UNIVERSITY OF SOUTHAMPTON

Bioengineering Research Group,

Faculty of Engineering and the Environment

Close to the Bone:

Investigations into Bone Tissue Mineralisation and Mechanobiology of Osteoporosis

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A dissertation submitted to the University of Southampton for the degree of

Doctor of Philosophy

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ABSTRACT

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<u>Doctor of Philosophy</u>

CLOSE TO THE BONE: INVESTIGATIONS INTO BONE TISSUE
MINERALISATION AND MECHANOBIOLOGY OF OSTEOPOROSIS

by Meadhbh Aíne Brennan

Osteoporosis is a metabolic skeletal disease characterized by low bone mass, depleted micro-architecture and reduced strength. The public health costs of osteoporosis relate almost entirely to the fractures that are the clinical manifestation of the disease and it presents a significant cause of morbidity in today's ageing population. Oestrogen deficiency during the menopause is the primary causative factor for postmenopausal osteoporosis and although much is known about the pathophysiology of the disease, including dysregulated bone cell function whereby more bone is digested than is formed; the underlying mechanisms involved have not yet been delineated. Recent studies have suggested that although overall strength is decreased following osteoporotic bone loss, the remaining bone tissue is stronger and stiffer, suggesting an alteration in bone tissue composition. Bisphosphonates are among drug treatments administered to tackle bone loss, however the incidence of osteoporotic fractures still remains high. Furthermore, the precise effect of drug treatment on bone tissue mineralisation is unknown.

The global aim of this thesis is to discern the alterations in the quantity and distribution of bone mineral during osteoporosis. Specifically, it is sought to test the hypotheses that bone mineral distribution is altered at a tissue level following oestrogen deficiency and bisphosphonate treatment and that oestrogen depletion alters normal mineralisation and mechano-responsiveness of bone cells. Quantitative backscattered imaging (qBEI) on a scanning electron microscope was used to examine individual bone trabeculae from the proximal femur of ovariectomised sheep (oestrogen deficient state), aged matched control sheep and sheep treated with the bisphosphonate Zoledronic acid. It was found that oestrogen deficiency caused significantly higher mineral heterogeneity within trabeculae (site speficic within the femur) and along a common osteoporotic fracture line. Bone mineralisation was diminished with prolonged oestrogen deficiency and

conversely was higher in older healthy sheep compared to younger control sheep. Furthermore, significantly lower mineral heterogeneity was found in OVX sheep treated with Zoledronic acid compared to untreated OVX sheep. These results indicate that changes in bone tissue mineralisation during oestrogen deficiency may be a contributing factor for reduced mechanical strength during osteoporosis, while drug induced increased homogeneity may contribute to the ability of Zoledronic acid to prevent fracture occurrence during oestrogen deficiency.

The next study aimed to delineate the mechanisms responsible for such altered mineral distribution. Osteoblast and osteocyte cells were pre-treated with oestrogen and the effects of oestrogen deficiency were evaluated by subsequently withdrawing oestrogen from cells, or blocking oestrogen receptors using an oestrogen antagonist, fulvestrant. Specifically, alkaline phosphatase expression was investigated using p-nitrophenyl phosphate (pNPP), proliferation by assessing DNA content, calcium production using alizarin red assay and apoptosis by measuring for caspase 3/7 activity. Although mineral production was significantly increased by oestrogen pre-treatment, a further increase in mineral production and apoptosis were observed following oestrogen withdrawal from cells. These observations increase our understanding of the mechanisms controlling bone formation and bone cell death and may aid in the development of enhanced therapeutics for the treatment of osteoporosis.

The final study of this thesis aimed to determine if the mechano-biological response of osteoblasts is impaired during oestrogen deficiency and whether changes in bone mineralisation may be related to altered bone formation in response to mechanical stimulation. Osteoblasts were pre-treated with oestrogen and subsequently oestrogen was withdrawn from cell cultures and their responses under fluid shear stress were evaluated. Firstly, daily loading cycles, using an orbital rotator, were applied to cells and mineralisation and cell viability (using alamar blue assay) were assessed after 7 and 14 days. In a separate experiment, following 2 and 7 days of oestrogen withdrawal, osteoblasts were exposed to 2 hours of shear stress in a custom designed parallel plate bioreactor. PGE_2 was quantified in cell culture conditioned media using an immunoassay kit. It was found that orbital fluid flow induced shear stress significantly increased mineral production by bone cells and that under an applied shear stress, mineral production was decreased during oestrogen withdrawal. It was also observed that mechanical loading and oestrogen are required in unison to promote mineral production.

PGE$_2$ release was significantly increased with applied laminar flow, but was decreased by oestrogen withdrawal.

Together, these studies provide evidence that bone cells become accustomed to levels of circulating oestrogen and that diminished oestrogen causes osteocyte apoptosis, increased osteoblast mineralisation and altered mechano-sensitivity. These changes might explain the decreased mean concentrations of mineral, together with increased mineral heterogeneity, from our earlier *in vivo* studies. Therefore, the results of the thesis provide a unique insight into why the tightly coupled mechanisms of matching bone's structure and composition to the loads it experiences are disrupted when levels of circulating oestrogen are depleted.

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DECLARATION OF AUTHORSHIP

I, Meadhbh Brennan, declare that the thesis entitled 'Close to the Bone: Investigations into Bone Tissue Mineralisation and Mechanobiology of Osteoporosis' and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

this work was done wholly or mainly while in candidature for a research degree at this University;

where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

where I have consulted the published work of others, this is always clearly attributed;

where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

I have acknowledged all main sources of help;

where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

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Nomenclature

ADP	Adenosine Diphosphate
AGP	Above Growth Plate
ALP	Alkaline Phosphatase
AMP	Adenosine Monophosphate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BGP	Below Growth Plate
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMDD	Bone Mineral Density Distribution
BMP	Bone Morphogenetic Protein
BMU	Bone Multi-cellular Unit
BRU	Bone Remodelling Unit
BSE	Backscattered Electron
BSU	Bone Structural Unit
Cbfa1	Core Binding Factor $\alpha 1$
CFU	Colony Forming Unit
CON	Control
CRT	Cathode Ray Tube
CX43	Connexin 43
DEXA	Dual Energy X-ray Absorptiometry
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
E	Young's Modulus
E_2	Estradiol
EBD	Electron Backscatter Diffraction
EDS	Energy Dispersive Spectroscopy
EDX	Energy Dispersive X-ray
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
FTIR	Fourier transform infrared spectroscopy
GL	Grey Level
HCL	Hydrochloric Acid
HRT	Hormone Replacement Therapy
HSC	Hematopoietic Stem Cell
IGF	Insulin-like Growth Factor

IR	Infrared
MCSF	Macrophage Colony Stimulating Factor
MSC	Mesenchymal Stem Cell
NBP	Nitrogen Containing Bisphosphonate
NCP	Non-collagenous Proteins
NO	Nitric Oxide
NOF	National Osteoporosis Foundation
OM	Optical Microscopy
OPG	Osteoprotegerin
OVX	Ovariectomised
PDGF	Platelet Derived Growth Factor
PGE$_2$	Prostaglandin E$_2$
PMMA	Polymethylmethacrylate
PS	Phosphatidylserine
PTH	Parathyroid Hormone
qBEI	Quantitative Backscatter Imaging
RANKL	Receptor Activator of Nuclear Factor-k B Ligand
ROI	Region of Interest
SEM	Scanning Electron Microscopy
SERD	Selective Oestrogen Receptor Down-regulator
SERM	Selective Oestrogen Receptor Modulator
TGF-β	Transforming Growth Factor-β
TUNEL	TdT-mediated dUTP Nick End Labeling
WD	Working Distance
WHO	World Health Organisation
Wt % Ca	Weight Percentage Calcium
Z	Mean Atomic Number
ZOL	Zoledronic Acid

1. Introduction

1.1. Osteoporosis

Bone is a dynamic, mineralised tissue that confers both mechanical and metabolic functions to the skeleton. Osteoporosis is a disease of the skeleton characterized by low bone mass, micro-architectural deterioration and increased bone fragility. It usually presents as clinical fractures which cause severe pain, deformity and in certain cases leads to secondary complications or death (Cummings and Melton, 2002; Johnell and Kanis, 2006). The most common fracture sites are the proximal femur, vertebrae, wrist and pelvis. Hip fractures have, by far, the most impact from a public health perspective as they are associated with the greatest bulk of the osteoporosis-related medical costs (Cooper *et al.*, 1993). Bone mass decreases with age independent of other risk factors (Wendlova and Pacakova, 2007), whereas osteoporotic fracture risk rises dramatically with age. It is estimated that one in two women and one in five men will suffer a fracture after the age of 50 (Van Staa *et al.,* 2001). In addition, approximately nine million osteoporotic fractures occur globally each year (Johnell and Kanis, 2006) and this figure is set to increase three fold by 2050 (Gullberg *et al.*, 1997).

Primary osteoporosis refers to both bone loss occurring in post-menopausal women (type I) and bone loss owing to the normal ageing process (type II). Post-menopausal osteoporosis (type I) is the most common form of the disease and is believed to initiate when oestrogen production is deficient following the menopause (Riggs *et al.,* 2002; Falahati-Nini *et al.,* 2000). This phase lasts for 4-8 years where an imbalance in the bone remodelling cycle leads to loss of predominantly trabecular bone. Type II osteoporosis is characterised by a slow, steady loss of bone mass in both trabecular and cortical bone. It is primarily due to a reduction in bone formation and typically occurs after 70 years of age. Men only experience type II osteoporotic bone loss as a result of a reduction in testosterone and oestrogen availability (Riggs *et al.,* 2002). Secondary osteoporosis can present in old or young individuals and is a secondary effect of medications or diseases including anorexia nervosa, cystic fibrosis, depression and diabetes mellitus type I (Lerner, 2006). It is well established that there is a macro-level decrease in mechanical properties during osteoporosis (Ederveen *et al.,* 2001; Bourrin *et al.,* 2002; Geusens *et al.,* 1996). This can be attributed to a decrease in bone mass since osteoporotic bone is characteristically less dense. However, an increase in strength has been demonstrated at a trabecular level during oestrogen deficiency (McNamara *et al.*, 2006). Mineral

concentration and its distribution within the bone tissue is a key determinant of the mechanical strength of bone (Currey, 1984b; Hernandez, 2008; Jaasma *et al.,* 2002; Render *et al.,* 2006; Ruffoni *et al.,* 2007), however research performed to discern the differences in mineralisation during osteoporosis has produced conflicting observations (Bohic *et al.*, 2000; Boyde *et al.*, 1998; Gadeleta *et al.*, 2000; McNamara *et al.,* 2006; Rohanizadeh *et al.*, 2000). In addition, studies have shown that mineral is more heterogeneously distributed within trabeculae during oestrogen withdrawal (Busse *et al.*, 2009; Roschger *et al.*, 2008) however, the exact trabecular regions where altered mineralisation occurs and the precise mechanisms by which such changes might arise, have not been distinguished. During oestrogen withdrawal bone removal by osteoclasts is increased (Richelson *et al.*, 1984). This leads to depleted bone architecture, as bone trabecular struts are perforated (Mosekilde, 1990) and an overall reduction in bone mass and strength (Watts *et al.*, 2010). Currently, there is a great deal of research being conducted into pharmaceuticals aimed at remedying the disease. Bisphosphonates are the most commonly used drug targeted at preventing osteoporotic bone loss and they act by inhibiting removal of bone tissue and therefore, retaining bone mass and trabecular architecture, however, fracture prevention still only lies at approximately 50-60% (Black *et al.*, 1996; McClung *et al.*, 2001).

1.2.   Objectives and Hypotheses

The aim of this thesis is to discern the alterations in the quantity and distribution of bone mineral during osteoporosis and to examine the cellular mechanisms responsible for such material-level changes. Specifically, mineral distribution within individual trabeculae and between distinct anatomical locations will be compared between ovariectomised sheep, a group treated with bisphosphonate and a healthy aged matched control group. Furthermore, the effects of oestrogen withdrawal on bone cells in terms of proliferation, mineral production, cell death and mechano-sensitivity will be investigated. To address these objectives 5 hypotheses have been defined which will be tested in order to provide a better insight into the mechanisms of altered mineralisation due to oestrogen deficiency.

Hypothesis 1: Bone mineral distribution is altered at a tissue level during oestrogen deficiency

Since bone strength is correlated with tissue stiffness (Fyhrie and Schaffler, 1994) and the degree of mineralisation (Currey, 1984b; Follet *et al.,* 2004), the increases in strength

and stiffness of trabeculae during oestrogen withdrawal (McNamara *et al.*, 2006) suggest an increase in mineral concentration may occur within trabeculae during osteoporosis. As bone with a high mineral content has diminished impact resistance (Currey, 1969) this may heighten the risk of fracture. Furthermore, increases in remodelling rates brought on by oestrogen deficiency vary considerably according to skeletal site (Baldock *et al.*, 1998), suggesting that oestrogen withdrawal may alter the distribution of bone tissue mineral between different skeletal locations.

Hypothesis 2: Mineralisation is altered with prolonged oestrogen depletion

The discrepancies between studies reporting increases, decreases, or no change in bone tissue mineralisation during oestrogen deficiency (Bohic *et al.*, 2000; Boyde *et al.*, 1998; Gadeleta *et al.*, 2000; McNamara *et al.,* 2006; Rohanizadeh *et al.*, 2000) may be due to variations in the duration of oestrogen deficiency, as it has been shown that there is an initial period of significant increase in bone turnover markers, followed by a waning of this response (Binkley *et al.*, 1998). In addition, it has been shown that healthy bone tissue mineralisation is altered with ageing (Currey *et al.*, 1996; Vajda and Bloebaum, 1999); however, it has not yet been investigated whether alterations in the degree of bone tissue mineralisation reported previously are prolonged with continued oestrogen deficiency, or are a transient characteristic caused by the initial diminished oestrogen levels. Together, these observations lead to the hypothesis that bone tissue mineralisation is altered over prolonged oestrogen depletion.

Hypothesis 3: Mineralisation is altered as a result of bisphosphonate treatment

 It has been observed that the diminutive increases in bone mineral density with bisphosphonate treatment cannot account for the large decreases in fracture occurrence observed with their administration (Liberman *et al.*, 1995; Riggs and Melton, 2002; Yao *et al.*, 2007). Since tissue mineralisation is dependent on functional remodelling (Tsubota *et al.*, 2009) and mineralisation kinetics (Akkus *et al.,* 2003; Ruffoni *et al.,* 2007) and bisphosphonates alter bone remodelling by inhibiting bone resorption (Carano *et al.*, 1990; Fleisch *et al.*, 1969), the distribution of bone tissue mineral may be altered by bisphohsphonate treatment.

Hypothesis 4: Oestrogen deficiency alters the normal bone mineralisation process by osteoblasts and osteocytes

 While there has been extensive research regarding the effects of oestrogen depletion on

bone resorbing cells, osteoclasts, whereby increased osteoclastic resorption occurs during osteoporosis (Heaney *et al.*, 1978; Lerner, 2006) and oestrogen treatment reduces bone resorption by osteoclasts (Oursler *et al.*, 1991), there has been much less focus on cells responsible for bone formation and mineral deposition; osteoblasts and osteocytes. Since both osteoblasts and osteocytes possess receptors for oestrogen (Bellido *et al.*, 1993) (Bord *et al.*, 2001; Braidman *et al.*, 2001), oestrogen withdrawal may have a direct impact on mineral production by these bone cells. In addition, it has been proposed that following cell death by apoptosis, mineral infilling of the remaining lacunae, a phenomenon referred to as micropetrosis (Boyde, 2003; Frost, 1960; Kingsmill and Boyde, 1998) may occur. Interestingly, oestrogen withdrawal in women results in a higher percentage of dead osteocytes in bone (Tomkinson *et al.*, 1997), possibly due to increased apoptosis. Together, these studies indicate that oestrogen withdrawal may cause alterations in mineral production by bone cells, which may be responsible for the changes that occur at a tissue level during osteoporosis. If validated, these experiments may give an important insight into the primary events of the bone loss cascade during oestrogen deficiency.

Hypothesis 5: The mechano-responsiveness of osteoblasts is impaired during oestrogen deficiency

In healthy bone, mechanical adaptation ensures efficient load bearing by means of a cellular process, which relies on a biological system that senses the applied mechanical loading and reacts accordingly. It has been proposed that bone's mechano-responsiveness fails during osteoporosis, but the precise mechanism by which this is manifested is not understood. Since mechanical stimulation increases mineral production (Chambers *et al.*, 1993; Robling *et al.*, 2000; Turner *et al.*, 1996), it may be that oestrogen depletion impairs the detection of mechanical loading, or impairs the normal response to the stimulus. Either mechanism may inhibit normal bone formation by bone cells in response to loading.

1.3. Thesis Structure

This thesis details the work completed for the duration of the candidates PhD studies. It begins by presenting an extensive literature review of the function, activities, characteristics, and constituents of healthy and osteoporotic bone, in addition to the mechanisms behind the quantitative backscatter imaging (qBEI) technique which is employed to quantify tissue mineralisation, in Chapter 2. Chapter 3 details a study which

investigates the effects of oestrogen deficiency on tissue mineralisation, thereby testing hypothesis 1. Chapter 4 addresses hypotheses 2 and 3 by evaluating the effects of prolonged oestrogen deficiency, healthy ageing and bisphosphonate treatment on tissue mineralisation. Chapter 5 is concerned with *in vitro* cell studies which test hypothesis 4 by investigating mineral production and apoptosis of bone cells undergoing oestrogen withdrawal. Chapter 6 examines whether the mechano-responsiveness of bone cells are impaired due to oestrogen withdrawal, thereby testing hypothesis 5 of the thesis. Chapters 3-6 each set out the background and rationale for each study, a detailed description of the materials and methods employed, as well as the results and an in-depth discussion of the findings. Finally, chapter 7 outlines the main findings of the thesis, together with recommendations for future work based on the subject of the thesis.

2. Literature Review

2.1. Bone Function

Bone is a highly specialised form of connective tissue that plays a vital supportive and protective role in the body. It provides an internal structural framework for mechanical support in addition to shielding internal organs and bone marrow from potential damage. It also presents sites of muscle attachment and levers to transmit forces generated by muscles and thereby permit locomotion. This complex living tissue also participates in metabolic homeostasis as it is a major source of inorganic ions, particularly calcium and phosphate (Murray, 1999).

2.2. Bone Morphology

Anatomically, there are two distinguishable types of bones in the human skeleton; flat bones, namely the scapula, mandible and ilium, and long bones which include the femur, tibia and humerus. A typical long bone consists of a central cylindrical shaft or diaphysis, an epiphysis at either extremity, as well as an intermediate metaphysis region. The growth plate, an expanding region of hyaline cartilage matrix and proliferative cells, separates the metaphyses from the epiphysis and represents a region where bone growth and elongation occurs (Murray, 1999). In a skeletally mature adult the cartilaginous growth plate becomes replaced by cancellous bone and the epiphysis becomes fused to the metaphysis. The epiphyses are covered by a layer of hyaline or articular cartilage which permits articulation of bones at a joint, thus enabling movement of the skeleton. The periosteum, a thin layer of fibrous connective tissue with an inner cellular film of undifferentiated cells, covers the outer surface of most bones and plays an important role in bone growth and fracture repair. The diaphysis of long bones contains an internal marrow cavity which is lined by a thin layer of osteoblastic and bone lining cells, the endosteum (Cowin, 2001). The blood supply to the cortex of long bones is primarily served by the medullary vessels (Trueta and Caladias, 1964). Haversian canals supply cortical bone with blood, while nutrients are supplied to trabecular bone through a canalicular system.

The outer shell (cortex) of all bones and the diaphyses of long bones consists of dense and highly calcified tissue, known as cortical bone, which plays a supportive and protective role within the body (Buckwalter *et al.*, 1996; Currey, 1984a; Favus, 2006). Trabecular bone tissue is encased within the outer cortical cortex and comprises of a highly connected system of vertical and horizontal struts, called trabeculae. Intermediate

spaces between trabeculae are filled with hematopoietic bone marrow. The majority of trabeculae are oriented along lines of recurrent mechanical stress (Von Meyer, 1867), while the remainder join perpendicularly to adjacent trabeculae in order to strengthen the ne2rk (Wolff, 1892). Trabecular bone dissipates internal stresses to the outer cortical shell (Lemaire *et al.,* 2004), see Figure 2.1, and also plays a role in vital metabolic functions (Murray, 1999).
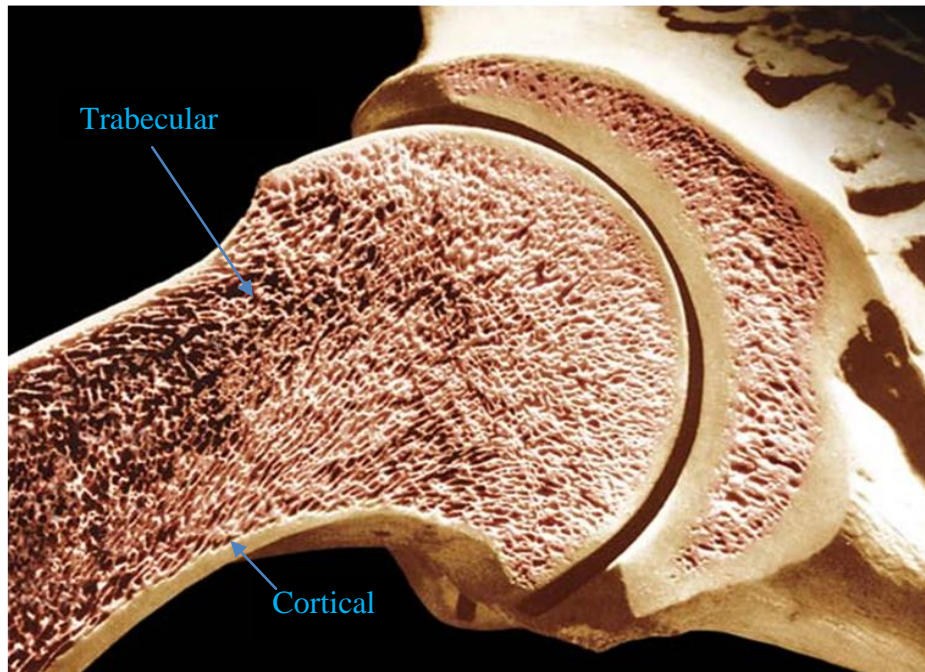
Figure 2.1: Structure of Cortical and Cancellous Bone (Fawcett, 2011; Poole and Compston, 2006; Ritchie *et al.*, 2009)

2.3. Bone Composition

Bone is composed of approximately 65% mineral, 35% organic matrix, cells and water. The organic matrix is composed of approximately 90% collagen and 10% non-collagenous proteins. The collagen in bone is primarily type I collagen, but trace quantities of type III, IV and VI are also present (Miller, 1969; Miller, 1984). Type I collagen serves as a scaffold and is thought to be an initiator of mineral deposition (Glimcher, 1989). Non-collagenous proteins such as osteocalcin, osteonectin, osteopontin and bone sialoprotein provide bonds between collagen fibrils, facilitate mineralisation and act as chemo-attractant to facilitate the binding of osteoclasts (Fantner *et al.*, 2005; Roach, 1994). Bone mineral presents as tiny crystals (in the order of 20x40x200Å) of impure carbonated hydroxyapatite, Ca_{10} $(PO_4)_6$ $(OH)_2$ and other

minerals including magnesium, potassium, manganese, silica, fluoride, iron, zinc and citrate, which are integrated into the crystal lattice or absorbed onto the Ca_{10} $(PO_4)_6(OH)_2$. The mechanical integrity of the skeleton, its load bearing strength and its capacity to control the mineral ion homeostasis are significantly influenced by the quantity of mineral present (Currey and Brear, 1990).

Collagen fibrils play a highly interactive role with mineral crystals (Landis *et al.*, 1996), they have been indicated as the primary foundation or nucleator for crystal deposition in bone tissue (Glimcher, 1959). The importance of collagen as a template for mineral deposition was demonstrated by studies on specimens suffering from osteogenesis imperfecta which is a set of diseases characterised by a defect in the subject's collagen. One study revealed defects in collagen structure resulted in an altered mineral-to-matrix ratio in addition to mineral composition (Camacho *et al.*, 1996). Cross-linking of collagen also has an effect on mineral deposition. It was shown that blocking the cross-linking of collagen in cultured osteoblasts reduces in the amount of mineral deposits (Gerstenfeld *et al.*, 1993). Collagen is not however the sole regulator for crystal deposition, this is evident as other tissues such as tendons, skin, and ligaments contain the same type I collagen but do not normally calcify. Several other inducer and inhibitors of bone mineralisation will be discussed in detail in section 2.4.1.

2.3.1   Hierarchical Structure of Bone

Bone is a composite material with a highly complex hierarchical structure. Its components and their organisation within bone give rise to an ideal combination of material properties bestowing bone with its high strength, fracture toughness and stiffness, as well as low weight. The structure of bone is illustrated at different structural levels in Figure 2.2 and detailed in Table 2.1.
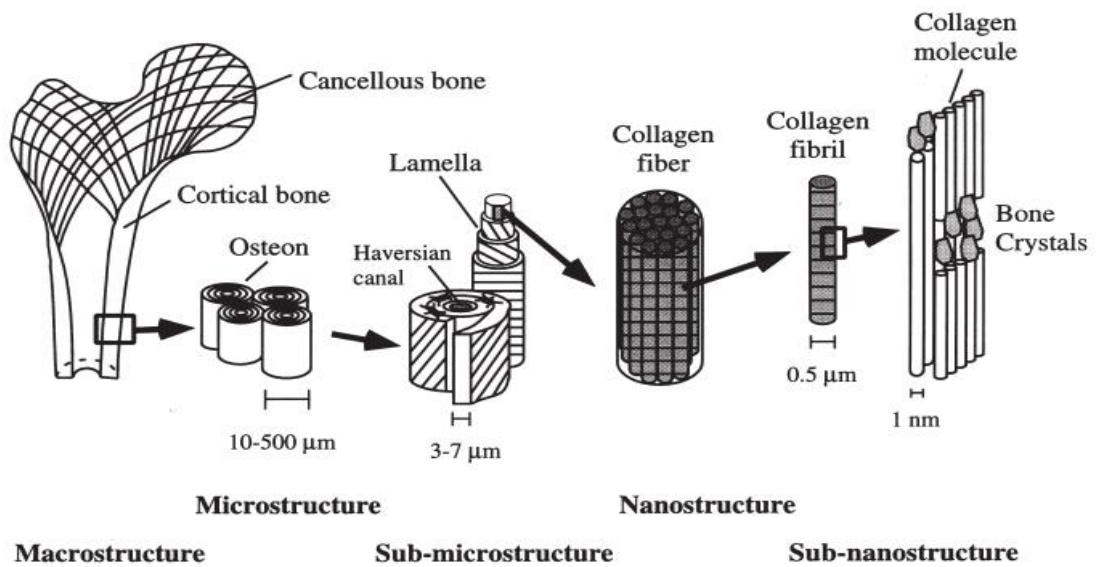
Figure 2.2 Hierarchical Structural Organization of Bone (Rho et al., 1998)

Collagen molecules, mineral crystals and water molecules are found at the sub-nanostructure level, where collagen fibrils are composed of collagen molecules arranged with apatite crystals distributed within the discrete spaces between the collagen molecules. At the nanostructure level, collagen fibrils are arranged into fibers. At the sub-microstructure level in cortical bone, mineralised collagen fibers are arranged into concentric parallel rings surrounding a haversian canal which together forms osteons. In trabecular bone, tissue lamellae are arranged in a less organized manner into individual trabeculae. At the macro-structural level bone type is distinguished as either cortical with densely packed osteons, or trabecular bone with a porous network of interconnecting trabecular struts (Rho *et al.*, 1998).

Table 2.1: Structural Hierarchy of Bone

Scale	Size Range	Components
Sub-nanostructure	<500nm	Apatite crystals, collagen and water molecules
Nanostructure	500-1000 nm	Mineral matrix and collagen fibrils arranged into fibers
Sub-microstructure	1-10μm	Single lamella, cement lines, canaliculi.
Microstructure	10-5000μm	Single trabeculae , osteons
Macrostructure	1-10cm	Cortical and cancellous bone

2.4. Bone Cells

A variety of cells reside within bone, such as osteoblasts, osteocytes, bone lining cells and the precursors of these cells, also referred to as osteoprogenitor cells, as well as osteoclasts. Osteoblasts are responsible for the synthesis and secretion of a new bone matrix, while osteoclasts resorb or destroy bone tissue. The function and activities of osteoclasts and osteoblasts are directly co-ordinated to maintain bone mass and they interact during skeletal development and throughout life.
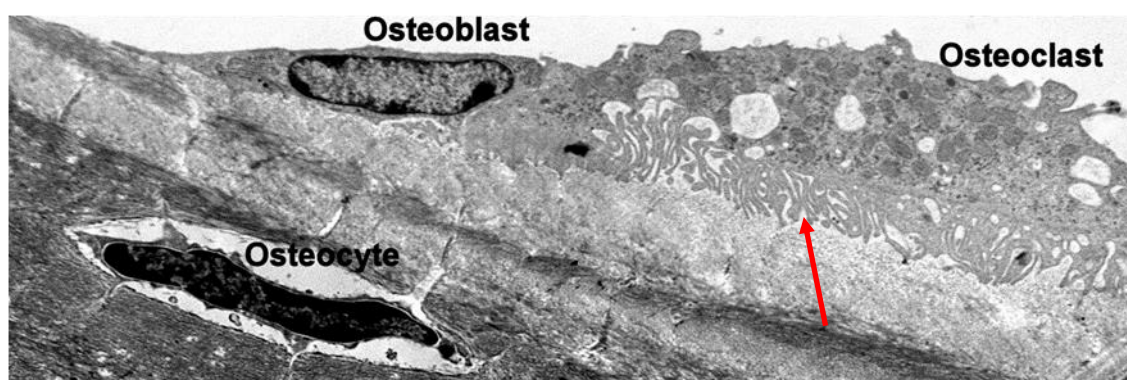
Figure 2.3 TEM image of an osteoblast, osteoclast, and osteocyte in situ within bone tissue. Arrow indicates the ruffled border of an osteoclast. Reproduced with permission from Dr. L.M. McNamara

2.4.1 Osteoblasts and Bone Lining Cells

Osteoblasts are of mesenchymal origin, once committed to the osteoblastic lineage osteoprogenitor cells proliferate and differentiate into pre-osteoblasts and ultimately mature osteoblasts (Okazaki *et al.*, 2002). Osteoblasts, who reside on the bone surface (see Figure 2.3) produce all the components of the bone matrix by secreting and synthesizing an unmineralised bone matrix, the osteoid, and subsequently mineralizing this matrix (Ducy *et al.*, 2000). It takes 5-10 days before the matrix has been 70% mineralised and complete mineralisation takes between 3-5 months. Active osteoblasts are cuboidal cells (15-30µm thick) and posses a large nucleus, abundant endoplasmic reticulum, gap junctions, a large golgi apparatus and secretory vesicles (Cowin, 2001).

Many substances have been identified that govern the activity of osteoblasts. Alkaline phosphatase (ALP) is secreted by osteoblasts and is vital for increasing local phosphate concentration and promoting mineralisation by removing nucleation inhibitors (Favus, 2006). Animals with defective alkaline phosphatase expression have been shown to

develop the condition hypophosphatasia, which manifests as significant decreases in mineral deposition (Narisawa *et al.*, 1997). Bone morphogenetic proteins (BMP) are a family of cytokines which induce differentiation of mesenchymal stem cells (MSCs) to osteoblasts *in vitro* (Barnes *et al.*, 1999) and have demonstrated increased bone formation *in vivo* in rats (Wang *et al.*, 1990). Osteonectin is a glycoprotein that binds to collagen, calcium and hydroxyapatite, suggesting it facilitates bone formation by acting as a nucleator for matrix mineralisation (Sikavitsas *et al.*, 2001). Transforming growth factor – β (TGF-β) stimulates extracellular matrix production by promoting collagen synthesis and increasing alkaline phosphate production (Barnes *et al.*, 1999). Insulin-like growth factor (IGF) stimulates osteoblast proliferation and collagen production *in vitro* (Gillery *et al.*, 1992). Osteoblast proliferation is also stimulated by platelet-derived growth factor (PDGF) and fibroblast growth factors (FGF) (Sikavitsas *et al.*, 2001).

Conversely, there are several inhibitors of tissue mineralisation. Osteopontin, a phosphorylated bone matrix sialoprotein prevents apatite formation and growth due to its high affinity for apatite crystals (Boskey *et al.*, 1993) and inorganic pyrophosphate blocks mineralisation by up-regulating the amount of osteopontin (Addison *et al.*, 2007). Osteocalcin, another non-collagenous protein, is thought to inhibit bone mineralisation as it has an affinity for calcium (Sikavitsas *et al.,* 2001). Furthermore, parathyroid hormone inhibits osteoblast function (Buckwalter *et al.,* 1996).

2.4.1.1 Mechanotransduction of Osteoblasts

In addition to responding to biochemical stimuli, osteoblasts are capable of mechanotransduction, which is the conversion of a physical force into a biochemical signal (Machwate *et al.*, 1995). Mechanical loading of the skeleton confers mechanical strain to bones, which in turn causes increased bone formation *in vivo* (Chambers *et al.*, 1993; Robling *et al.*, 2000; Turner *et al.*, 1996) and *in vitro* (Sittichockechaiwut *et al.*, 2009). Indeed even after just 96 hours after a mechanical stimulus *in vivo*, mineralisation of newly formed bone matrix has been demonstrated *in vivo* (Forwood *et al.*, 1996).

