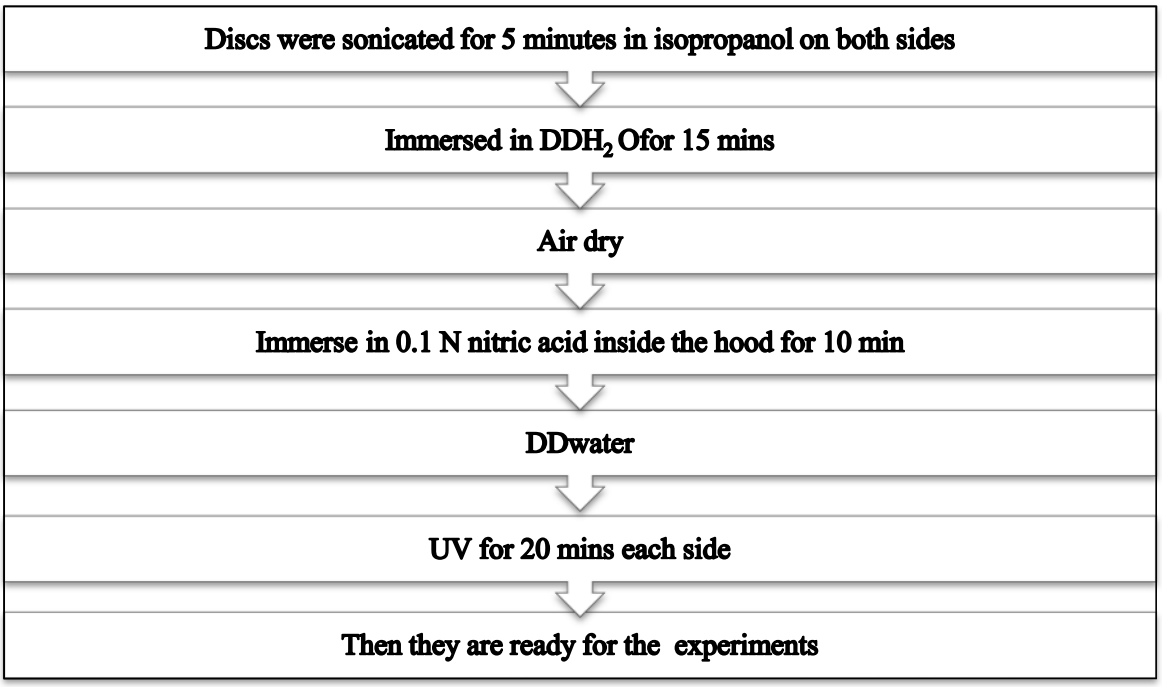


Figure 6.2: Illustrated diagram summarising the preparation steps of Ti discs for the cell culture experiments.

6.2.2 Assessment of Cell Proliferation

The effects of low doses (100nM and 10nM) of ALE and PAM on hMSCs' proliferation were measured by seeding $1x10^4$ cells per well in a 24 well plate. The cells' numbers were serially measured at different points of time days 1, 3, 7, and 14 using alamar blue assay as described in section (2.4).

6.2.3 Cell attachment

This test was performed to analyse whether BPs may promote cellular attachment to the substrate. Cells were grown in GM and incubated at 37ºC at 5% CO_2 for 24 hours. After that, the media was removed and replaced with fresh growth media. Cells were then counted using Alamar blue assay as discussed in 2.4.

6.2.4 Cell retention

In order to understand how well the cells retain the substrate following BP treatment, a retention test was carried out. The cells grew and were left for 4 and 24 hs to attach to the substrate. The media was then discarded, and the substrate was washed 3X with PBS; each wash lasted for 5 min in an orbital shaker followed by placing fresh growth media. Cells were then counted using Alamar blue as discussed in section 2.4.

6.2.5 Cell Migration

Cell migration assays (Cultrex, 3465.24k) have been used to assess the migration of cells toward the Ti surfaces in the presence of BPs. Briefly, hMSCs were cultured in serum-free media 24 h prior to the assay. The next day, 10 X 10^4 cells per well (in triplicate) were seeded onto an 8µm pore polymer membrane in an insert chamber to facilitate cell migration. A 500µl aliquot of GM was placed at the bottom of the chamber, and the cells were incubated at 37ºC and 5% CO_2 for 4.5 hours. The membranes were washed three times with a wash buffer and incubated with a cell dissociation solution containing calcien AM at 37°C for 60 minutes. Two 1000µl aliquots were removed from each well, and the fluorescence intensity (excitation ʎ= 530 nm, emission ʎ= 590 nm) of each was measured on a plate reader (BioTeK FLX800).

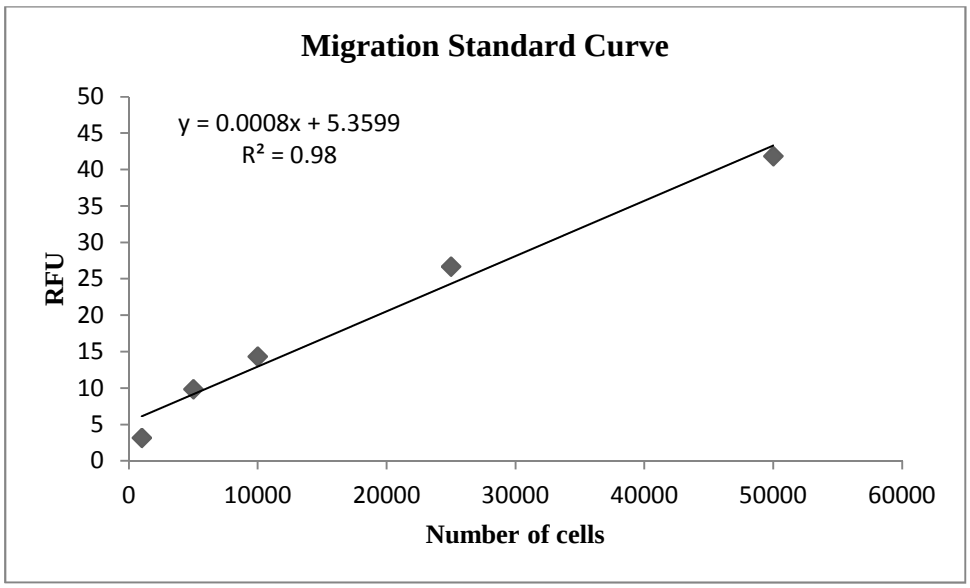


Figure 6.3: A standard curve of a migration assay.

The figure shows a line graph of a five-point standard curve with the mean reference fluorescence uptake plotted against cell numbers. Values were gained by plating a known cell number in a 24-well plate. The cells were then incubated with calcien AM stains for 60 mins, then the fluorescence intensity of each was measured on a plate reader as stated in (2.8). Each point represents the mean of three replicates (n=3)..

6.2.6 Assessment of Cells Mineralisation

The hMSCs' osteogenic differentiation was assessed by seeding 10,000 cells /well in a 24 well plate in OM. Cell cultures were maintained by changing the media every 2-3 days. The osteogenic markers were evaluated as described in the next sections

6.2.6.1 Quantification of Calcium Deposition

The total calcium formed by cells was examined at day 21 post seeding with a colorimetric quantichrom kit (BioAssay Systems) according to the manufacturer's instructions, as described in section (2.5.1).

6.2.6.2 Quantification of ECM Collagen

The total amounts of ECM formed by hMSCs were measured at day 14 post seeding using the Sirocel collagen assay kit according to manufacturer's instructions (Biocolor) as described in section (2.6). The total amount was measured from three replicate as it was difficult to measure form a single well due to the small quantities produced by small cells number.

6.2.6.3 Alkaline Phosphatase Activity

The ALP activity was assessed at day 7 post seeding using the colorimetric SensoLyte® pNPP Alkaline Phosphatase Assay Kit (Cambridge Bioscience) according to the manufacturer's instructions as described in section (2.5.3).

6.2.7 Vinculin and Actin staining

To analyse the effects of low BP doses on cell adhesion and attachment, the expression of the focal adhesion proteins, vinculin and F-actin, were studied using fluorescently labelled markers. The cells were allowed to mature in 24-well plates and incubated with ALE and PAM in OM for 24 h. The next day, the medium was discarded and the cells were washed with PBS and fixed with 4% paraformaldehyde for 20–30 minutes. The cells were then rinsed with PBS, blocked with 10% goat serum in PBS for 30 minutes at room temperature, and incubated with anti-vinculin antibody (Abcam) at 1:200 dilution and left at 4°C overnight. The next day, after several PBS washes, the cells were incubated with goat anti-mouse IgG (Life Technologies) at a 1:200 dilution for 1 h at room temperature in the dark. The cells were washed three times in PBS (5 min each) and stained with 4′, 6-diamidino-2-phenylindole (DAPI) for nuclear visualisation. For F-actin, the hMSCs were stained with phalloidin-iFluor 488 in PBS (Abcam, 1:1000). Finally, the cells were washed and viewed using a Leica–DMIRB fluorescence microscope equipped with

COOLSNAP Monochrome Camera. Images were collected and processed with image J Imaging System.

6.2.8 Scanning electron microscopy

A scanning electron microscope (SEM) was used to analyse cell morphology in this study. In order to prepare the samples to be imaged, the samples were washed three times with PBS and then fixed in 3% glutaraldehyde (Agar Scientific, UK) in 0.1-M cacodylate buffer (CAB) for 24 h at 4˚C. The next day, the fixative was removed; the cells were then dehydrated in a series of graded ethyl alcohols for 10 min at each concentration, and dried with hexamethyldisilazane (TAAB Ltd, UK) in foil cups for 2–5 min with subsequent removal and drying in a fume hood for at least an hour. The dried samples were mounted on stubs, coated with gold, and viewed using a FEI XL30 FEG Scanning Electron Microscope (FEI, Eindhoven, Netherlands).

6.3 **Results**

6.3.1 Surface properties

As shown in table 1, surface roughness was analysed where the mean the of R_a value=0.63µm (n=5) and the contact angle was performed with a mean of 69.1 (n=9).

Table 6.1: surface roughness and contact angle of Ti surface.

	Mean	± Standard deviation
Surface roughness	0.63µm	±0.004 µm
Contact angle	69.1°	± 3.7°

6.3.2 Proliferation

Human MSC proliferation in GM was examined over 1, 3, 7 and 14 days. ALE (100nM and 10nM) stimulated significant cell proliferation in all cells on days 1, 3, 7 and 14 (Figure 6.4 (A)). Also, cells that had been treated with 100nM PAM showed significant proliferation when compared to control cells which were treated with GM only. Cells treated with 10nM PAM promoted cell proliferation but it was not statistically significant (Figure 6.4 (B)).