Shear stress induced by fluid flow reflects an indirect effect of applied mechanical strain that may act on osteoblasts *in vivo* and has been used extensively for *in vitro* investigations of mechanotransduction in osteoblasts. Intracellular calcium signalling is an immediate response to mechanical stimuli, an elevation in intracellular calcium signalling has been observed seconds after mechanical loading in osteoblastic cells (Huo *et al.,* 2008). Calcium signalling is required for the expression of bone matrix proteins such as osteopontin (You *et al.,* 2001) and for numerous other essential functions such as

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proliferation and differentiation (Berridge *et al.,* 2000; Zayzadoon *et al.,* 2006). Osteoblasts can disseminate mechanically induced intercellular calcium signalling via two different mechanisms. The first entails activation of plasma membrane receptors, generated by a soluble mediator (the extracellular paracrine signal adenosine triphosphate –ATP), which causes release of intracellular calcium stores. Alternatively, osteoblasts can also propagate intercellular calcium signalling by direct intercellular communication using gap junctions, causing the release of intracellular calcium stores (Jorgensen *et al.*, 2000). ATP is a high energy molecule that stores energy and is present in the cytoplasm and nucleoplasm of every cell. Adenosine monophosphate (AMP) can be formed by ATP synthesis by combining two molecules of adenosine diphosphate (ADP), and it has been shown that AMP production by osteoblasts is increased with application of shear stress *in vitro* (Reich *et al.*, 1990).

Under applied shear stress *in vitro*, bone cells produce increased quantities of a number of other biochemicals in addition to intracellular calcium, including prostaglandin E_2 (PGE$_2$), nitric oxide (NO) and osteopontin (Bakker *et al.*, 2001; Batra *et al.*, 2005; Donahue *et al.*, 2003; McGarry *et al.*, 2005b; Smalt *et al.*, 1997). Mineral matrix production has also been shown to be increased under applied loading (Sikavitsas *et al.*, 2003; Van den Dolder *et al.*, 2003). It has been demonstrated that prostaglandins induce the production of AMP in mesenchymal bone marrow cells (Scutt *et al.*, 1995); therefore the up-regulation of AMP with shear stress could occur indirectly via a mechanism involving increased prostaglandin production with shear stress. Wnt proteins are a family of secreted proteins and Wnt/Lrp5 signalling is required for normal proliferation and functions of osteoblasts. The down regulation of Wnt inhibitors by osteocytes is dependent on mechanical stimulation (see section 2.4.3.1), therefore mechanical loading indirectly regulates bone mass by Wnt/Lrp5 signalling (Johnson *et al.*, 2004; Krishnan *et al.*, 2006).

In spite of the extensive evidence that mechanical stimulation of bones *in vivo* increases bone formation (Chambers *et al.*, 1993; Robling *et al.*, 2000; Turner *et al.*, 1996) and also that unloading due to disuse or microgravity leads to loss of bone mass (Collet *et al.,* 1997; Jee *et al.,* 1983) and mineral content (Le Blanc *et al.*, 1985; Tilton *et al.*, 1980), *in vivo* experiments cannot distinguish between direct and indirect effects of strain. Therefore, several *in vitro* experiments have investigated the sensing mechanisms of mechano-transduction in osteoblasts by comparing direct mechanical strain such as tension and compression of the cell attached to a substrate versus indirect effects of

strain such as shear stress. When shear stress was compared with unidirectional linear strain applied to osteoblasts *in vitro*, it was found that mechano-responsiveness, in terms of NO and PGE_2 release, was rapidly increased in cells exposed to shear stress, however cells subjected to linear strain showed no increase in the production of these biochemicals (Smalt *et al.*, 1997). However, other studies have shown that when bone cells were subjected to cyclic strains on a plastic substrate, PGE_2 increased with direct strain and the amount of PGE_2 released by cells was dependent on the strain magnitude (Murray and Rushton, 1990). Interestingly a different study found that although shear stress increased NO and PGE_2 to a much greater degree than which was found with substrate strain, collagen I production was significantly increased with direct strain, whereas shear stress decreased collagen I production (McGarry *et al.,* 2005b). Other studies have shown that shear stress is the major mechanism of osteopontin up-regulation by osteoblasts compared with direct mechanical strain (Owan *et al.*, 1997).

Several studies have focused solely on fluid flow and have differentiated between the consequential shear stress, streaming potentials and chemo-transport. Studies have shown that the primary mechanism for mechano-transduction, in terms of NO and PGE_2 release, is shear stress, when compared with chemo-transport or streaming potentials (Bakker *et al.,* 2001) (Donahue *et al.,* 2003). This conclusion was made on the basis that the up-regulation of these biochemicals due to shear stress, was the same as when the viscosity of the media was increased and flow rate was decreased in order to achieve the same shear stress (Bakker *et al.,* 2001). Jacobs *et al* discovered that oscillatory flow was a much less potent up-regulator of NO and PGE_2 than either steady or pulsatile flow and concluded that the underlying cause was the net reduction in fluid flow in oscillatory flow, indicating that chemo-transport does indeed play a role in the mechano-responsiveness of cells to fluid flow (Jacobs *et al.*, 1998). Donahue et al investigated the role of chemotransport further by keeping the shear stress constant by altering the flow rate and viscosity. They found that with constant shear stress, decreasing the flow rate, which deprives the cells of nutrients, caused decreased NO, PGE_2 and intracellular calcium mobilization (Donahue *et al.,* 2003). This study suggested that although chemo-transport does not elicit a response alone, it modulates the effect of shear stress on cells, which is in contrast to the findings of Bakker *et al* (Bakker *et al.,* 2001).

The mechanisms by which osteoblasts sense mechanical stimuli are still to be fully elucidated. There is a growing body of evidence however that cytoskeleton-integrin interactions are involved. It was shown *in vitro* that the increased expression of proteins

linked to mechano-transduction (COX-2 and c-fos) by shear stress occurs by a mechanism whereby the reorganization of actin filaments into contractile stress fibers occurs which involves the recruitment of β_1-integrins and α-actinin to focal adhesions (Pavalko *et al.*, 1998). Blocking the development of actin stress fibers with cytochalasin D inhibited the shear stress induced gene expression in osteoblasts (Pavalko *et al.*, 1998). Furthermore, it has been demonstrated that the signal transduction mechanism for the mechanical activation of osteopontin is dependent on microfilaments of the cytoskeleton since cytochalasin D, which disrupts actin microfilaments, inhibited mechanical activation of osteopontin expression (Toma *et al.*, 1997). In summary, the mechanotransduction of osteoblasts involves a cascade of events including ATP and calcium signalling, the release of NO and PGE_2 and Wnt/Lrp5 signalling, which leads to the formation of new bone tissue. As an osteoblast completes their lifespan, which lasts approximately two months, they give rise to both bone lining cells and osteocytes (Parfitt, 1994).

The surface of quiescent bone is covered by a layer of elongated bone lining cells lying on a 1-2µm layer of un-mineralised collagen matrix (Manolagas, 2000). These cells are critical in the bone resorption process, as osteoclasts are unable to attach to the un-mineralised collagenous layer, it is thought that bone lining cells secrete collagenase that digest the matrix to permit osteoclast attachment (Cowin, 2001). In addition, bone lining cells contract to permit osteoclast access the underlying mineralised bone (Murray, 1999).

2.4.2 Osteoclasts

Osteoclasts are large multi-nucleated cells whose primary role is to digest the bone matrix in a process known as bone resorption. Osteoclasts cover less than 1% of the bone surface in adolescents, hence are comparatively scarce cells, and are temporarily found in resorption cavities or Howship's lacunae on the bone surface during bone resorption (Watanabe *et al.*, 1995). Osteoclasts are derived from circulating mononuclear precursors in hemopoietic tissues (Udagawa *et al.*, 1990). Briefly, a hematopoietic stem cell (HSC) differentiates through the colony-forming unit (CFU) for granulocytes and macrophages to the pre-osteoclast and multinucleated cell and finally a mature active resorbing osteoclast is formed (Bar - Shavit, 2007). Mature osteoclasts possess up to 50 nuclei and range in diameter from 20 - 100µm (Roodman, 1996). These highly motile cells contain multiple cellular adhesions, cytoskeleton proteins, granules, vacuoles and an abundance of mitochondria throughout the cytoplasm (Horne, 1995).

Osteoclastogenesis is a diverse physiological pathway dependent on various conditions and signalling factors. There are several factors that promote osteoclast differentiation and resorption and prevent osteoclast apoptosis. These include the Receptor Activator of Nuclear factor-kB Ligand (RANKL) and the macrophage colony-stimulating factor (M-CSF). Conversely, factors that inhibit osteoclast differentiation and resorption include BMP-2, TGF-β, calcitonin and oestrogen (Cowin, 2001; Filvaroff and Derynck, 1998).
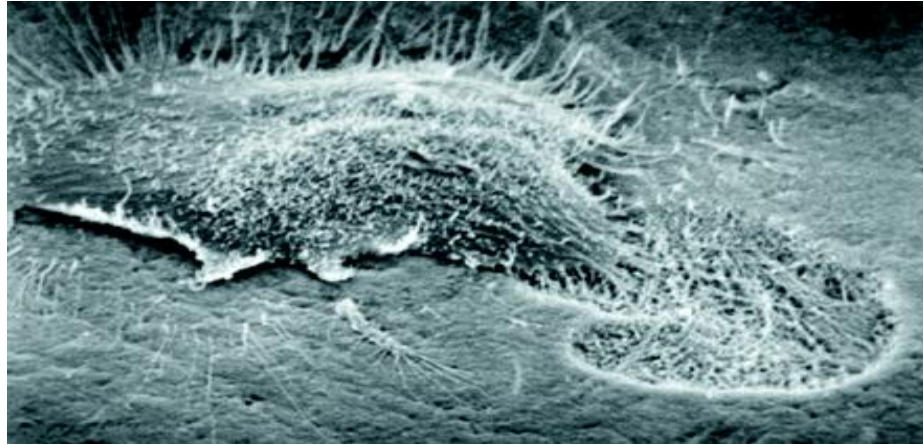
Figure 2.4: Scanning electron micrograph of an osteoclast resorbing bone (Poole and Compston, 2006)

Several factors are thought to initiate resorption including micro-damage to the bony matrix (Lee *et al.*, 2002; Martin, 2002). Following the retraction of bone lining cells from the surface of bone, the exposed mineralised matrix acts as a chemo-attractant for osteoclast recruitment to the site (Murray, 1999). The resorptive cycle is a complex multi-step process including attachment to the mineralised bone surface, polarization, formation of a sealing zone, dissolution of both the mineral and organic components of the matrix, removal of the degradation products and finally cell apoptosis or their transition into an inactive phase once again (Salo *et al.*, 1997). The ruffled border is the most remarkable morphology feature of the osteoclast and is highlighted by the arrow in Figure 2.3. It is a region of widespread plasma membrane folding, formed by the fusion of acidic intracellular vesicles (Palokangas *et al.*, 1997), appearing only when the osteoclast has attached to the bone surface. This border is the cells' resorptive organelle, secreting acids and proteolytic enzymes that degrade bone. The sealing zone is rich in microfilaments and devoid of organelles. It completely surrounds the ruffled border, permitting attachment of the osteoclast to the underlying bone matrix and the formation

of a cellular microenvironment (Teitelbaum, 2000) in which the pH is about 3.5 (Cowin, 2001; Vaananen and Horton, 1995).

An image of an osteoclast resorbing bone is presented in Figure 2.4. The dissolution of crystalline hydroxyapatite crystals occurs by secretion of hydrochloric acid (HCL) into the sealed extracellular microenvironment through the ruffled boarder (Vaananen *et al.*, 2000). Following solubilisation of the mineral component of the bone matrix several proteolytic enzymes digest the collagen and other proteins of the organic bone matrix and the degradation products are removed through vesicles from the ruffled border where they are deposited into the extra-cellular space (Salo *et al.*, 1997). The resorption process releases calcium, phosphate and other ions from bone for homeostasis and instigates the structural remodelling, which adjusts skeletal architecture to mechanical loads (Bilezikian *et al.*, 2002). The life cycle of an osteoclast *in vivo* has yet to be fully understood, however their life span is up to six weeks, after which it migrates to the marrow space where it undergoes apoptosis (Marks and Seifert, 1985).

2.4.3   Osteocytes

Osteocytes are the most abundant cell in mature bone representing more than 90% of the total number of bone cells. They reside singularly within the mineralised bone matrix in spaces called osteocyte lacunae (see Figure 2.3) and are derived when osteoblasts become embedded in their secreted osteoid. Osteocytes lose much of the organelle of their predecessors and acquire elongated dendritic processes, which are extensions of their plasma membrane. Osteocytes communicate with other embedded osteocytes, bone lining cells and osteoblasts through canaliculi or channels within the bone matrix. They also retain a nutrient supply and dispose of waste through this lacunar-canalicular network (Cowin, 2001). Osteocyte cell processes are connected to one another and to cells on the bone surface via gap junctions (Li *et al.*, 2000), which are transmembrane channels that connect the cytoplasm of adjacent cells, allowing communication between cells (Xia *et al.*, 2010). Gap junction formation is facilitated by a group of proteins known as connexins (Goodenough *et al.*, 1996) and in osteocytes, connexin 43 (CX43) is found on the membrane of the cell body as well as on the cell processes (Jones *et al.*, 1993). These highly sensitive and communicative cells have the ability to sense changes in the levels of hormones circulating within interstitial fluid (Manolagas, 2000), induce signals of bone resorption by activating death signalling pathways during apoptosis (Kogianni *et al.*, 2008) and communicate with bone lining cells through their gap

junctions to stabilize bone mineral by maintaining an appropriate local ionic environment (Cowin, 2001).

2.4.3.1 Mechanotransduction of Osteocytes

The application of mechanical strain during physiological loading creates a pressure difference that drives fluid flow through the lacunar-canalicular network. In this way, mechanical strain translates into flow induced shear stress imposed on cells. Osteocytes are mechanosensory cells as they can detect changes in mechanical strains (Bonewald, 2002) and they relay this information to osteoblasts through biochemical signalling. Several signalling pathways are involved in the osteocyte mechano-responsiveness. The dentritic processes of osteocytes are mechano-sensitive and induce the opening of gap junctions and hemichannels (Burra *et al.*, 2010) to allow for cell signalling. Osteocytes have been shown to release intracellular calcium as an immediate response to mechanical loading *in vitro* (Ajubi *et al.*, 1999; Taiji *et al.*, 2009). PGE_2 is an anabolic agent that regulates bone metabolism and increases bone mass in animals (Harada *et al.*, 1995). PGE_2 release has been shown to be facilitated by the translocation of Connexin 43 to the membrane surface of osteocytes in response to strain and the formation of Connexin 43 hemichannels (Cherian *et al.*, 2005), allowing signalling between cells. The opening of such hemichannels in the gap junction has also been shown to be adaptively controlled by the magnitudes of mechanical stimulation (Siller-Jackson *et al.*, 2008). It has been shown that osteocytes are more sensitive than osteoblasts to flow induced shear stress *in vitro*, with respect to the release of prostaglandin PGE_2 (Klein-Nulend *et al.*, 1995a). Other evidence of osteocyte extracellular signalling is the release of NO in response to mechanical strain (Zaman *et al.*, 1999). Interestingly, in a separate study, it has been shown that mechanically stimulating a single osteocyte *in vitro*, increased production of NO was found, and in particular the production of NO by surrounding osteocytes was increased also. This demonstrates that a single osteocyte can dissipate a mechanical stimulus to its surrounding osteocytes through soluble extracellular signalling factors (Vatsa *et al.*, 2007).

Osteocytes also play a role in the regulation of bone's response to parathyroid hormone (PTH). Targeted deletion of the PTH/PTHrP receptor, a G-protein coupled receptor, in mice resulted in the failure of a bone anabolic response to PTH treatment (Barry *et al.*, 2009) and deletion of osteocyte specific $G_s\alpha$ resulted in osteopenia and increased osteocyte density, highlighting the key role of osteocyte G-protein signalling in the regulation of bone mass (Barry, 2009). In addition, it has been suggested that PTH

signalling also crosstalks with other signalling pathways such as Wnt/β-catenin signalling pathway (Rhee *et al.*, 2009). Furthermore, sclerostin, an osteocyte-specific glycloprotein, inhibits bone formation by blocking Wnt signalling through the Lrp5 receptor that is required to initiate bone formation. Mechanical stimulation *in vivo* reduces sclerostin levels, thereby indirectly increasing bone formation by osteoblasts (Robling *et al.*, 2008). Also, it has been shown that when NO is inhibited during application of shear stress, the enhanced expression of Wnt target genes by shear stress is suppressed in osteocytes, suggesting that NO plays an important role in shear stress induced Wnt production.

The mechanism by which osteocytes sense fluid flow induced shear stress has not yet been fully elucidated, but it has been proposed that flow induces a drag force on filaments that attach osteocyte cell processes to the canalicular wall (You *et al.*, 2001) (You *et al.*, 2004), which is relayed to the central actin filament bundle of the cell process. It has been proposed that the pericellular space is regularly interrupted by underlying collagen fibrils that adhere to the cell process membrane through integrin attachments (McNamara *et al.*, 2009; Wang *et al.*, 2007). Integrins are heterodimeric transmembrane proteins that bind both to the extracellular matrix outside the cell and also to the actin cytoskeleton via the β domain on the inside of the cell. α actinin is a key protein responsible for linking actin filaments to integrin cytoplasmic domains (Pavalko and Burridge, 1991). Therefore, it is believed that integrins also facilitate considerable amplification of bone tissue strains at the cell level and consequently lead to cell signalling (Han *et al.*, 2004; McNamara *et al.*, 2009; Wang *et al.*, 2007; You *et al.*, 2004; You et al., 2001).

The glycocalyx, a pericellular matrix, has also been shown to play an important role in osteocyte mechanoresponsiveness (Reilly *et al.,* 2003). PGE$_2$ release by osteocytes under fluid shear stress was shown to be significantly abrogated following degradation of the glycocalyx (Reilly *et al.,* 2003). Glycocalyx degradation had no effect on intracellular calcium signalling however, revealing that the calcium and PGE$_2$ responses occur via different pathways (Reilly *et al.,* 2003). Finally, primary cilia, which are solitary, microtubule-based organelles that project from the surface of cells into the extracellular milieu, have been shown to act as mechanosensors in both osteoblast and osteocyte cells (Malone *et al.*, 2007). In this study, increased PGE$_2$ production, osteopontin mRNA levels and cyclooxygenase 2 (COX2) gene expression was observed with applied shear stress, but when primary cilia were abrogated, no increase in any of

the aforementioned levels were found (Malone *et al.*, 2007). In summary, osteocytes play a pivotal role in sensing mechanical stimuli and responding via various signalling mechanisms, they play an important role in the controlling bone mass and anabolic responses to hormones, in addition to regulating calcium homeostasis.

2.5. Bone Remodelling

Remodelling is a fundamental property of bone which functions to renew aged, dead and hyper-mineralised bone, and restore bone following micro-damage (Martin, 2002), through the coupled activities of osteoclast and osteoblasts. Immature (woven) or primary bone present during infancy is remodelled at 2-3 years and thus replaced by secondary bone. Secondary bone in turn is remodelled periodically assuming normal bone turnover; cortical bone is remodelled every 20 years whilst trabecular bone is replaced every 1-4 years (Cowin, 2001). Bone is removed from regions of the skeleton where mechanical loads are low and conversely bone is deposited and micro-architecture is adapted in regions under repeated high mechanical strains. Finally, remodelling grants bone the capacity for mineral homeostasis as it alters the concentration of essential minerals such as calcium in the interstitial fluid (Cowin, 2001).
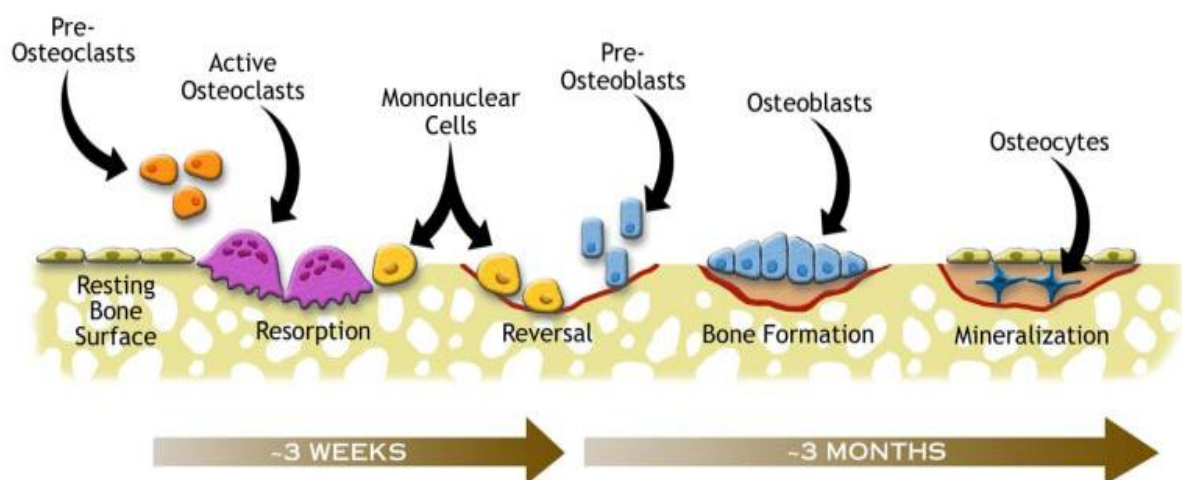
Figure 2.5 Bone Remodelling Cycle (Ann Arbor, 2005)

Remodelling of bone occurs in discrete packets within the matrix that are geographically and chronologically separate from other remodelling packets. This individual operational unit of cells is called a bone multi-cellular unit (BMU), first described by frost in 1973 (Frost, 1973). A BMU involves oteoclast resorption, (see Figure 2.4) followed by osteoblast formation of new bone called a bone structural unit (BSU) (Murray, 1999). The life cycle of a BMU consists of six successive steps; resting, activation, resorption,

reversal, formation and mineralisation, see Figure 2.5. The life span of a BMU ranges from 6-9 months. Approximately 3-4 million new BMU are initiated each year and on average 1 million BMU's are active at any given moment (Weinstein and Manolagas, 2000).

2.6. Apoptosis

Cells can either die by apoptosis or necrosis. Necrosis is the premature death of cells caused by external factors to the tissue, such as poisons, trauma, or infections and is characterized by cytoplasm and membrane swelling (Bonfoco *et al.*, 1995). Apoptosis is the naturally occurring process of programmed cell death. The term apoptosis was first coined to describe the distinctive morphological changes that a cell undergoes during cell death that differs from necrosis (Kerr *et al.*, 1972). These include cytoplasmic shrinkage, nuclear condensation and changes to the mitochondrial and cell membranes. Protrusions or blebs form on the cell membrane, referred to as membrane blebbing, which detach from the cell, forming apoptotic bodies, as depicted in Figure 2.6. Apoptosis is in stark contrast to that of necrosis, where the internal organelles and cell lyse without any formation of apoptotic bodies. Apoptosis is a tightly regulated process, genetically controlled, hence the term 'programmed cell death' with precise biochemical qualities (Hengartner and Horvitz, 1994; Wyllie, 1980). Apoptotic bodies and the dying cell are phagocytosed by macrophages. Apoptotic cells do not induce an inflammatory response *in vivo* whereas necrotic cells are phagocytosed by macrophages and cause an inflammatory response *in vivo*. There are also forms of cell death intermediate to apoptosis and necrosis which may have characteristics of both types. In addition, following apoptosis *in vitro*, secondary necrosis ensues where the apoptotic cells release their cytoplasmic contents into the cell culture media following loss of membrane integrity (Riss and Moravec, 2004).

In healthy bone, similar to the coupled resorption and formation processes of the remodelling cycle, the birth and death of bone cells are inexorably linked. Following resorption, osteoclasts either return to the resting stage or undergo apoptosis (Hughes and Boyce, 1997). Osteoblasts, on the other hand have three fates, approximately 5% become bone lining cells, 30% become osteocytes and 65% die by apoptosis (Jilka *et al.*, 1998; Parfitt, 1994; Weinstein and Manolagas, 2000). The coupled balance between proliferation and apoptosis dictates the quantity and life span of osteoclasts and osteoblasts and the amount of respective remodelling they perform, thereby regulating bone mass and strength.

- Change in refractive index
- Cell shrinkage
- Chromatin condensation
- Membrane blebbing
- Nuclear fragmentation
- Apoptotic body formation
- Phagocytosis by surrounding cells
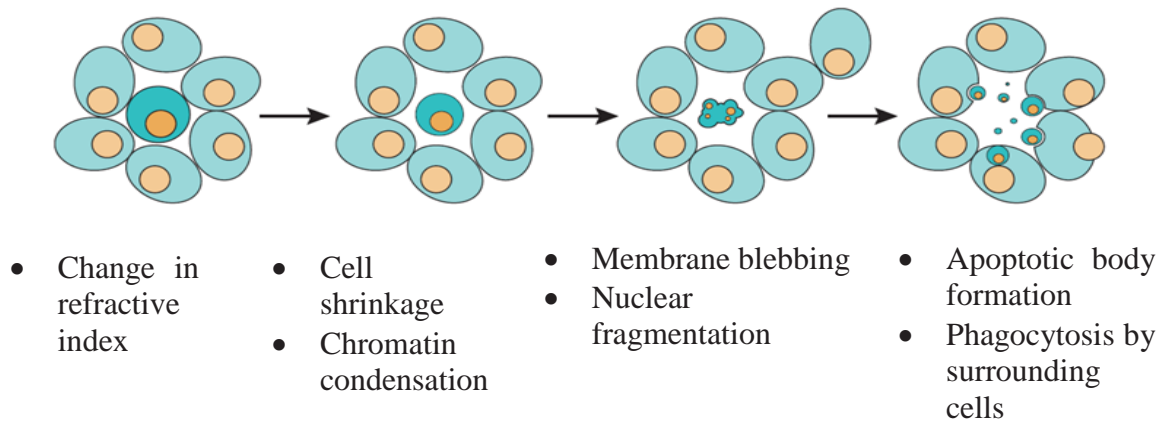
Figure 2.6 Morphological characteristics of apoptosis (Promega, 2011)

Apoptosis is a tightly regulated process with various steps involved; therefore, there are multiple experimental methods available to detect and quantify apoptosis. The cysteine aspartic acid-specific protease (caspase) family are early markers of apoptosis as they play important roles as effectors of apoptosis of mammalian cells (Nicholson and Thornberry, 1997). Caspases can be detected using luminescent, fluorescent, or colorimetric techniques and their detection is employed extensively in the measurement of apoptosis (Bradford *et al.*, 2010; Plotkin *et al.*, 1999; Tan *et al.*, 2006).

Apoptosis can also be detected by measuring changes in the cell membrane. Healthy cells have an asymmetry of phospholipids on the inner and outer side of the cell membrane, however there is a alteration in this symmetry during cell death. Phosphatidylserine (PS) becomes abundant on the outer surface of the membrane and this change in phospholipid asymmetry can be detected using Annexin V, which is a phospholipid binding protein with a high affinity for PS. Annexin V is then conjugated to fluorescein isothiocyanate (FITC) which can be detected using fluorescent microscopy or flow cytometry (Claro *et al.*, 2011; Duus *et al.*, 1994). Another common means of detecting apoptosis is by fluorometric or colorimetric TUNEL (TdT-mediated dUTP Nick End Labeling) that detect DNA fragmentation, which occurs during apoptosis (Plotkin *et al.,* 1999; Sun *et al.*, 2006; Weinstein *et al.*, 1998).

2.7. **Pathophysiology of Osteoporosis**

According to the American Association of Clinical Endocrinologists, osteoporosis is a disorder which is characterized by compromised strength and an increased risk of fracture (Watts *et al.*, 2010). Bone fracture occurs when the applied load exceeds the

load-carrying behaviour of the bone, which in material mechanics encompasses the strength, stiffness, and toughness of the material (Vable, 2002). Bone strength reflects bone mass and the quality of the constituent material. Therefore, in order to understand why bones break it is necessary to examine changes in bone mass and bone quality, which include the composition, the degree of micro-damage and the micro-architecture of the tissue material. The quality of the bone tissue and hence the overall strength is also affected by the rate of bone renewal (Felsenberg and Boonen, 2005; Robling *et al.*, 2006).

As described in section 2.5 normal bone turnover entails a balance between bone resorption and bone formation, by the concerted efforts of osteoclasts and osteoblasts, in order to maintain healthy bone with neither a loss of bone mass nor an increase in damage accumulation. A perturbation of this co-ordinated cellular activity occurs during osteoporosis. Firstly, the activation frequency of BMU, which is the statistical probability that bone remodelling will occur on any bone surface at any given moment (Riggs and Parfitt, 2005), is increased during osteoporosis. Secondly, bone resorption supersedes bone formation. The increased frequency of BMU and the disparity between the resorption and formation phases is evident by the increase in calcium and osteocalcin levels in the urine of patients with this metabolic disease (Lerner, 2006). This has major consequences that manifest as low bone mass and micro-architectural deterioration of bone tissue, as demonstrated in Figure 2.7.
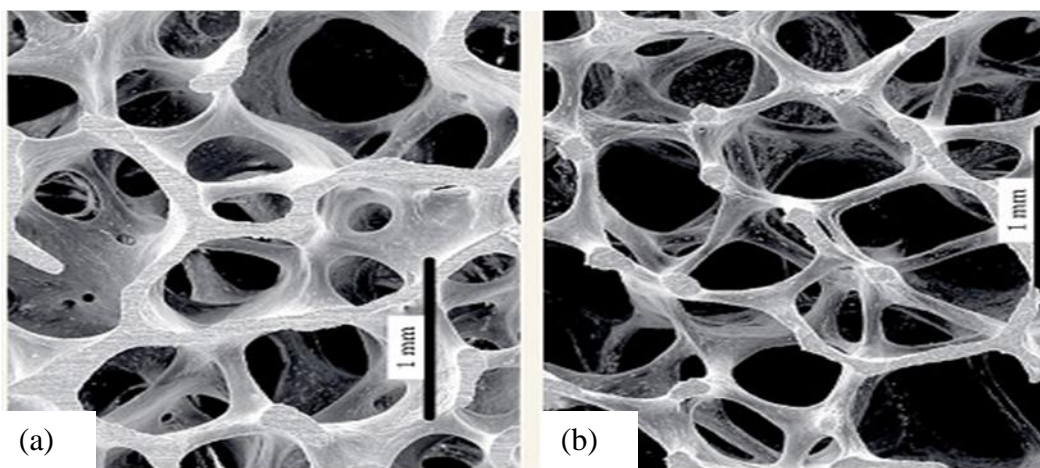
Figure 2.7 A scanning electron micrograph of (a) normal bone versus (b) osteoporotic bone with depletion of interconnecting struts (Ritchie *et al.*, 2009)

Diminishing levels of circulating oestrogen following the menopause increases the rate of bone removal (Riggs et al., 2002). This occurs because oestrogen withdrawal prolongs the life-span of osteoclasts and therefore there is a net increase in the number of resorption cavities, and an increase in the depth of resorption pits, which together can lead to a complete perforation of the trabecula (Mosekilde, 1990). The resultant diminished bone architecture reduces the overall strength of bone which is demonstrated in a study that investigated the trabecular architecture of patients with the same bone mass, with and without vertebral fractures (Aaron *et al.*, 2000). This study reported that those with fractures had four times the number of broken trabeculae than patients without fracture, in spite of having the same bone mass. The effect of oestrogen depletion on osteoblast activity is less well known, however the main products of osteoblast activity, collagen and mineral, play an important role in determining bone strength. The degree of bone mineralisation (Follet *et al.,* 2004) and the distribution of this mineral within the bone tissue (Ciarelli *et al.*, 2003), both influence bone strength. Oestrogen deficiency is thought to increase (Boyde *et al.,* 1998), decrease, or have no impact on mineral concentration, while the distribution of mineral is significantly altered as a consequence of oestrogen withdrawal. Human osteoporotic bone also has significantly less collagen cross-links compared to age and gender matched controls, highlighting the important role of collagen in the maintenance of healthy tissue. This suggests that impaired collagen cross-linking could contribute to reduced strength of bone tissue in individuals with osteoporosis (Oxlund *et al.*, 1996).

Disruption of normal apoptosis has been linked to osteoporosis; experiments on murine osteoclasts showed oestrogen treatment increased apoptosis from approximately 0.5% to 2.7% (Hughes *et al.*, 1996). The larger erosion pits in oestrogen deficient bone could therefore be contributed to by the loss of this apoptosis induction effect which would lead to a longer lifespan of osteoclasts. Conversely, osteoblasts and osteocyte apoptosis increases with age (Almeida *et al.*, 2007) and oestrogen deficiency (Emerton *et al.* 2010) *in vivo*.

2.7.1 Epidemiology

Osteoporosis is a worldwide dilemma whose prevalence in today's ageing society means that is an immense global, economic and social burden. It is estimated that 30-50% of women and 15-30% of men will suffer an osteoporotic fracture in their lifetime (Randell *et al.*, 1995) and irrespective of the fracture type sustained, fracture sufferers are 50-100% more likely to have another fracture of a different type in their lifetime

(Klotzbuecher *et al.*, 2000). In the year 2000 there were an estimated nine million osteoporotic fractures globally, with fracture occurrence more prevalent in Europe than in any other continent (Johnell and Kanis, 2006). It is estimated that 179,000 men and 611,000 women will suffer a hip fracture each year in Europe and that the financial impact of all osteoporotic fractures is calculated provisionally as being €25 billion (Melton *et al.,* 2003). Our improved health care and better lifestyles permit longer life expectancy but means that consequently the prevalence of osteoporosis is ever increasing. Globally, the 323 million individuals over the age of 65 in 1990 will increase to a predicted 1555 million by 2050. Solely, this demographic trend could result in an increase in the worldwide incidence of hip fractures from 1.7 million in 1990 to an estimated 6.3 million in 2050 (Cooper *et al.*, 1992). In summary, it is clear from the statistics presented that osteoporotic fractures are a significant cause of disability and morbidity, particularly in developed nations, and an immense economic burden on our health systems. Finding adequate interventions to remedy this disease is vital and it is imperative that an understanding of the underlying causative factors of bone tissue changes in osteoporosis is achieved.

2.7.2 Diagnosis

Osteoporosis is suspected in most low trauma fractures over the age of 45 and bone mineral density (BMD) measurement by dual-energy X-ray absorptiometry (DEXA) scanning is the clinical standard diagnostic tool. According to the world health organisation (WHO), when bone mineral density is 2.5 standard deviations below the mean for normal Caucasian women, the presence of osteoporosis is diagnosed (WHO, 1994). Arresting the disease before the occurrence of a fracture results in a significant financial saving (Brecht *et al.*, 2004), however it has been suggested that BMD diagnostics are underutilized in the majority of European countries as a result of lack of availability of densitometers, personnel trained to perform scans and reimbursement (IOF, 2001). The rationale for employing BMD as a indicator of osteoporosis are that DEXA scans can be easily performed in situ and epidemiologic studies have linked decreasing BMD with increased fracture risk (Marshall *et al.*, 1996). However, BMD measurements are by no means optimal for the detection of patients at high risk of fracture, as although they have high specificity, they have low sensitivity (WHO, 1994). The risk of fracture is very high with low BMD values and the risk of fracture doubles with each standard deviation reduction in the T-score of the BMD, regardless of the site of bone measured in DXA (Marshall *et al.,* 1996). However, a high percent of fractures

occur in individuals with normal BMD (Cummings *et al.*, 2002). Likewise, BMD cannot fully characterize the efficacy of osteoporosis medications since fracture reductions of up to 50% have paralleled little or no gain in BMD (Sarkar *et al.*, 2002). There are also a plethora of risk factors independent of low bone density including age, previous fragility fracture, smoking, alcohol intake $\geq$ 3 units/day and body mass index $\leq$19 (Poole and Compston, 2006). From a mechanical point of view the failings of DXA are that although it measures bone mass at the whole bone level, it is not capable of ascertaining the strength, stiffness, or toughness of the material which also have a huge impact on the fracture risk of bone.

2.7.3 Pharmacological Interventions

Due to the major impact osteoporosis has on society, the prevention and management of this disease is of paramount importance. As a consequence of extensive investigations into osteoporosis, there are currently a wide range of pharmaceuticals available targeted at preventing bone loss and preventing further fractures. These include oestrogens, Selective Oestrogen Receptor Modulators (SERMs), calcitonin, parathyroid hormone, calcium, vitamin D, anabolic agents and most popularly, biphosphonates (Boivin and Meunier, 2003). The administration of oestrogen to female patients, known as Hormone Replacement Therapy (HRT), was the most popular osteoporotic treatment in the 1980's as it significantly reduced skeletal bone loss (Komulainen *et al.*, 1999) and fracture incidence (Turner *et al.*, 1994) by countering the acceleration in bone turnover following the menopause (Benhamou, 2007). However HRT prescription for postmenopausal osteoporosis is rapidly declining due to its association with the development of breast cancer (Colditz *et al.*, 1995; Lindsay *et al.*, 1996). Raloxifene was the first SERM to be marketed for osteoporosis treatment; it has oestrogenic actions on bone tissue, such as prevention of bone loss and increasing bone mass and conversely has anti-oestrogenic actions on the uterus and breast. Therefore, Raloxifene is administered for reducing the risk of breast cancer in postmenopausal women with osteoporosis and has proved effective at increasing BMD in the spine and femoral neck and reducing the risk of fractures (Ettinger *et al.*, 1999).

Most therapeutic agents prescribed for osteoporosis are targeted at inhibiting bone resorption, however a few agents have recently been developed that are aimed at bone formation. Teriparatide, a portion of human parathyroid hormone (PTH), is a potent anabolic agent which enhances bone formation, however it is currently only administered to tackle the most severe postmenopausal cases (Benhamou, 2007). It has

demonstrated a 65% reduction in vertebral fractures (Neer *et al.*, 2001) and has achieved increased cortical thickness, trabecular connectivity and increased portion of plates compared to mechanically weaker rods (Jiang *et al.*, 2003). PTH increases BMD and bone mineral content in ovarectomized monkeys (Brommage *et al.*, 1999) and reduces fracture risk of osteoporosis patients when administered alone or in combination with anti-resorptive medications (Vestgaard *et al.*, 2007). Similarly, strontium ranelate boosts bone formation and also inhibits bone resorption (Meunier *et al.*, 2004; Seeman *et al.*, 2006).

2.7.3.1 Bisphosphonates

Biphosphonates are the most widely employed pharmaceutical for treatment and prevention of osteoporosis (Bauss and Schimmer, 2006). They are highly effective inhibitors of bone resorption, thereby increasing the mass and mechanical resistance of bone. The efficacy of this drug cohort has been shown to depend on treatment duration, dosage, frequency, animal model and bisphosphonate type. This class of agent includes alendronte, etidronate, pamidronate, clodronate, (Gomez and Xiao, 2009) and zoledronate acid.

Bisphosphonates inhibit bone resorption by selectively adsorbing to mineral surfaces, from where they are subsequently ingested by osteoclasts, and thereby modulate osteoclast function. The P-C-P backbone, common to all bisphosphonates, is composed of two phosphate groups covalently bonded to a carbon and it is the reason this class of drug is called bisphosphonates. The P-C-P moiety, together with the hydroxyl act as a 'bone hook' and thereby allowing rapid attachment of bisphosphonates to bone mineral surfaces (Russell, 2007). The internalization of bisphosphonates into osteoclasts has been demonstrated *in vivo* by fluorescently labelling the drug and its uptake was demonstrated in intracellular vacuoles (Sato *et al,* 1991). Bisphosphonates are categorized into two main groups according to whether or not they contain nitrogen. Non-nitrogen containing biphosphonates (non-NBP) cause osteoclastic cell death by apoptosis (Frith *et al.*, 1997). Nitrogen containing biphosphonates (NBP) inhibit bone resorption by perturbing the cytoskeleton necessary for maintaining the ruffled border that facilitates osteoclastic bone resorption (Murakami *et al,* 1995; Rodan and Reszka, 2002). They also interfere with protein prenylation and thus the signalling of critical regulatory proteins, thereby affecting cellular activity and survival (Russel, 2007).

The first generation of biphosphonates, etidronate, a non-NBP, has shown to preserve bone mass in post-menopausal women (Herd *et al.*, 1997). Ibandronate, a highly potent

NBP was shown to increase cancellous bone mass and improve bone strength in animals (Smith *et al.,* 2003) and post-menopausal women (McClung *et al.*, 2004). Similarly, alendronate, a NBP, increases bone density and strength in animals (Spadaro *et al.*, 2006) and postmenopausal women (Boivin *et al.*, 2000). Finally, risendronate, a NBP, increases bone mass and preserves trabecular micro-architecture in animals (Borah *et al.*, 2002) and postmenopausal women (Borah *et al.*, 2004).

Although bisphosphonates are currently the most popular pharmaceuticals administered to treat osteoporosis, current drug treatments are by no means a solution to the dilemma of osteoporosis. Usually, bisphosphonates are administered orally however, disadvantages include low bioavailability and gastrointestinal intolerability. It has therefore been suggested, following animal (Smith *et al.,* 2003) and human (Reid *et al.*, 2002) trials, that bisphosphonates should be administered by intermittent intravenous injections in order to improve patient compliance. An intravenous formulation of Ibandronate, administered over 15-30 seconds every three months, is approved in the UK for postmenopausal osteoporosis (Poole and Compston, 2006). Furthermore, osteonecrosis of the jaw is an extremely rare (64 cases as of 2004) complication associated with bisphosphonate treatment (Ruggiero *et al.*, 2004). It is a severe bone disease whereby the death of bone cells occurs because of decreased blood flow, and leads to severe bone loss and collapse of regions of the maxilla and the mandible bone. Its symptoms include pain, loose teeth and exposed bone. The disease is most prevalent in bisphosphonate treated patients who have undergone invasive dental procedures. The precise cause is unknown; however, it is thought that bisphosphonates might interfere with the body's ability to heal bone following dental procedures such as tooth extractions (Reid and Cundy, 2009).

2.7.3.2 Zoledronic Acid

Zoledronic acid (ZOL), marketed under the brand name Zometa by Novartis, Switzerland, is a potent, third generation NBP. *In vivo* animal trials analysing the efficacy of this drug have produced promising findings. In the rat model of postmenopausal bone loss, ZOL administered as subcutaneous injections for ten days decreased bone resorption and produced a dose dependent increase in trabecular bone volume and architectural integrity (Alexander Pataki *et al.*, 1997). In addition, ZOL administration to ovariectomised adult rhesus monkeys increased bone mineral density (BMD) of the spine and radius in a dose dependent manner and reduced bone turnover (Binkley *et al.*, 1998). Human trials have revealed an increase in BMD of

postmenopausal women treated with this pharmaceutical (Reid *et al.,* 2002), as well as a significant reduction in fracture occurrence (Black *et al.*, 2007).

2.8. Quantification of Mineral Content in Bone

Determining the variations of mineral content of trabecular bone on a microscopic scale can yield important insights into mechanical strength, integrity and remodelling activities. Bone mineral composition may be analysed by employing a variety of analytical techniques such as gravimetric analysis, energy dispersive spectroscopy, scanning electron imaging, backscatter electron imaging, x-ray diffraction, as well as spectroscopic methods such as infrared, raman, and nuclear magnetic resonance.

Gravimetric analysis can be used to assess the water content, mineral content and carbonate content of bone. Generally the bone is crushed and the percentage water, mineral, and carbonate per weight is established. 'Ashing' is a gravimetric analysis technique and the residue or ash content obtained when bone is heated to extreme temperatures may be used to quantify the total amount of mineral within a bone sample. Heating bone to these temperatures removes all the organic components of bone such as matrix proteins, water, and bone cells and therefore only the inorganic or mineral components of bone are left in the ash content (Cowin, 2001). A temperature of 550°C is recommended for ashing procedures by the Royal Society of Chemistry (Watson, 1994). Ash content analysis has been used extensively to assess the mineral content in bone (Aerssens *et al.*, 1997; Kolosova *et al.*, 2002; Pointillart *et al.*, 1995; Thorp and Waddington, 1997). Gravimetric analysis offers reproducible quantitative data, however it does not provide information on the quality of the mineral. Another disadvantage is the long ashing times required, approximately 10 hours, and the destructive nature of the technique, rendering samples useless for further assessment. Moreover, any information regarding the location of the mineral distribution is lost during this technique.

Fourier transform infrared spectroscopy (FTIR) may be utilised to provide qualitative and quantitative information regarding components in bone that possess IR active vibrations, therefore it can analyse the mineral phosphate, matrix proteins and lipids. It has been used extensively to analyse the mineral content of bone (Boskey *et al.*, 2005; Marcott *et al.*, 1998). The advantage of employing this technique for analyzing bone mineral is that it can map alterations in mineral properties at a spatial resolution of 20μm (Paschalis *et al.*, 1997). However, there are disadvantages associated with FTIR such as the requirement of having particles less than $2\mu m$ in order to avoid light scattering. Secondly, FTIR instruments measure interferograms, not spectra, therefore fourier

transforms must be performed to produce a spectra and the execution of this can affect the results (Vagenas *et al.*, 2003). Raman microprobe spectroscopy has also been used to analyse bone minerals (Carden and Morris, 2000). This IR technique relies on inelastic scattering, called Raman scattering of light from a laser in the visible, near ultraviolet, or near infrared range. The primary disadvantage of this technique is the interference from the fluorescent elements of the organic matrix (Cowin, 2001).

Quantitative backscatter electron imaging (qBEI) has been demonstrated as an extremely effective tool for measuring microscopic mineral content variations in bones as the BE image intensity (grey level) has been shown to have a strong positive correlation with ash (mineral) content (Crofts *et al.*, 1994). In addition, one can calibrate the grey level scale in order to produce comparable results and the grey level can be related to weight percentage calcium (Roschger *et al.*, 1998). qBEI is therefore the chosen analytical technique for mineral content quantification in this thesis and is described in detail in section 2.9.

2.9. QBEI

Quantitative Backscatter Electron Imaging (qBEI) is a Scanning Electron Microscope (SEM) technique which permits compositional and topographical analysis of specimens. A SEM is a high resolution instrument that forms an image by producing a beam of high energy electrons and examining the interactions between the incident electrons and the specimen of interest. The SEM can achieve a magnification and resolution of 500,000x and 0.4nm respectively. It can acquire compositional information by producing images whose contrast is based on the constituent elements and compounds of the sample. Also, characteristic x-rays produced when the electron beam hits the sample can be used to identify and image, specific elemental distributions (from boron to plutonium) in a specimen (Goldstein *et al.*, 2003). The development of electron backscatter diffraction (EBSD) in the SEM permits the attainment of crystallographic information of the material (Randle, 1995).

A basic schematic of the principle components of a typical SEM is presented in Figure 2.8 (a). The SEM generates a beam of electrons in a vacuum using an electron gun which thermionically (using a heated electronic conductor) emits electrons from a tungsten or lanthanum hexaboride cathode towards an anode at an energy ranging from 1keV to 30 keV. The electrons are passed through a series of apertures including electromagnetic condenser lenses which collimate the beam and objective lens for

focusing, allowing adjustment of the final beam diameter, called the probe diameter or spot size.

When the electron beam strikes the specimen the electrons will scatter through the sample within a defined area called the interaction volume. This electron beam–specimen interaction causes the formation and scattering of electrons which can be classified as either elastic scattering or inelastic scattering. Elastic scattering occurs between the incoming electrons from the electron beam and the nucleus of the target atoms. This gives rise to backscattered electrons that are of particular importance in this thesis. Inelastic scattering occurs between the incident beam and the loosely bound electrons of the conduction band, or the tightly bound valence electrons and nucleus. This occurrence gives rise to secondary electrons, x-rays and auger electrons, as illustrated in Figure 2.8 (b) (Goldstein *et al.*, 2003).

Secondary electrons (SE) are low energy electrons (10-50eV) which are detected by a scintillator-photomultiplier system know as the Everhart-Thornley detector. When produced deep within the interaction volume they do not possess adequate energy to escape and are absorbed by the specimen. Therefore, only secondary electrons close the surface of the specimen will escape the interaction volume. The secondary electrons emitted can go in any direction and therefore a Faraday cage surrounds the secondary electron detector and exerts a positive pull on weakly negative secondary electrons towards the detector. As the beam scans the specimen and the interactions are detected, an image is sequentially formed presenting the information of interest.

2.9.1 Backscatter Image Formation

Backscattered electrons (BSE) arise due to elastic collisions between the incoming electrons in the primary beam and the nucleus of the target atoms. Backscattered electrons are produced deep within the sample however as they have much higher energies than secondary electrons they are able to escape from deeper within the interaction volume, see Figure 2.8 (d). As their generation region is larger than those of secondary electrons they give less spatial resolution than SE. However, because they have higher energies they are less affected by charge-up and sample damage. Specimen charge-up is when negatively charged electrons collect locally on the surface of the specimen, which prevents normal emission of SE and causes abnormal contrast and distorted images.
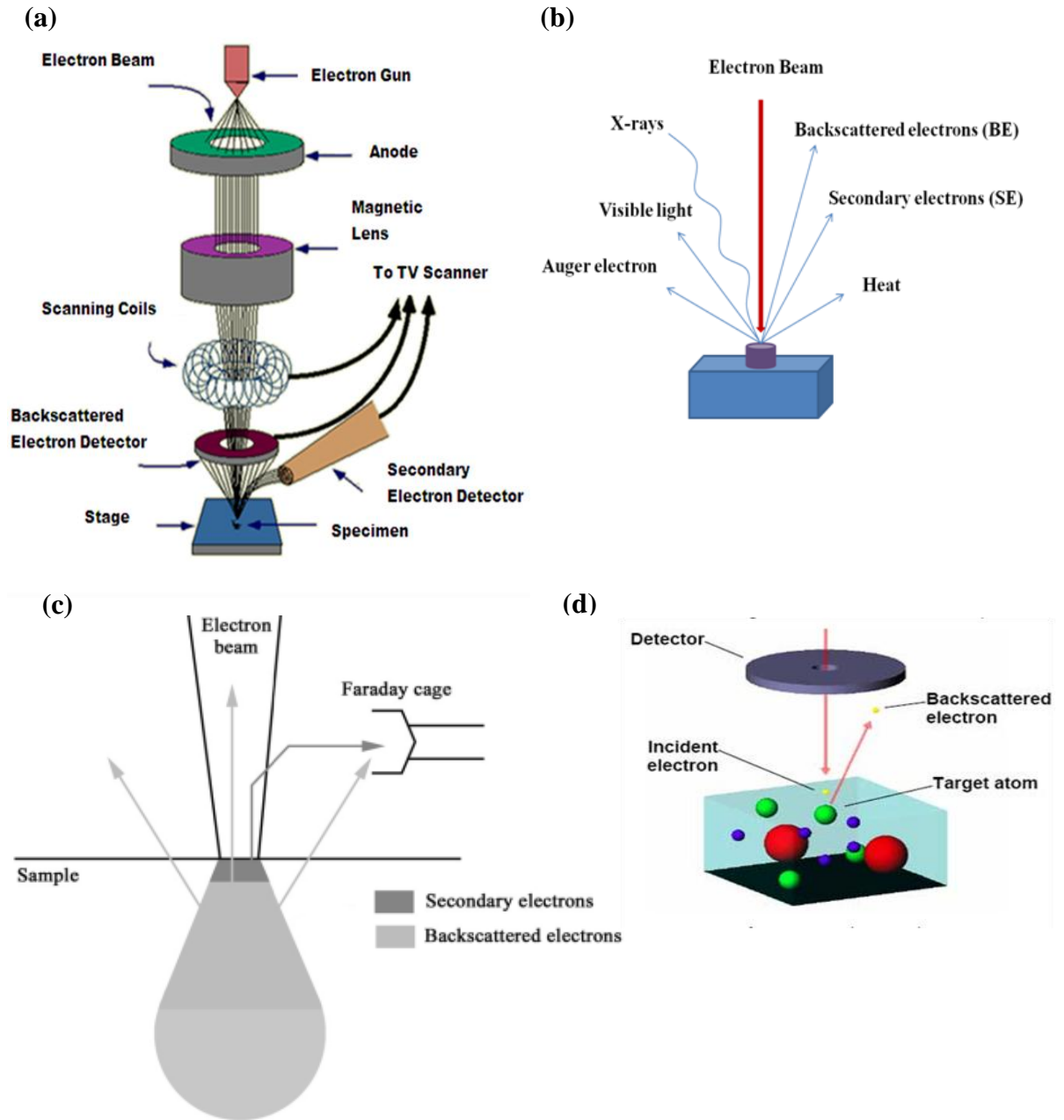
Figure 2.8: (a) Basic components of a SEM (Iowa State University, 2009). **(b)** Electron beam-specimen interactions in a SEM. **(c)** Backscattered electrons detection (Corp., 2009). **(d)** Interaction volumes of secondary and backscattered electrons (Smith College, 2009).

The backscatter detector is located within the specimen chamber above the specimen in a 'doughnut' shape configuration with the incident beam passing through its hole or centre, as depicted in Figure 2.8 (a) and (d). The backscattered electron image can either provide compositional or topographical sample information. Addition of a paired semiconductor detector gives compositional information, while subtraction provides information regarding the specimen topography. This thesis is solely concerned

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with compositional information of the mineralised bone specimens. The intensity of the backscattered image is related to its mean atomic number (Z). Regions of the material that are composed of larger, heavier atoms will backscatter more electrons thus generating brighter grey tones in the backscatter image. Conversely, lighter atoms will produce darker grey tones in the image. In this way, an image grey level (GL) can be generated which provides compositional-based information (Goldstein et al., 2003).

2.9.2 Image Quality

There are a number of interdependent SEM parameters under operator control that can impact the quality of the qBEI obtained. These include the probe current, the spot size, the acceleration voltage and the working distance. Once the incident beam current passes through the anode, its current decreases drastically and it is referred to as the probe current. It affects the smoothness of the image; decreasing the probe current will reduce the chance of charge-up and also reduce the spot size (JEOL, 2009). The smaller the spot size the higher the magnification and resolution, but the greater the chance of specimen damage.

The accelerating voltage (voltage applied to the filament) employed in SEM imaging usually ranges from 5kV to 20kV. Increasing the accelerating voltage decreases the spot size and theoretically should increase the resolution of the image as there is a decrease in the spherical aberration of the system. Spherical aberration is the failure of the lens system to image central and peripheral electrons at the same point, thus creating more than one focal point which leads to un-sharp image formation. However, the relationship between accelerating voltage and resolution depends on the atomic number of the specimen of interest because increasing the accelerating voltage also increases the interaction volume due to the higher energy of the electrons in the incident beam; see Figure 2.9 (a) and (b). Consequently, the backscattered electrons are emitted from a larger area of the specimen and this has the effect of reducing the resolution of the image, much less so in specimens of higher atomic number however. Therefore, increasing the accelerating voltage increases the resolution in specimens with high atomic number and decreases the resolution in specimens with low atomic number, such as biological samples. Several disadvantages accompany an increase in the accelerating

voltage which includes a higher possibility of specimen damage, lack of detail on specimen surface, and increased possibility of charge-up.
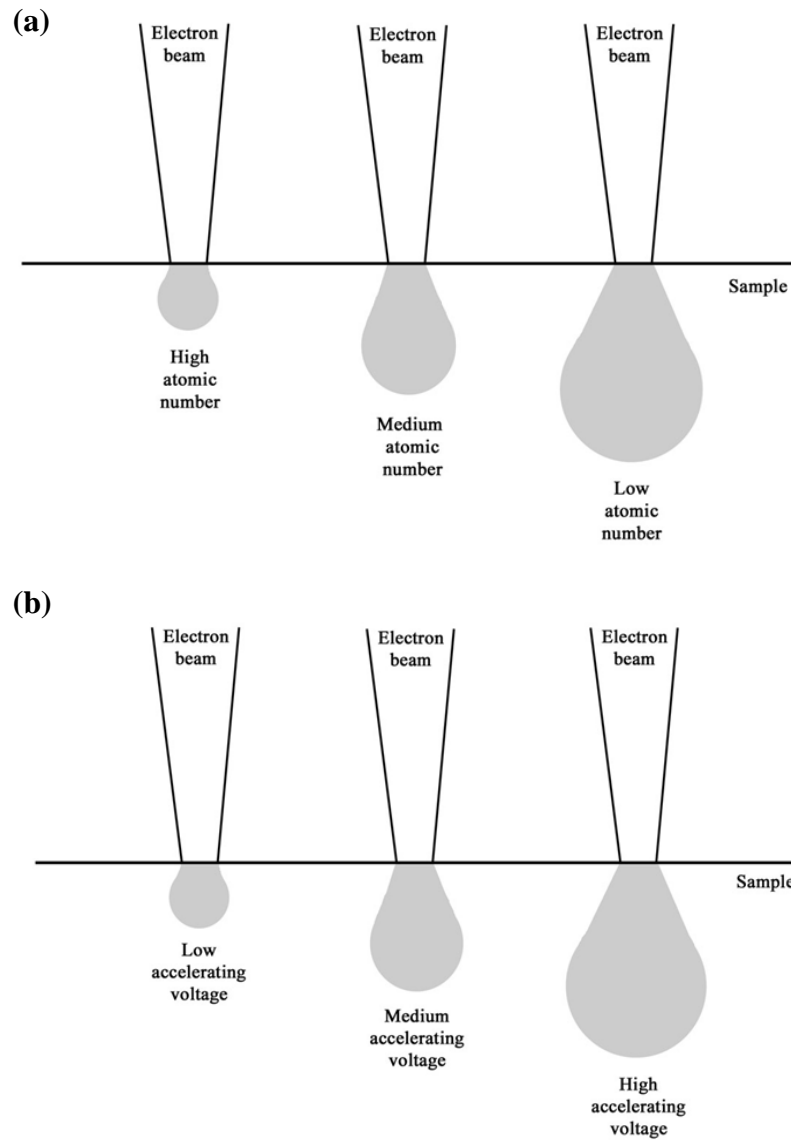
Figure 2.9 (a) Effect of atomic number on the interaction volume **(b)** Effect of the acceleration voltage on the interaction volume (Smith College, 2009).

The depth in the sample that appears to be clearly focused is known as the depth of field (*h*). Figure 2.10 (c) illustrates this concept. The working distance (WD) is the distance between the specimen of interest and the closest condenser lens. Decreased working distance results in a decreased depth of field as the specimen is scanned with a wider

cone of electrons. It also has the effect of improving the resolution as the effects of spherical aberration are reduced and the spot size is reduced. Depth of field can therefore be improved by increasing the working distance, decreasing the objective lens apertures and decreasing the magnification of the image (Starink, 2008).
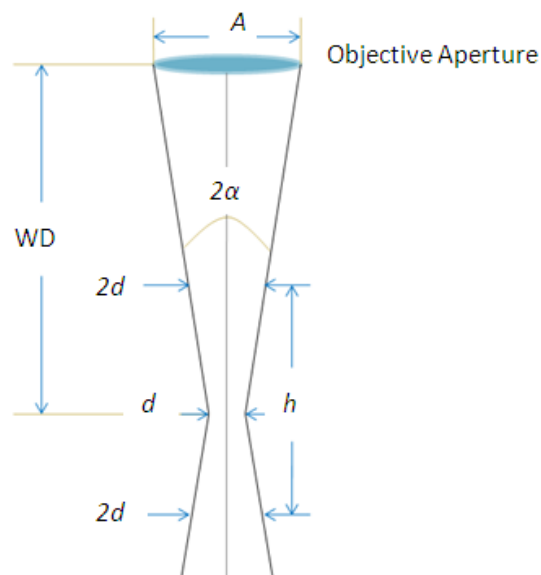
Figure 2.10 Effect of working distance (WD) and objective aperature diameter (A) on the depth of field (h).

2.9.3 Quantitative Calibration

Since different materials are differentiated by different intensities, or grey levels ranging from 0-255, in a backscatter image, it is essential to employ a method of calibrating this GL scale to ensure accuracy and reproducibility of the quantitative measurements between specimens. Two means of GL scale calibration have been used predominantly in qBEI throughout the bone tissue mineral literature. The first employed polymers with high atomic numbers synthesized by Davy et al, (thermoset dimethylacrylate esters derived from the reaction of halogenated phthalic acids and glycidyl methacrylate) (Davy, 1994) to calibrate the GL scale (Boyde *et al.*, 1995; Boyde *et al.*, 1999). A second technique related the qBEI grey levels to weight percentage calcium content (wt % Ca) (Roschger *et al.,* 1998; Roschger *et al.*, 1995). Carbon (Z=6) and aluminium (Z=13) standards were imaged and the brightness and contrast were altered to C and AL grey levels of 25 and 225 respectively. This produced a calibration line relating atomic number to grey level, see Figure 2.11 (a). Secondly, a standardization line of BE grey

levels versus calcium concent, see Figure 2.11 (b), was developed by imaging pure osteoid (0 wt % Ca) and hydroxyapatite (39.86 wt % Ca) under the calibrated GL conditions (Roschger *et al.,* 1998; Roschger *et al.,* 1995). Energy Dispersive X-ray Analysis (EDX) and GL analysis were performed at the same positions on the same bone samples and a clear linear correlation between GL and wt % Ca was revealed (Roschger et al., 1995). This technique has been employed fruitfully by various research groups in bone mineral content investigations (Gupta *et al.*, 2005), (Bloebaum *et al.*, 1997), (Burr *et al.*, 2003; Sutton-Smith *et al.*, 2008) and is the calibration method utilised throughout the present work.
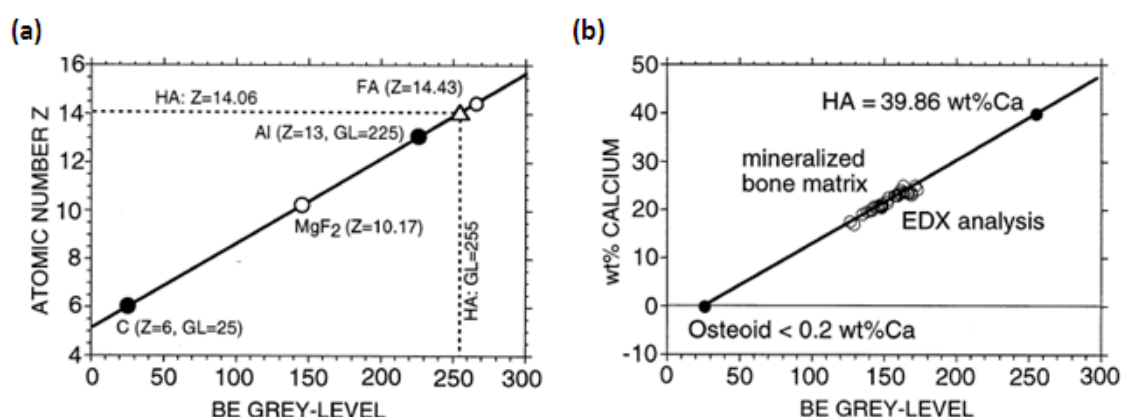
Figure 2.11 (a) Calibration line (y=5.1352+0.035024*x) of grey levels (GL) into atomic numbers (Z) for the calibration of the GL scale. **(b)** Standarization line (*y*= -4.332 + .1733*x*) of BE grey levels and calcium concentrations (wt % Ca) (Roschger *et al.,* 1995)

2.10. Summary

Chapter 2 has presented a detailed overview of the structure, composition and functions of bone, with particular focus given to the cellular constituents of bone, during health and osteoporosis. In summary, bone is a dynamic tissue that plays supportive and metabolic functions and permits movement. Bone tissue is continually being renewed throughout life by the coupled activities of bone resorbing osteoclasts and bone forming osteoblasts, in order to remove old and damaged bone. Bone is remodelled in accordance with the mechanical loads imposed on it and both osteocytes and osteoblasts are capable of sensing mechanical stimuli and producing a biochemical response. During osteoporosis increased bone remodelling as a consequence of oestrogen withdrawal results in depleted bone architecture, low bone mass, reduced strength and increased risk of fracture. The mineral content of bone tissue is a governing factor for bone strength

and is thought to be altered during osteoporosis, which could contribute to increased fracture risk. Osteocyte apoptosis is also thought to increase during osteoporosis, this may lead to a depleted osteocyte mechano-sensing network and altered mineral distribution. Bisphosphonates are the primary drug prescribed in the treatment of osteoporosis and although an increase in bone density is achieved by the inhibition of bone resorption, fracture risk still remains high. Experimental methods employed to quantitatively assess bone mineral content of *ex vivo* bone tissue include gravimetric analysis, fourier transform infrared spectroscopy (FTIR) and quantitative backscattered imaging (qBEI). In the following chapters a detailed investigation of altered tissue mineralisation during oestrogen is presented. This is followed by *in vitro* cell culture studies during static and applied loading conditions in Chapters 5 and 6, which aim to understand the cellular mechanism which may be responsible for such changes.

3. Site Specific Increase in Bone Mineral Heterogeneity during Oestrogen Deficiency

3.1. Introduction

The overall mechanical strength and fracture resistance of bone is determined by both the bone mass and the quality of the bone tissue (Judex *et al.*, 2003). Bone quality encompasses many features of the bone tissue, in particular (1) tissue micro-architecture, (2) the degree of micro-damage and (3) tissue composition. It is now well established that, during oestrogen deficiency, bone mass and trabecular micro-architecture are significantly degraded by way of trabecular thinning, micro-fracture and loss of trabecular connectivity (Compston *et al.*, 1989; Lane *et al.*, 1998; Parfitt, 1987). There is also evidence that the extent of micro-damage within bone tissue is increased during oestrogen deficiency (Dai *et al.*, 2004). It is less clear, however, how tissue composition is affected by oestrogen deficiency. Bone is a composite material, composed of an inorganic phase (predominantly the mineral calcium phosphate) and an organic phase (collagen, non-collagenous proteins and cells). The mechanical behavior of bone tissue is determined by the quantity and mechanical integrity of each of these phases. In particular the mineral concentration is a key determinant of the mechanical strength of bone (Currey, 1984b; Ruffoni *et al.,* 2007). Previous research to discern differences in mineral concentration between oestrogen deficient and normal bone tissue has produced many conflicting observations; some studies report a decrease (Gadeleta *et al.*, 2000; Loveridge *et al.*, 2004), whilst others reveal an increase (Boyde *et al.,* 1998; Dickenson *et al.*, 1981; McNamara *et al.,* 2006), or no change in mineral concentration (Bohic *et al.*, 2000; Ciarelli *et al.*, 2003; Rohanizadeh *et al.*, 2000). Variations in experimental methods or animal model may explain such discrepancies. Alternatively, the distribution of bone tissue mineral in oestrogen deficient bone might vary depending on the structural level or the anatomical location from which bone was chosen for analysis.