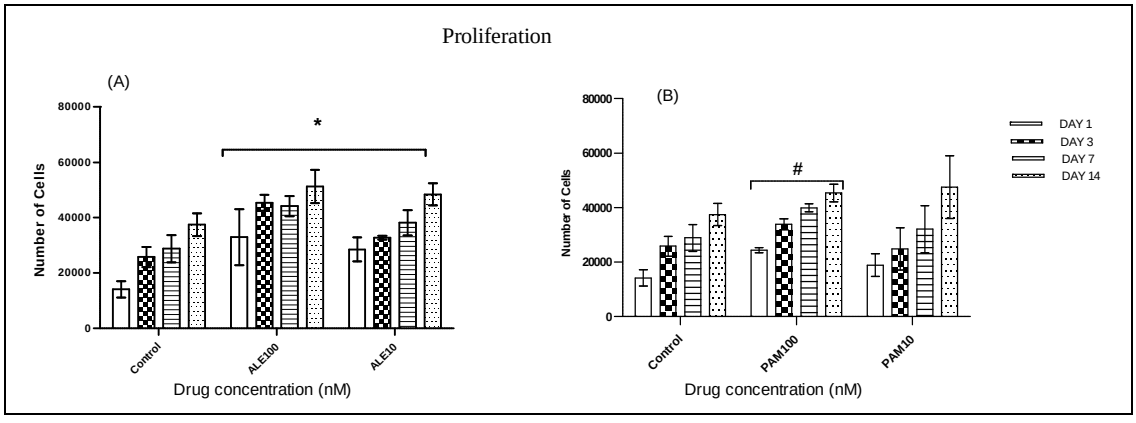


Figure 6.4 The effects of low doses of ALE and PAM (100 nM and 10 nM) on hMSC proliferation on Ti surfaces.

Proliferation were assessed at different time points. (A): All groups treated with ALE (100nM and 10nM) had shown significant cell proliferation when compared to the control group that was treated

with GM only. (B): The group treated with 100nM showed significant cell proliferation compared to the control group that treated with GM only (values= mean±SD; * and # P<0.05).

6.3.3 Attachment and retention

The effect of ALE and PAM on cells attachment after 24 hours of incubation in GM was examined. The data showed there was no significant effect between treated groups compared to the control group that was treated with GM only as shown in figure (6.5). Cells retention was analysed at 4 and 24 hours posts seeding by quantifying the cells number and there was no significant changes as shown in figure (6.6).

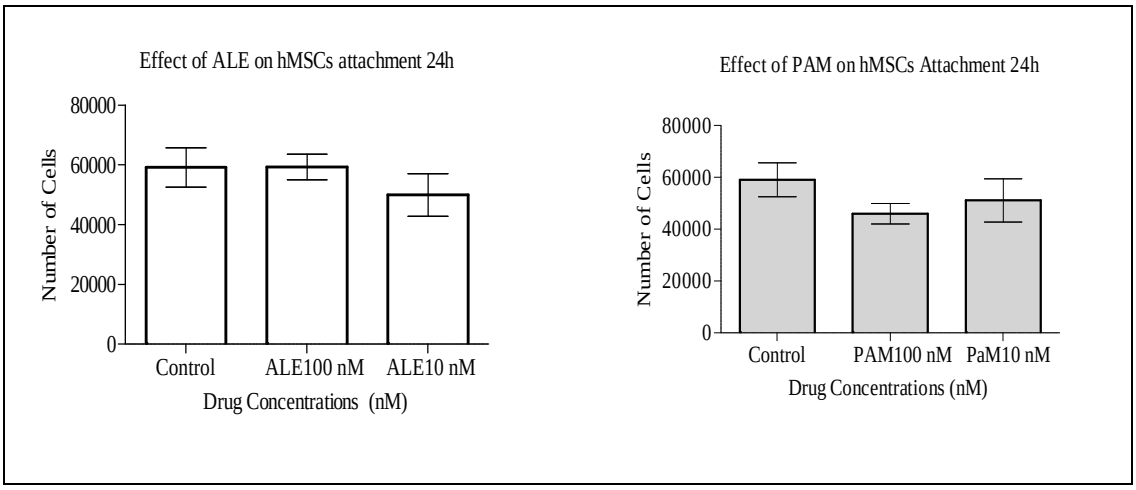


Figure 6.5 Evaluation of hMSCs attachment following BPs treatment

Attachment was assessed after 24 hours of culture in growth media. There was no significant changes between treated groups compared to the control group that was treated with GM only (values= mean±SD).

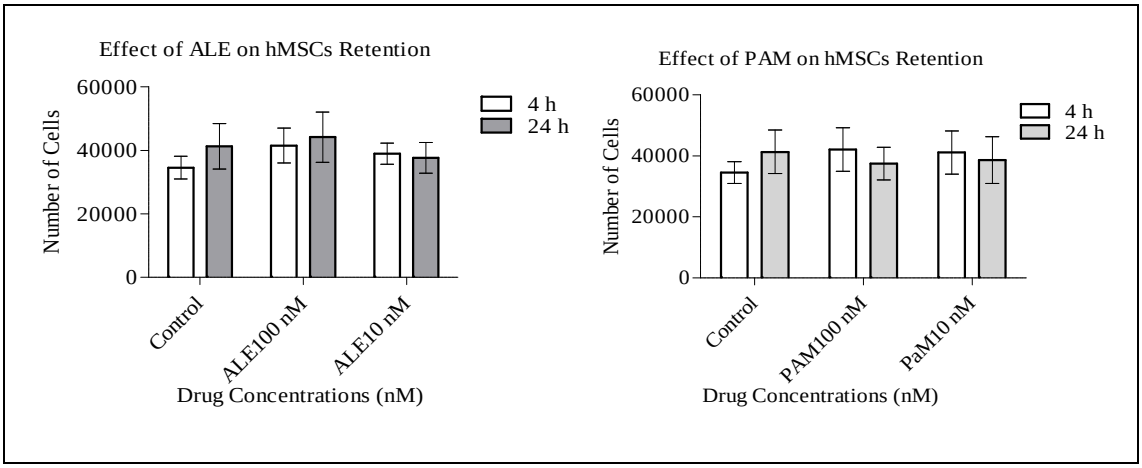


Figure 6.6 Evaluation of hMSCs retention following BPs treatment

Cells retention was assessed after 4 and 24 hours of culture in growth media. There was no significant changes between treated groups compared to the control group that was treated with GM only (values= mean±SD).

6.3.4 Migration

Cells after 4.5 hours at 37ºC at 5% CO_2, were observed to have migrated toward the substrate. However, cells with low doses of BPs (100 nM and 10 nM) showed that these drugs significantly enhanced cell migration toward the titanium surface when compared to the control group that had been treated with OM only (Figure 6.7).

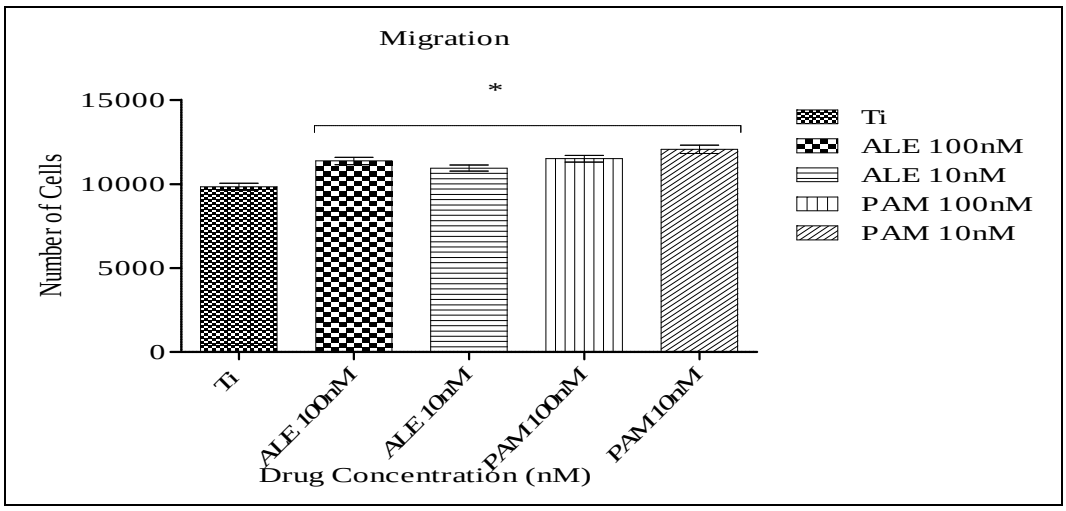


Figure 6.7: The effect of ALE and PAM on cells migration

Significant hMSC migration was observed after 4.5 hour incubation at 37ºC at 5% CO2 . Significant differences were observed between the cells treated with the low dose of drugs and the control group (OM only). Each column represents the mean±SD. * P< 0.05 treated group vs. control (Ti).

6.3.5 Mineralisation

6.3.5.1 Quantification of Calcium Deposition

The effect of ALE and PAM on calcium deposition on Ti surface was evaluated at day 21 post seeding. The results of this experiment are illustrated in figure (6.8). Data showed that both low dose of ALE and PAM (100nM and 10nM) significantly stimulated hMSC osteogenesis following drug treatment for 3 weeks when compared to cells that had been treated with OM only.

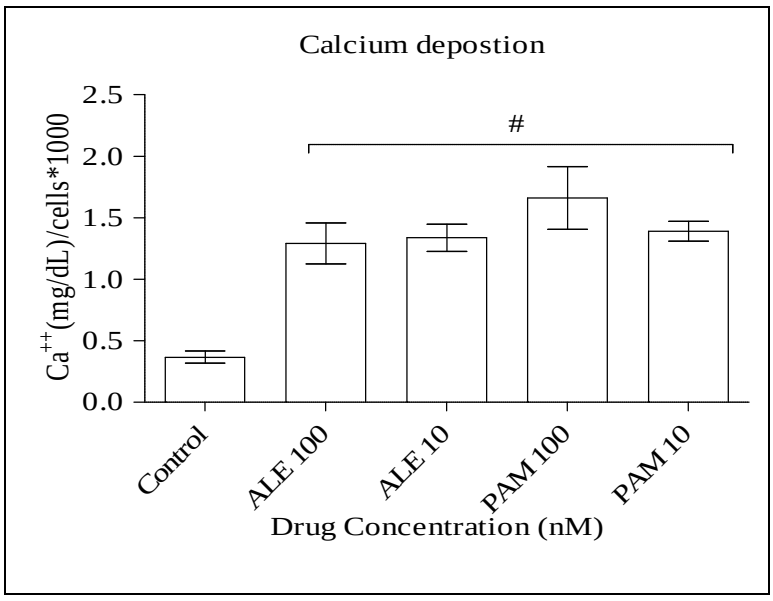


Figure 6.8: Quantification of total calcium deposition

Cells were divided into five groups with each group treated with different ALE and PAM concentration (100 nM and 10 nM). At day 21, calcium deposition was analysed. The results showed that BPs significantly stimulated hMSC mineralisation following drug treatment for 21 days when compared to cells that had been treated with osteogenic media only.. Each column represents the mean±SD. #=p<0.05, treated group vs. control (OM).

6.3.5.2 Quantification of ECM Collagen Deposition

The effects of low dose of ALE and PAM response after 21days post seeding on type I collagen deposition were analysed. The results of this experiment are illustrated in figure (6-9). The data shows that the low dose of PAM (100nM and 10nM) significantly stimulated the cells to deposit more collagen compared to the group that had been treated with OM only at day 21. Cells that had been treated with ALE (10 nM) showed less deposited collagen compared to the control group treated with OM only at day 21 but this was not significant, as shown in figure (6.9).