Trabecular bone is a hierarchically organised structure, formed by an interconnecting network of trabeculae, which are comprised of collagen fibrils organised into parallel tissue lamellae. While most studies have assessed the mechanical integrity of volumes of trabecular bone, changes at all levels of organisation likely contribute to fracture risk. In a previous study, micro tensile testing of individual trabeculae from the tibia of ovariectomised rats showed that although the *overall* bone mass and bone strength was reduced, the *remaining* individual trabeculae were approximately 40–90 % stronger and

stiffer than trabeculae from sham operated controls (McNamara *et al.,* 2006; McNamara *et al.,* 2005). Preliminary studies suggested that these changes in tissue properties were related to micro structural changes in tissue mineralisation (McNamara *et al.,* 2006; McNamara *et al.,* 2005). A recent study corroborated this by reporting increased calcium content within individual trabeculae during oestrogen deficiency (Busse *et al.*, 2009). Studies have shown that mineral is more heterogeneously distributed in oestrogen deficient trabeculae (Busse *et al.,* 2009; Roschger *et al.*, 2008). However, these studies have not yet distinguished precisely where mineralisation differences arise within trabeculae, e.g. whether tissue mineral content is altered at superficial surfaces, or whether such changes are ubiquitous throughout trabeculae. It is known that bone mineral is heterogeneously distributed across different anatomical regions of the healthy skeleton (Nazarian *et al.*, 2007), due to normal variations in functional remodelling (Tsubota *et al.*, 2009) and tissue mineralisation kinetics (Akkus *et al.,* 2003; Ruffoni *et al.* 2007). Furthermore, remodelling activity is variable across anatomical locations of the proximal femur (Tsubota *et al.,* 2009). Although it has been shown that increased remodelling activity during oestrogen deficiency (Bell, 1996; Caverzasio *et al.*, 2008) is not uniform across sites of the distal femur (Baldock *et al.*, 1998), it is not yet known whether such variations alter the normal distribution of bone tissue mineral between anatomical locations. In this study, quantitative backscattered electron imaging (qBEI) with scanning electron microscopy (SEM) was used to test the hypothesis that the distribution of bone tissue mineral is altered during osteoporosis. Specifically, the objective of the study was to discern the difference in mean calcium content and the density distribution of calcium at a tissue level between healthy and osteoporotic sheep trabecular bone. Furthermore, it was sought to quantify whether changes are ubiquitous across all trabecular bone tissue from the proximal femur, or are limited to discrete anatomical locations within the proximal femur. This chapter presents an edited version of previously published work (Brennan *et al.*, 2011).

3.2. Materials and Methods

3.2.1 Animal Groups and Trabecular Bone Origins

Animal bone tissue was used in this study in order to obtain statistically relevant samples sizes of both osteoporotic models and healthy controls, which would be very difficult to obtain from human subjects. Although some non human female primates experience a cessation of the reproductive cycles similar to menopause in humans several elements inhibit their usefulness for research including expense of purchase, ease of handling and

possibility of disease transmission to humans. The link between human menopause and oestrogen deficiency is well established (Butler and Santoro, 2011; Longcope *et al.*, 1986; Richelson *et al.*,1984). For this reason, the most common approach to generate an animal model for studying the menopause involves a surgical procedure known as ovariectomy, which involves disruption of normal ovarian function and thereby induces oestrogen deficiency systemically. Ovariectomy has been applied in rats, monkeys and other mini-pigs as an osteoporotic model (Borah *et al.,* 2002; Gadeleta *et al.,* 2000; McNamara *et al.,* 2006; Rohanizadeh *et al.,* 2000). Although small animal models, such as rats, are easy to maintain, inexpensive and have an accelerated ageing process compared to larger animals, bone from such models differs from human bone in terms of cell biology, mechanical properties and hormone production and regulation. Sheep have been utilised successfully by other researchers as models for osteoporosis (Chavassieux *et al.*, 2001; Turner *et al.*, 1995a; Turner *et al.*, 1995b). The sheep has a bone remodelling cycle similar to humans (3-5 months) (Canalis *et al.*, 2007) and experiences bone loss during osteoporosis similar to humans (Turner *et al.*, 1995a). Their ease of handling, vast availability and lack of variability, introduced through diet and exercise, make them an ideal model for human osteoporosis. The ovariectomised sheep has been shown to be a valid model for analysing the alterations in trabecular architecture that accompany osteoporosis (Newton *et al.*, 2004).

The bone specimens analysed in this study originated from an ovine osteoporosis model, acquired from a collaborative project (Bone for Life) between the Trinity Centre for Bioengineering, Trinity College, Dublin and the Department of Anatomy in the Royal College of Surgeons in Ireland (RCSI), Dublin, which has been described previously (Brennan *et al.*, 2009; Kennedy *et al.*, 2008a; Kennedy *et al.*, 2009a; Kennedy *et al.*, 2009b; Kennedy *et al.*, 2008b). Bone tissue analysed in this study came from either; (1) skeletally mature mixed breed ewes that underwent ovariectomy to induce an oestrogen deficient state (OVX, n=7) or (2) an aged-matched control group (CON, n=5). All surgery was performed following ethical approval by the Ethics Committee in the School of Veterinary Science in University College Dublin and under an animal licence granted by the Irish Department of Health. The serum estradiol and progesterone levels in these sheep for the duration of the 12 month study are presented in Appendix 2, illustrating that ovariectomy successfully diminished circulating serum hormone levels. Animals were maintained at pasture where no difference in feeding or activity levels were observed between groups, however this was not directly monitored or

quantitatively assessed. Animals in the current study were sacrificed at 12 months post-ovariectomy and bones were harvested and frozen at -20°C.

3.2.2 Specimen Preparation

Bone tissue samples, consisting of the proximal extremity of the left femur, were defrosted and thoroughly washed by extracting the marrow and fatty deposits with a water jet and were then left to air dry. Five individual trabeculae were excised from three regions of the left proximal femora (greater trochanter, lesser trochanter and femoral head) of animals from each of the CON and OVX cohorts; see Figure 3.1 (a). The sample sizes are presented in Table 3.1. Trabeculae were randomly chosen and excised with a scalpel and forceps with the aid of a 3x magnifying glass. Excised samples were embedded into polymethylmethacrylate (PMMA) solution in glass vials that was polymerised to form a solid structure in order to permit ease of handling and to guarantee spatial continuity and contiguity for qBEI analysis. Firstly, a polymer base was prepared in the 10mm diameter vials to ensure samples did not come into contact with the surface of the glass. Under the protection of a fume hood, MMA solution was pipetted into the glass vials to an approximate 10mm height. The vials were capped securely and placed in an oven at 55°C for approximately seven hours or until they are adequately hardened, but the top surface of the polymer was still tacky to touch with the forceps. Heating the MMA causes it to polymerise and harden into PMMA. Trabeculae were placed vertically on top of the base of PMMA and the polymer was pipetted into each vial to completely immerse the specimens. Vials were placed in a vacuum dessicator for two hours to expel air from the medium. The vials were then capped tightly and placed in an oven at 50°C for approximately 18 hours. The vials containing the vertically standing specimens embedded in a polymerised resin were placed in a freezer at -20°C to facilitate the subsequent removal of the glass by fracture.

A precision diamond cutting machine (Minitom, Struers, Denmark) with saw rotation of 400rpm was used to expose a cross-section through the trabeculae. Samples were ground and polished using a polishing wheel (Labopol -21, Stuers, Denmark) fitted with silica carbide paper, ensuring constant water irrigation throughout, and then by using diamond suspension of decreasing particle size down to 0.25 μm. Extremely smooth surfaces are a prerequisite for qBEI as scratches and polishing reliefs could generate a topographical contrast that would interfere with the qBEI material contrast. In order to provide a conductive path for electrons for SEM analysis and prevent charge up on the specimen

surface of non-conductive specimens, such as biological samples, samples were coated with a thin layer, approximately 30nm, of carbon by vacuum evaporation (Edwards 306 Auto Unit, Crawley, U.K.).

3.2.3 Scanning Electron Microscopy and qBEI

In order to ascertain the mineral content of the bone specimens, quantitative backscatter images were acquired of the cross section of each trabecula using an SEM (JSM 5910, JEOL, Welwyn Garden City, U.K.), fitted with a backscattered detector. Following chamber venting, loading of specimens and chamber evaluation, the incident beam was turned on for analysis. The following operating settings were applied: electron acceleration voltage of 15kV, beam current of 1.2nA and objective lens aperature diameter of 30µm. The magnification was kept the same for all images (200x), which resulted in a pixel resolution of 0.66µm/pixel and images comprising 1280 x 901 pixels..
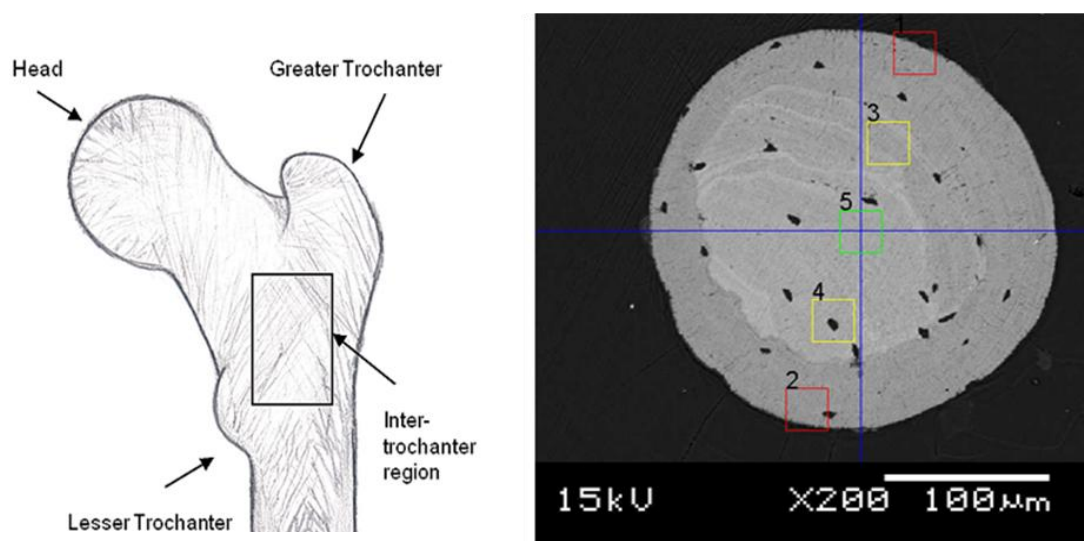
Figure 3.1 (a) Schematic depicting anatomical regions from which single trabeculae were excised from sheep bone; Greater Trochanter (GT), Lesser Trochanter (LT) and Femoral Head (H). Note also the inter-trochanteric region which is a primary fracture site in osteoporosis is also depicted. **(b)** qBEI image of trabecular cross section; tissue lamellae are clearly evident. Spatial distribution of calcium was analysed in specific regions of interest (ROI); Superficial (Boxes 1, 2), intermediate (Boxes 3, 4) and (Box 5) deep regions. For each trabecula eight superficial, eight intermediate and eight deep ROI were analysed.

Quantitative backscattered electron images were acquired with the samples at a fixed working distance of 12mm; see Figure 3.1 (b). In order to calibrate the grey level scale range, pure carbon and aluminium standards (Agar Scientific, Stansted, U.K.) were included in each scan. The standards were imaged in the qBEI mode and the contrast and

brightness settings were altered until carbon measured 25 on the grey level (GL) scale and aluminium measured 225. Calibrations were checked after every image capture and were recalibrated if necessary

3.2.4 Bone Mineral Density Distribution Analysis

Each qBEI image was analysed to determine the GL intensities of each pixel using a custom MATLAB script. An image histogram of pixel count versus grey level was produced (with pixels due to PMMA embedding material threshold out). Pixel count was presented as a percentage of total pixel count and therefore expressed as a percentage of total bone area (% bone area). A linear relationship between the grey levels of the backscattered image and the local calcium content was established previously (Roschger *et al.*, 1998). This relationship was employed to calculate bone mineral content (*wt%*Ca) from the GL measurements (*GL*) according to equation 3.1

$$wt \% \, Ca = -4.332 + (0.1733 \times GL) \tag{3.1}$$

This relationship was used to determine the bone mineral content (wt % Ca) of individual trabeculae excised from three regions of the proximal femora (greater trochanter, lesser trochanter and femoral head) of animals from each cohort. A frequency distribution of the calcium content of single trabeculae, known as the bone mineral density distribution (BMDD), was used to compare the distribution of bone tissue mineral between normal and osteoporotic bone similar to previous methods (Busse *et al.,* 2009; Roschger *et al.,* 1998). Histograms of wt % Ca were derived from the high-resolution qBEI images (0.66 μm), which produced histograms with a resolution of 0.17 wt % Ca. A Gaussian curve-fit was superimposed on the BMDD histograms since the data was normally distributed. The mean calcium content of each trabecula, μ_{Ca}, was calculated according to equation 3.2

$$\mu_{Ca} = \frac{\sum (wt \% \, Ca) \times (\% \, bone \, area)}{100} \tag{3.2}$$

The heterogeneity of calcium within each trabecula was quantified by calculating the full width at half maximum (FWHM) from the BMDD histogram. The FWHM is the distance between points on the Gaussian curve at which the function reaches half its maximum value (Figure 3.2). To assess inter-trabecular variation, the standard deviation, σ, and coefficient of variation ($c_v = \sigma / \mu_{Ca}$) for three anatomical regions (greater

trochanter, lesser trochanter, femoral head) of every sheep was computed. In order to evaluate inter-group comparisons, the percentage differences of μ_{Ca}, σ and c_v between anatomical regions were calculated.

3.2.5 Spatial Distribution of Calcium within Trabeculae

BMDD analysis provides important information regarding the mean calcium content and heterogeneity of bone tissue mineral but does not distinguish where precisely mineralisation differences arise within trabeculae. To attain spatial information on mineral distribution within trabeculae, calcium content was quantified at specific regions of interest (ROI) through the cross section of trabeculae. Each qBEI was analysed using a custom MATLAB script, which allowed the operator to manually select the surface of a trabecula and then automatically analyse mineral content in the superficial region, the intermediate region and the deep region within each trabecula, see Figure 3.1 (b). The distinction and differentiation of superficial, intermediate and deep regions through the cross section of rod-like and plate-like trabeculae can be subjective. Therefore, regions of interest (ROI) were automatically generated to avoid operator bias. Two opposite ROI on the surface of a trabecula (superficial region), two intermediate ROI (midway between the trabecular surface and centre) and one core (deep) ROI were automatically defined at equidistant locations. These ROI were of the same size (38 x 38 pixels) and were defined proportional to the diameter of each cross section, to allow comparison of trabeculae with one another regardless of thickness or shape and also remove any bias that might be introduced in a manual method. This method generated five ROIs through the cross section of trabeculae and this was performed four times per trabecula to generate eight superficial, eight intermediate, and four deep ROIs, in total, per trabecula. Histograms of GL were plotted for each ROI, a threshold was applied to remove GL data from PMMA embedding material and the pixel count (frequency) was weighted accordingly, and since the data was normally distributed, a Gaussian curve fit was superimposed on the histograms (see Figure 3.2). The mean wt % Ca for each level (superficial, intermediate, and deep) was calculated. The percentage difference of wt % Ca between the deep and superficial regions of trabeculae was computed and compared between CON and OVX groups to assess the influence of oestrogen deficiency on the spatial distribution of calcium within single trabeculae.
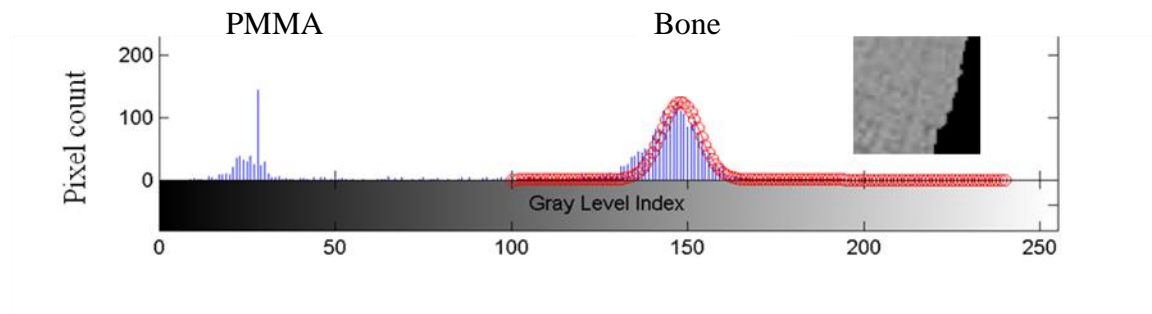
Figure 3.2 Histrogram of pixel count versus grey level for a typical region of interest (ROI) (pictured inset) in the superficial region of trabeculae, with a Gaussian curve fit applied. The peaks on the left represent the PMMA embedding material (black in the ROI), which was removed from analysis by applying a threshold to each image.

3.2.6   Statistical Analysis

General linear model (GLM) ANOVA's were applied to statistically analyse wt % Ca parameters between groups. A p value of ≤0.05 was considered statistically significant. The mean calcium content (μ_{Ca}) of normal and OVX bone within regions was compared, as well as the pooled mean calcium content between CON and OVX (i.e. combined data from all anatomical regions). Next, mineral heterogeneity (FWHM) within trabeculae was compared between normal and OVX bone tissue. The spatial distribution of mineral within trabeculae (% difference between superficial and deep regions) was compared between normal and OVX bone tissue. Sheep were defined as random factors nested within either CON or OVX groups while femora regions and trabecular regions were fixed factors, nested within the sheep from which they arose. Therefore, sample size for the purpose of statistically comparing OVX and CON refers to sheep numbers. Tukey's method for pair wise comparisons, with a family error rate of 0.05, was employed to determine statistical differences between distinct anatomical locations within femora and between trabecular regions. Finally, Student's t-tests were employed to compare CON and OVX sheep by assessing the % difference of the standard deviation (σ) and coefficient of variation (c_v) of mineralisation between regions.

3.3.   Results

The results of this study are presented in detail below. Each section addresses a specific

question related to the global hypothesis, which is "The distribution of bone tissue mineral is altered during osteoporosis".

3.3.1 Does Oestrogen Deficiency Alter the Mean Calcium Content of Bone Tissue?

Pooled mean calcium content data from all trabeculae in all three regions of the proximal femur revealed no significant difference in μ_{Ca} between CON and OVX groups. Similarly, no difference in μ_{Ca} was found between these two groups within any of the specific femoral regions, i.e. lesser trochanter, femoral head and greater trochanter. These data are presented in Table 3.1.

3.3.2 Does Oestrogen Deficiency Alter the Mineral Heterogeneity of Trabeculae?

BMDD analysis revealed significantly higher FWHM, a measure of heterogeneity, within trabeculae from the greater trochanter of the OVX group compared to CON (3.57 ± 0.68 vs. 3.17 ± 0.36, p<0.04), see Figure 3.3. However, the higher heterogeneity in OVX animals compared to CON was site specific, as no significant differences were observed in the FWHM from trabeculae of the head and lesser trochanter regions between groups. These data are presented in Table 3.1.
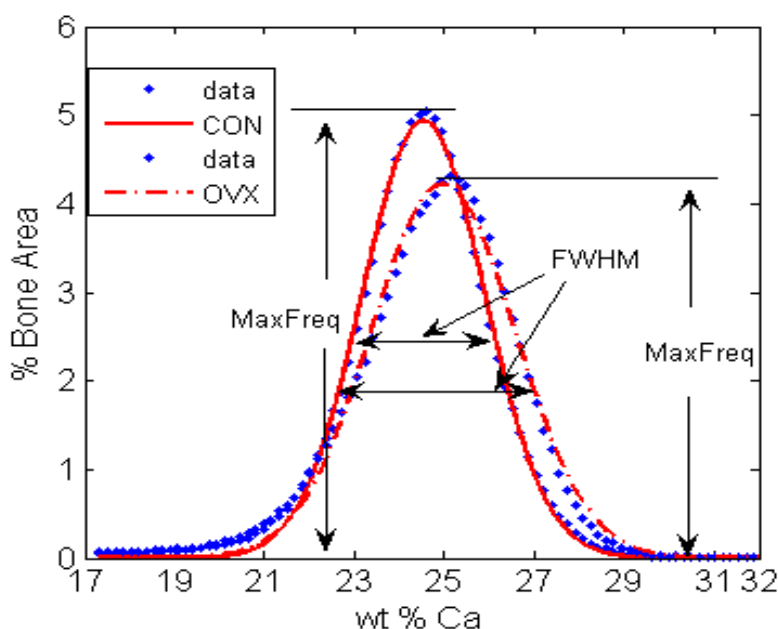
Figure 3.3 Bone mineral density distributions (BMDD) through the entire cross section of representative CON (n=25) and OVX trabeculae (n= 30) in the greater trochanter of proximal femora. A Gaussian curve fit (red) was applied to the data points (blue). The mineralisation profiles in superimposition reveal significantly higher (p<0.04) full width at half maximum (FHWM) and a non-significant shift towards a higher mineralisation range in OVX trabeculae.

Table 3.1: Mean weight percentage calcium (wt % Ca), full width at half maximum (FWHM) and sample sizes (n) for bone trabeculae from the Greater Trochanter (GT), Femoral Head (H) and Lesser Trochanter (LT) regions of control (CON) and OVX bone. Data is presented as mean ± standard deviation. * indicates different from CON LT (p<0.01), + higher than LT (p<0.01), ++ higher than H (p<0.02), and ** FWHM differs between groups.

		Mean	p	FWHM	p	n Sheep	Trabeculae
GT	CON	24.46 ± 1.09 *	0.65	3.17 ± 0.36	0.04	5	25
	OVX	24.71 ± 1.06 +, ++		3.57 ±0.68	**	6	30
H	CON	23.67 ± 1.26	0.88	3.60 ± 0.31	0.31	4	20
	OVX	23.78 ± 1.48		3.84 ± 0.72		7	35
LT	CON	23.29 ± 0.95	0.76	3.86 ± 0.61	0.34	5	25
	OVX	23.40 ± 1.68		3.96 ± 0.61		5	25
Pooled data	CON	23.80 ± 1.20		3.54 ± 0.53		5	70
	OVX	24.0 ± 1.49	0.65	3.78 ± 0.74	0.10	7	90

3.3.3 Does Oestrogen Deficiency Alter the Spatial Distribution of Mineral within Trabeculae?

For both CON and OVX groups, the highest mineral content was in the core of trabeculae with a lower calcium content measured in the outermost surface (superficial region), see Figure 3.4. For bone tissue from the lesser trochanter of CON animals, calcium content was significantly higher in the deep region of trabeculae compared to the superficial region (p=0.01). In the OVX group, calcium content was significantly higher in the deep region compared to superficial region for all three femoral regions; the greater trochanter (p<0.01), head (p<0.01) and lesser trochanter (p<0.01). In OVX animals, calcium content was higher in intermediate ROIs compared to superficial ROIs in the head region (p<0.02). These data are presented in Table 3.2. The % difference of calcium content between the deep and superficial regions was statistically higher in the OVX group compared to CON in the proximal femur when data from all regions were pooled (p<0.04). When regions were analysed separately, these differences were detected in the head region (p<0.05), but not in the greater or lesser trochanters, see Figure 3.5.
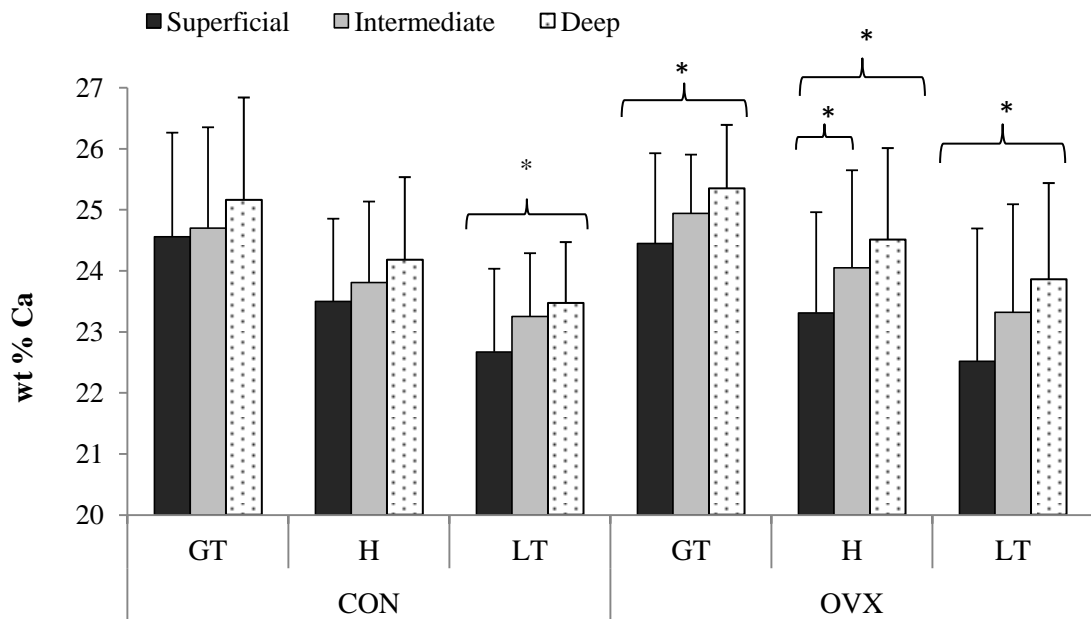
Figure 3.4 Spatial distribution of calcium between superficial, intermediate and deep ROIs, in the greater (GT), head (H) and lesser trochanter (LT) regions of the proximal femora of control sheep (CON, n=5) and ovariectomised sheep (OVX=7). * indicates statistical significance between trabecular regions under horizontal brackets ($p \leq 0.02$).

Table 3.2: Spatial distribution within trabeculae from the greater trochanter (GT), head (H) and lesser trochanter (LT) regions of control (CON) and ovariectomized (OVX) sheep. Wt % Ca from the superficial (S), intermediate (I) and deep (D) trabecular regions is compared between groups. Data is presented as mean ± standard deviation. * indicates significant difference ($p<0.05$)

		S	I	D	Pairwise comparison		n	
					S - D	S - I	Sheep	Trabeculae
GT	CON	24.56 ± 1.70	24.70 ± 1.65	25.16 ± 1.68	0.07	0.86	5	25
	OVX	24.45 ± 1.47	24.94 ± 0.96	25.35 ± 1.04	0.01*	0.06	6	30
H	CON	23.50 ± 1.36	23.81 ± 1.32	24.18 ± 1.35	0.16	0.67	4	20
	OVX	23.31 ± 1.64	24.05 ± 1.60	24.51 ± 1.50	0.01*	0.02*	7	35
LT	CON	22.67 ± 1.36	23.25 ± 1.04	23.47 ±0.99	0.01*	0.09	5	25
	OVX	22.52±2.17	23.32±1.77	23.86 ± 1.75	0.01*	0.13	5	25
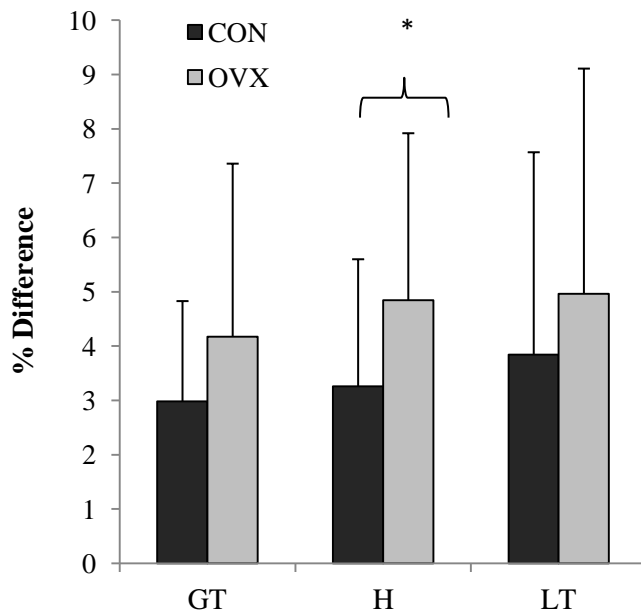
Figure 3.5 Comparisons between CON (n=5) and OVX (n=7) groups for the % difference between superficial and deep regions of trabeculae in the greater trochanter (GT), lesser trochanter (LT) and head (H) regions of proximal femora. Increased % difference by the OVX group was significant in the head region (p<0.05). * indicates significance between groups.

3.3.4 Does Oestrogen Deficiency Alter the Mineral Distribution across Anatomical Locations?

Significant differences in μ_{Ca} were found across anatomical regions of the femur in both CON and OVX groups. In the CON group, μ_{Ca} was significantly higher in bone tissue from the greater trochanter compared to the lesser trochanter (p<0.01). In OVX sheep, μ_{Ca} in the greater trochanter region was significantly higher than that of the lesser trochanter (p<0.01) and head (p<0.02) regions, see Figure 3.6.

Inter-trabecular variation: The standard deviation (σ) of mineralisation between trabeculae of the lesser trochanter was significantly higher than that of the greater trochanter in the OVX group (1.36 ± 1.13 vs. 0.36 ± 0.29, p<0.05). Similarly, the coefficient of variation (c_v) was higher in the lesser trochanter compared to the greater trochanter (0.06 ± 0.05 vs. 0.01 ± 0.01, p< 0.05). No significant difference existed between the two regions in the CON group.

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Inter-group variation: The % difference in mean mineral content (μ_{Ca}) between anatomical regions (greater and lesser trochanter) was not significantly different when comparing CON and OVX groups (p<0.42), see Figure 3.7. The % difference in the standard deviation (σ) of trabecular mean mineral content between the greater and lesser trochanters was statistically higher for OVX compared to CON (77.11 ± 11.70 vs. 45.64 ± 23.70, p<0.03). The % difference in coefficient of variation (c_v) between the greater and lesser trochanters was also statistically higher for OVX compared to CON (77.77 ± 11.16 vs. 48.16 ± 24.80, p< 0.04).
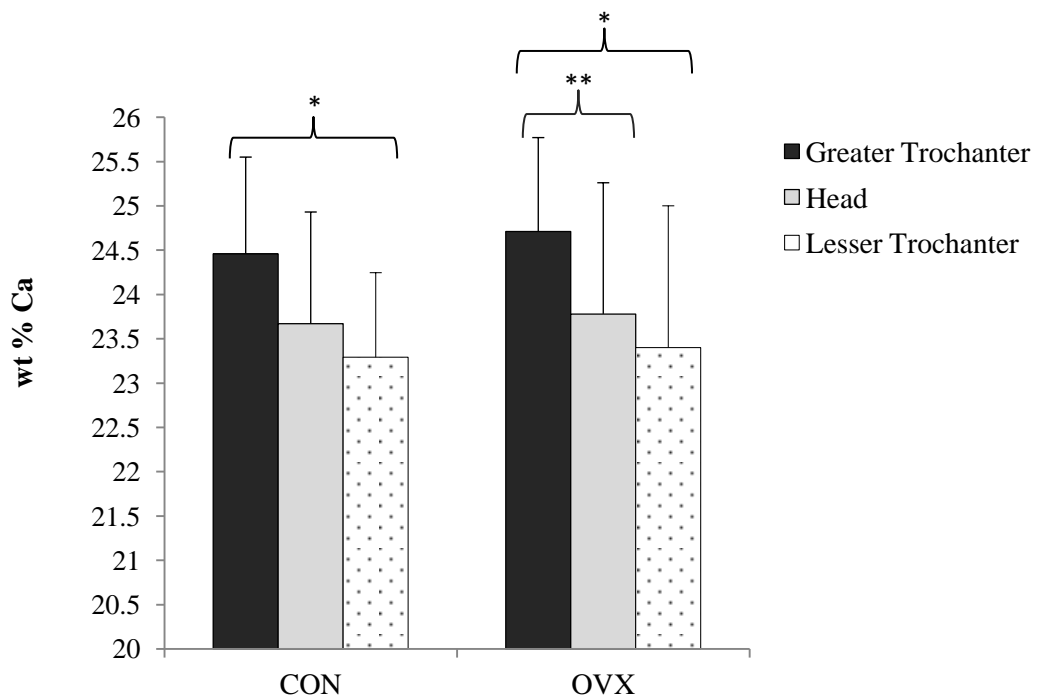
Figure 3.6 Comparison of mean calcium content (wt % Ca) between anatomical regions within the proximal femur of CON (n=5) and OVX (n=7) sheep.* and ** signify statistical difference between femoral regions indicated by the horizontal brackets, (p<0.01, and p≤0.02) respectively.
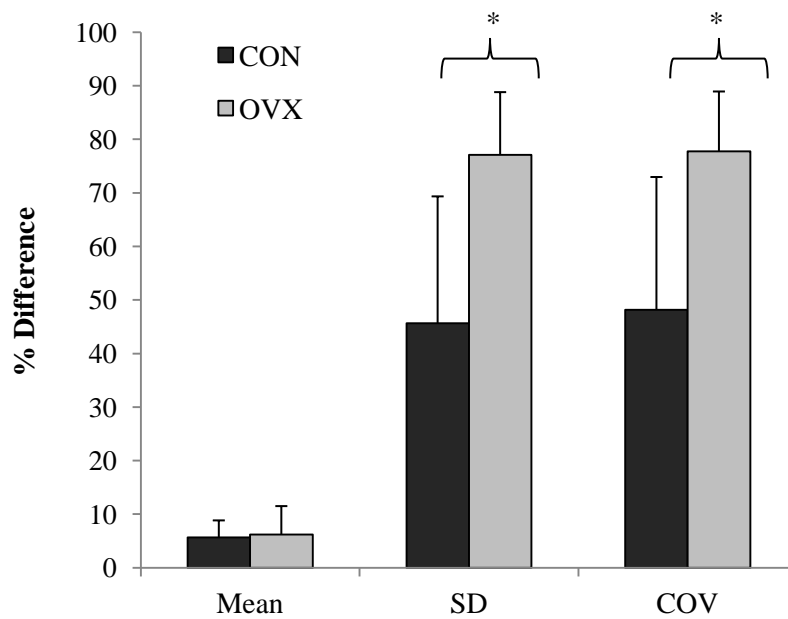
Figure 3.7 Comparisons between CON (n=5) and OVX (n=7) groups for the % difference of trabecular mineralisation between the greater and lesser trochanters. The differences were significant (p<0.05) for standard deviation (σ) and coefficient of variation (cυ). The % difference in mean calcium content μ_{Ca} was not significantly different. * indicates statistical significance between groups.

3.4. Discussion

This study provides direct evidence that oestrogen deficiency alters the distribution of bone tissue mineral compared to healthy controls, both on the trabecular level, and across anatomical locations. Although bone tissue mineral distribution is heterogeneous in healthy bone, the current study reveals that this heterogeneity is significantly more pronounced in oestrogen deficient animals. Increases in heterogeneity within trabeculae were site specific, occurring in the greater trochanter, whereas the % difference in mineral content between superficial and deep region was significantly increased in the femoral head. Finally, regional differences in the variability (σ , c_v) of mineral content between the greater and lesser trochanters were significantly more pronounced during oestrogen deficiency.

It should be noted that there were some limitations associated with this study. Firstly, ovariectomy, or removal of the ovaries, was used as a model of osteoporosis which causes dramatic lowering of circulating oestrogen levels (see Appendix 2), whereas during the menopause the cessation of the ovary functions is a more gradual process.

Plasma oestrogen levels during the menstrual cycle of females of reproductive age ranges from 50-200 pg/mL (Longcope and Pratt, 1978), levels for females from 42 years of age ranges from 60-80 pg/mL (Randolph *et al.,* 2004), and levels for postmenopausal women are less than 40 pg/mL (Randolph *et al.,* 2004). However, sheep plasma oestrogen levels for control animals are in the range of 2pg/mL, which are rather less than human oestrogen concentrations prior to the menopause. Nevertheless, the ovariectomised sheep model has been an accepted means of assessing the effects of osteoporosis on bone tissue in many previous studies (Chavassieux *et al.*, 2001; Turner *et al.,*1995a; Turner *et al.,* 1995b) and has shown to experience bone loss during oestrogen deficiency similar to humans (Turner *et al.*, 1995a). Secondly, ewes, unlike humans, have twenty oestrous cycles and experience a short period (1-2 months) of oestrogen deficiency annually (Newton *et al.*, 2004). Therefore, while comparisons between each sheep group can provide an insight into the role of oestrogen deficiency in bone mineralisation, the limitations in the animal model should be considered when making comparisons with human tissue. It must be noted however that calcium content values that were measured in sheep bone tissue (22-26%) are comparable with ranges reported from control (18-28%) and osteoporotic human bone tissue (16-26%) (Gupta *et al.*, 2005; Roschger et al., 1998; Roschger *et al.*, 2001).

Five trabeculae from each of the three skeletal sites of each sheep were used for analysis and selection may have been biased towards larger trabeculae due to limited availability and the difficulties associated with excising them for analysis. Furthermore, as the preparation method is destructive, it was not possible to track changes in specific trabecular regions over the course of time. Therefore, the intermediate region of trabeculae from OVX bone might not correspond to the intermediate region that existed prior to increased surface remodelling characteristic of oestrogen deficiency. However, our analysis method enabled selection of equidistant regions relative to the diameter of each trabecula, rather than setting a predefined distance for intermediate and deep regions, ensuring that the chosen region was indeed the intermediate layer of the newly remodelled OVX trabeculae. It is expected that differences in tissue heterogeneity would be more pronounced with larger sample sizes and in human osteoporotic bone compared to healthy subjects that do not experience an oestrogen deficient period. Finally, there are important limitations associated with the method to calculate wt % Ca from grey level intensities acquired from qBEI images that should be noted. Although carbon and aluminium have been employed extensively as calibration standards for qBEI on bone

samples (Grabner *et al.*, 2001; Misof *et al.*, 2003; Roschger *et al.,* 1998; Sutton-Smith *et al.*, 2008) channelling contrast can occur due to their crystalline nature. Two approaches were used to reduce this effect; (i) the angle of electron beam incidence was kept constant throughout all experiments and (ii) mechanically polished carbon and aluminium were used as standards to reduce the contrast channelling effect (Finch, 1936; Reimer, 1998). The method to calculate wt % Ca from grey level intensities was developed by calibrating backscattered images using quantitative energy dispersive x-ray (EDX) analysis (Roschger et al., 1998). Although this method has been employed extensively to relate grey levels to calcium content of bone tissue (Busse *et al.,* 2009; Gupta *et al.,* 2005; Misof *et al.,* 2003; Seitz *et al.*, 2010; Sutton-Smith *et al.*, 2008; Vajda, 1996), Monte Carlo simulations of electron trajectories have shown that the 3D x-ray production volume for calcium greatly exceeds the excitation volume of backscattered electrons (Howell and Boyde, 2003). Furthermore, there is evidence that EDX-standardized BSE images tend to give a lower mineral content value of bone than when measured with traditional ash measurements (Vajda *et al.*, 1998). However, an identical approach was used to compare both CON and OVX groups and, as such, these limitations are expected to influence each group equally. Therefore, the relative differences observed between groups are believed to be an accurate representation of tissue level changes as a result of oestrogen deficiency. Alternative analysis techniques such as synchrotron μCT could be used in future to assess mineral distribution ex vivo within bone tissue in 3D, overcoming the challenges of sample size and tissue preparation (Nuzzo *et al.*, 2002).

It is interesting that no differences were detected from the pooled mean mineralisation data of all trabeculae from all regions of normal and OVX tissue. This is consistent with previous studies, which found no difference in the mineral content of osteoporotic bone compared to control (Bohic *et al.,* 2000; Ciarelli *et al.,* 2003; Loveridge *et al.*, 2004; Rohanizadeh *et al.,* 2000). Increased mineral heterogeneity within trabeculae during oestrogen deficiency is in agreement with other studies (Busse *et al.,* 2009; Roschger *et al.,* 2001). Our results reveal, for the first time, that changes in mineral heterogeneity in the femur during oestrogen deficiency are dependent on anatomical location. In addition, this study shows that oestrogen deficiency results in a higher variability of mineral distribution between regions of the proximal femora and also causes specific alterations in the spatial distribution of mineral between trabecular regions. These findings corresponded to no changes in the *mean calcium content* of trabeculae, or the %

difference of mean calcium content between anatomical locations during oestrogen deficiency, highlighting that such important effects of oestrogen deficiency on mineral distribution may be undetectable by studies focusing solely on mean mineral content of bone tissue during oestrogen deficiency (Bohic *et al.,* 2000; Boyde *et al.,* 1998; Ciarelli *et al.,* 2003; Dickenson *et al.,* 1981; Gadeleta *et al.,* 2000; McNamara *et al.,* 2006; Rohanizadeh *et al.,* 2000).

The spatial distribution of mineral within trabeculae from control bone tissue was as expected; these trabeculae had a more mineralised core, as this tissue was older and secondary mineralisation had persisted for longer (Grynpas, 1993), whereas the surface regions are more frequently renewed and would be less mineralised (Ciarelli *et al.,* 2003; Gadeleta *et al.,* 2000). It is interesting that oestrogen deficiency altered the spatial distribution of calcium within trabeculae in the proximal femur, specifically by means of a greater difference in mineralisation between the superficial and deep regions of trabeculae from the head region. This might be attributed to increased surface remodelling characteristic of osteoporosis (i.e. higher turnover rates) which results in the formation of new superficial tissue (Parfitt *et al.*, 1983). As such, increased heterogeneity may be a transient characteristic, and the normal heterogeneity might be restored by complete mineralisation of new bone over time. In addition, since osteocytes possess oestrogen receptors (Batra *et al.*, 2003); direct modulation of osteocyte secondary mineralisation might occur when levels of circulating oestrogen are deficient. It is known that osteocyte apoptosis is up-regulated during oestrogen withdrawal (Kousteni *et al.*, 2001; Tomkinson *et al.*, 1997) and, as such, infilling of the remaining cavities, known as micropetrosis (Boyde, 2003; Frost, 1960; Kingsmill and Boyde, 1998), might occur. The studies outlined in Chapter 5 and 6 are thus conducted to delineate if these mechanisms contribute to the differences observed in the current study.

The precise implications of differences in mineralisation between trabecular regions, on the mechanical properties of oestrogen deficient bone are unknown. Assuming previously published relationships between calcium content and ash fraction (Vajda *et al.,* 1998) and between ash fraction and mechanical strength (Hernandez *et al.,* 2001), an increase as small as 0.5% (wt % Ca) between trabecular regions would equate to an increase in strength of 4.5% between the regions. A greater concentration of calcium in the deep regions with a lower concentration of mineral in the superficial regions would be beneficial under bending loads, whereby the superficial regions would be capable of withstanding greater strain without fracture, thereby protecting the whole trabeculae

from fracture. However, it is also likely that there is a critical level for differences in mineralisation between regions, and beyond such a level, these differences become detrimental to the mechanical integrity of the trabeculae. Alternatively, these alterations in spatial distribution of mineral may be a compensatory mechanism following bone loss during oestrogen withdrawal. Further experiments are required to delineate this problem.

The site specific nature of increased mineral heterogeneity may occur as a result of local changes in bone remodelling activity or mechanical loading during oestrogen deficiency. While previous studies on the same sheep cohort have reported variations in trabecular micro-architecture between anatomical regions of the vertebrae of healthy sheep (Kennedy *et al.*, 2009c), site-specific changes in bone remodelling activity or tissue micro-architecture of OVX sheep were not identified (Kennedy, 2007). However, a previous study has demonstrated site specific variations in bone loss and remodelling activity in ovariectomised rats; while bone loss was significant in the diaphysis and metaphysic, the epiphysis appeared to be immune to bone loss and the time course of remodelling activity varied considerably between regions (Baldock *et al.,* 1998). One other study reported that oestrogen deficiency amplifies the variability of mineralisation in alveolar bone of the jaw, and that this is likely due to alterations in active bone remodelling in response to forces generated during mastication (Ames *et al.*, 2010). These observations suggest that the alterations in mineral heterogeneity observed in the current study may occur as a result of changes in local factors, such as cellular activity and mechanical loading during oestrogen deficiency. The studies outlined in Chapter 5 and 6 will investigate these factors further. These findings also highlight the fact that it is imperative to assess bone tissue from different regions when evaluating the effects of oestrogen deficiency on trabecular bone.

It is well established that low bone mass and micro-architectural deterioration occur during oestrogen deficiency and are primarily responsible for the reduced bone strength and increased fracture risk, characteristic of osteoporosis. As bone mineral content is a determinant of bone strength (Currey, 1984b; Ruffoni *et al.,* 2007), alterations in tissue mineral distribution within trabeculae and across anatomical locations, might also be indicative of reduced mechanical integrity. Indeed, previous computational studies have predicted that increased heterogeneity within trabeculae results in significantly increased stiffness (Van der Linden *et al.*, 2001), and adaptive modelling will likely occur leading to trabecular thinning, bone loss and trabecular perforation (Mulvihill *et al.*, 2008; Van der Linden *et al.*, 2004). Increased heterogeneity between skeletal sites may indicate

zones of the bone that are more affected by oestrogen deficiency and therefore represent weak points. In fact, computational studies have predicted that regions of bone that have more heterogeneous material properties are at an increased risk of fracture (Hernandez, 2008; Jaasma *et al.,* 2002; Renders *et al.,* 2008). It is most interesting that significant alterations in tissue mineral distribution occurred between the greater trochanter and lesser trochanter (% difference σ and c_v), which is coincident with the most common osteoporotic fracture site known as the inter-trochanteric fracture line. Taken together with our results, it is proposed that alterations in tissue mineral distribution may be a contributing factor for weakened bone at increased risk of fracture, at this site during osteoporosis.

3.5. Conclusion

This study provides evidence that the distribution of tissue-level mineral is altered during osteoporosis, which may alter tissue-level mechanics. In particular, our results suggest that these changes are anatomically distinct and do not occur ubiquitously throughout the proximal femur. These findings highlight the fact that methods evaluating the mean mineral content of bone tissue are insufficient to detect oestrogens impact on tissue mineral distribution and also indicate that local factors might have a significant impact on bone tissue during oestrogen depletion. Furthermore, in addition to the well established changes in bone mass and architecture during oestrogen deficiency, alterations in tissue mineral distribution in ovariectomised animals may be a contributing factor for reduced mechanical strength. Heterogeneous regions may be indicative of weakened zones at both the trabecular and anatomical levels that contribute to a reduction in mechanical strength, and render osteoporotic bones more susceptible to fracture. Therefore, these findings are of potential importance in understanding the underlying mechanisms of altered mechanics during osteoporosis. Whether these findings are a transient effect of oestrogen deficiency or whether they are sustained with prolonged oestrogen deficiency has not yet been evaluated. In addition, since this study indicates that altered mineral distribution may contribute to increased fracture risk during oestrogen deficiency; it is interesting to postulate that the beneficial effects of bisphosphonates may lie in the ability of these drugs to normalise the tissue mineral distributions back to that of controls. These questions will, therefore, be investigated in Chapter 4. In Chapters 5 and 6, whether changes in bone cell activities as a direct effect of oestrogen withdrawal are responsible for the tissue-level alterations in mineral concentration that were observed in this Chapter will be examined.

4. Effects of Ageing, Prolonged Oestrogen Deficiency and Zoledronate on Bone Tissue Mineral Distribution

4.1. Introduction

One of several bone quality characteristics known to govern the mechanical strength of bone is the quantity of mineral, together with its distribution within the bone tissue matrix (Currey, 1984b; Follet *et al.,* 2004). The degree of bone mineralisation is influenced by the frequency of bone remodelling and mineral deposition rates. Increases in the degree of bone tissue mineralisation have been shown to be associated with a significant enhancement of overall bone strength (Vose and Kubala, 1959). However, beyond approximately 66% mineralisation, further elevations in mineral content lead to brittleness and decreased bone mechanical strength (Bonfield and Clark, 1973; Currey, 1969). Although bone mineral content is generally believed to increase with ageing (Currey *et al.*, 1996; Reid and Boyde, 1987; Vajda and Bloebaum, 1999), there are conflicting observations of altered bone mineralisation as a consequence of ageing. Studies on healthy human bone which found increased mineralisation with ageing (Currey *et al.,* 1996; Reid and Boyde, 1987; Vajda and Bloebaum, 1999), indicate a material level alteration in tissue properties with ageing, whilst others that demonstrate no correlation (Roschger *et al.,* 1998; Roschger *et al.*, 2003) (Bloebaum *et al.*, 2004; Boivin and Meunier, 2002), signifying that changes in healthy bone over time are purely structural and that the material that constitutes the volume of bone remains relatively unchanged.

Oestrogen deficiency, occurring following the menopause, has been established as the primary causative factor in postmenopausal osteoporosis. A marked increase in bone turnover rates occurs during osteoporosis (Balena *et al.*, 1993) and leads to low bone mass and strength, depleted bone architecture, and increased risk of fractures (Cummings and Melton, 2002). Previous studies quantifying trabecular bone mineralisation have found increases (Boyde *et al.,* 1998; McNamara *et al.,* 2006), decreases (Gadeleta *et al.*, 2000; Yao *et al.*, 2006; Yao *et al.*, 2007), or only slight alterations in mineral content (Bohic *et al.,* 2000; Brennan *et al.,* 2011; Ciarelli *et al.,* 2003; Rohanizadeh *et al.,* 2000) as a consequence of oestrogen deficiency. These discrepancies may be explained by the duration of oestrogen depletion under investigation, as it has been shown that after the onset of oestrogen deficiency,

biological and structural alterations occur immediately, but these responses wane over time (Binkley *et al.,* 1998; Smith *et al.,* 2003). Bone mass deterioration and increases in bone turnover markers have been shown to stabilize at about nine months post ovariectomy in monkeys (Binkley *et al.*, 1998). A further study on ovariectomised monkeys noted a rapid phase of bone loss lasting approximately 8-12 months following ovariectomy, and by 16 months post-ovariectomy, a trend toward normalization of markers and stabilization of bone mass was observed (Smith *et al.,* 2003). However, the impact of such time dependent changes in remodelling and bone mass on bone tissue mineralisation has not been elucidated. In chapter 3, increased mineral heterogeneity within trabeculae, as well as alterations in mineral distribution along the inter-trochanteric fracture line in the proximal femur of sheep following 12 months of oestrogen deficiency was demonstrated. However, whether these changes are sustained with prolonged oestrogen deficiency is unknown.

Presently biphosphonates are the most widely used pharmaceutical used to treat osteoporosis. Bisphosphonates act to inhibit bone resorption by selective absorption to bone mineral surfaces and subsequent internalization by osteoclasts, where they interfere with osteoclast function. They reduce bone turnover by decreasing activation frequency and markers of bone remodelling and thereby prolong the secondary mineralisation of bone (Balena *et al.,* 1993). Bisphosphonate treatment has been shown to increase bone mineral density (BMD), mechanical strength and reduce the incidence of bone fractures (Balena *et al.,* 1993; Black *et al.,* 1996; McClung *et al.,* 2001). Zoledronic acid is a third generation, nitrogen containing bisphosphonate (NBP) (Green *et al.*, 1994). NBPs perturb the cytoskeleton necessary for maintaining the ruffled border that facilitates osteoclastic bone resorption (Rodan and Reszka, 2002). In animal models of osteoporosis, Zoledronic acid has been shown to prevent bone loss and increases bone mineral density and trabecular bone volume (Glatt, 2001; Pozzi *et al.*, 2009). Zoledronic acid suppresses bone resorption and increases bone mineral density (Leal *et al.*, 2010; Reid *et al.,* 2002) and has been shown to inhibit fractures of the vertebrae and the hip in clinical trials on postmenopausal women (Black *et al.*, 2007). It has been postulated that the small increases in BMD (3-8%) with bisphosphonate treatment (Riggs and Melton, 2002; Yao *et al.,* 2007), can only explain a small portion of the significant reduction in the incidence of bone fractures observed with this drug treatment (Liberman *et al.,* 1995; Riggs and Melton, 2002). Therefore, it is likely that other changes in the tissue composition might also occur. Whether bisphosphonate treatment counteracts the

increased mineral heterogeneity occurring during oestrogen deficiency (Brennan *et al.*, 2011), thus contributing to reduced fracture incidence, has yet to be delineated. In this study, quantitative backscattered imaging (qBEI) was used to quantify bone mineral density distributions (BMDD) within trabeculae from proximal femora of an ovine model of osteoporosis that underwent oestrogen deficiency for 31 months, an ovariectomised group administered with Zoledronic acid and aged-matched controls. The hypothesis that bone tissue mineralisation is altered during normal ageing and over the course of prolonged oestrogen deficiency will be tested by comparing BMDD parameters with previously reported data from sheep that underwent oestrogen deficiency for the shorter duration of 12 months (n=7) and their aged matched counterparts (n=5) (Brennan *et al.,* 2011). Furthermore, the effects of Zoledronic Acid treatment on BMDD parameters and site specific mineral distribution within the proximal femur will be assessed.

4.2. Materials and Methods

4.2.1 Bone Samples

Bone tissue originated from the ovine osteoporosis model described in Chapter 3. Unlike the OVX and CON sheep sacrificed after 12 months for the study presented in Chapter 3, the bone tissue examined in this chapter originated from OVX and age-matched CON sheep that were maintained for a further 19 months. In addition, 20 months post-ovariectomy, four OVX animals were randomly assigned to a Zoledronic acid (NovartisPharma, Switzerland) treated group (ZOL). Each animal received a 5mg dose of ZOL in 100 ml of saline infused over 30 minutes via an indwelling jugular catheter per week for 5 weeks. Animals were sacrificed at 31 months post-ovariectomy and bones were harvested and frozen at -20°C. 15 individual trabeculae (5 from each of the three proximal femora regions - greater trochanter, lesser trochanter and femoral head) were excised from each animal in the CON (n=5), OVX (n=5) and ZOL (n=4) groups. Bone trabeculae were prepared as described in chapter 3, by embedding in PMMA blocks, surface polishing and finally coating the surface with a thin layer of carbon.

4.2.2 Quantitative Backscattered Electron Imaging (qBEI)

The bone mineral density distribution (BMDD) of each trabeculae was determined from quantitative backscattered images (qBEI) of the cross section through trabeculae, as published previously (Brennan *et al.*, 2011) and described in Chapter 3. Briefly, a Scanning Electron Microscope (JSM 5910, JEOL, Welwyn Garden City, U.K.) fitted

with a backscattered detector was operated at an electron acceleration voltage of 15kV and a beam current of 1.2nA. Backscattered images were acquired with samples at a fixed working distance (12mm) and magnification (200x). Pixel resolution was 0.66μm/pixel and images comprised pixel dimensions of 1280 x 901. The grey level scale range was calibrated using pure carbon and aluminium standards (Agar Scientific, Stansted, U.K.). Bone mineral density distribution (BMDD) parameters including the mean calcium concentration (μ_{Ca}), the median, the peak height, and the full width at half maximum (FWHM), as well as the spatial distribution of the mean wt % Ca in the superficial, intermediate, and deep regions of interest (ROI) within trabeculae, and the percentage difference in wt % Ca between ROI, were quantified as detailed in Chapter 3 and were compared between OVX, ZOL and CON groups.

4.2.3 Statistical Analysis

General linear model (GLM) ANOVA's were applied to statistically analyse BMDD and spatial distribution parameters between groups. A p value of ≤0.05 was considered statistically significant. The difference in BMDD parameters; the mean calcium concentration (μ_{Ca}), median, FWHM, and peak height, was evaluated between groups by comparing pooled measurements (i.e. combined data from all femora regions), in addition to comparing data from distinct regions (greater trochanter, head and lesser trochanter). To evaluate the effects of ageing, parameters of BMDD were compared between CON sheep cohorts that were sacrificed 12 months following experiment initiation (n=5) with those at 31 months (n=5). Similarly, the effects of prolonged oestrogen deficiency were assessed by comparing sheep that were oestrogen deficient for 12 months (n=7) with sheep that were oestrogen deficient for the longer duration of 31 months (n=5). To evaluate the effects of oestrogen deficiency and Zoledronate treatment in the 31 month cohort, mineralisation parameters of OVX, ZOL and CON groups were compared within femoral regions (intra) and also regions (intra-regional) were compared within sheep groups. The spatial distribution of mineral within (intra) trabeculae; superficial, intermediate and deep mineral concentration, as well as the % difference between superficial and deep regions, was compared between sheep cohorts. The spatial distribution between (inter) trabeculae was also compared within sheep groups. In the GLM, age and osteoporosis status (OVX, ZOL, and CON) were fixed factors. Sheep were defined as random factors nested within OVX, ZOL, or CON groups, while femora regions and trabecular regions were fixed factors, with trabeculae

nested within the sheep from which they arose. Tukey's method for pair wise comparisons, with a family error rate of 0.05, was employed to determine statistical differences between distinct anatomical locations within femora and between trabecular regions. One-way ANOVAs were used to compare regional % differences between sheep groups.

4.3. Results

4.3.1 Effects of Ageing on Bone Mineralization

When data was pooled from all regions within the proximal femur, see Figure 4.1, the peak height of wt % Ca distributions was significantly lower in the 31 month control group compared to the 12 month control cohort (4.57 ± 0.64 vs. 4.24 ± 0.65, $p<0.04$), whereas the FWHM was significantly higher (3.54 ± 0.53 vs. 3.84 ± 0.68, $p<0.03$) with ageing. When femoral regions were analysed separately, in the greater trochanter of control proximal femora, significantly lower peak height of the distributions (5.02 ± 0.56 vs. 4.26 ± 0.61, $p<0.01$), together with higher FWHM (3.17 ± 0.36 vs. 3.84 vs. 0.60, $p<0.01$) were found in the 31 month control group compared to the 12 month control group. In the lesser trochanter region, significantly higher mean wt % Ca (23.29 ± 0.95 vs. 24.67 ± 1.20, $p<0.01$) were observed in the 31 month control group compared to the 12 month controls, as signified in Figure 4.2. No difference in mean wt % Ca existed with ageing in the Head region.

4.3.2 Effects of Prolonged Oestrogen Deficiency on Bone Mineralization

When sheep that were oestrogen deficient for 12 months were compared with a group that were oestrogen deficient for 31 months, an apparent lower mean wt % Ca was found in the 31 month group when data was pooled from all regions within the proximal femur (24.02 ± 1.49 vs. 23.26 ± 1.86, $p<0.07$), as indicated by Figure 4.1, although this was not statistically significant. However, when mineral distributions within discrete regions of OVX femora were compared, the duration of oestrogen deficiency significantly impacted mineralisation. In the greater trochanter region, significantly lower mean wt % Ca was observed in the 31 month group compared to the 12 month group (24.71 ± 1.06 vs. 22.86 ± 1.03, $p<0.01$), see Figure 4.2. Furthermore, the peak of the distribution was significantly lower (4.76 ± 0.98 vs. 3.84 ± 0.38, $p<0.01$), and conversely the FWHM higher (3.57 ± 0.68 vs. 4.29 ± 0.48, $p<0.01$) in sheep that were oestrogen deficient for 31 months compared to those that were oestrogen deficient for 12 months, as indicated in

Table 4.1. No significant difference in mineral distribution parameters was found in the head or lesser trochanter regions.
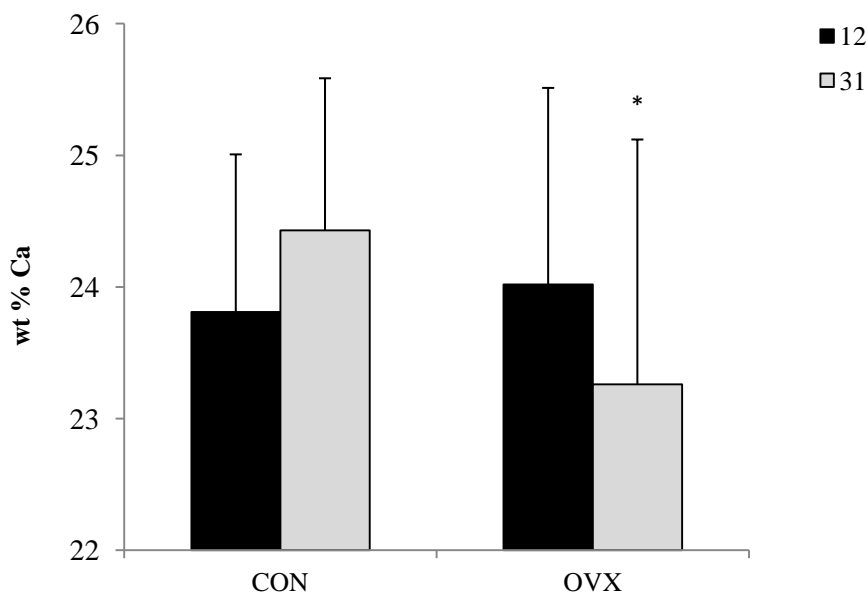
Figure 4.1 Effect of normal ageing: presented as 12 month CON sheep (n=5) compared to 31 month CON sheep (n=5), and prolonged oestrogen deficiency: presented as 12 month OVX (n=7) compared to 31 month OVX (n=5), on mean mineralisation (wt % Ca) when data from all regions of the proximal femur was pooled. * indicates OVX at 31 months is significantly lower compared to age matched controls
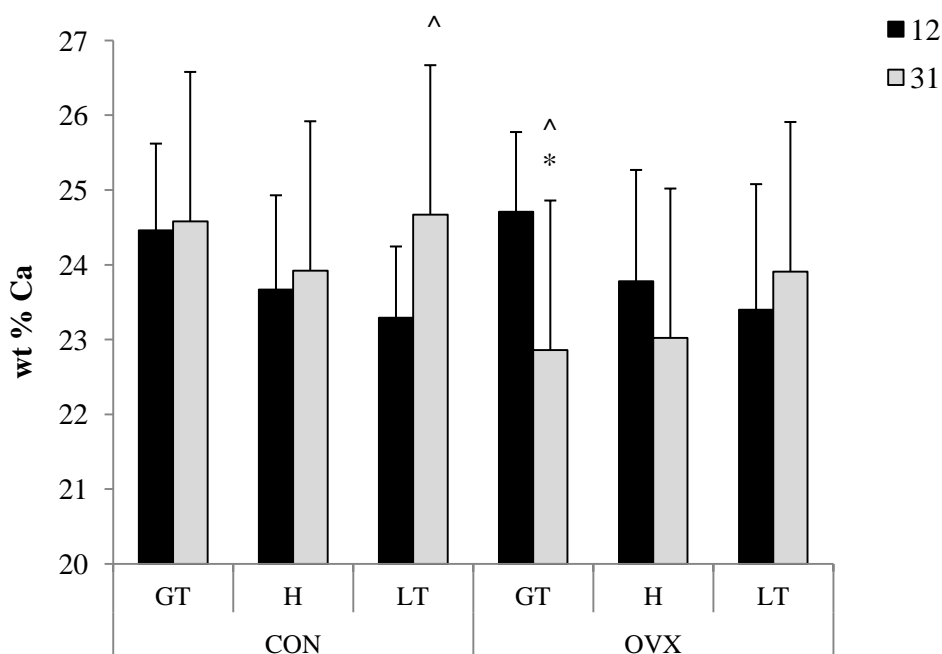
Figure 4.2 Effect of ageing (CON) and prolonged oestrogen deficiency (OVX) on mean mineralisation (wt % Ca) in the greater trochanter (GT), head (H), and lesser trochanter (LT) regions of the proximal femur. ^ indicates significant differences between 12 and 31 months of the same region, * indicates significantly lower than age-matched controls in GT. See Table 4.1 for sample sizes with respective proximal femoral regions

4.3.3 Effect of Oestrogen Deficiency on Mineralisation of Femoral Regions

Intra-regional: Comparing OVX with CON at 31 months, when BMMD data from trabeculae in all three regions of the proximal femur was pooled, there was significantly lower mean wt % Ca (23.26 ± 1.86 vs. 24.43 ± 1.16, $p<0.01$) in OVX animals compared to CON, as indicated in Figure 4.1. When trabeculae were analysed within each region, as depicted in Figure 4.2, significantly lower mean (22.86 ± 1.03 vs. 24.58 ± 1.18, $p<0.02$) and peak (3.84 ± 0.38 vs. 4.26 ± 0.61, $p<0.04$) values were found in the greater trochanter of OVX compared to CON. No significant difference in BMDD parameters existed between groups in the Head or Lesser Trochanter regions.

Inter-regional: In CON sheep, the FHWM of the lesser trochanter was significantly lower than that of the head region (3.63 ± 0.58 vs. 4.09 ± 0.79, $p<0.03$). In the OVX group, at 31 months, the peak height of the BMDD histogram was significantly higher in the head region compared to the greater trochanter (4.65 ± 0.83 vs. 3.84 ± 0.38, $p<0.01$), and conversely the FWHM was significantly lower in the head region compared to the greater trochanter (3.49 ± 0.61 vs. 4.29 ± 0.48, $p<0.01$), see Table 4.1.

The % difference in mean mineralisation between regions of the proximal femur is presented in Figure 4.3. OVX showed significantly higher mineral variability between the greater trochanter and head regions compared to control sheep (8.75 ± 5.18 vs. 3.96 ± 3.19, $p<0.05$). Similarly, the % difference between the lesser trochanter and the head region was significantly more in OVX compared to CON (13.48 ± 5.94 vs. 4.77 ± 4.23, $p<0.02$).

Table 4.1: BMDD parameters of oestrogen deficient (OVX), age-matched controls (CON), and Zoledronate treated sheep (ZOL). Results are presented as Mean ± SD. Values sharing a letter are significantly different to each other. p values; a<0.02, b<0.04, c<0.03, d-f <0.01

	Mean		Median	Peak		FWHM		n	
								Sheep	Trabeculae
Greater Trochanter									
OVX	22.86 ± 1.03	a	23.06 ± 1.15	3.84 ± 0.38	b f	4.29 ± 0.48	d	5	25
ZOL	23.46 ± 0.30		23.57 ± 0.48	4.04 ± 0.43		3.96 ± 0.45		4	20
CON	24.58 ± 1.18	a	24.80 ± 1.36	4.26 ± 0.61	b	3.84 ± 0.60		5	25
Head									
OVX	23.02 ± 2.31		23.21 ± 2.47	4.65 ± 0.83	f	3.49 ± 0.61	d	5	25
ZOL	23.65 ± 1.93		23.84 ± 2.08	4.32 ± 0.70		3.79 ± 0.60		4	20
CON	23.92 ± 0.95		24.32 ± 1.07	4.04 ±0.71		4.09 ± 0.79	c	4	20
Lesser Trochanter									
OVX	23.91 ± 2.00		24.20 ± 2.23	4.23 ± 0.89		3.96 ± 0.88		5	25
ZOL	23.20 ± 0.78		23.32 ± 0.85	4.19 ± 0.41		3.63 ± 0.44		4	20
CON	24.67 ± 1.20		24.90 ± 1.35	4.38 ± 0.63		3.63 ± 0.58	c	5	25
Pooled regional data									
OVX	23.26 ± 1.86	e	23.49 ± 2.03	4.22 ± 0.79		3.94 ± 0.74		5	75
ZOL	23.60 ± 1.30		23.80 ± 1.42	4.24 ± 0.54		3.79 ± 0.51		4	60
CON	24.43 ± 1.16	e	24.70 ± 1.28	4.24 ± 0.65		3.84 ± 0.68		5	70

4.3.4 Zoledronic Acid Reduces the Variability of Mineral between Anatomical Locations

Intra-regional: Comparing ZOL with OVX at 31 months, no statistical difference was found when trabeculae from all regions were pooled, or evaluated within distinct femoral regions. Similarly, when ZOL and CON were compared, no significant difference was found.

Inter-regional: In stark contrast to the significant differences in BMDD parameters between femoral regions found in OVX and CON groups (section 4.3.3), no difference existed in any BMDD parameters (mean, median, peak, FWHM) between any region (i.e. Greater Trochanter, Head, Lesser Trochanter) of proximal femora, as indicated in Table 4.1.