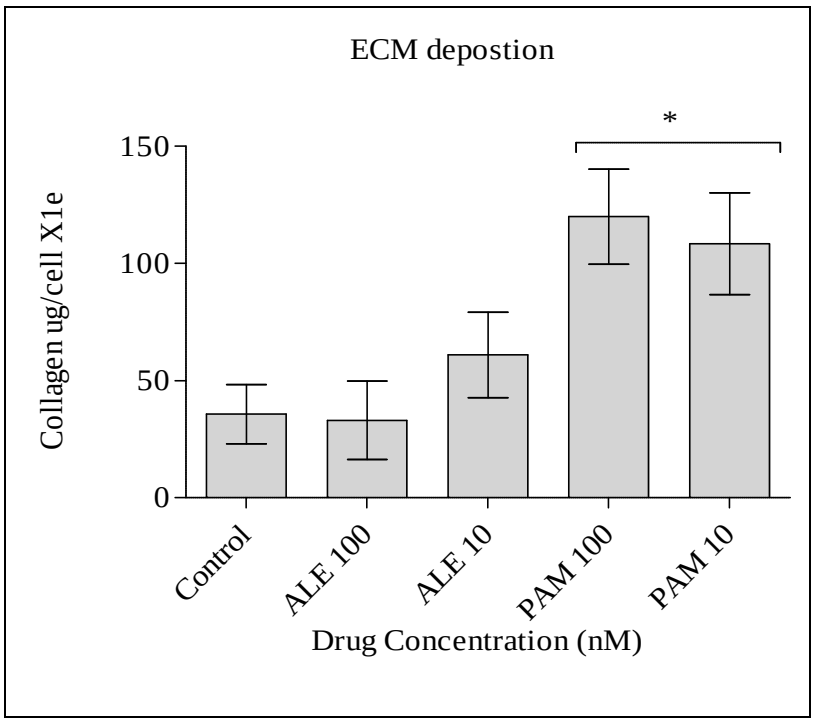


Figure 6.9: Quantification of ECM collagen deposition

Cells were divided into five groups with each group treated with different ALE and PAM concentration (100 nM and 10 nM). At day 14, extracellular collagen formation was analysed. The results showed that pamidronate (PAM) significantly stimulated hMSCs to produce collagen following treatment with drugs for 14 days when compared to cells that had been treated with osteogenic media only. No significant changes were observed in cells treated with Alendronate when compared to the control. Each column represents the mean±SD. *=p<0.05, treated group vs. control (OM).

6.3.5.3 Alkaline Phosphatase Activity

The effect of ALE and PAM on the ALP activity was measured at day 7 post seeding. The results show that the group treated with ALE (100nM and 10nM) at day 7 was significantly stimulated by the ALP activity compared to the control groups treated with OM only, as shown in figure (3-9). Similarly, the group treated with PAM (100nM and 10nM) at day 7 was significantly stimulated by the ALP activity compared to the control groups treated with OM only, as shown in figure (6.10).

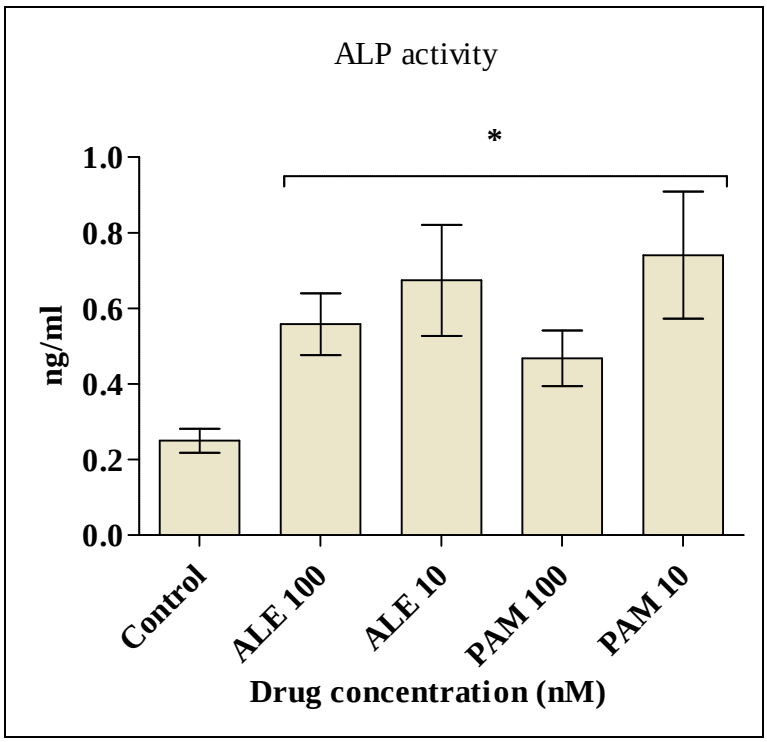


Figure 6.10: The effect of ALE and PAM on ALP activity

ALP activity after 7 days incubation was significantly increased in all cells treated with the lower dose of BPs drug when compared to the control group treated with osteogenic media only. Each column represents the mean±SD. *=p<0.05, treated group vs. control (OM).

6.3.6 Vinculin and Actin

Cell morphology was analysed by labelling cells with phalloidin to visualise internal F-actin structures and vinculin. Data showed that after 24 h of culture, those cells treated with ALE and PAM, exhibited clear differences in cell appearance and spread. These finding were supported by SEM images (Figure 6.11 and 6.12).

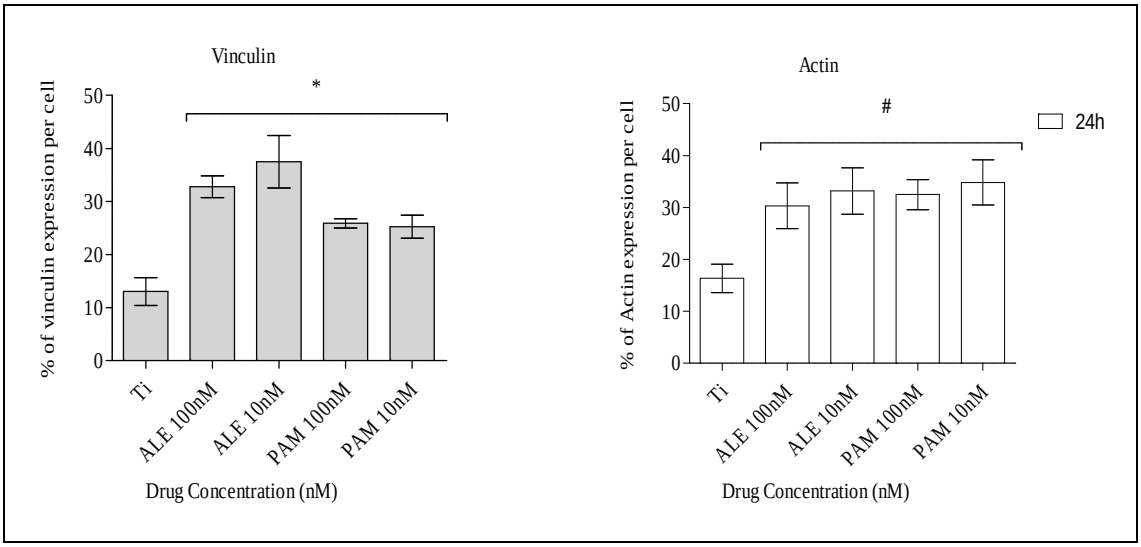


Figure 6.11: Actin and Vinculin expression analysis after 24 h of culture in OM with and without drugs. Groups that been treated with low dose of ALE and PAM showed a significant increase in Actin and Vinculin expression compared to the control group that was treated with osteogenic media on the Ti surface.

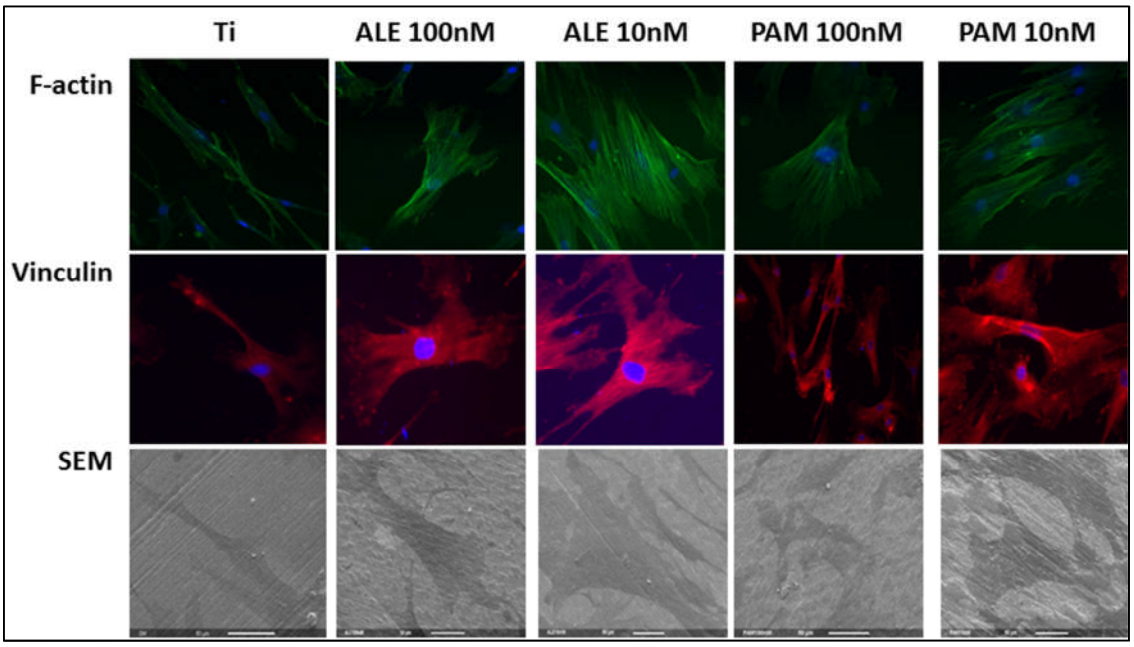


Figure 6.12: The effect of ALE and PAM on cell morphology

Florescent microscopy images showing the effect of low dose of drugs on cell organisation and spreading on Ti surfaces after 24 h. Actin expression appeared more abundant in cells treated with the ALE and PAM (100 nM and 10 nM). Likewise, vinculin expression appeared more abundant in treated cells. Images were taken using X 40 objective. Scale bar=50 µ M. SEM images were found to correlate with fluorescent images.

6.4 **Discussion**

Bone remodelling is a complex process requiring several cellular processes; bone cell recruitment, differentiation, bone synthesis and angiogenesis to stimulate blood flow (Seeman, 2009). Good biocompatibility and rapid osseointegration are essential elements of implant success and stability (Goriainov et al., 2014, Plecko et al., 2012, Quirynen et al., 2014, von et al., 2014). The previous chapters suggest that the low dose of ALE and PAM have a direct effect on hMSCs osteogenic behaviour and proliferation. In this chapter, we investigated whether low doses of BPs enhanced proliferation and osteogenic differentiation of hMSCs on Ti surfaces. hMSCs were used as they are the first cells to colonise implant surfaces and offer a target for enhancing osseointegration (Davies, 2003).

Topographical and chemical modifications of implant materials play integral roles in cellular migration, attachment, adhesion and proliferation of hMSCs (Logan et al., 2015, Wall et al., 2009, Wang et al., 2008). The ability of BPs to promote hMSCs proliferation on Ti surfaces is a crucial factor in implant success. In this study, we observed that over time, low doses of BP increased cell density at Ti surfaces. This observation may influence bone formation and improve osseointegration. In addition, these findings suggest that ALE and PAM exert effects on stem cell density. Furthermore, BPs appear to have a significant impact on hMSCs migration towards Ti surfaces. This effect is not just due to cell proliferation, our data indicates that BPs have an effect at the cellular level by accelerating cell migration towards Ti surfaces. These results are supported by early adhesion and cell spreading (Figure 4 & 5).

SEM images showed that the ALE and PAM exerted effects on hMSC morphology; cells were perfectly spread and adhered to Ti surfaces. This was confirmed by the expression

of focal adhesion proteins; vinculin spreading is considered a parameter of the interaction between the cell and implant material (Ezzell et al., 1997, Logan and Brett, 2013). A firm attachment and spread of hMSCs is an important factor for differentiation to osteoblasts, which over time become mature and produce fibronectin (Terheyden et al., 2012). From these findings, we believe that the ALE and PAM may have stimulatory effect on chemotactic behaviour toward substrates such as Ti.

To evaluate BPs on hMSC mineralisation, both early and late osteogenic markers such as calcium, collagen type I and alkaline phosphatase activity were assessed. It has been shown that in a clinical setting, following implantation, several cell types migrate towards the implant surface including endothelial, osteoblast and stem cells (Khalil et al., 2011). This is a very important step for osteoinduction and over time, this accumulation at the implant surface, will enhance implant success and implant stability (Javed et al., 2013, Truhlar et al., 2000).

Our data have shown that BPs exert stimulatory effects on hMSC osteogenic differentiation; cells treated with drugs produce more calcium when compared to the control group. These results were supported by elevation of ALP activity for both drugs after 7 days in culture. However, collagen type I is the main component of the extracellular matrix and is produced by osteoblasts and other cells derived from hMSCs (K.Gelsea, 2003). In this work, PAM stimulated higher collagen formation on Ti surface than ALE; this could be that PAM is clinically more potent than alendronate. The clinical success of any implant is based on its ability to anchor the endosseous to the surrounding bone (Chrcanovic et al., 2015).

This research confirms that low concentrations of ALE and PAM enhance the hMSC osteogenic response *in vitro,* and therefore could have translational applications *in vivo;*

enhancing osseointegration and clinical outcomes for Ti and other bone implants. We found that systemic application of low drug doses, which is clinically, almost 1000 times less than clinical doses and coating concentrations, has a largely beneficial effect on hMSC proliferation and osteogenic differentiation on Ti surfaces. Previous reports have shown that coating with BPs inhibits the osteolysis process (Abtahi et al., 2012, Stadelmann et al., 2008). In contrast to these reports, in our study, we have assessed hMSC migration, proliferation and osteogenic differentiation and used recognised assays and biomarkers to explore this phenomenon.

Apart from BP, different materials have also been used to stimulate bone regeneration and formation such as guided bone regeneration (GBR) (Donos et al., 2004, Elgali et al., 2015). Systemic application of this drug in conjunction with regenerative material (GBR) may synergistically enhance the bone remodelling process and accelerate wound healing. These findings may enhance bone remodelling and wound healing in the peri-implant area.

In conclusion, we have demonstrated that low doses of BPs stimulate hMSCs proliferation and osteogenic differentiation on Ti surfaces. These findings have promising implications in the systemic application of low doses of these BP drugs towards improved implant osseointegration and successful clinical outcomes. We believe that the systemic application of drugs could be pivotal in accelerating osseointegration and the bone healing process.

7 General Discussion

7.1 Overview of the thesis

The research presented in this thesis was based on the hypothesis that a low dose of BPs potentially enhanced proliferation and osteogenic differentiation of hMSCs. This research was conducted on the basis of three primary observations, which led to the thesis question. The first and primary observation was the enhancement effect of these BPs, including ALE and PAM, on the osteogenic and proliferative behaviour of hMSCs. The effect of these drugs has previously been analysed using several cell types, such as osteoclasts, osteoblasts, keratinocytes and fibroblasts (Hughes et al., 1995, Fromigue and Body, 2002, McLeod et al., 2014, Migliario et al., 2013). The second observation was that *in vitro* findings suggest that the BPs may alter cell functions and commitment to differentiation into mature cells by accelerating or slowing down the growth rate, as well as via involvement in some cell signalling pathways. The human body is a complex environment, and the cells considered as the major building units, hMSCs, represent the primary source of most of its cells (Hass et al., 2011, Bianco et al., 2008). The third observation was the effect of the low dose of BPs on the behaviour of different cell types. This has previously been measured by studying proliferation rate and molecular changes, including upregulation or downregulation of gene expression (Abtahi et al., 2012, Corrado et al., 2010, Kim et al., 2011).

However, the present study primarily focused on the effects of two types of commonly used BPs (ALE and PAM) on hMSC proliferation and osteogenic differentiation. An attempt was made to understand the mechanism by which hMSCs are activated by these agents and how the drug may have a long term effect on these cells. Moreover, their

effects on phenotypic changes and hMSC proliferation and osteogenic differentiation on Ti was also studied (Figure 7.1).

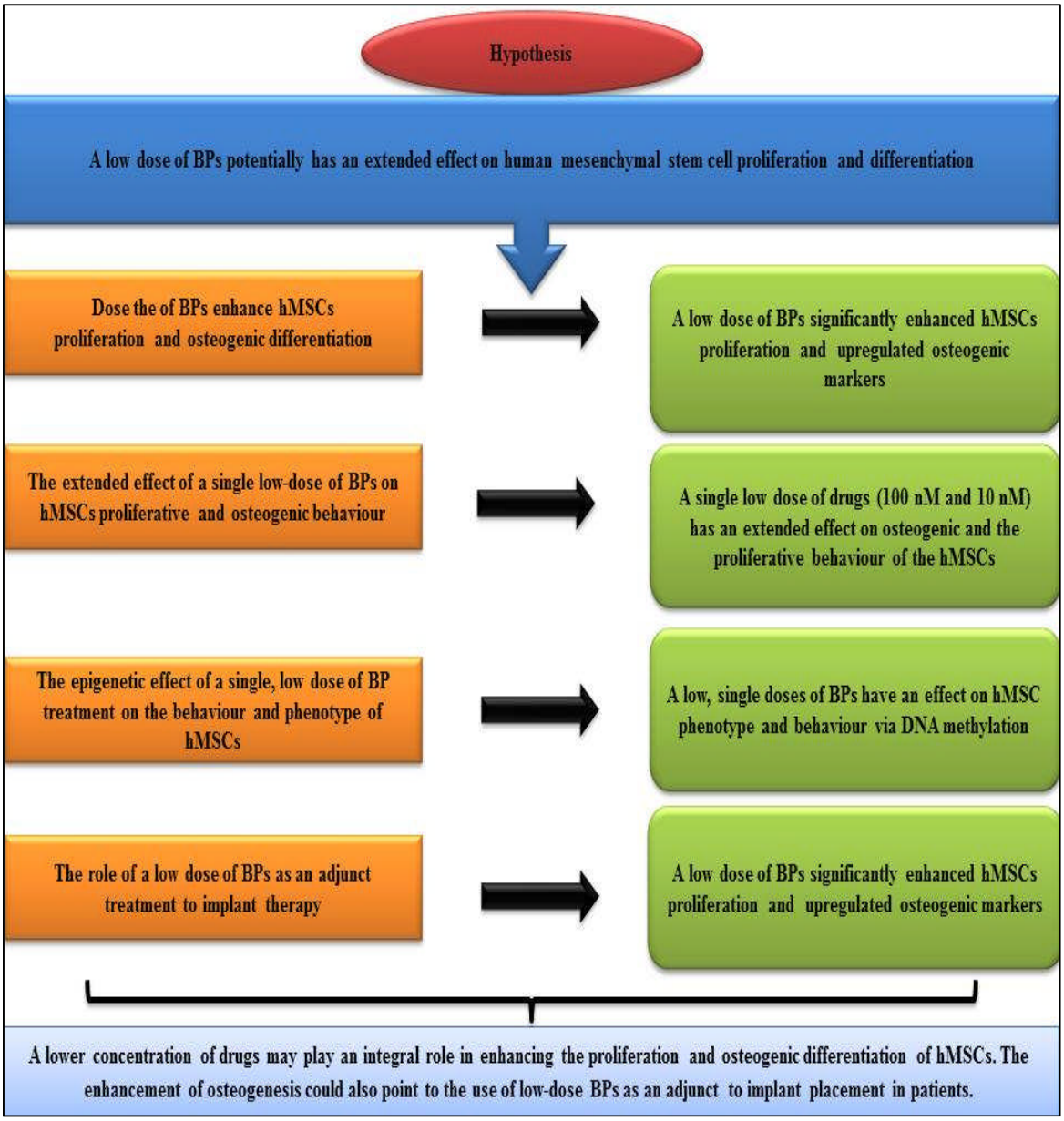


Figure 7.1: Thesis flow diagram

7.2 Effect of BPs on hMSCs

The effects of BPs (ALE and PAM) on the cellular metabolic activity of hMSCs were investigated using a descending dose range of 100µM to 10nM. Constant changes were

observed at the high dose, but this dramatically changed when we used low doses, such as 100nM and 10nM. The data suggest that the low dose of BPs was directly committed to, and accelerated, hMSC differentiation to the osteoblastic lineage. Clinically, ALE and PAM have been systemically administered to control the excessive activities of osteoclasts (Drake et al., 2008). Osteoclasts and osteoblasts are dependent on each other via intracellular signalling, which subsequently affects their role in the bone-remodelling cycle (Mundy, 1995, Teti, 2013, Matsuo and Irie, 2008). This interaction determines the balance between bone resorption and bone formation (Martin and Sims, 2005).

The phenotypic difference between hMSCs in the *in vitro* and *in vivo* environments and between different species may vary as a result of some changes stemming from the use of different growth conditions that mimic the *in vivo* setting, which may alter some of the cells' characteristics. However, it has also been shown that hMSCs in the two different environments share some common features, including the ability to proliferate and differentiate to other lineages and in the expression of their most common surface markers (Baksh et al., 2007). Our findings showed that hMSC populations were highly homogeneous in their proliferative and osteogenic differentiation following BP drug treatment. Moreover, the proliferative effect of the drugs was observed at the low dose (100nM or10nM), which was almost 1000x lower than the actual clinical dose. However, it remains unclear as to how ALE and PAM enhanced the rate of cell proliferation, although we believe this effect may have been due to the activation of some growth factors, such those of the ß-FGF and BMPs families, or as a result of interference with some of cell signalling pathways, such as the MAPK pathway (Giuliani et al., 1998, Fromigue and Body, 2002, Zhang and Liu, 2002).

The osteogenic differentiation of hMSCs is a complex process that goes through different stages, from as early as day 4 to day 28, and different markers represent each stage (Nakamura et al., 2009). Chapter 3 showed that culturing hMSCs with BPs (ALE or PAM) may directly stimulate these cells towards their osteoblast lineage. In addition, some data suggest that these drugs target some of the key osteogenic markers, including ALP, calcium deposition and collagen, and accelerate the osteogenic differentiation process. As mentioned previously, ALP is an early osteogenic marker and plays an integral role in bone matrix calcification (Orimo, 2010). This marker is induced by local and systemic triggers, for example, Hall et al. found that zinc affects ALP via increasing the half-life of the ALP of osteoblast-like cells (Hall et al., 1999). Systemically, it has been found that other factors, such as PTH, stimulate ALP *in vitro* (Tian et al., 2011). Overall, these findings suggest that BPs may locally stimulate ALP activity after 7 days of incubation.