The % difference in mean mineralisation between regions of the proximal femur is presented in Figure 4.3. The mineral variability between the lesser trochanter and head (6.97 ± 4.58 vs. 13.48 ± 5.94, $p<0.05$) was significantly less in ZOL treated sheep compared to OVX sheep.
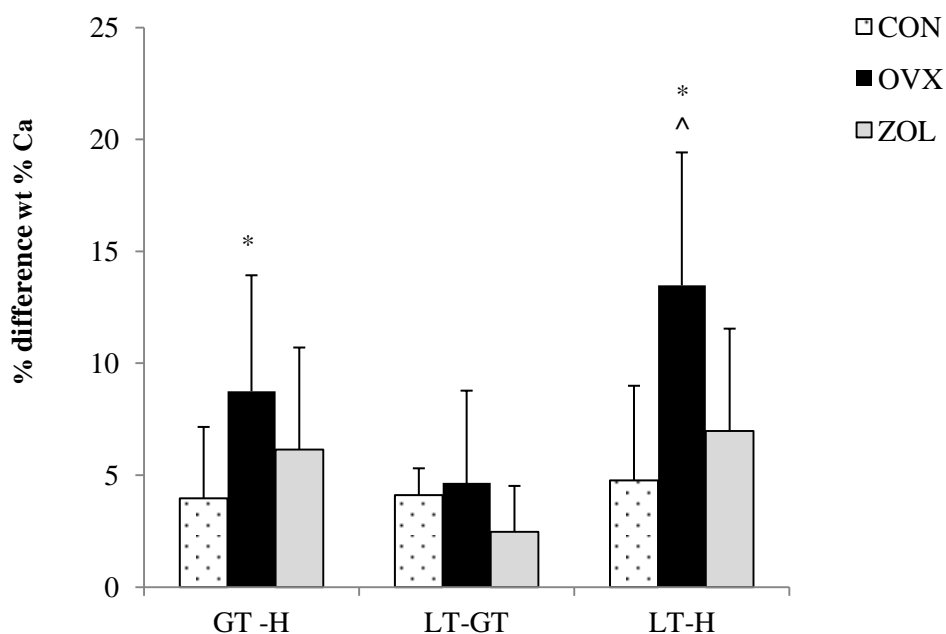
Figure 4.3 % difference of mean calcium concentration between regions (greater trochanter, head and lesser trochanter) of CON (n=5), OVX (n=5) and ZOL (n=4) groups (31 month cohorts). * indicates significantly higher compared to controls. ^ indicates higher compared to ZOL.

4.3.5 Effect of Oestrogen Deficiency on Mineralisation of Trabeculae

Intra-trabecular: When experimental groups were compared within trabecular ROI (intra-trabecular) it was found that in the greater trochanter, OVX was significantly lower than CON in the superficial (22.04 ± 1.30 vs. 23.80 ± 1.41, $p < 0.04$), intermediate (22.81 ± 1.26 vs. 24.84 ± 1.40, $p < 0.02$), and deep (23.37 ± 1.13 vs. 25.54 ± 1.18, $p < 0.01$) ROI.

Inter-trabecular: When bone tissue from sheep, that underwent oestrogen deficiency for 31 months, and age-matched controls, was analyzed, it was observed that for all groups, the highest mineral content was in the core of trabeculae with a lower calcium content measured in the superficial region, as illustrated in Figure 4.4. For bone tissue from the greater trochanter of CON animals, calcium content was significantly lower in the superficial region of trabeculae compared to both the intermediate region (23.80 ± 1.41 vs. 24.84 ± 1.40, $p < 0.01$) and the deep region (23.80 ± 1.41 vs. 25.54 ± 1.18, $p < 0.01$). The mean mineral concentration in the intermediate region was also significantly lower than the deep region ($p = 0.02$). In the head region of CON animals, the superficial region was significantly lower than the intermediate (23.05 ± 1.11 vs. 23.72 ± 1.3, $p < 0.04$) and the deep region (23.05 ± 1.11 vs. 24.22 ± 1.42, $p < 0.01$). In the lesser trochanter of the CON group, calcium concentration was significantly lower in the superficial region to that of the intermediate (23.86 ± 1.30 vs. 24.89 ± 1.25, $p < 0.01$) and the deep region (23.86 ± 1.30 vs. 25.25 ± 1.20, $p < 0.01$). In the greater trochanter of the OVX group, the calcium concentration was significantly lower in the outermost superficial region of trabeculae compared to the intermediate (21.94 ± 1.29 vs. 22.81 ± 1.26, $p < 0.01$) and to the deep region (22.54 ± 1.29 vs. 23.37 ± 1.13, $p < 0.01$). The intermediate region was also significantly lower than the deep region ($p < 0.04$). No significant difference was seen between trabecular ROI in the head region of OVX sheep. In the lesser trochanter of the OVX cohort, the superficial was significantly lower than the intermediate region (22.44 ± 2.14 vs. 23.88 ± 1.55, $p < 0.01$) and the deep region (22.44 ± 2.14 vs. 24.24 ± 1.51, $p < 0.01$).
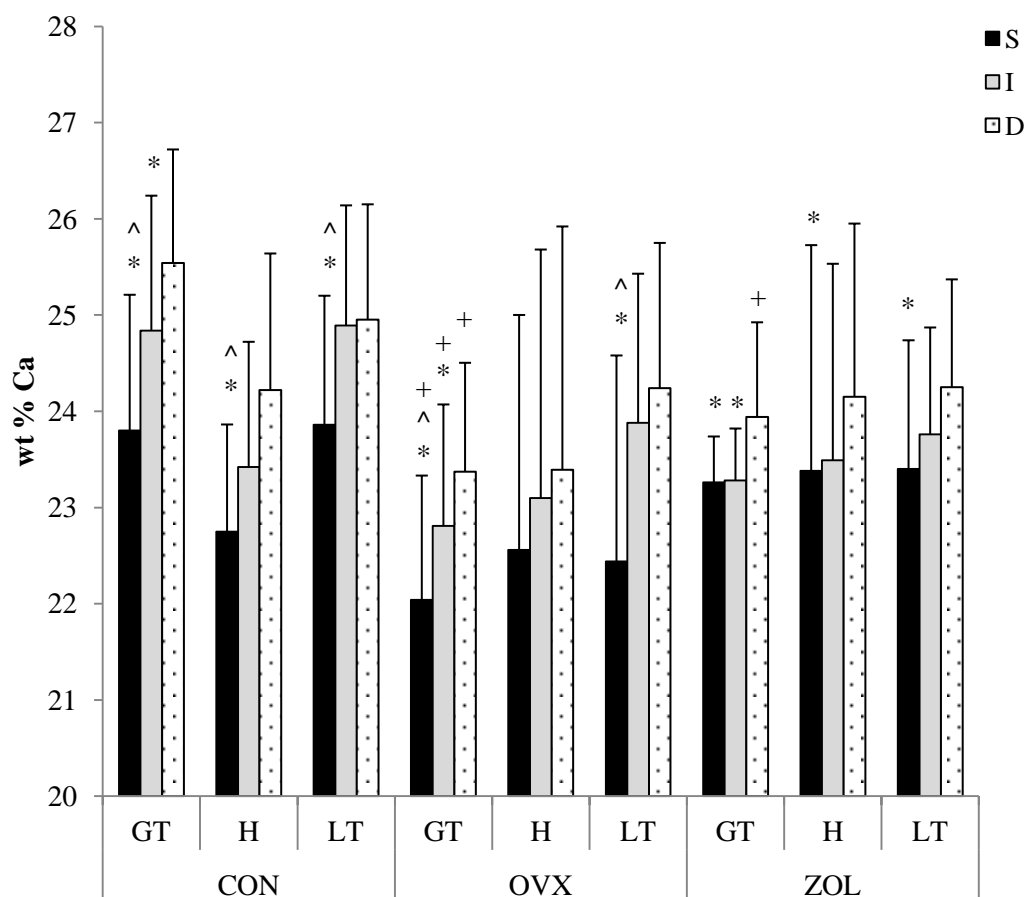
Figure 4.4 Intra-trabecular spatial distribution of calcium (wt % Ca) between superficial, intermediate, and deep ROI in the greater trochanter (GT), head (H) and lesser trochanter (LT) regions of the proximal femur from the 31 month sheep cohort. * indicates significantly different to deep ROI within the same femoral region of the indicated group and ^ indicates significantly different to intermediate ROI. + indicates significant difference to the same ROI of the CON group. See Table 4.1 for respective sample sizes within each ROI.

4.3.6 Zoledronic Acid Alters the Spatial Distribution of Mineral within Oestrogen Deficient Trabeculae

Intra-trabecular: Comparing groups within trabecular regions of interest (intra-trabecular), it was observed that in the greater trochanter, the calcium content in the deep ROI of the ZOL group was significantly lower than that of the CON group (23.94 ± 0.98 vs. 25.54 ± 1.18, $p<0.04$).

Inter-trabecular: In contrast to the mineral distribution in OVX sheep, in ZOL sheep there was no statistical difference between the superficial and intermediate ROI within trabeculae, in any of the greater trochanter, head, or lesser trochanter regions, as illustrated in Figure 4.4. In the greater trochanter region, the mean mineral concentration

in the deep region was significantly higher compared to the superficial region (23.26 ± 0.48 vs. 23.94 ± 0.98, $p<0.02$) and also compared to the intermediate region (23.28 ± 0.54 vs. 23.94 ± 0.98, $p<0.02$). The superficial ROI was lower than the deep ROI in the head (23.38 ± 2.35 vs. 24.15 ± 1.81, $p<0.03$) and lesser trochanter (23.40 ± 1.34 vs. 24.25 ± 1.12, $p<0.01$) of the ZOL group.

In the lesser trochanter the % difference in mean mineral concentration between superficial and deep ROI (S-D) was significantly lower in the ZOL group compared to the OVX group (3.97 ± 2.39 vs. 8.25 ± 4.33, $p<0.05$), as depicted in Figure 4.5. Likewise, when comparing % difference of mean mineralisation between the superficial and intermediate ROI (S-I), ZOL was significantly lower compared to OVX (2.01 ± 1.80 vs. 6.65 ± 4.27, $p<0.03$). There was also a significant decrease in ZOL compared to CON in the lesser trochanter region (2.01 ± 1.80 vs. 4.36 ±1.99, $p<0.01$).
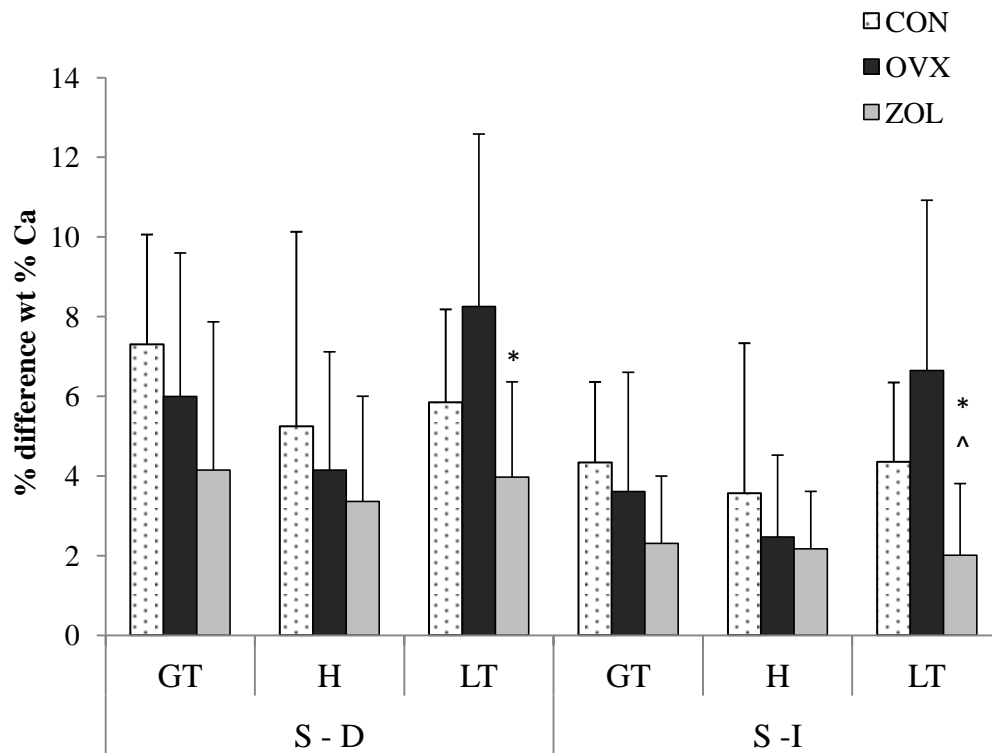
Figure 4.5 % difference of mean calcium concentration between the superficial and deep (S-D) and between the superficial and intermediate (S-I) trabecular regions of CON (n=5), OVX (n=5) and ZOL (n=4) sheep from the 31 month group. *indicates significantly lower than OVX while ^ indicates significant lower than CON.

4.4. Discussion

This study found significantly higher mineralisation and mineral heterogeneity in the proximal femora of the 31 month control group compared to 12 month controls. In contrast, when sheep that were oestrogen deficient for 12 months were compared with those oestrogen deficient for 31 months, it was observed that sheep with more prolonged oestrogen deficiency had lower mineralisation but higher mineral heterogeneity. BMDD parameters (mean, median, peak, FWHM) differed significantly between femora regions in CON and to a greater extent OVX. Furthermore, the OVX group had more mineral variability between anatomical locations compared to control sheep. However in contrast, ZOL treatment of oestrogen deficient sheep significantly lessened the tissue mineral variability, both at a trabecular level and between femoral regions, compared to the OVX group. Together, these indicate that ZOL treatment acts to reverse the higher heterogeneity occurring during oestrogen deficiency and restore mineralisation levels closer to control values.

A limiting factor of this study which could have contributed to the lower mineral content seen at 31 months compared to 12 months post-ovariectomy, could be that the 12 month OVX group were sacrificed in November, whereas the 31 month cohort were sacrificed in June. Sheep are considerably less active in the winter months, therefore remodelling rates would be decreased and bone tissue would likely be older and more mineralised, compared to the summer months. However, in spite of the seasonal differences, significantly higher mineralisation was observed in the 31 month control group compared to the 12 month controls, therefore the reduction found in OVX animals over prolonged oestrogen deficiency (31 months) compared to the 12 month OVX group, cannot be attributed to the seasonal differences at sacrifice. As stated in Chapter 4, another aspect that could affect differences in mineralisation between groups is the automated method of defining regions of interest within trabeculae according to trabecular diameter at analysis and not prior to the increased remodelling characteristic of oestrogen deficiency. However, significantly lower mineralisation was found in all ROI of OVX trabeculae compared to CON, which would only be expected to reduce further if ROI were defined before oestrogen deficiency. Finally, the small sample size of ZOL sheep (n=4) could be a limiting factor of this study. However, in spite of this, with zoledronate treatment of oestrogen deficient sheep, clear, statistically significant alterations in mineral distributions were found, compared to oestrogen deficiency alone.

The finding of higher mineralisation in more aged healthy sheep (31 months CON versus 12 month CON) affirm previous findings in monkey (Grynpas *et al.*, 1993) and human subjects (19-96 years)(Kingsmill and Boyde, 1998). This can be attributed to increased mean tissue age; since newly formed collagen fibrils accumulate mineral over time, older bone will contain more mineral than newly formed bone. The higher heterogeneity with ageing in healthy bone tissue demonstrated in the current study corresponds with studies on healthy human proximal femur (Bloebaum *et al.*, 2004). The reduction in mineralisation demonstrated with prolonged oestrogen deficiency (sheep sacrificed 12 versus 31 months post-ovariectomy) is interesting and cannot be explained by ageing since it follows the opposite pattern to the changes occurring in CON animals. Indeed, after 31 months of oestrogen deficiency, significantly less mineralisation compared to age matched controls was found, whereas the previous study, presented in Chapter 3, found no differences in mean mineralisation between OVX and CON groups after 12 months of oestrogen deficiency (Brennan *et al.*, 2011). These findings therefore reveal the importance of duration in assessing the effects of oestrogen deficiency on bone mineralisation and may explain discrepancies regarding the effect of oestrogen deficiency between previous studies.

The lower mineralisation observed with 31 months of oestrogen deficiency compared to controls is likely due to the reduction in mineralisation in oestrogen deficient animals over time, in combination with increasing mineralisation in CON animals as they age. Increased rates of bone turnover associated with oestrogen deficiency (Wronski *et al.*, 1989) could explain this lower mineralisation. Sites of bone with high turnover will comprise newer packets of bone that have a low mineral content because there is a greater chance of resorption before full mineralisation is achieved. This study concurs with others from literature which evaluated the impact of long term oestrogen depletion compared to controls (Gadeleta *et al.*, 2000; Loveridge *et al.*, 2004). Following two years of ovariectomy, FTIR analysis revealed significant lower mineralisation in trabecular bone of vertetrae from skeletally mature monkeys (Gadeleta *et al.*, 2000). Similarly, a further study on osteoporotic female patients (72-90 years) affirms our findings of reduced mineralisation with long term oestrogen deficiency (Loveridge *et al.*, 2004). The calcium content of sheep that were oestrogen deficient for 31 month reported in this study range from 22-24 wt % Ca, whereas age-matched controls were higher and range from 23-26 wt % Ca. These values are within ranges reported for control (18-28%) and osteoporotic human bone tissue (16-26%) (Roschger *et al.*, 1998; Roschger *et*

al, 2001; Gupta *et al.,* 2005). After 31 months of oestrogen deficiency, the mineral content was significantly lower compared to control age matched control sheep. Several studies have reported that increased mineral content correlates with improved strength and stiffness (Yao *et al.,* 2006; Currey, 1969; Follet *et al.,* 2004). Furthermore, the degree to which the strength and stiffness would be affected by the reductions in mineral content as a consequence of OVX was estimated by first relating mineral content to ash content (calcium occupies 39.9% of stoichiometric HA by weight) (Vajda *et al.,* 1998) and then utilizing published relationships between ash fraction and compressive strength (Hernandez *et al.,* 2001). By these means, it was estimated that even decreases in calcium concentration as low as 2% (wt % Ca) observed with oestrogen deficiency can reduce bone strength by 19% and may therefore contribute to the reduced fracture resistance of oestrogen deficient bone tissue.

ZOL did not differ significantly compared to OVX or CON in terms of mean mineralisation; indeed, this group appeared to be intermediate to both. ZOL treatment was administered for 11 months to sheep that had been oestrogen deficient for 20 months previously; therefore perhaps with longer treatment durations ZOL would continue to increase mineralisation to control levels. Indeed, a study on the effects of Alendronate on osteoporotic women demonstrated significantly increased mean mineralisation after 3 years of treatment but no significance was revealed after only two years (Roschger *et al.,* 2001).

The most striking observation of this study is that ZOL administration to oestrogen deficient sheep reduced calcium heterogeneity that arose during oestrogen deficiency, whereby ZOL reduced the % difference of the mean mineralization between anatomical locations and also between trabecular regions of OVX sheep. These alterations could occur due to the well established reduction in remodelling activity associated with bisphosphonate treatment. In addition, altered mineral heterogeneity may occur as a direct consequence of modulation of mineralisation by cells, independent of remodelling inhibition. Interestingly, recent studies have shown a direct uptake of the bisphosphonate risedronate by osteocytes, which was concentrated within newly embedded osteocytes (Roelofs *et al.,* 2010). Thus, bisphosphonates may have the ability to directly alter osteocyte functions such as secondary mineralisation and thereby contribute to altered mineral heterogeneity. Furthermore, a recent study observed that Zoledronic acid has an anabolic affect on bone (Gamsjaeger *et al.,* 2011). It showed that Zoledronic acid exerts effects on matrix quality, including increased mineralisation and mineral

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maturity/crystallinity, as well as decreased GAG content, independent of decreased bone turnover (Gamsjaeger *et al.,* 2011). The increased homogeneity between skeletal sites found in the current study may indicate that this drug acts to modulate bone cell activity in response to mechanical loading.

The finding of lower mean mineralisation in OVX sheep compared to controls after 31 months was confirmed in all trabecular regions. Higher mineral content found towards the core of trabeculae was expected and is consistent with findings in human iliac bone (Ciarelli *et al.,* 2003). Lower trabecular mineral heterogeneity with bisphosphonate treatment concurs with studies that revealed higher homogeneity of mineral distribution with Alendronate treatment of osteoporotic females (Boivin *et al.,* 2000; Roschger *et al.,* 2001) and minipigs (Roschger *et al.*, 1997). Indeed, ZOL also had lower variability of mineral within trabeculae compared to control sheep, albeit to a lesser degree than when ZOL was compared to the OVX group. However, since ZOL treatment was administered to sheep following a period of osestrogen deficiency, overall mineralization of the ZOL group did not differ compared to CON, as stated previously. Interestingly, since mineralisation is positively correlated to tissue stiffness, the results presented in this chapter correlate with a further study which showed that Zoledronic acid treatment reduced heterogeneity of elastic modulus distribution of oestrogen deficient bone tissue (Yao *et al.,* 2007).

The results of this study may suggest that in addition to the overall material properties of bone tissue, including the strength and stiffness, material property distribution and therefore the stress concentrations within the constituent tissue, may be an important factor governing the fracture resistance of bone. Regions of bone with more heterogeneous mineralisation have been associated with impaired mechanical integrity and increased fracture risk (Ciarelli *et al.*, 2003) and past studies have suggested that variations in the mineral heterogeneity of individual trabeculae can impact on the fracture toughness of the bone (Keaveny and Hayes, 1993). Computational studies have predicted that higher mineral heterogeneity within trabeculae causes increased stiffness (Van der Linden *et al.*, 2001) and that higher trabecular stiffness leads to bone loss and trabecular perforation (Mulvihill *et al.,* 2008). Furthermore, it has been predicted computationally that regions of bone with higher mineral heterogeneity have increased risk of fracture (Hernandez, 2008; Jaasma *et al.*, 2002; Renders *et al.*, 2008). That the overall effect of ZOL on bone tissue is beneficial is clear from the consistent decreases in fracture occurrence detailed in the numerous clinical trials with ZOL

(Black *et al.,* 2007; Lyles *et al.,* 2007; Rizzoli *et al.,* 2009). Therefore, it is postulated that the ability of ZOL to homogenize mineral distribution within trabeculae and between anatomical locations in the compromised trabecular architecture of oestrogen deficient bone is a factor attributing to reduced fracture occurrence with this drug.

In conclusion, in spite of the extensive studies in literature highlighting the importance of trabecular bone for the mechanical integrity and fracture resistance of the hip, there is a lack of understanding regarding the fundamental changes in mineral concentration and its distribution during healthy ageing, oestrogen deficiency, and bisphosphonate treatment. This study is the first to quantify the effects of the duration of oestrogen deficiency on bone tissue mineralisation and it has been highlighted that long term oestrogen deficiency reduces the calcium concentration of ovine bone tissue whereas mineralisation is higher in older healthy sheep. The possible cellular mechanisms responsible for altered tissue mineralisation will be investigated in Chapters 5 and 6. In particular, in Chapter 6 the effects of oestrogen withdrawal for different durations on the mechano-responsiveness of osteoblasts will be investigated. This will determine if altered mechano-sensitivity could be responsible for the decreases in mineralisation with increasing durations of oestrogen deficiency found in the current chapter. Most interestingly, it has been demonstrated that ZOL treatment reversed the effects of OVX on mineral heterogeneity and led to a homogeneous calcium distribution both within trabeculae and between femoral regions. It is proposed that these changes in mineral distribution may contribute to the ability of Zoledronic acid treatment to prevent fracture occurrence during oestrogen deficiency.

5. Mineralisation and Apoptosis of Bone Cells following Oestrogen Withdrawal

5.1. Introduction

Over 70 years ago, oestrogen deficiency during the menopause was established as the primary contributing factor for postmenopausal osteoporotic bone loss, and it was found that oestrogen treatment improved the calcium balance in postmenopausal women (Albright *et al.*, 1941). Since then, it has been shown extensively that bone loss and fractures in postmenopausal women can be prevented by oestrogen treatment (Ettinger *et al.*, 1985; Lindsay *et al.*, 1976; Lufkin *et al.*, 1992). During normal physiology, bone tissue is continually being renewed by the concerted efforts of osteoclast and osteoblast cells (Hill, 1998). A perturbation of the normal bone renewal processes, where excessive resorption, without adequate new bone formation, occurs at the onset of oestrogen deficiency (Balena *et al.,* 1993; Richelson *et al.,* 1984). Such cellular changes lead to increased bone turnover that manifests in osteoporotic bone as reduced bone mass, deterioration of bone's micro-architecture and an overall reduction in bone strength (Compston *et al.*, 1989; Lane *et al.*, 1998; Parfitt, 1987). These structural and biomechanical alterations during oestrogen withdrawal impair the ability of the skeleton to perform fundamental mechanical functions and lead to increased fracture risk (Cummings and Melton, 2002).

As discussed in chapter 3, higher strength, stiffness (McNamara *et al.,* 2006; McNamara *et al.,* 2005), and mineral concentration (Busse *et al.,* 2009) have been observed in bone tissue trabeculae during the initial stages of oestrogen withdrawal compared to control groups. However, other studies have reported no change (Bohic *et al.,* 2000; Ciarelli *et al.,* 2003) or lower mineralisation (Gadeleta *et al.,* 2000) as a consequence of oestrogen deficiency. Higher mineral heterogeneity following oestrogen withdrawal was demonstrated (Chapter 3) and it has also been shown that prolonged oestrogen deficiency causes significantly reduced tissue mineralisation (Chapter 4). Bone mineralisation contributes to bone strength (Ruffoni *et al.,* 2007) and it has been predicted that increased mineral heterogeneity is a governing factor for fracture risk (Mulvihill *et al.,* 2008; Van der Linden *et al.,* 2001), however the cellular mechanisms responsible for such bone tissue level changes during oestrogen withdrawal have not yet been distinguished.

During the normal bone formation process osteoblasts secrete and synthesize the un-mineralised bone matrix, the osteoid, and subsequently mineralise this osteoid matrix (Ducy *et al.*, 2000). They play a seminal role in bone regeneration after removal of aged or damaged tissue, which is essential for the maintenance of the structural integrity and metabolic capacity of the skeleton. Osteocytes reside in lacunae and are derived from osteoblasts that become embedded in their secreted mineralised matrix (Cowin, 2001). They are believed to have the ability to modify the mineral content of their surrounding matrix, by either dissolving matrix and mineral from their perilacunar regions (Baud, 1962; Bélanger, 1963) or by producing new mineralised tissue, which is known as secondary mineralisation (Baud, 1968; Baylink, 1971). Osteoblasts and osteocytes possess receptors for oestrogen (Bellido *et al.,* 1993; Bord *et al.,* 2001; Braidman *et al.,* 2001), hence their function, in particular tissue mineralisation, may be affected when oestrogen production is deficient during postmenopausal osteoporosis (Hoyland *et al.*, 1999).

The beneficial effects of oestrogen (Ettinger *et al.*, 1985; Lindsay *et al.*, 1976; Lufkin *et al.*, 1992) and the detrimental effects of oestrogen withdrawal (Cummings and Melton, 2002) on bone strength and fracture resistance are evident, however, the cellular mechanisms controlling such altered tissue alterations are less clear. It is known that oestrogen causes reduced osteoclastic resorption (Oursler *et al.,* 1991), by both inhibiting osteoclast attachment and by inducing apoptosis, a form of programmed cell death (Hughes *et al.,* 1996; Saintier *et al.*, 2006). Oestrogen deficiency, on the other hand, increases the number of osteoclasts, prevents apoptosis, and enhances their resorbing activities (Jilka *et al.*, 1992; Pacifici, 1996). However, the effects of oestrogen and oestrogen deficiency on osteoblasts and bone formation have yielded conflicting results: studies show that oestrogen decreases osteoblast proliferation (Robinson *et al.,* 1997), whereas oestrogen deficiency increases osteoblast proliferation (Modrowski *et al.*, 1993). Conversely, other studies demonstrate significantly increased cell proliferation with oestrogen treatment (Chen *et al.*, 2002; Rao *et al.*, 2003). Furthermore, previous studies have revealed that expression of alkaline phosphatase (ALP), a glycoprotein found on the surface of osteoblasts, and osteocalcin (OC), a protein secreted by osteoblasts that regulates calcium ion homeostasis, are stimulated, inhibited, or unresponsive to oestrogen (Chen *et al.,* 2002; Harris, 1996; Robinson *et al.,* 1997). It has also been demonstrated *in vitro* that continuous oestrogen treatment enhances (Patlas *et al.*, 2005), or has no effect (Rao *et al.,* 2003) on mineral production by osteoblasts.

Recent evidence suggests that alterations in osteoblast activity may be the first event that occurs following oestrogen withdrawal and that altered osteoclast activity and consequential increased bone resorption may be later events in the osteoporotic bone loss cascade (Jilka *et al.*, 1996; Krum *et al.*, 2008; Manolagas *et al.*, 2002). Based on these studies and the observed increases in strength, stiffness and mineral content in the initial stages following oestrogen withdrawal (Busse *et al.,* 2009; McNamara *et al.,* 2006; McNamara *et al.,* 2005), it is hypothesized that the loss of oestrogen stimulates osteoblastic mineral production activity.

Osteoblasts have an average life span of approximately 3 months, after which they either differentiate into osteocytes or bone lining cells, or die by apoptosis (Parfitt, 1994). Osteocytes also die by apoptosis and it has been demonstrated that an increase in osteocyte apoptosis occurs in hip fracture patients compared to healthy controls (Delgado-Calle *et al.,* 2011), following drug induced oestrogen withdrawal in women (Tomkinson *et al.,* 1997) and also in the ovariectomised mouse model (Kousteni *et al.*, 2001). Furthermore, it has been shown that there are more empty lacunae in hip fracture patients (Delgado-Calle *et al.,* 2011) and in a sheep model of osteoporosis, compared to healthy controls (Zarrinkalam *et al.*, 2011). Such increases in osteocyte cell death may result in an indirect alteration in bone mineralisation as mineral infilling of empty osteocyte lacunae, a phenomenon referred to as micropetrosis has been demonstrated (Frost, 1960). The exact mechanisms involved in micropetrosis are not understood, however it has been postulated that osteocytes may hyper-mineralize their surrounding matrix during death by apoptosis (Boyde, 2003; Frost, 1960; Kingsmill and Boyde, 1998). Although it has been shown that oestrogen treatment prevents drug induced osteoblast and osteocyte apoptosis *in vitro* (Kousteni *et al.,* 2001; Pantschenko *et al.*, 2005), whether apoptosis occurs as a direct consequence of oestrogen withdrawal *in vitro* has not yet been investigated.

The objective of this study is to elucidate how alterations in mineral distribution are initiated during oestrogen depletion. Specifically, two separate hypotheses by which oestrogen deficiency might alter bone tissue mineralisation are tested; (1) oestrogen deficiency directly alters mineral production by bone cells and (2) oestrogen deficiency increases bone cell apoptosis and thereby leads to a micropetrosis type response wherein osteocytes hyper-mineralise their surrounding matrix. Using *in vitro* cell culture, osteoblast - like cells (MC3T3-E1) and osteocyte-like cells (MLO-Y4) were pre-treated with oestrogen for varying durations. Cellular proliferation, alkaline phosphatase

activity, mineralisation and apoptosis were evaluated in these cells when exposed to either oestrogen withdrawal or an oestrogen antagonist, Fulvestrant, which blocks oestrogen receptors.

5.2. Materials and Methods

5.2.1 Cell Culture

Two bone cell lines, MC3T3-E1 osteoblast-like cells and MLO-Y4 osteocyte-like cells, were used in this study. MC3T3-E1 osteoblast-like cells are a good model for osteoblast studies as they express high amounts of alkaline phosphatase, produce mineral and have the ability to differentiate into osteocytes (Sudo *et al.*, 1983). MC3T3-E1 osteoblast-like cells were maintained in α-MEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Murine derived MLO-Y4 cells (a gift from Prof. Lynda Bonewald, San Antonio) possess many similar characteristics with primary osteocytes such as low expression of alkaline phosphatase and production of numerous dendritic processes (Kato *et al.*, 1997). MLO-Y4 cell cultures were maintained in α-MEM supplemented with 2.5% foetal bovine serum (FBS) and 2.5% calf serum (CS), 2 mM L-glutamine, 100 U/ml penicillin/ 100 μg/ml streptomycin in culture flasks coated with 0.15mg/ml collagen (type I from rat tail, C3867 Sigma Aldrich), according to standard methods for maintaining MLO-Y4 cells.

5.2.1.1 Oestrogen Pre-treatment

Estradiol is the primary type of oestrogen produced by the ovaries before the menopause. Therefore, in the present study, *in vitro* administration of 17β-estradiol, a naturally occurring oestrogen derived from cholesterol, was used to simulate normal conditions when circulating oestrogen is available to bone cells. *In vitro* addition of 17β-estradiol at a concentration of $1x10^{-8}$ M was used in the studies outlined below, both as a pre-treatment and an experimental treatment, at a concentration that has been employed previously in studies on the effects of oestrogen on bone cell biology (Chen *et al.*, 2002; Rao *et al.*, 2003). MC3T3-E1 and MLO-Y4 cells were cultured at 37 °C in a humidified 5% CO_2 environment under three separate conditions (A) untreated cells maintained in aforementioned appropriate media without addition of estradiol, (B) cells pre-treated for 2 days with 17β-estradiol ($1x10^{-8}$ M) and (C) cells pre-treated for 14 days with 17β-estradiol ($1x10^{-8}$ M). Cells were passaged as required and media was replenished every 3-4 days.

5.2.1.2 Oestrogen Deficiency Experiments

The effects of oestrogen deficiency on MC3T3-E1 and MLO-Y4 cells were evaluated by either (1) withholding oestrogen from oestrogen pre-treated cells (oestrogen withdrawal) or (2) by blocking oestrogen receptors with Fulvestrant (ICI 182,780). Fulvestrant is an oestrogen receptor (ER) antagonist used to treat postmenopausal women who are suffering from oestrogen receptor-positive metastatic breast cancer. Fulvestrant competes with endogenous oestrogen for binding to the oestrogen receptor (Curran and Wiseman, 2001) with a binding affinity that is 89% that of 17-β estradiol (Wakeling and Bowler, 1987), and thereby inhibits oestrogen signalling through the oestrogen receptor (Kansra *et al.*, 2005; Wakeling, 1993; Wakeling, 2000). This compound has been used previously to mimic oestrogen deficiency in human osteoblasts *in vitro* (Foo *et al.*, 2007).

MC3T3-E1 were plated in triplicate at a density of 3×10^4 cells/ml in 24 well plates in osteogenic media supplemented with 50μg/ml ascorbic acid, 10mM/L β-glycerophosphate and 10nM/L dexomethasome. For MLO-Y4 cultures, 24 well plates were coated with 0.15mg/ml collagen (type I from rat tail, C3867 Sigma Aldrich), dissolved in 0.02M acetic acid. Cells were plated in triplicate at a density of 1.5 x 10^4 cells/ml, in collagen coated tissue culture plates with appropriate media. Both MC3T3-E1 and MLO-Y4 cells were subsequently cultured under separate experimental conditions; **(1) Sustained oestrogen exposure (E2):** cells were cultured in their appropriate media (osteoblast osteogenic media or osteocyte appropriate media respectively) with the addition of 17β-estradiol ($1x10^{-8}$ M), **(2) Reduced oestrogen exposure (E1):** cells were cultured in their appropriate media with the addition of $1x10^{-10}$ M 17β-estradiol, this concentration of oestrogen has also been used extensively to evaluate the effects of oestrogen (Rao et al., 2003; Robinson *et al.*, 1997), as it is within normal physiological ranges for normal serum levels of females (Stricker *et al.*, 2006), **(3) Oestrogen withdrawal (E-):** cells were cultured in their appropriate media without the addition of oestrogen, **(4) Oestrogen inhibition (F):** cells were cultured in their appropriate media with the addition of $1x10^{-7}$ M Fulvestrant, **(5) Oestrogen inhibition with reduced oestrogen exposure (E1+F):** cells were cultured in their appropriate media with the addition of $1x10^{-10}$ M 17β-estradiol and $1x10^{-7}$ M Fulvestrant, **(6) Oestrogen inhibition with sustained oestrogen exposure (E2+F):** cells were cultured in their appropriate media with the addition of $1x10^{-8}$ M 17β-estradiol and $1x10^{-7}$ M Fulvestrant. This concentration has been used previously to block the effects of

oestrogen on bone cells (Robinson et al., 1997). All cells were maintained at 37 °C in a humidified 5% CO_2 environment and were cultured for 1, 4, 7 and 14 days. Mediums were replenished every 3-4 days. Experimental groups are presented in Table 5.1.

5.2.1.3 Osteocyte Apoptosis Experiments

To evaluate the effects of oestrogen deficiency on cell death and more specifically osteocyte apoptosis, MLO-Y4 cells that were pre-treated with 17β-estradiol for 14 days were plated in 96 well plates at a density of 4 x 10^4 cells/ml on collagen (type I, rat tail) coated tissue culture plates in appropriate media. Cells were subsequently cultured under the following conditions for 24 hours; **(1) Sustained oestrogen exposure (E2):** cells cultured in their appropriate media with the addition of $1x10^{-8}$ M 17β-estradiol, **(2) Reduced oestrogen exposure (E1):** cells were cultured in osteocyte appropriate media with the addition of $1x10^{-10}$ M 17β-estradiol **(3) Oestrogen withdrawal (E-):** Osteocyte appropriate media without the addition of oestrogen, **(4) Oestrogen inhibition (F):** cells cultured in their appropriate media with the addition of $1x10^{-7}$ M Fulvestrant, **(5) Oestrogen inhibition with sustained oestrogen exposure (E2+F):** cells cultured in their appropriate media with the addition of $1x10^{-8}$ M 17β-estradiol and $1x10^{-7}$ M fulvestrant, see Table 5.1. The **E1+F** group was eliminated in order to facilitate reduction of test groups, required due to constraints imposed on sample numbers by the apoptosis protocol. It was chosen instead of **E2+F** since it matched the pre-treated oestrogen concentration.

After 24 hours of culture, each group was divided in two and half of the cell cultures were treated with Etoposide, to promote apoptosis. This drug was employed to determine if oestrogen deficient cells are more susceptible to induced apoptosis compared with cells exposed to oestrogen. Etoposide is a potent and widely used chemotherapeutic agent, which acts by inhibiting the enzyme topoisomerase II and aids in re-ligation of the DNA strands. It therefore promotes DNA strand breakage and errors in DNA synthesis and consequently induces apoptosis. Etoposide was prepared as a stock solution (1mM) in dimethylsulphoxide (DMSO). Final concentration when added to culture media was 50μM. All compounds were purchased from Sigma Aldrich, St. Louis, MO, USA unless otherwise stated.

Table 5.1 Pre-treatments, experimental conditions and assays performed in this study

	Cell lines	Pre-treatment	Experimental conditions		Experimental Assays
Study 1: Mineralisation	MC3T3-E1 osteoblast	14 days oestrogen	**E-** (osteogenic media)		Cells assayed at 1, 4, 7 and 14 days for:
	MLO-Y4 osteocyte	2 days oestrogen	**E1** ($1x10^{-10}$ M 17β-Estradiol)		Proliferation (DNA hoechst)
			E2 ($1x10^{-8}$ M 17β-Estradiol)		ALP activity (ρNPP)
			F ($1x10^{-7}$ M Fulvestrant)		Mineralisation (Alizarin red)
		Untreated	**E1+F**		
			E2+F		
Study 2: Osteocyte Apoptosis	MLO-Y4 osteocyte	14 days oestrogen	**E-** (osteogenic media)	With Etoposide	Cells assayed after 4, 12, 24, 30, 48 and 72 hours for**:**
			E1 ($1x10^{-10}$ M 17β-Estradiol)		
			E2 ($1x10^{-8}$ M 17β-Estradiol)		
			F ($1x10^{-7}$ M Fulvestrant)	Without Etoposide	Apoptosis (Caspase 3/7)
			E2+F		Cytotoxicity

5.2.2 Cellular Assays

5.2.2.1 Hoechst 33258 for DNA Quantification

In order to assess the effects of oestrogen deficiency on cellular proliferation, measurements of DNA content were performed after 1, 4, 7 and 14 days of culture. Cells were washed in sterile PBS and lysed in their plates in 500 µL molecular grade water by three freeze/ thaw cycles (-80˚C, 25˚C). The lysate was assayed for DNA content using a fluorescent dye Hoechst 33258. A standard curve was generated using 0 to 500ng DNA of calf thymus DNA with 0.1 µg/mL of Hoechst dye (Sigma Aldrich), see Appendix 3. Fluorescent assay buffer (100 mM Tris HCl, pH 7.4, with 10 mM EDTA and 2 M NaCl) was added to each well of a 96-well plate at 50 µL/well. Standards (50µL) and samples were added to each well in triplicate. Finally 100µL Hoechst 33258 dye solution (0.1µg/mL) was added to each well. The plate was incubated for 10 minutes in the dark at room temperature, and then fluorescence (Ex360 nm / Em 460 nm) was read on a Microplate Reader (Wallac 1420 VICTOR 2 TM, Perkin-Elmer, Boston,MA,USA).

5.2.2.2 Alkaline Phosphatase Expression

Both the nucleus and cytoplasm of osteoblasts contain the glycoprotein alkaline phosphatase (ALP), which is expressed by active osteoblasts and can therefore be employed as a marker for osteoblast activity. ALP facilitates mineralisation by producing vesicles that act as sites for nucleation (Cowin, 2001) and by hydrolyzing inhibitors of mineral deposition (Favus, 2006). ALP activity was quantified using an ALP Colorimetric Assay Kit (Abcam). ALP enzyme converts the *p*-nitrophenyl phosphate (*p*NPP) substrate to an equal amount of coloured *p*-Nitrophenol (*p*NP). Colorimetric absorbance was measured at 405 nm using a micro-plate reader. Relative colorimetric readings were converted to ALP activity by creating a standard ALP curve, as depicted in Appendix 3.

5.2.2.3 Alizarin Red S Staining for Mineralisation

Mineralisation was quantified after 7 and 14 days of culture. Cells were washed with sterile PBS solution and fixed with 10% formalin for 10 minutes. Cells were thoroughly washed with distilled water and stained with 40mM Alizarin Red-S in deionized water (pH 4.2) for 15 minutes at room temperature on an orbital rotator. Alizarin red stain binds to mineral nodules present. Cultures were rinsed three times with distilled water to remove any unbound stain. Cells were subsequently de-stained using 100nM cethylpyridium chloride in deionized water for 20 minutes on an orbital rotator to

solubilise bound dye. The absorbance of the extracted stain was measured at a wavelength of 550 nm. Relative photometry units were converted to µmole/well of alizarin red by creating a standard curve, as illustrated in Appendix 3. Alizarin red measurements were expressed as µmole/well of calcium since one mole of alizarin red bind to two moles of calcium (Norgaard *et al.*, 2006). Mineral production was subsequently normalized to quantity of DNA (µmole Ca/ng DNA).

5.2.2.4 Apoptosis Quantification

Cultures were assessed for apoptosis after 4, 12, 24, 30 and 48 and 72 hours of culture, with/without Etoposide administration using the ApoTox- Glo Triplex Assay (Promega). Apoptosis was quantified by measuring caspase 3/7 activity. These members of the cysteine aspartic acid-specific protease (Caspase) family are early markers of apoptosis as they play important roles as effectors of apoptosis of mammalian cells (Nicholson and Thornberry, 1997). 50µL of the caspase 3/7 reagent was added to each test well and briefly mixed by orbital shaking for 30 seconds. Wells were incubated for 60 minutes at room temperature. The reagent causes cell lysis and following caspase cleavage, a substrate for luciferase (aminoleciferin) is released which causes the production of light. Luminescence, which is directly proportional to apoptosis induction, was measured on a micro-plate reader.

5.2.3   Statistical Analysis

Experiments were conducted in triplicate and repeated giving n=6 per group. Data were expressed as a mean ± standard deviation. Statistical differences between groups were determined using an ANOVA crossed factor model, defined using the general linear model ANOVA function. Comparison between treatments and pre-treatments were made using the Tukeys-Kramer multiple comparison test (Minitab 16). Statistically significance was defined as $p \leq 0.05$.

5.3.   Results:

5.3.1   Does Oestrogen Deficiency alter Osteoblast DNA Content *in vitro*?

5.3.1.1 Day 1 and Day 4 DNA Results

No significant difference in DNA content was found between treatment groups and controls (**E2**), in previously untreated cells, or those pre-treated for 14 days, either at day 1 or day 4, as depicted in Figure 5.1. In cultures pre-treated for two days however, significantly higher DNA content was found with reduced oestrogen deficiency (**E1**) and

oestrogen withdrawal (**E-**), compared to continued oestrogen treatment (**E2**), at day 4 (1371 ± 257 vs. 769 ± 130, p<0.01) and (1385 ± 359 vs. 769 ± 130, p<0.01) respectively, as depicted in Figure 5.1.

Effect of Oestrogen Pre-treatment on DNA Content: At day 4, in the **E-**, **F**, and **E2+F** groups, DNA content was significantly lower in cultures that were pre-treated for 14 days compared with untreated cells (590 ± 361 vs. 1375 ± 242, p<0.01), (841 ± 363 vs. 1503 ± 574, p<0.01), and (643 ± 389 vs. 1405 ± 690, p<0.01) respectively. Also at day 4, in the **E2+F** group, DNA content was significantly lower in cultures pre-treated for two days compared to untreated cultures (583 ± 155 vs. 1405 ± 690, p<0.04). In the **E-** group, cells pre-treated for 14 days had significantly lower DNA content compared to cells pre-treated for two days (590 ± 361 vs. 1385 ± 359, p<0.03).
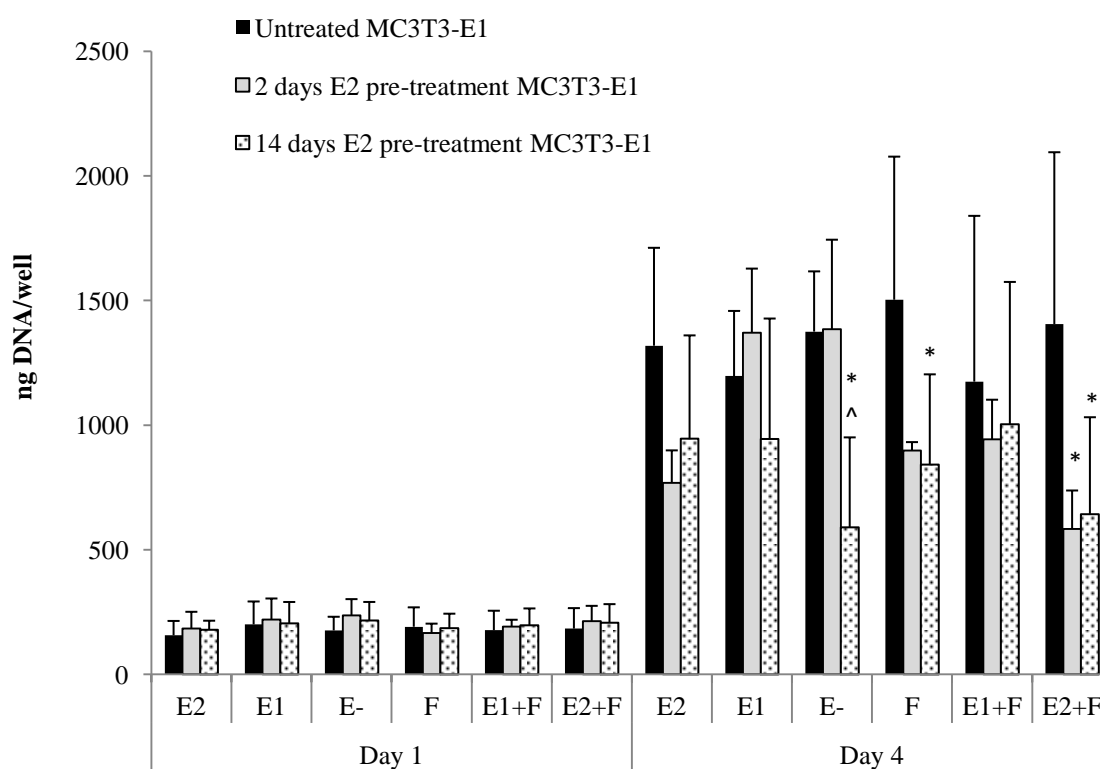
Figure 5.1 Comparison of DNA, quantified using Hoechst 33258, between continued oestrogen treatment (**E2**), reduced oestrogen concentration (**E1**) and oestrogen deficiency by withdrawal of oestrogen (**E**-), or by blocking oestrogen receptors (**F**),(**E1+F**) and (**E2+F**) at day 1 and day 4, n=6 per group, at each time point. Groups that share a letter are significantly different to each other, p values for a and b<0.01. ∗ indicates significantly different to previously untreated cultures, while ^ indicates significantly different to two days E2 pre-treatment, within the same treatments and time points.

5.3.1.2 Day 7 and Day 14 DNA Results

When DNA content was compared between treatment groups and controls (**E2**) at day 7 and day 14, no significant difference was found, in previously untreated cells, or those with either 2 or 14 days of oestrogen pre-treatment, as indicated by Figure 5.2.

Effect of Oestrogen Pre-treatment on DNA Content: At day 7, cultures pre-treated with oestrogen for two days had significantly higher DNA content compared to untreated cultures in the **E1** and **E1+F** groups (4485 ± 1067 vs. 1408 ± 161, p<0.05) and (4815 ± 541 vs. 1453 ± 393, p<0.04) respectively, as illustrated in Figure 5.2. Also at day 7, in the **E-** group, cells pre-treated for 14 days had significantly lower DNA content, compared to cells pre-treated for 2 days (1288 ± 679 vs. 4644 ± 698, p<0.05). No significant effect of pre-treatment on DNA content was found at day 14.

Figure 5.2 Comparison of DNA, quantified using Hoechst 33258, between continued oestrogen treatment (**E2**), reduced oestrogen concentration (**E1**) and oestrogen deficiency by withdrawal of oestrogen (**E-**), or by blocking oestrogen receptors (**F**),(**E1+F**) and (**E2+F**) at day 7 and day 14. n=6 per group, at each time point. ∗ indicates significantly different to previously untreated cultures, while ^ indicates significantly different to two days E2 pre-treatment, within the same treatments and time points.

5.3.2 Does Oestrogen Deficiency alter Osteoblast ALP Expression *in vitro*?

5.3.2.1 Day 1 and Day 4 ALP Results

No difference in ALP production, between treatment groups (**E1, E-, F, E1+F and E2+F**) compared to oestrogen treatment controls (**E2**)**,** were observed at day 1 or day 4 in either previously untreated cultures or cultures pre-treated with oestrogen for two days. However, in cultures that were pre-treated for 14 days, significantly higher ALP production was observed when oestrogen was withdrawn from cells (**E-**), compared to continued oestrogen treatment (**E2**) at day 4 (0.82 ± 0.26 vs. 0.39 ± 0.19, $p<0.01$), as indicated in Figure 5.3.

Effect of Oestrogen Pre-treatment on ALP Production by Osteoblasts: At day 1, in all treatment groups (**E2, E1, E-, F, E1+F and E2+F**), 14 days pre-treatment and 2 days pre-treatment were significantly higher compared to previously untreated cultures (p values <0.01). Furthermore, in all treatment groups, cultures pre-treated for 2 days produced significantly more ALP compared to cultures pre-treated for 14 days (p values <0.01). By day 4, cultures pre-treated with oestrogen for 14 days were significantly higher compared to untreated cultures in all groups (p values<0.05), except in **E1**. In addition, culture pre-treated for 14 days were significantly higher compared to cultures pre-treated for only 2 days in all treatment groups (p values <0.04).
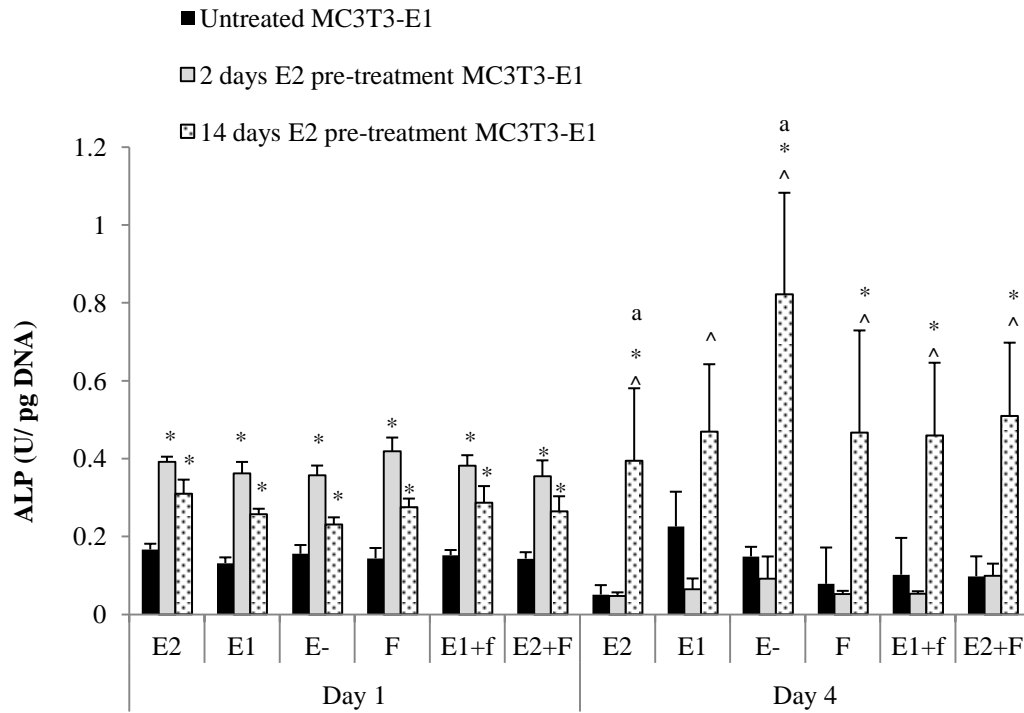
Figure 5.3 Comparison of ALP expression by osteoblasts between continued oestrogen treatment (**E2**), reduced oestrogen concentration (**E1**) and oestrogen deficiency by withdrawal of oestrogen (**E-**), or by blocking oestrogen receptors (**F**),(**E1+F**) and (**E2+F**) at day 1 and day 4. n=6 per group, at each time point. Groups that share a letter (a) are significantly different to each other, p<0.01) * indicates significantly different to previously untreated cultures, while ^ indicates significantly different to two days **E2** pre-treatment, within the same treatments and time points.

5.3.2.2 Day 7 and Day 14 ALP Results

In untreated cells, significantly lower ALP activity was found in the oestrogen blocker group (**E2+F**) compared to the oestrogen treatment controls (**E2**) at day 7 (0.06 ± 0.01 vs. 0.60 ± 0.18, p<0.04), as indicated in Figure 5.4. At day 14, significantly higher ALP production was found in the **F** group compared to controls (**E2**) (1.21 ± 0.07 vs. 0.56 ± 0.12, p<0.03).

In cultures pre-treated for two days, ALP production in the oestrogen withdrawal group (**E-**), was significantly higher compared to continued oestrogen treatment (**E-**) at day 7 (0.59 ± 0.13 vs. 0.13 ± 0.06, p<0.04). In cultures pre-treated for 14 days, no difference between any group and controls was observed at day 7, but at day 14, oestrogen

withdrawal from cells (**E-**) resulted in significantly higher ALP activity, compared to continued oestrogen treatment (**E2**) (2.92 ± 0.70 vs. 1.12 ± 0.38, p<0.01).

Effect of Oestrogen Pre-treatment on ALP Production by Osteoblasts: At day 7, there is no significant effect of pre-treatment on ALP activity. At day 14, in (**E2, E-, F, E1**+**F** and **E2**+**F**) treatment groups, ALP activity was significantly higher in cultures pre-treated for 14 days, compared to those with 2 days of pre-treatment (p values <0.01). Furthermore, in the **E-** and **E2**+**F** groups, ALP activity was significantly higher in cells pre-treated for 14 days, compared with previously untreated cells (p values <0.04). Finally, in the **F** and **E2**+**F** groups, ALP was significantly lower in cells pre-treated for two days compared with untreated cells (p<0.01).
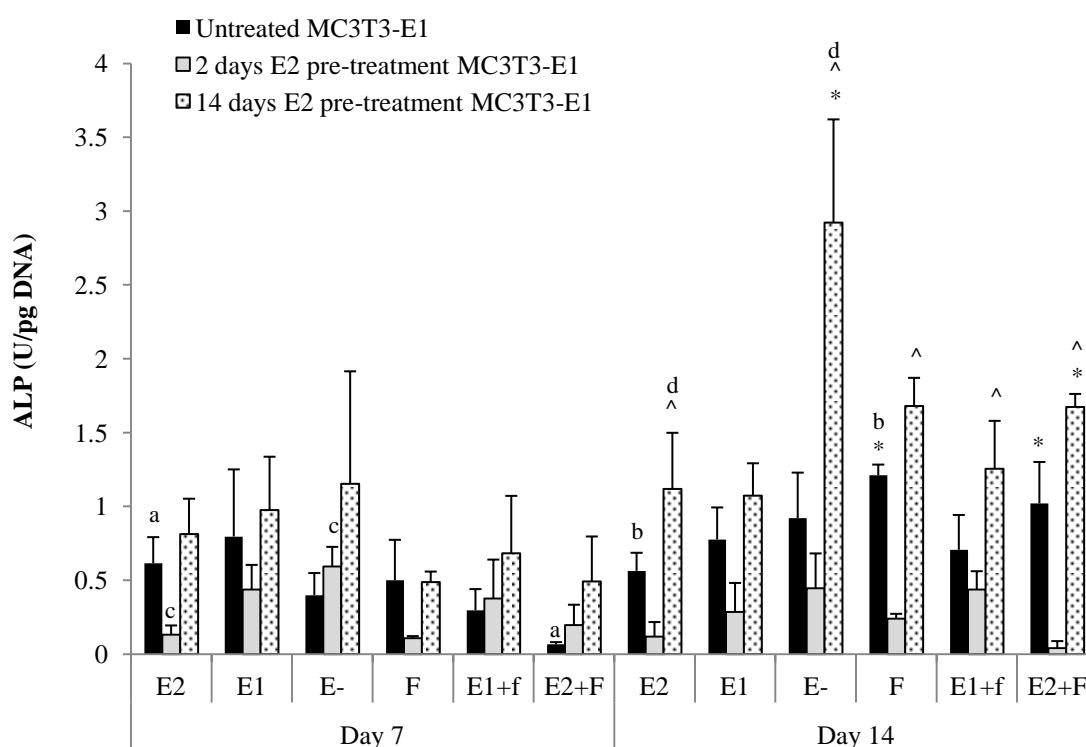
Figure 5.4 Comparison of ALP expression by osteoblasts between continued oestrogen treatment (**E2**), reduced oestrogen concentration (**E1**) and oestrogen deficiency by withdrawal of oestrogen (**E-**), or by blocking oestrogen receptors (**F**),(**E1**+**F**) and (**E2**+**F**) at day 7 and day 14. n=6 per group, at each time point. Groups sharing the same letters are significantly different to each other, a<0.04, b<0.03, c<0.04, d<0.01.* indicates significantly different to previously untreated cultures, while ^ indicates significantly different to two days **E2** pre-treatment, within the same treatments and time points

5.3.3 Does Oestrogen Deficiency alter Osteoblast Mineralisation *in vitro*?

Osteoblast mineralisation results at day 7 and day 14 from cultures without oestrogen pre-treatment, with 2 days pre-treatment and with 14 days pre-treatment are presented in detail in Figure 5.5. When treatment groups were compared, in previously untreated cultures and those pre-treated for two days with oestrogen, no significant differences compared to continued oestrogen treatment (**E2**) were found. In cultures that were pre-treated for 14 days with oestrogen, withdrawal of oestrogen (**E-**) resulted in significantly higher mineral production compared to continued oestrogen treatment (**E2**) at day 14 (459.12 ± 170.56 vs. 247.69 ± 164.28, p<0.01). Conversely, blocking oestrogen receptors with (**F)** and (**E2+F)** treatments, caused significantly lower mineralisation, compared with continued oestrogen (**E2**) at day 14 (52.40 ± 39.71 vs. 247.69 ± 164.28, p<0.03) and (64.40 ± 32.63 vs. 247.69 ± 164.28, p<0.05) respectively.

Effect of Oestrogen Pre-treatment on Mineral Production by Osteoblasts: The effect of pre-treatment on subsequent mineral production by MC3T3-E1 cells is also presented in Figure 5.5. At day 7, in the oestrogen withdrawal group (**E-**), cultures pre-treatment for 14 days had significantly higher mineral production compared to untreated cultures and those with 2 days pre-treatment (109.95 ± 67.83 vs. 22.89 ± 13.88, p<0.02) and (109.95 ± 67.83 vs. 17.08 ± 5.40, p<0.03) respectively. In the oestrogen blocker group (**E2+F**), cultures pre-treatment for 14 days also had higher mineralization compared to untreated cultures (86.58 ± 62.44 vs. 9.52 ± 1.22, p<0.05).

At day 14, in the continued oestrogen group (**E2**), reduced oestrogen group (**E1**) and oestrogen withdrawal group (**E-**), pre-treatment for 14 days caused higher mineral production compared to untreated cultures (247.69 ± 164.28 vs. 23.96 ± 9.57 , p<0.04), (269.69 ± 172.25 vs. 36.26 ± 11.36, p<0.04) and (459.12 ± 170.56 vs. 25.84 ± 8.34, p<0.01) respectively. Furthermore, in the oestrogen withdrawal group (**E-**), cultures pre-treated for 14 days also had higher mineralization compared to 2 days of oestrogen pre-treatment (459.12 ± 170.56 vs. 94.31 ± 71.89, p<0.01). Interestingly, blocking oestrogen receptors with fulvestrant (**F, E1+F, and E2+F**) abrogated the enhanced mineral production due to oestrogen pre-treatment, as no significant difference was found in any of these groups when comparing pre-treatments.
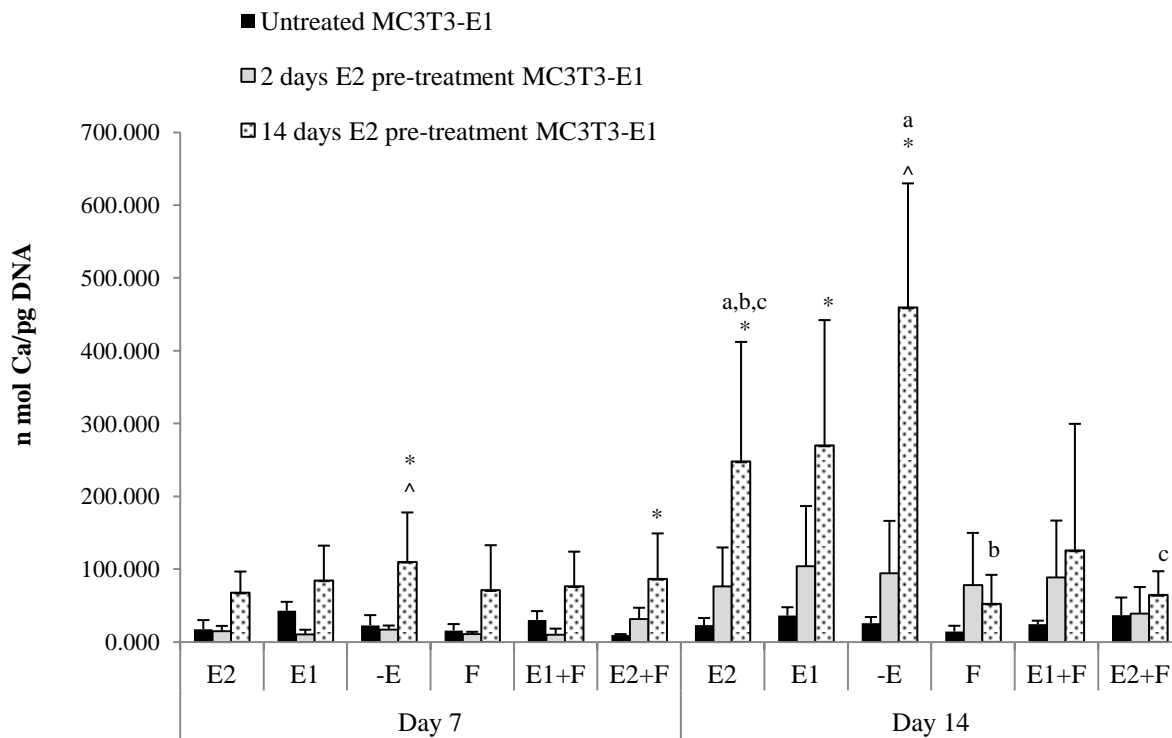
Figure 5.5 Mineral production by osteoblasts, after 7 and 14 days, either without prior oestrogen pre-treatment, or with 2, or 14 days of oestrogen pre-treatment. Continued oestrogen treatment control (**E2**) is compared with lowering oestrogen concentration (**E1**), oestrogen withdrawal (**E-**), and blocking oestrogen receptors (**F, E1+F, E2+F**). n=6 per group, at each time point. Groups sharing the same letters are significantly different to each other, a<0.01, b<0.03, c<0.05. ∗ indicates significantly different to previously untreated cultures, while ∧ signifies significantly different to cells with two days of pre-treatment, of the same subsequent treatment group and time point.

5.3.4 Does Oestrogen Deficiency alter Osteocyte DNA Content *in vitro*?

5.3.4.1 Day 1 and Day 4 DNA Results

In cells that were previously untreated, or those with two days of oestrogen pre-treatment, no significant difference was found between treatment groups and oestrogen treated controls (**E2**) at either day 1 or day 4, as depicted in Figure 5.6. In cells that were pre-treated for 14 days with oestrogen, significantly less DNA content was found in the oestrogen blocker group (**E2+F**) compared with controls (**E2**), at day 4 (819.60 ± 380.71 vs. 1315.25 ± 53.64, p<0.01).

Effect of Oestrogen Pre-treatment on DNA Content by Osteocytes: No significant effect of pre-treatment on DNA content was found in any of the subsequent treatment groups at day 1, or day 4.
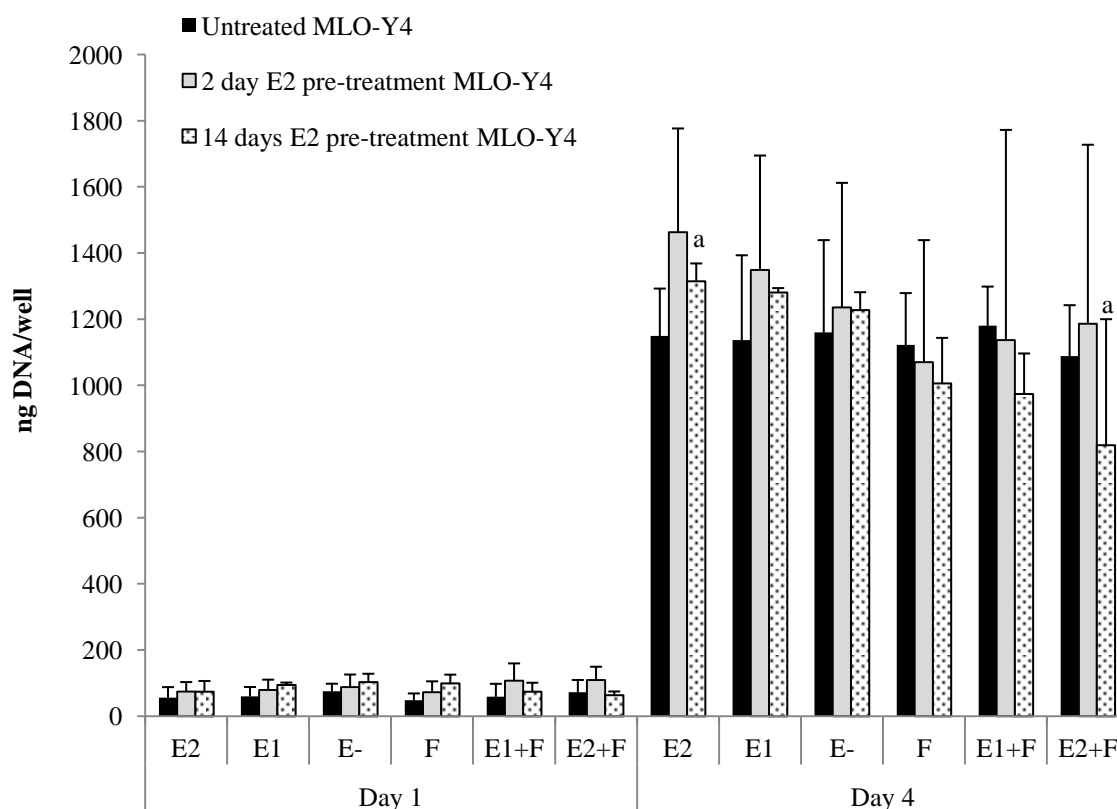
Figure 5.6 Comparison of DNA content by osteocytes, quantified using Hoechst 33258, between continued oestrogen treatment (**E2**), reduced oestrogen concentration (**E1**) and oestrogen deficiency by withdrawal of oestrogen (E-), or by blocking oestrogen receptors (**F**),(**E1+F**) and (**E2+F**) at day 1 and day 4. n=6 per group, at each time point. Groups that share a letter (a) are significantly different to each other, $p<0.01$.