However, the findings presented in Chapter 3 reveal that hMSCs treated with a low concentration of BPs produce more ALP and may represent an additional stimulator of osteogenic differentiation. Similarly, this effect was observed and confirmed by significant deposition of calcium and collagen at a later stage. A high dose (100µM) of ALE and PAM significantly decreased osteogenic differentiation, due to inhibition of cell proliferation. Pan et al. found that high doses of BPs increase cell mineralisation (Pan et al., 2004), but the results of the present study found that a high dose limit cell mineralisation. However, our results did correlate with those of other previous studies, despite the type or the dose used (Huang et al., 2016, Abtahi et al., 2012). In the majority of these previous studies, either primary osteoblasts or an osteoblast cell line were used, whereas we ventured further, up to the source of these cells, the hMSCs, which gives a

clear indication that the effect occurs at a more central level. It is unclear as to whether that effect was exerted only on osteogenic commitment, or whether other lineages, such as adipocytes, myocytes and neurons, may also have been affected.

However, in addition to BPs, several reports have revealed that osteogenic differentiation of hMSCs can be enhanced and accelerated by adding growth factors or by co-culturing with other bone cells, such as osteoblasts (Birmingham et al., 2012, Hanada et al., 1997). However, in the present study we highlighted the fact that BPs stimulate hMSC osteogenic differentiation without the addition of any growth factors. Furthermore, the results provide an indication that hMSCs may be regulated by chemical factors that may be directly or indirectly required for their differentiation. In general, ALE was more effective than PAM in the stimulation of osteogenesis differentiation and proliferation in hMSCs, a finding that it is in accordance with those of previous reports (Walsh et al., 2004, DiMeglio and Peacock, 2006). We also showed that both drugs had an effect on additional osteogenic markers, and this effect was studied over a long time-frame, providing a wider perspective on drug effect. It remains unclear as to why ALE had this influential effect on the osteogenic mechanism, but we believe it may be related to the chemical structure and absence of a methyl group from its chemical formula. However, treating cells with a dose that mimics the actual *in vivo* dose showed some apoptotic effect, and limited the cells' growth rate. This might serve as an indication as to why these drugs lead to some clinical complications, such as osteonecrosis of the jaw, but the mechanism behind this effect is still not yet understood (Estefania et al., 2006, Marx and Tursun, 2012).

Type I collagen is one of the most important components of the extracellular matrix and plays a crucial role in the structural and biochemical support of cells within the bone

matrix (Hay, 2013). The present study provides an additional perspective on how low doses of ALE and PAM may be involved in ECM synthesis, as shown in Chapter 3, Figures (3-11&3-12). We were also subsequently interested in the effects of ALE and PAM on osteogenic gene expression. The data indicated that, in the presence of ALE and PAM, hMSCs tended to differentiate significantly more than did those cultured in osteogenic medium only, via upregulation of osteogenic genes. The data also showed that ALE significantly upregulated Runx2, OPN, Wnt5a, ALP, COL 1a1 and OC, although these results correlate with those of few previous reports (Fu et al., 2008, Xiong et al., 2009). However, we investigated a greater number of osteogenic genes, and over a longer time interval, to confirm whether this effect only targeted selective genes, as well as whether it was consistent or intermittent. Moreover, as each gene is involved in different cellular osteogenic activities, this finding indicates that ALE and PAM have multifunctional effects. The effect of ALE on osteogenic genes was noticed at different points, which provides additional confirmation that its role in osteoblastogenesis is multifunctional and multifactorial. However, PAM showed a similar effect on some genes, such as OPN, Wnt5a and OC, also indicating that ALE exerts a greater effect on gene expression than PAM.

Stem cell mobilisation is a process whereby these cells can be recruited and introduced to blood stem cells (Cottler-Fox et al., 2003). This is similar to the process of enhancement of stem cell proliferation and release as a response to injury and inflammation. It has been reported that some agents, such as non-steroidal anti-inflammatory drugs (NSAIDs), induce stem cell mobilisation without affecting cell proliferation and differentiation (Butler and Rafii, 2013, Bensinger et al., 2009). In this study, we have showed that a low dose of BPs had an effect on proliferation and differentiation *in vitro*.

7.3 **Behavioural effect of BPs on hMSCs**

The findings described in Chapter 3 led us to investigate whether a single low dose can have an extended effect on cell behaviour, as studied via the same parameters, and whether that effect may remain in evidence even after cell passage. In addition, we investigated this effect on epigenetic changes using the DNA methylation approach, something that had not previously been proposed. These findings may answer the question regarding an association between BPs and hMSC epigenetic phenotype. hMSC behaviour can be affected by several internal and external factors; internally via growth factors and cytokines, and externally via intracellular interaction, as well as by mechanical and physical stimuli (Chen et al., 2016, Ge et al., 2009, Sundelacruz and Kaplan, 2009, Tan et al., 2015).

A single, low dose of both ALE and PAM showed an extended effect on hMSC proliferation over different time intervals, as stated in Chapter 4. This indicates that the cells are sensitive to these external stimuli, and they reacted and accommodated to this trigger via an increase in their proliferation rate. In general, cell proliferation primarily occurs in the first phase of cell division, namely, the gap phase (G1) (Cooper, 2000). It is influenced and controlled by several factors, such as growth factors, as well as cyclin-dependent kinases (CDK). We have observed that ALE and PAM have an early effect on cell proliferation; this may indicate that these agents were involved in, and had direct control over, the G1 phase. However, the mechanism is not yet clearly understood and requires further investigation. However, we believe that the effect may be related to a signalling pathway that controls cell proliferation, such as the MAPK signalling pathway, as stated in Chapter 4. These findings are supported by the results of several previous

studies that showed that cell proliferation has a multifactorial controller (Graham et al., 1990, Kim et al., 2008).

The finding of a behavioural effect on both early and late markers in osteogenic differentiation showed that both ALE and PAM may directly control and accelerate this phenomenon. These results were in accordance with previous studies that showed that different parameters have an effect on ALP activity, collagen and osteocalcin (Potier et al., 2007, Hanada et al., 1997, Salasznyk et al., 2004). However, we evaluated the effect even after cell passaging, as well as withdrawal of the dose across a longer time-frame, which provides a wider a perspective with regard to this effect. The temporary effects of the low dose (100nM or10nM) of both drugs on enhancing osteogenic markers were significantly greater compared with the control group, as stated in Chapter 4. These findings correlate with several previous observations, whereby osteogenic behaviour was enhanced by external mechanical and physical stimulation (Chen et al., 2016, Ge et al., 2009, Sundelacruz and Kaplan, 2009). Together with our findings, this provides a clear indication that the modification of cell behaviour and commitment is a possible approach that will have promising clinical implications, particularly in stem cell therapy and the field of tissue engineering. Moreover, the data suggest that BPs have a synergistic effect on increasing hMSC osteogenesis, via stimulation of both early and late osteogenic markers.

7.4 The epigenetic effect of a single, low dose of BP treatment on the behaviour and phenotype of hMSCs

The hMSCs showed a high potential for clinical application to treat a number of diseases, including musculoskeletal, cardiovascular and metabolic bone diseases (Marcacci et al., 2007, Horwitz et al., 2002). However, it has been proposed that epigenetic changes

control hMSC phenotype and behaviour via different mechanisms, including histone modifications, chromatin remodelling and DNA methylation. The latter is associated with most of the epigenetic changes (Weinhold, 2006). Our data showed that the BPs showed significant involvement in, and alteration of, DNA methylation, which subsequently affects cell behaviour. It is possible that the epigenetic changes pre-programmed the cells following the single low-dose application. It is challenging to address the extent of this effect on DNA methylation to direct the cells to their desired direction and function. The mechanisms by which DNA methylation directs cells to a certain lineage is not yet fully understood. However, it could occur via a disruption between transcriptional factors and their target sites in the binding process.

hMSCs differentiate to a variety of cell types, including the adipocyte, osteoblast and chondrocyte. We believe that there is a need for epigenetic mechanisms to regulate cell differentiation; therefore, the cell will maintain the mature phenotype. This regulatory action occurs via controlling gene expression, for example it has been reported that DNA methylation controls the expression of some osteogenic genes, including ALP, OC, Osterix and OP (Arnsdorf et al., 2010, Lee et al., 2006, Penolazzi et al., 2004). Our data support these findings via the control of several hMSC gene probes that play a crucial role in the cell cycle and in cell differentiation. However, external factors, such as chemical compounds including 5-azacytidine, have shown a stimulatory effect on hMSC differentiation towards the osteoblast lineage (Locklin et al., 1998). In this project, the BPs showed promising results with regard to controlling hMSC proliferation. In addition, we believe that it is not only DNA methylation that is involved, but that other mechanisms, such as chromatin remodelling and histone modifications, may also be affected, making the process more complex (Gordon et al., 2011, Shen et al., 2003) .

A growing body of evidence has shown that stem cells respond to external stimuli, whether it be mechanical, physical, environmental or chemical (Chen et al., 2016, Ge et al., 2009). These types of responses may enable stem cells to memorise the effects (Yang et al., 2014). With this in mind, our findings showed enhanced stem cell proliferation and differentiation even after removal of the trigger, which is a clear indication that the cells memorised this effect.

We observed that BPs were widely involved in the methylation of many gene probes, such as MAPK8IP1 and SPRY4; it is believed that these genes are involved in cell division and growth. Interestingly, our data revealed that the low dose of PAM also simultaneously had an effect on methylation of RUNX family promoters, including RUNX2 and RUNX3. RUNX2 plays an important role during the hMSC osteoblastic differentiation process (Afzal et al., 2005, Franceschi and Xiao, 2003, Lian et al., 2003). In general, we found a greater number of hypermethylated gene probes than hypomethylated probes. As these significant changes were shown *in vitro*, it is still necessary to confirm whether a similar effect translates to the actual *in vivo* environment.

Finally, considering all these findings together, a single low dose of BPs remains an important step in showing a potentially crucial regulatory factor of epigenetic mechanisms. The current project mainly focused on the study of DNA methylation alteration following BP treatment. We also examined methylation distribution in chromosomes, genes transcript structure and genome islands. This was in order to assess whether there is a link between BP dose and DNA methylation distribution within the genome, which will also be a target for future research in this area.

7.5 **Effect of Low Dose Bisphosphonates on hMSC Osteogenic Response to Titanium**

Since implants have come to be used in the medical field, and commonly in the dental field, a successful outcome has become a challenge, and primarily depends on the success of a phenomenon known as osseointegration (Al-Hezaimi et al., 2014, Jayesh and Dhinakarsamy, 2015, PI., 2005, Quirynen et al., 2014). Successful osseointegration is affected by several factors, which have been discussed in section (1.3.3). hMSCs play an integral role in initiation of this phenomenon, whereby it is believed that they are the first cell type to adhere to the implant surface (Davies, 2003). Titanium was always considered as the best type of dental implant material, due its biocompatibility (Saini et al., 2015). However, although it had been used for decades, there remained a chance of failure; therefore, modifications to the titanium surface, either chemical, via implant coating, or physical, via an increase in the surface area and roughness, have been carried out in the majority of latter studies (Smeets et al., 2016, Barfeie et al., 2015).

As discussed in section (1.3.6), several types of implant coating materials and techniques have been proposed, but each type remains associated with clinical limitations (Xuereb et al., 2015). One proposed type is that of BPs, although there have been few reports describing the use of implants coated with BPs (Greiner et al., 2008, Jakobsen et al., 2007, Jakobsen et al., 2009). However, implant success remains a drawback with this approach, as a result of a decrease in osteolysis around the implant and the high concentration that has been used. In addition, the effect of BPs on osteoclasts may interfere with the long-term success of the implant. Moreover, use of a high dose may increase the risk of bone microdamage (Mashiba et al., 2000).

The findings described in Chapters 3 and 4 revealed that the application of a low dose is a promising approach with regard to enhancing implant success rate. Our results suggest that low doses of ALE and PAM should support and enhance hMSC proliferation and osteogenic differentiation. This effect will accelerate the healing process and will subsequently improve the osseointegration process, as BPs may help in obtaining an effective seal around the implant, which will interfere with mucosal formation between bone and the implant surface.

Furthermore, in conjunction with other factors, including growth factors, this effect may increase cell migration and activation of the healing process. hMSCs are considered suitable for endogenous repair, due to the enhancement of osteogenic differentiation, which may be related to their capacity to produce some growth factors and cytokines. A good example is that hMSCs express BMPs, which play an integral role in bone formation and repair (Nixon et al., 2007). Therefore, some studies have reported the use of these types of bone growth factors (Facca et al., 2011, Tachi et al., 2011); however, there remains a risk of their unwanted overproduction, which may interfere with long-term success. Moreover, it has been reported that hMSCs are active during inflammation and wound repair, and are also involved in an increase in proliferation rate (Malhotra et al., 2016). The outcome of this activity is to accelerate the wound-healing process and to reduce excessive scar production.

In addition to BPs, it has been proposed that other drugs, such as antibiotics (AB), are used with implants, either via coating or by systemic administration. The primary aim of AB use is to inhibit adhesion and colonisation of bacteria at the titanium surface, which is considered to be a key player in infection and peri-implantitis (Ferraris and Spriano, 2016,

Godoy-Gallardo et al., 2016). AB will disrupt colonisation of the bacteria and subsequently inhibit biofilm.

Topical application of BPs to the implant placement site has been proposed in order to improve screw fixation (Tengvall et al., 2004), but this approach has a limitation, in that there is a high chance that the drug will be flushed away by the blood. However, systemic application of BPs at the titanium surface might be more effective, because it will directly accelerate the osseointegration process. Furthermore, local delivery of BPs has long been used with regard to titanium coating, primarily to inhibit the osteolysis process (Abtahi et al., 2012, Jakobsen et al., 2009, Greiner et al., 2008). However, our data showing that systemic application, using a dose of 1000X less than the actual *in vivo* dose, may be more beneficial without resulting in side effects for the patient.

hMSC migration and initial adhesion to the titanium surface are key factors in osseointegration and implant success (Banik et al., 2016). We found that the low doses of ALE and PAM significantly increased hMSC migration. In addition, the cells were well spread on the titanium surface, as shown via a study of focal adhesion protein and described in Chapter 5. However, the exact mechanisms by which the BPs enhanced hMSC migration and adhesion are not yet fully understood. These findings, together with a study of osteogenic markers, are promising with regard to the use of drugs, particularly BPs, as adjunct therapy to facilitate osseointegration and enhance the healing process.

7.6 **Conclusion**

In summary, the findings presented in this thesis indicate the effects of ALE and PAM on the osteogenic and proliferative behaviour of hMSCs. The study proposes that low doses of ALE and PAM promoted hMSC proliferation and osteogenic differentiation. The

differential osteogenesis of hMSCs is a reflection of an extended effect of the drugs on different osteogenic markers. It was observed that this effect was permanent, even after passaging of these cells. The enhancement of hMSC osteogenic differentiation occurred through various cellular mechanisms. The data have a potential clinical implication with regard to different applications, such as tissue engineering or drug delivery. In the transplantation field, the low dose of BPs may be used in *ex vivo* expansion of hMSCs to commit the cells to the desired progenitor cell type. In addition, the effect of the drugs may delay cellular aging of these cells, and provides a promising approach in the tissue engineering and regenerative medicine field. The epigenetic findings provided full clarification that ALE and PAM have an effect on epigenetic changes that occur during osteogenic differentiation. These changes provide promising evidence in support of the future success of regenerative medicine-based approaches, and are crucial for the hMSC phenotype in the long-term. Furthermore, these finding suggest that an understanding of the phenotypic changes that occur during the life cycle of hMSCs could provide a clear vision with regard to the success of using these cells in the medical field and an understanding the underlying mechanisms of these external triggers. Finally, further to this if drugs can be used to change the epigenetic profile of the cells and manipulate the way that cells behaves we need to start considering techniques that alter the epigenetic profile of cells in such a way as to enhance the regenerative capacity of the cells via either chemical or physical or combined stimuli to create the epigenetic change desired for particular applications.

7.7 **Implications and Future directions**

While this study adds to the field, further research is still required to investigate the effect of BPs on hMSC behaviour, focusing on some of the cell signalling pathways, such as the

MAPK and ERK pathways. In addition, the effect of BPs on hMSC commitment to other cell lineages must be identified. The interaction of these cells also remains to be elucidated, as does whether the latter contributed to our findings, since it was discovered that stem cells are centrally involved in tissue repair, particularly that of bone tissue. The next phase is to translate these results to an actual *in vivo* study using animal models. However, it will be a challenge to ascertain whether the osteogenic effects of BPs on hMSCs *in vitro* would be translated to an *in vivo* model and result in stronger bone with more mineral deposition, as the surrounding structure may have an effect involving cell growth and phenotype in the *in vivo* environment. As was observed *in vitro*, *in vivo* experiments may confirm whether the therapeutic application of drugs may represent an adjunctive tool in enhancing bone regeneration and tissue repair in conjunction with hMSCs. The primary implication of Ti can be divided into short- and long-term therapeutic usage. However, the use of this type of material is primarily associated with short therapeutic usage, in order to evaluate the behaviour of bone cells and for the design of bone-related tissue engineering materials.

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8 Appendix

8.1 Supplementary tables

Table 8:1:The top 25 most significantly differentially methylated (hypermethylated and hypomethylated) probes in the 100nM PAM group vs the control group.

Probe ID	Island status	Gene name	Genomic features	P value	Adj. p val
cg22867714	S_Shore	VWF	Body	5.63E-10	0.000223
cg08066417	OpenSea	CDH5	5'UTR	1.42E-09	0.000281
cg01749142	S_Shore	AKT1	TSS1500	2.14E-09	0.000282
cg06991955	Island	TSC2	Body	6.35E-09	0.000629
cg02184338	Island			1.07E-08	0.000667
cg00472393	Island	C6orf167;MIR548H3;C6orf167	5'UTR;Body; first exon	1.29E-08	0.000667
cg09605254	Island	FAM91A1	TSS200	1.33E-08	0.000667
cg08254315	OpenSea			1.35E-08	0.000667
cg23713156	Island	WDR45L;WDR45L	5'UTR; first exon	1.56E-08	0.000685
cg23307708	Island	PPP2CA	TSS200	2.17E-08	0.00086
cg05966498	Island	DAP;DAP	First exon ;5'UTR	2.52E-08	0.000906
cg18855356	OpenSea	CROCCL1	TSS200	2.85E-08	0.000941
cg19826439	Island			3.89E-08	0.001084
cg17896229	N_Shore	PROKR2	First exon	4.27E-08	0.001084
cg18399183	OpenSea	METTL7A	TSS1500	4.48E-08	0.001084
cg14284424	N_Shore			4.73E-08	0.001084
cg03782202	Island	HOXD11	TSS1500	4.82E-08	0.001084
cg08244085	Island			5.26E-08	0.001084
cg18224395	S_Shore	RCN3	Body	5.39E-08	0.001084
cg26057840	N_Shore			5.64E-08	0.001084
cg20733436	Island	TMED7-TICAM2;TICAM2;TMED7-TICAM2	Body;5'UTR;body	5.84E-08	0.001084
cg19187155	Island	NMUR1	TSS200	6.56E-08	0.001084
cg02699780	Island	DYNLL1;SFRS9	TSS1500; first exon	6.64E-08	0.001084
cg27098574	Island	BCAR1;BCAR1;BCAR1;BCAR1;BCAR1;BCAR1;BCAR1;BCAR1	Body;5'UTR; first exon;body;body;5'UTR;body;body; first exon	6.83E-08	0.001084

Table 8.2: The top 25 most significantly differentially methylated (hypermethylated and hypomethylated) probes in the 10nM PAM group vs the control group.

Probe ID	Island status	Gene name	Genomic features	P value	adj. p val
cg20733436	Island	TMED7-TICAM2	Body;5'UTR;body	2.00E-10	5.00E-05
cg02184338	Island			2.52E-10	5.00E-05
cg23307708	Island	PPP2CA	TSS200	4.32E-10	5.18E-05
cg08244085	Island			6.54E-10	5.18E-05
cg01749142	S_Shore	AKT1;AKT1	TSS1500;TSS1500	7.52E-10	5.18E-05
cg14126493	Island	F12	Body	7.84E-10	5.18E-05
cg18788524	Island	SEC22B	First exon	1.10E-09	5.74E-05
cg03729288	Island	NEURL4	TSS200;TSS200	1.16E-09	5.74E-05
cg07237882	Island	HNRNPA0	TSS1500	1.79E-09	7.52E-05
cg19826439	Island			1.90E-09	7.52E-05
cg20678233	Island	C7orf52	Body	2.11E-09	7.59E-05
cg03901462	Island	NXF1;NXF1;NXF1;NXF1	first exon ; first exon ;5'UTR;5'UTR	2.42E-09	7.63E-05
cg22867714	S_Shore	VWF	Body	2.58E-09	7.63E-05
cg05349077	S_Shore	PIGQ;PIGQ	Body	2.85E-09	7.63E-05
cg06159435	Island	H2AFX	TSS200	3.20E-09	7.63E-05
cg00300090	Island	SETMAR;SETMAR	First exon n;TSS200	3.24E-09	7.63E-05
cg18072802	Island	GSX2;GSX2	5'UTR; first exon	3.28E-09	7.63E-05
cg17154315	Island	ZFPM2;ZFPM2	5'UTR; first exon	3.79E-09	8.00E-05
cg08254315	OpenSea			3.88E-09	8.00E-05
cg24542492	Island	STRADA	5'UTR	4.04E-09	8.00E-05
cg09605254	Island	FAM91A1	TSS200	4.28E-09	8.08E-05
cg18865796	Island	TMEM205;CCDC159;TMEM205;CCDC159;TMEM205	TSS1500; first exon ;TSS1500;5'UTR;TSS1500	4.94E-09	8.89E-05
cg25595571	Island	BAG5;BAG5;BAG5;BAG5;C14orf153	TSS1500;5'UTR; first exon;TSS1500;TSS1500	5.38E-09	9.27E-05
cg21767207	Island	PSENEN;U2AF1L4;U2AF1L4	TSS200;TSS200;TSS200	5.81E-09	9.58E-05

Table 8.3: Top 25 differentially methylated regions in the 100nM PAM group compared to the control group.

Sequence name	Freq.	Mean beta fc	Transcript type	Gene name	CpGs
chr6:13710980-13712555	8	-0.00619	protein_coding	RANBP9	7702
chr19:15559628-15560731	10	0.00373	protein_coding	WIZ	24407
chr19:19005959-19007311	6	0.02183	protein_coding	CERS1	24577
chr6:153451486-153452320	5	0.015364	protein_coding	RGS17	8632
chr2:238535538-238536869	12	0.00907	retained intron	LRRFIP1	4002
chr2:7005449-7006627	11	0.004783	protein_coding	CMPK2	2548
chr1:111746277-111746873	3	0.035121	processed_transcript	DENND2D	1428
chr16:49315302-49316197	6	0.012543	protein_coding	CBLN1	20530
chr22:45705512-45706530	11	0.004729	retained_intron	FAM118A	27527
chr10:135075438-135075522	3	0.018599	retained_intron	ADAM8	14847
chr19:49468454-49468917	5	-0.01374	protein_coding	FTL	25376
chr18:12376968-12378044	10	0.022031	protein_coding	AFG3L2	22874
chr16:1664086-1664582	9	0.018467	protein_coding	CRAMP1L	19890
chr22:37915443-37916157	4	-0.03663	protein_coding	CARD10	27344
chr13:44453183-44453867	13	0.002997	processed_transcript	CCDC122	17683
chr22:38851318-38851884	7	0.024692	protein_coding	KCNJ4	27378
chr3:196730265-196730609	4	-0.00077	Antisense	MFI2-AS1	5293
chr10:121355708-121356513	4	-0.00309	processed_transcript	TIAL1	14627
chr7:143058944-143059659	9	0.030698	nonsense_mediated_decay	FAM131B	10119
chr1:229761129-229762071	6	-0.00542	protein_coding	TAF5L	2330
chr3:183948058-183948800	7	-0.00462	protein_coding	VWA5B2	5166
chr11:45921959-45922184	3	0.037896	retained_intron	MAPK8IP1	15352
chr17:19290353-19291120	8	-0.08387	retained_intron	MFAP4	21580
chr17:21360346-21360741	5	0.018072	lincRNA	RP11-728E14.2	21611

Table 8.4: Top 25 differentially methylated regions in the 10nM PAM group compared to the control group.

Sequence name	Freq.	Mean beta fc	transcript type	gene name	CpGi
chr17:71188271-71189320	11	0.028827	nonsense_mediated_decay	COG1	22343
chr5:171682568-171682651	3	-0.12734	processed_pseudogene	KLF3P1	7353
chr16:49314323-49316197	8	0.02579	protein_coding	CBLN1	20530
chr15:25414716-25415399	7	-0.08606	processed_pseudogene	TMEM261P1	18914
chr19:30016136-30016478	3	0.016306	retained_intron	CTC-525D6.1	24668
chr7:157404440-157404705	3	-0.0091	protein_coding	AC005481.5	10326
chr3:129325693-129325717	3	0.03223	protein_coding	PLXND1	4903
chr2:24346215-24346760	5	0.010196	protein_coding	PFN4	2648
chr17:19265555-19266474	13	0.014639	nonsense_mediated_decay	B9D1	21579
chr13:113299697-113300120	4	-0.05575	protein_coding	C13orf35	17946
chr2:25896178-25896320	3	0.008803	retained_intron	DTNB	2669
chr8:134584145-134584692	5	0.013734	processed_transcript	ST3GAL1	11172
chr12:52400650-52401214	5	0.009296	protein_coding	GRASP	16635
chr13:31773952-31774536	5	0.013702	protein_coding	B3GALTL	17626
chr6:13711723-13712174	5	0.011875	protein_coding	RANBP9	7702
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chr11:63706312-63706491	3	0.02164	nonsense_mediated_decay	NAA40	15534
chr22:45405061-45406130	13	0.010738	retained_intron	PHF21B	27520
chr7:98030280-98030482	4	0.027452	processed_transcript	BAIAP2L1	9804
chr3:183948058-183948322	6	0.001072	protein_coding	VWA5B2	5166
chr17:1394477-1394841	4	0.013153	protein_coding	MYO1C	21230
chr1:235812840-235813452	5	0.023646	protein_coding	GNG4	2370
chr2:119599545-119600002	5	0.135539	processed_transcript	EN1	3298
chr5:171614719-171615938	11	0.016362	protein_coding	STK10	7352

8.2 **Scientific output**

Publications

N.R. Alqhtani, N.J. Logan, S. Meghji, R. Leeson, P.M. Brett , Low dose effect of bisphosphonates on hMSCs osteogenic response to titanium surface in vitro. Bonr(2017), doi: 10.1016/j.bonr.2017.02.002

N.R. Alqhtani, Ross J, V.S Dhillon, M. Shahid, S. Meghji, R. Leeson, P.M. Brett , Single low dose bisphosphonate treatment has an effects on epigenetic and enhances osteogenesis in hMSCs. (Submitted)

Conference abstract

"The Effect of Bisphosphonates on hMSCs Proliferation and Osteogenic Differentiation" - International Association for Dental Research (IADR) - Dubrovnik, Croatia, 10^{th}-13^{th} September 2014. Poster Presentation.

"Single Low dose Bisphosphonate Treatment has Long Term Effects on, and Enhances Osteogenesis in hMSCs" -9th UK Mesenchymal Stem Cell meeting,- Manchester, UK 3rd December 2015. Poster presentation.

"In vitro Small Dose Effect of Bisphosphonates on hMSCs osteogenic response to Titanium surface" -4th International Conference Strategies in Tissue Engineering- Wurzburg, Germany,10^{th}-12^{th} June 2015. Poster presentation.

"Stem Cell Behaviour Following a Single Dose of Bisphosphonate Treatment" –Saudi Student Conference 9 (SSC9), Birmingham, UK 13^{th} -14^{th} February, 2016. Poster Presentation.

"Bisphosphonates as an adjunct to Titanium Implant therapy"- IADR- Seoul, South Korea 22^{nd}-25^{th} June 2016. Poster Presentation.

Presentations

"Osteonecrosis of Jaw: A Study of the Mechanism and Potential Therapy" UCL Eastman Dental Institute, London, 10^{th} October 2013. Oral presentation.

"Bisphosphonates effect on hMSCs osteogenic response, proliferation and migration toward Titanium surface"SSC8, London, 31 January 2015. Oral presentation.

"Single low dose bisphosphonate treatment has long term effects on, and enhances osteogenesis in hMSCs" UCL Eastman Dental Institute, London, – PhD Research Symposium, 11^{th} May 2016. Oral presentation.

The Effect of Bisphosphonates on hMSCs Proliferation and Osteogenic Differentiation

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Introduction

Bisphosphonates are chemical analogues related to pyrophosphate; they are well-known inhibitors of osteoclasts activity. Over the last 40 year they been used in the clinic to treat various bone diseases characterized by excessive bone resorption such as osteoporosis, malignant bone diseases and hypercalcaemia of malignancy. However, human mesenchymal stem cells (hMSCs) show regenerative properties and are able to differentiate in to different types of human cell such as osteoblasts. The effects of bisphosphonate on hMSCs proliferation and osteogenic differentiation are still unclear. The aim of this study is to investigate the effect of bisphosphonates on hMSCs proliferation and osteogenic differentiation.

Material and Methods

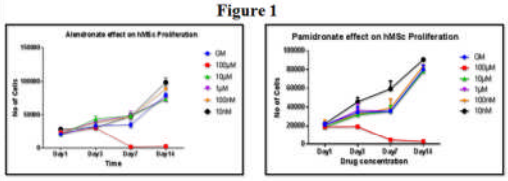
In this study the effect of two types commonly used BPs (alendronate and pamidronate) on hMSCs proliferation and osteogenic differentiation were investigated. Cell proliferation, using alamar blue assay and osteogenic differentiation, using Von Kossa staining assay ,calcium assay and collagen assay were studied.

Results

Proliferation

hMSCs were incubated with medium containing different concentrations of bisphosphonate drugs, both Alendronate and Pamidronate, for 1,3,7 and 14 days, at 100µM there was a cytotoxic effect on cells proliferation on day 7 and day14. Whereas the cells were inhibited at in day 3 ($P < 0.05$). Interestingly, small dose of pamidronate (10 nM) shows a significant stimulation of hMSCs proliferation on day 3, 7 and 14 ($p<0.05$). Also the alendronate shows a significant stimulation on day 14.(Figure 1)

Figure 1



ECM Calcium deposition

Osteogenic differentiation was assayed by using calcium assay kit. After 14 and 21 days of incubating cells with the bisphosphonates (Alendronate and Pamidronate), we found that both drugs have a significant inhibition of hMSCs osteogenic differentiation at 100 µM concentration. Interestingly, small doses of alendronate and pamidronate (10µM, 1µM ,100nM and 10 nM) show significant stimulation of hMSCs osteogenic differentiation. These results correlate with von Kossa staining. (Figure 2&3).

Figure 2

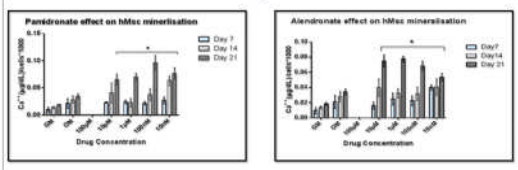
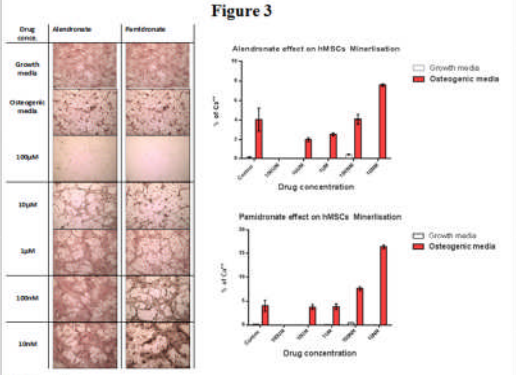


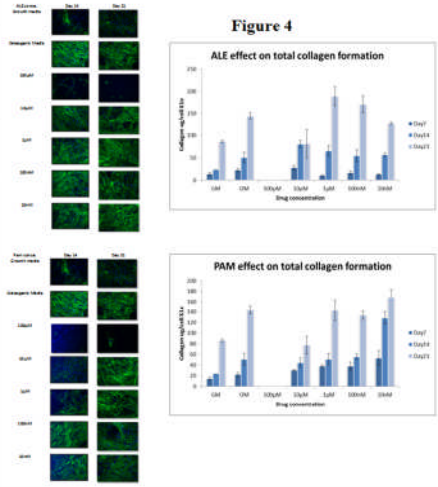
Figure 3



ECM collagen

The total ECM collagen formed by cells was measured at 7,14 and 21 days. The results shows that a small concentration of pamidronate (10nM) significantly increase ECM collagen deposition. The group that been treated with alendronate shows also significant stimulation of ECM collagen deposition at 1 µM and 100nM.(Figure 4)

Figure 4



Conclusion

These experiments show that low concentrations of BP drugs may play an integral role of enhancing hMSCs proliferation and osteogenic differentiation. Furthermore, bisphosphonates have a direct effect on osteoblast mineralisation. These findings suggest that bisphosphonates may more than compensate for the established effects of osteoclasts in bone density in osteoporosis patients.

Single Low dose Bisphosphonate Treatment has Long Term Effects on, and Enhances Osteogenesis in hMSCs

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Background

Bisphosphonates (BPs) are analogues of pyrophosphate. They have been used to treat bone diseases characterized by excessive bone resorption such as osteoporosis and malignant bone diseases. It is well known that bisphosphonates inhibit bone resorption by interfering with osteoclasts activity. In this study we have investigated the extended effect of a single low dose of BPs on hMSCs proliferative and osteogenic behaviour.

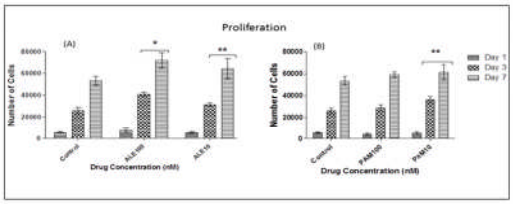
Material and Methods

In this study the effects of a single low dose of two types BPs (alendronate and pamidronate) on hMSCs behavior were investigated. hMSCs were plated at a density of 5×10^5 cells/75cm^2 flasks containing MSC growth media with 10% FCS, 2 mmol L-glutamine and 100U/ml each of penicillin/streptomycin and incubated at 37ºC in humidified 5% CO2, after 24h the medium was changed with growth media containing BPs at either 100nM and 10nM and incubated for further 24h. Then, cells were then washed and trypsinized and sub-cultured again without further exposure to BPs. The cell cultures were assayed for proliferation and osteogenic differentiation using alamar blue, alkaline phosphatase , hydroxyapatite and calcium assays..

Results

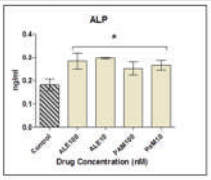
Proliferation

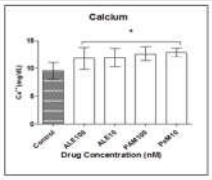
Human MSC proliferation in GM was examined over 1, 3, and 7 days. ALE (100nM and 10nM) stimulated significant cell proliferation in all cells on days 3 and 7 . Also, cells that had been treated with 10nM PAM on days 3 and 7 showed significant proliferation when compared to control cells which were treated with GM only. Cells treated with 100nM PAM promoted cell proliferation but it was not statistically significant.



Mineralisation

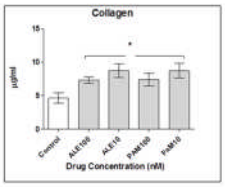
Markers that were linked to early and late stage osteogenesis were analysed. These markers included calcium, collagen type I and alkaline phosphatase. The data suggests that treating cells with a single low dose of drugs (100nM & 10nM) permanently changes the osteogenic behaviour of the hMSCs even after passaging the cells. Data showed that both BP drugs significantly stimulated calcium formation following after 3 weeks when compared to cells that had been treated with osteogenic media only. Furthermore, data showed that at day 7 there were a significant increases in alkaline phosphatase activity





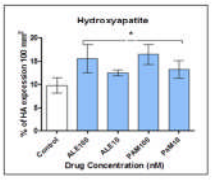
Extracellular Collagen formation

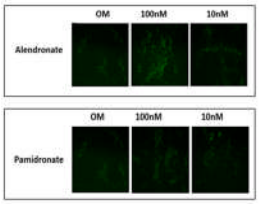
Extracellular matrix collagen was assayed with sirocel collagen assay kit from biocolor according to the manufacture's instruction. . The results showed that both ALE and PAM significantly stimulate hMSCs to produce more collagen following a single low dose drugs (100nM &10nM)for 14 days compared to the other group that being treated with osteogenic media only.



Hydroxyapatite

A Leica – DMIRB fluorescence microscope was used to visually examined hydroxyapatite nodule. Results showed that after 14 days of culture on OM following a single dose treatment; The group treated with ALN and PAM there were clear difference in the appearance and formation of hydroxyapatite nodule compare to the control group that had been treated with OM only.





Conclusion

The enhancement of osteogenesis might point to the use of low dose BPs as an adjunct to implant placement in patients. The permanent nature of the changes suggests a possible epigenetic mechanism operating

Acknowledgment

This research project was supported by Sattam bin Abdul-Aziz University, Ministry of Higher Education of Saudi Arabia.

In vitro Low Dose of Alendronate and Pamidronate promote hMSCs Osteogenic Differentiation.

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Introduction

In the last decades, titanium implant has been successfully used to replace the missing teeth. This success is mainly depending on their osseointegration; coating dental implant with deferent types of bone induced material has showed a positive effect. These materials include, bone stimulating factors, hydroxyapatite (HA) and calcium phosphate, and titanium/titanium nitride. However, human mesenchymal stem cells (hMSCs) shows regenerative properties and able to differentiate to different types of human cells such as osteoblasts. We previously reported that small dose of bisphosphonate may play an integral role of enhancing hMSCs proliferation and osteogenic differentiation. The aim of this study is to investigate wither the small dose of bisphosphonates may induce proliferation and osteogenic differentiation of hMSCs on Ti surface. This will enhance bone quality around the implant. Also, to achieve a better osseointegration and to accelerate wound healing around the implant surface.

Material and Methods

In this study we investigated the small dose effect of two types of BPs (alendronate and pamidronate) on hMSCs osteogenic response to Ti surface. hMSCs were plated in 1.9-cm2 wells containing MSC growth media with 10% FCS, 2 mmol L-glutamine and 100U/ml each of penicillin/streptomycin and incubated at 37ºC in humidified 5% CO2. Next day medium were changed with osteogenic media or growth media containing two different concentrations (100nM & 10nM) of the drugs. We assayed for cell proliferation using alamar blue assay and for the cells mineralisation using calcium assay and alkaline phosphates (ALP) activity. The total ECM collagen formed by cells was measured. Also, to analyse cell morphology, focal adhesion protein Vinculin and F-actin was labelled with fluorescent markers.

Results

Proliferation

hMSCs were incubated with the GM contains different concentration of bisphosphonates drug both Alendronate and Pamidronate for 1,3,7 and 14 days, at 100nM there was a significant stimulation on cells proliferation on day 7 and day14.(Figure 1)

Figure 1

Mineralisation

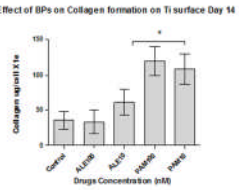
Osteogenic differentiation was assayed using calcium assay kit. After 21 days of growing up the cells on Ti desics with the bisphosphonates (Alendronate and Pamidronate), we found that both drugs have a significant stimulation of hMSCs osteogenic differentiation at 100 nM and 10nM concentration. (Figure 2)

Figure 2

Extracellular Collagen formation

Extracellular matrix collagen was assayed with sirocel collagen assay kit from biocolor according to the manufacture's instruction. . The results showed that PAM significantly stimulate hMSCs to produce more collgen following the treatment with drugs for 14 days compared to the other group that being treated with osteogenic media only.(Figure 3)

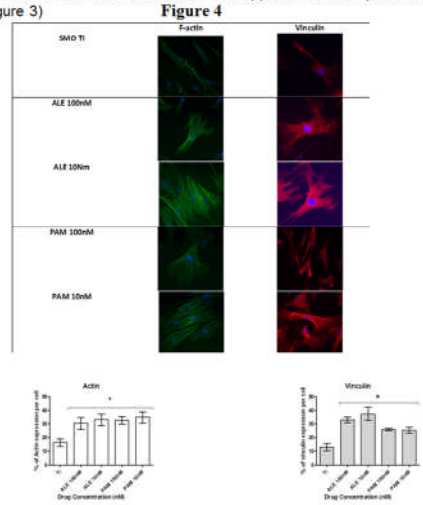
Figure 3



Immunocytochemistry and Cell morphology

A Leica – DMIRB fluorescence microscope was used to analyse cell morphology by labelling F-actin and Vinculin. Results show that after 24 h of culture on OM with and without drugs. The group treated with ALN and PAM there were clear difference in the appearance and spread of the cells. (Figure 3)

Figure 4



Conclusion

These experiments show that lower concentration of drugs may play an integral role of enhancing hMSCs osteogenic differentiation on titanium surface. This is due to a bisphosphonate may have a direct stimuli on osteoblasts mineralisation or bisphosphonate may enhance the wettability of titanium surface.

Acknowledgment

This research project was supported by Sattam bin Abdul-Aziz University, Ministry of Higher Education of Saudi Arabia.

Bisphosphonates as an adjunct to Titanium Implant therapy

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Introduction

Titanium implants have been successfully used to replace missing teeth for over 30 years. The success of the implant depends on successful osseointegration; coating dental implants with different types of bone inducing material has shown positive effects on osseointegration. Human mesenchymal stem cells (hMSCs) possess regenerative properties with the ability to differentiate into different cell types and are the first osteogenic cells to colonise an implant surface. Previously other groups have coated implants with BPs to reduce osteolysis at the site of implant placement and we have previously reported that low dose bisphosphonate effects hMSCs osteogenic differentiation.

Aims

The aim of this study is to investigate whether a low dose bisphosphonate treatment might induce proliferation, migration and osteogenic differentiation of hMSCs on Ti surfaces as a way of enhancing the rate and degree of osseointegration

Materials and methods

In this study we investigated the low dose (100nM&10nM) effect of two types of BP (alendronate and pamidronate) on hMSCs proliferation, migration and osteogenic response to Ti surface. We assayed for cell proliferation using alamar blue assay and for the cells mineralisation using calcium, collagen type I and alkaline phosphatase (ALP). Cell migration assays were used to assess migration of cells toward the Ti surfaces with and without BPs.

Results

Proliferation
hMSCs were incubated with the GM contains different concentration of bisphosphonates drug both Alendronate and Pamidronate for 1,3,7 and 14 days, at 100nM there was a significant stimulation on cells proliferation on day 7 and day14.(Figure 1)

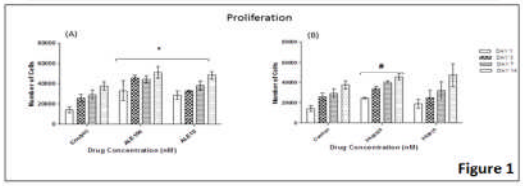

Figure 1

Migration
Cells after 4.5 hours at 37ºC at 5% CO2, were observed to have migrated toward the substrate. However, cells with low doses of BPs (100 nM and 10 nM) have shown that these drugs significantly enhanced cell migration toward titanium surface when compared to the control group that been treated with OM only (Figure 2).

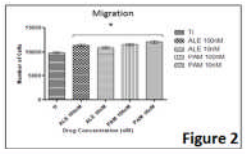

Figure 2

Mineralisation
Data showed that both BP drugs significantly stimulated hMSC osteogenesis following drug treatment for 3 weeks when compared to cells that had been treated with osteogenic media only (Figures 3 (A) and (B)). Furthermore, data showed that treating hMSCs with ALE and PAM for 7 days caused significant increases in alkaline phosphatase activity (Figure 3(C)).

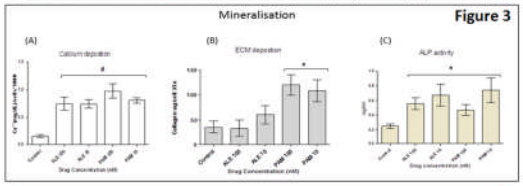

Figure 3

Immunocytochemistry and cell morphology
Data showed that after 24 h of culture, those cells treated with ALN and PAM, exhibited clear differences in cell appearance and spread. These finding were supported by SEM images(Figure 4).

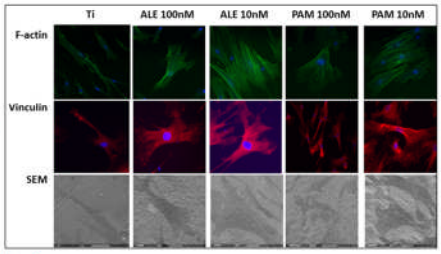

Figure 4

Conclusions

The results of these experiments show that low doses of BPs positively affect hMSCs *in vitro*, and if used systemically as an adjunct to implant therapy, might present a means of enhancing hMSCs osteogenic differentiation on Ti surfaces *in vivo*, hence increasing osseointegration of implants. Therefore the systemic administration of BPs could be pivotal in accelerating osseointegration and the bone healing process following implant placement and might allow the use of implant therapy in patients currently counter indicated.

Acknowledgements

This research project was supported by Sattam bin Abdul-Aziz University, Ministry of Higher Education of Saudi Arabia

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