5.3.4.2 Day 7 and Day 14 DNA Results

When treatment groups were compared with oestrogen treated controls (**E2**), no significant difference was found in either previously untreated cells, or those with 2 or 14 days pre-treatment.

Effect of Oestrogen Pre-treatment on DNA Content by Osteocytes: In all treatment groups, significantly less DNA content was found in cells that were pre-treated for 14 days, compared with those pre-treated for two days (p values <0.05) at day 7, as indicated in Figure 5.7. Furthermore, in (**E2, E1, E-, F, E2+F**) groups, significantly

more DNA content was found in cells pre-treated with oestrogen for two days, compared with previously untreated cells (p values <0.01). At day 14, in the **E2** control group, cells with two days pre-treatment had significantly more DNA content than previously untreated cells (p<0.01).
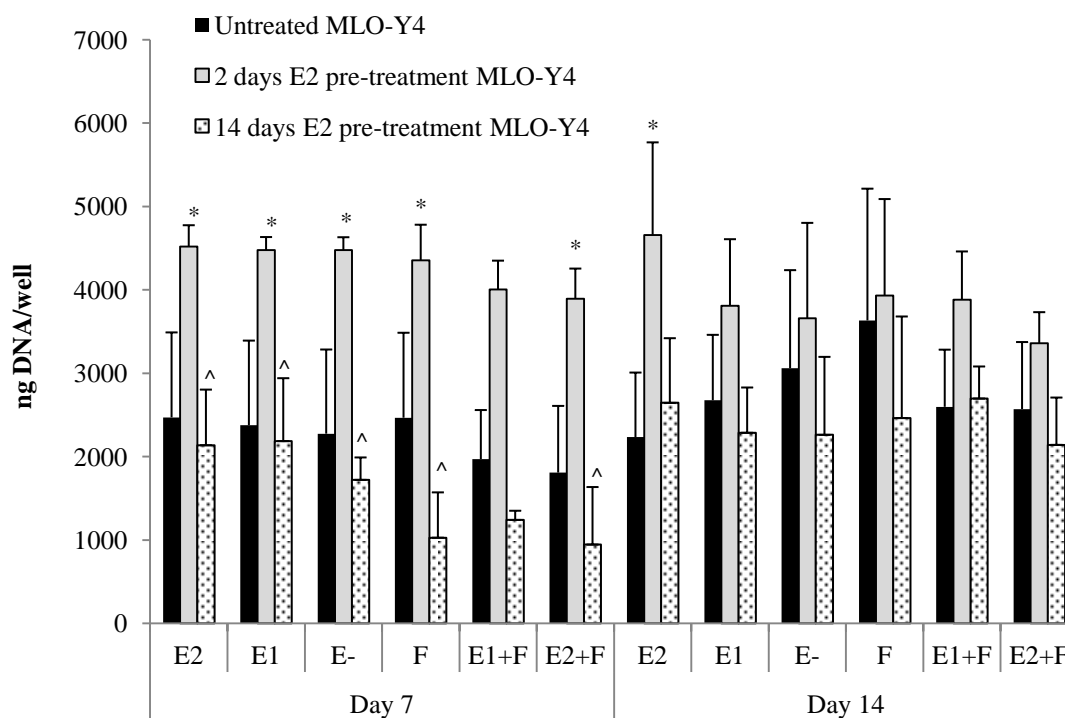
Figure 5.7 Comparison of DNA content in osteocyte cultures, quantified using Hoechst 33258, between continued oestrogen treatment (**E2**), reduced oestrogen concentration (**E1**) and oestrogen deficiency by withdrawal of oestrogen (**E-**), or by blocking oestrogen receptors (**F**),(**E1+F**) and (**E2+F**) at day 7 and day14. n=6 per group, at each time point. * indicates significantly different to previously untreated cultures, while ^ indicates significantly different to two days E2 pre-treatment, within the same treatments and time points.

5.3.5   Does Oestrogen Deficiency alter Osteocyte ALP Expression *in vitro*?

5.3.5.1 Day 1 and Day 4 ALP Results

In untreated cultures, ALP expression was significantly lower in the oestrogen withdrawal (**E-**) group compared to cells treated with oestrogen (**E2**) at day 1 (0.033 ± 0.003 vs. 0.046 ± 0.003, p<0.01), as depicted in Figure 5.8. Conversely, cells treated with the oestrogen blocker (**F**) showed significantly higher ALP expression compared to

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controls (**E2**) (0.06 ± 0.01 vs. 0.05 ± 0.01, p<0.01). Similar to day 1, the oestrogen withdrawal (**E-**) group had significantly less ALP production compared to oestrogen treatment (**E2**) (0.04 ± 0.01 vs. 0.04 ± 0.01, p<0.04).

In cultures that received two days of oestrogen pre-treatment, by day 4, cells treated with the oestrogen blocker (**F**) and (**E1**+**F**) had significantly more ALP production compared to continued oestrogen treatment (**E2**) (0.05 ± 0.01 vs. 0.04 ± 0.01, p<0.01) and (0.05 ± 0.01 vs. 0.04 ± 0.01 p<0.04) respectively. In cultures that were pre-treated for 14 days with oestrogen, by day 4, cultures treated with the oestrogen blocker (**E2+F**) showed significantly higher ALP production compared to continued oestrogen treatment (**E2**) (0.06 ± 0.01 vs. 0.04 ± 0.01, p<0.01).

Effect of Oestrogen Pre-treatment on ALP Production by Osteocytes: At day 1, in the continued oestrogen group (**E2**), reduced oestrogen group (**E1**), oestrogen deficiency group (**E-**) and the oestrogen blocker groups (**F**) and (**E1+F**), cells pre-treated for 14 days produced significantly less ALP (p values <0.01) compared to previously untreated cultures. In the **E1**, **E-**, **F**, **E1+F** and **E2+F** groups, cultures pre-treated with oestrogen for two days produced significantly less ALP at day 1 compared to untreated cultures (p values <0.01). In the **E1+F** and **E2+F** groups, 14 days pre-treatment was significantly higher than 2 days pre-treatment (p<0.01) and (p<0.01) respectively. At day 4, in the **E2+F** group, ALP production was significantly higher cultures that were pre-treated for 14 days compared with both, cells pre-treated for 2 days and untreated cells.
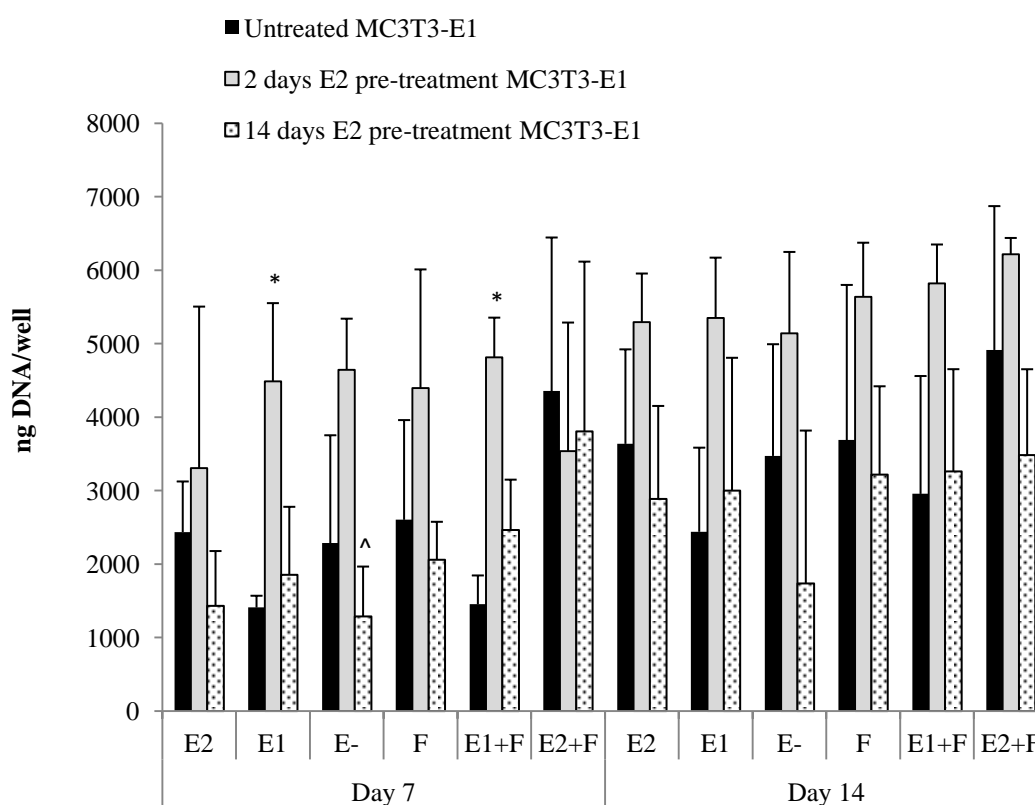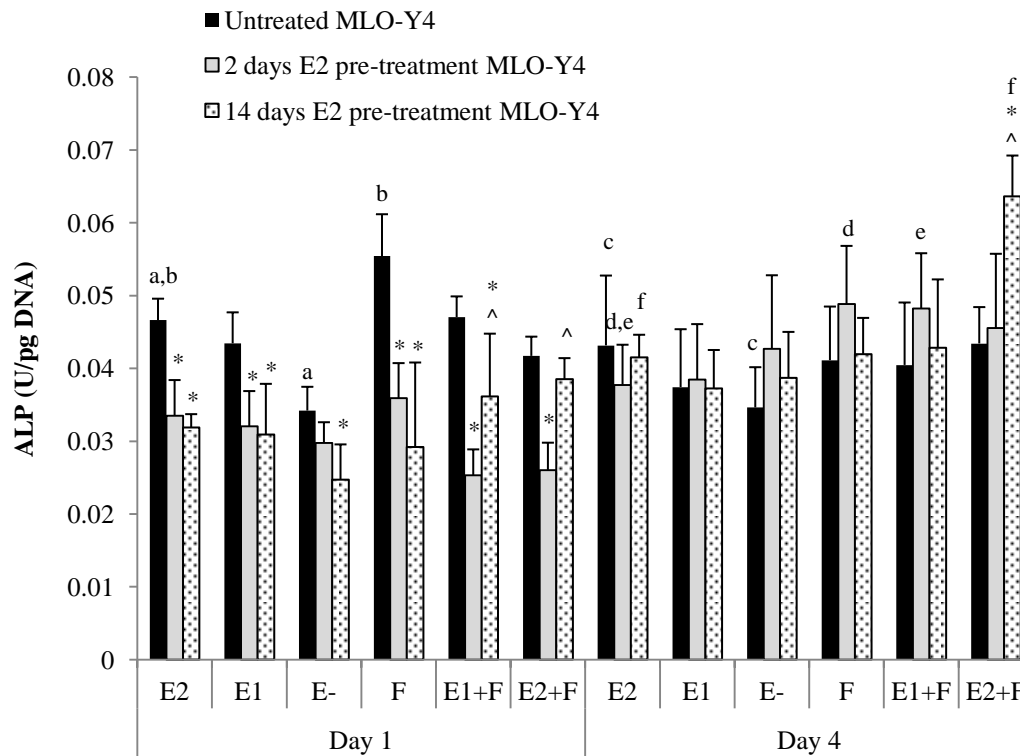
Figure 5.8 Comparison of ALP expression by osteocytes, between continued oestrogen treatment (**E2**), reduced oestrogen concentration (**E1**) and oestrogen deficiency by withdrawal of oestrogen (**E-**), or by blocking oestrogen receptors (**F**),(**E1+F**) and (**E2+F**) at day 1 and day 4. n=6 per group, at each time point. Groups that share a letter are significantly different to each other, p values are; a<0.01, b<0.01, c<0.04, d<0.01, e<0.04, f<0.01* indicates significantly different to previously untreated cultures, while ^ indicates significantly different to two days **E2** pre-treatment, within the same treatments and time points.

5.3.5.2 Day 7 and Day 14 ALP Results

In untreated cultures, no difference between any groups compared to controls (**E2**) was observed at day 7. However, at day 14, ALP production was significantly lower in the oestrogen blocker groups (**F**) and (**E2+F**), compared to controls (**E2**) (0.04 ± 0.01 vs. 0.07 ± 0.01, p<0.01) and (0.04 ± 0.03 vs. 0.07 ± 0.01, p<0.05), as depicted in Figure 5.9. In cultures pre-treated for two days with oestrogen, no significant difference was found between groups compared to controls at either day 7, or day 14. In cultures pre-treated for 14 days, significantly more ALP production was observed in oestrogen blocker groups (**F**) and (**E2+F**), compared to continued oestrogen treatment (**E2**), at day 7 (0.14 ± 0.01 vs. 0.07 ± 0.02, p<0.01) and (0.14 ± 0.01 vs. 0.07 ± 0.02, p<0.01) respectively. No difference compared to controls was found at day 14.

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Effect of oestrogen pre-treatment on ALP production by osteocytes: At day 7, in all treatment groups, (**E2, E1, E-, F, E1+F and E2+F**), ALP was higher in cultures that were pre-treated for 14 days compared to both untreated cultures (p values <0.01) and those pre-treated for only 2 days (p<0.01). Similarly at day 14, cultures pre-treated with oestrogen significantly increased ALP production of all subsequent treatment groups (**E2, E1, E-, F, E1+F, E2+F**), compared to both untreated (p<0.01) and 2 days pre-treated (p<0.01) groups.
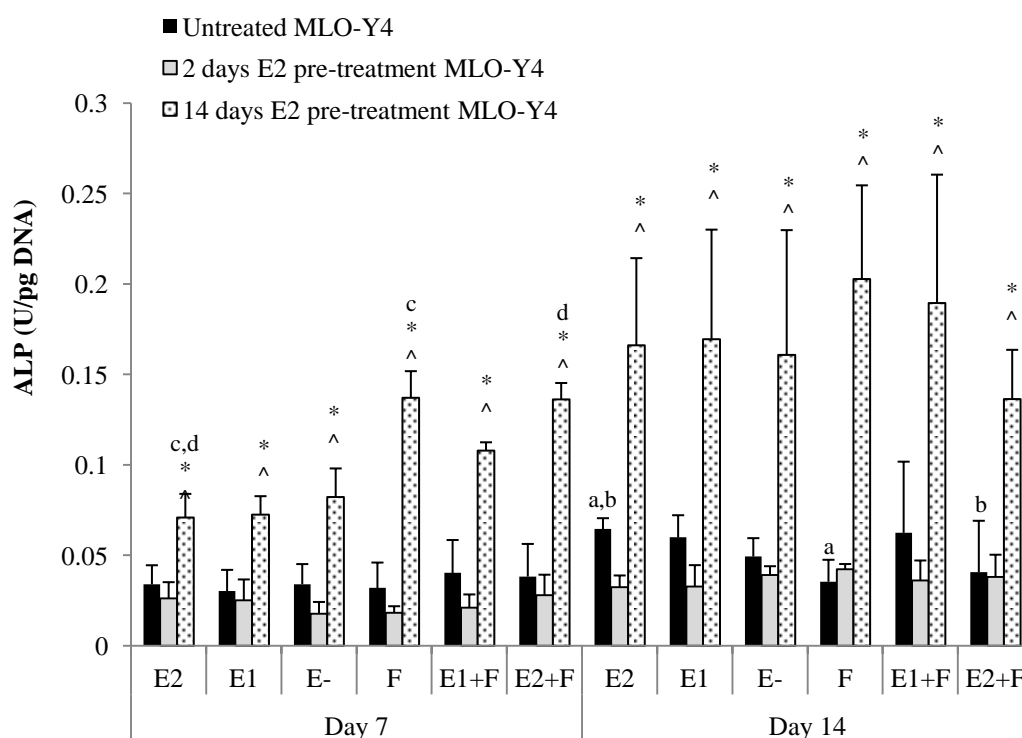
Figure 5.9 Comparison of ALP expression by osteocytes between continued oestrogen treatment (**E2**), reduced oestrogen concentration (**E1**) and oestrogen deficiency by withdrawal of oestrogen (**E-**), or by blocking oestrogen receptors (**F**),(**E1+F**) and (**E2+F**) at day 7 and day 14. n=6 per group, at each time point. Groups that share a letter are significantly different to each other, p values are; a<0.04, b<0.05, c<0.01, d<0.01. * indicates significantly different to previously untreated cultures, while ^ indicates significantly different to two days **E2** pre-treatment, within the same treatments and time points.

5.3.6 Does Oestrogen Deficiency alter Osteocyte Mineralisation *in vitro*?

Osteocyte mineralisation results at day 7 and day 14 from cultures without oestrogen pre-treatment, with 2 days pre-treatment, and with 14 days pre-treatment are presented in detail in Figure 5.10. No difference in mineral production was found between any

subsequent treatment groups (**E1, E-, F, E1+F and E2+F**) compared with continued oestrogen treatment (**E2**), at either day 7, or day 14 in either untreated cells, or those with 2, or 14 days of pre-treatment. However, at day 14, cultures without oestrogen (**E-**), had significantly higher mineral production compared to reduced oestrogen concentration (**E1**), in previously untreated cultures and cells that were pre-treated for 2 days (68.16 ± 23.85 vs. 35.16 ± 18.58, $p<0.05$) and (24.50 ± 5.51 vs. 17.70 ± 4.75, $p<0.01$) respectively.

Effect of Oestrogen Pre-treatment on Mineral Production by Osteocytes: At day 7, in the oestrogen withdrawal group (**E-**) and the oestrogen blocker groups (**E1+F**) and (**E2+F**), mineral content was significantly higher in previously untreated cultures compared to cultures pre-treated for two days (32.88 ± 15.55 vs. 7.04 ± 2.96, $p<0.05$), (37.05 ± 13.41 vs. 9.85 ± 4.11, $p<0.4$) and (37.77 ± 12.46 vs. 7.22 ± 1.85, $p<0.04$), as depicted in Figure 5.10. At day 7, in the reduced oestrogen concentration (**E1**), oestrogen withdrawal (**E-**) and oestrogen blockers (**E1+F**) and (**E2+F**) group, significantly more mineral production was observed in cultures that were pre-treated for 14 days compared to those pre-treatment for 2 days (40.68 ± 27.93 vs. 7.17 ± 2.94, $p<0.04$), (53.83 ± 6.04 vs. 7.04 ± 2.96, $p<0.01$), (79.55 ± 41.64 vs. 9.85 ± 4.11, $p<0.01$) and (65.43 ± 42.40 vs. 7.22 ± 1.85, $p<0.01$) respectively.

At day 14, in the oestrogen withdrawal group (**E-**), mineral production by previously untreated cultures was significantly higher compared to two days of oestrogen pre-treatment (68.16 ± 25.85 vs. 24.50 ± 5.51, $p<0.01$). In the continued oestrogen (**E2**), reduced oestrogen (**E1**), oestrogen withdrawal (**E-**) and oestrogen blocker groups (**E1+F**) and (**E2+F**), mineralisation was significantly higher in cultures pre-treated with oestrogen for 14 days compared to 2 days (67.65 ± 28.29 vs. 17.70 ± 4.75, $p<0.01$), (77.40 ± 50.76 vs. 16.88 ± 5.40, $p<0.01$), (105.66 ± 50.20 vs. 24.50 ± 5.51, $p<0.01$), (69.75 ± 41.49 vs. 19.18 ± 4.84, $p<0.02$) and (70.70 ± 33.18 vs. 21.17 ± 3.65, $p<0.02$).
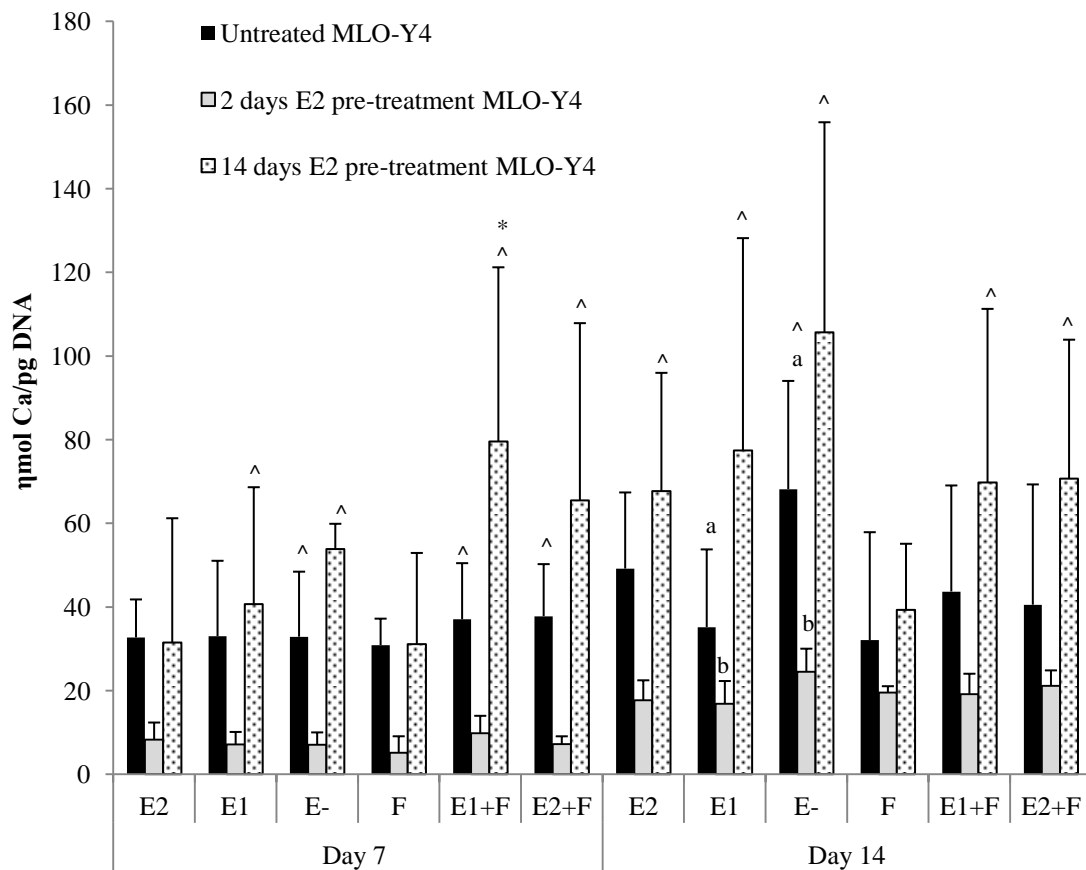
Figure 5.10 Mineral production by osteocytes without prior oestrogen pre-treatment, or with 2, or 14 days of oestrogen pre-treatment after 7 and 14 days. Continued oestrogen treatment control (**E2**) is compared with lowering oestrogen concentration (**E1**), oestrogen withdrawal (**E-**), and blocking oestrogen receptors (**F, E1+F, E2+F**). n=6 per group, at each time point. Groups sharing the same letters are significantly different to each other. Respective p-values are; a<0.05, b<0.01. ∗ indicates significantly different to previously untreated cultures, while ^ signifies significantly different to two days of oestrogen pre-treatment, of the same subsequent treatment group and time point.

5.3.7 Does Oestrogen Deficiency alter Apoptosis by Osteocytes *in vitro*?

The effects of decreased oestrogen concentration (**E1**), oestrogen withdrawal (**E-**), or blocking oestrogen receptors (**F**), (**E1+F**), (**E2+F**), on osteocyte apoptosis, and apoptosis induced using an apoptotic agent, Etoposide, as measured by caspase 3/7 activity, is presented in Figure 5.11. A significantly higher incidence of apoptosis was observed by 24 hours, when oestrogen was withdrawn (**E-**) from osteocytes, compared to continued oestrogen administration (**E2**) (110.5 ± 6.42 vs. 100, p<0.05). When apoptosis was further enhanced at 24 hours using etoposide this caused significantly more apoptosis, compared to cells not treated with Etoposide, in the oestrogen withdrawal group (**E-**) by the 30 hour time point (122.91 ± 24.13 vs. 98.46 ± 12.24,

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p<0.02). Conversely, there was no significant difference with etoposide treatment after just six hours with any other treatment group.

By 24 hours of Etoposide treatment (48 hour time point) it was found that cells treated with Etoposide showed significantly more apoptosis in all treatment groups ($p<0.01$). 48 hours after Etoposide administration (72 hours experimental time point), significantly less apoptosis was observed in the Etoposide treated cultures in all groups ($p<0.01$), except for the oestrogen withdrawal (**E-**) group, compared to untreated cultures. In addition, apoptosis in cultures without Etopside treatment was significantly lower in the oestrogen withdrawal group (**E-**) compared to continued oestrogen treatment (**E2**) ($p<0.01$).
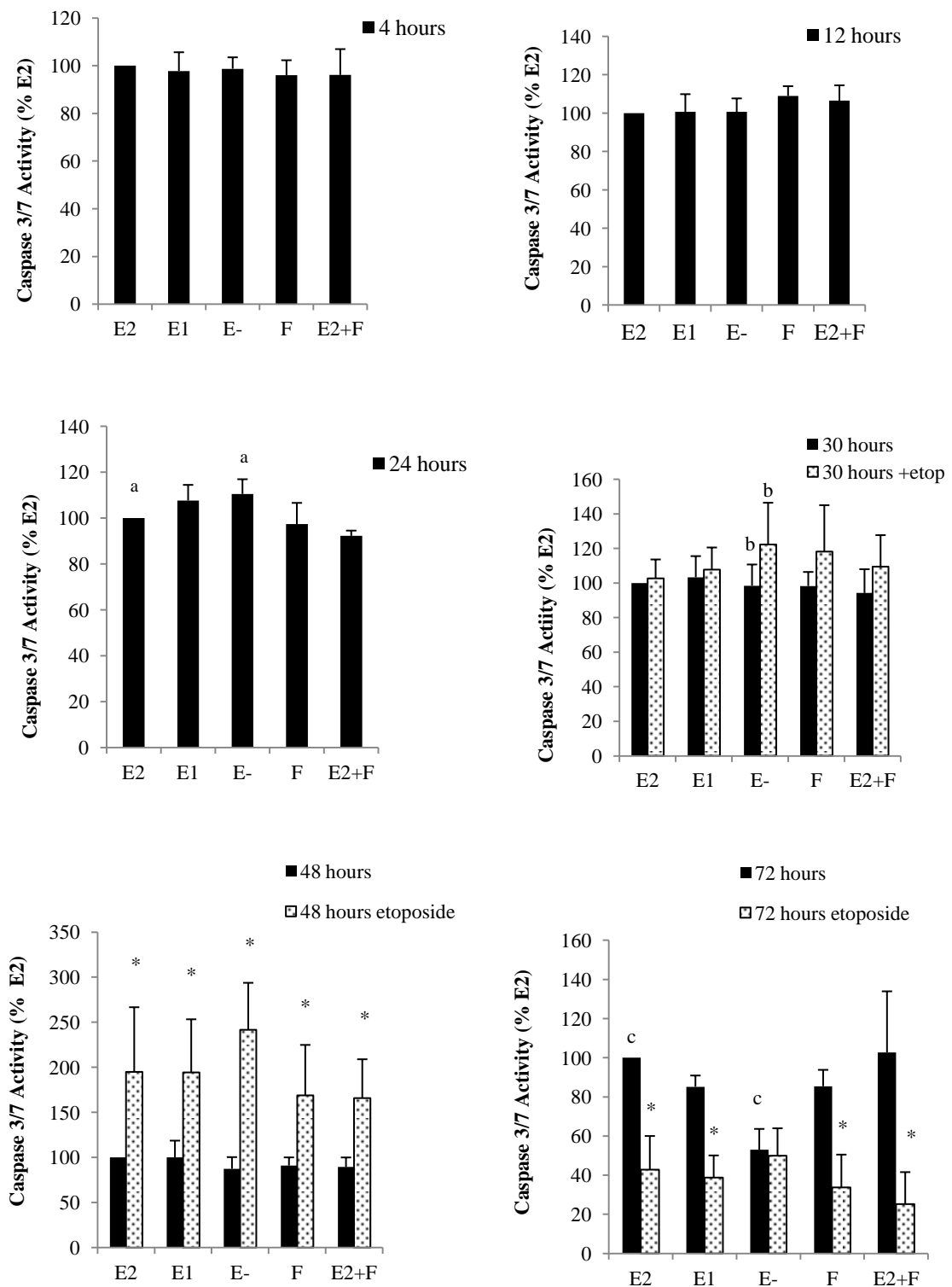
Figure 5.11 Apoptosis, measured by caspase 3/7 activity, of oestrogen pre-treated osteocytes during decreased oestrogen concentration (**E1**), oestrogen withdrawal (**E-**), and blocking oestrogen receptors (**F, E2+F**), compared to % control (continued oestrogen administration: E2). n=6 per group, at each time point. Groups sharing letters are significantly different to one another, p-values are as follows; a<0.05, b<0.02, c<0.01 * indicates significantly different to cultures without etoposide.

5.4. Discussion

This study provides direct evidence that oestrogen deficiency alters bone cell biology *in vitro*. Specifically, it was observed that changes in cell number, mineral production and apoptosis of osteoblast and osteocyte cells occur when oestrogen is withdrawn following a period where cells become accustomed to oestrogen. Oestrogen pre-treatment of both osteoblasts and osteocytes caused significantly higher ALP and mineral production compared to previously untreated cultures. Our studies also reveal that the duration of exposure to oestrogen plays an important role in proliferation and cell matrix production; while oestrogen pre-treatment for 14 days resulted in higher ALP activity and tissue mineralisation compared to untreated cells, such an effect was not observed in cells pre-treated for only 2 days. Most interestingly, oestrogen withdrawal from osteoblasts caused significantly more mineralisation compared with cells that continued to receive oestrogen. In addition, the current study shows that oestrogen withdrawal from osteocyte-like cells causes a significantly higher rate of apoptosis, both spontaneously and when apoptosis was artificially induced by administering Etoposide. Together, these findings highlight the impact of duration of oestrogen administration on bone cell function, and suggest that osteoblastic cells responded to oestrogen withdrawal by increasing apoptosis and mineral production.

It must be noted that there are a number of limitations associated with the current study. Firstly, MC3T3-E1 and MLO-Y4 cells are both cell lines, therefore, a primary culture of osteoblasts and osteocytes may be more appropriate for studying the effects of oestrogen deficiency on bone cells. The MC3T3-E1 cell line has been used previously in many osteoblastic bone cell studies however (Fatokun *et al.*, 2008; Yamamoto *et al.*, 1995). In addition, it is extremely difficult to attain osteocytes from human tissue due to their locations in the body. MLO-Y4 cells have been shown to display many characteristics of primary osteocytes and have been employed extensively in bone cell investigations (Cherian *et al.,* 2005; Kato *et al.*, 1997; Xia *et al.,* 2010). Furthermore, 2D monolayer cultures are suboptimal since cells *in vivo* reside in 3D matrices and interact with various other cell types. In spite of these limitations, important findings regarding oestrogen deficiency on the apoptosis of osteocytes and the bone formation capabilities of osteoblasts and osteocytes *in vitro* were observed.

The higher ALP activity and mineral production that was observed in both osteoblasts and osteocytes when pre-treated with oestrogen, compared to untreated cells, is in agreement with studies that show the enhanced effect of oestrogen on ALP production

and mineral production (Patlas *et al.,* 2005). It has been demonstrated *in vivo* that oestrogen retains bone mass, not only by acting on osteoclasts, but also by stimulating bone formation by osteoblasts (Chow *et al.*, 1992; Edwards *et al.*, 1992). Indeed, it has been suggested that alterations in osteoblast activity may be the first event that occurs following oestrogen withdrawal and that altered osteoclast activity and consequential increased bone resorption may be later events in the osteoporotic bone loss cascade (Manolagas *et al.*, 2002). This idea is in contrast to the widespread opinion that osteoporosis occurs primarily as a consequence of altered osteoclast activity due to oestrogen withdrawal. There are several lines of evidence to support this alternative concept. Firstly, Krum et al have showed *in vitro* that oestrogen's action on osteoclast apoptosis is mediated by Fas ligand production by osteoblasts (Krum *et al.*, 2008). Furthermore, it was shown in an osteopenia mice model with altered osteoblastogenesis that altered osteoclast activity was secondary to impaired osteoblast function, as osteoclast activity in ex vivo was restored to normal following addition of osteoblast cells from normal mice (Jilka *et al.*, 1996). If this theory is substantiated, the alterations in osteoblast activity observed in the current study as a direct result of oestrogen withdrawal could have an important impact on the initiation of altered bone remodelling in osteoporosis.

The increased ALP activity and mineral production that was found with oestrogen withdrawal is in agreement with *in vivo* studies. One study shows mineralised bone formation was significantly higher in OVX rats compared to sham operated rats and OVX rats treated with oestrogen (Yokose *et al.*, 1996). In addition, a study on osteoporotic females showed significantly higher mineralisation in osteoporotic bone compared to controls (Dickenson *et al.,* 1981) and drug induced oestrogen suppression caused significantly increased mineralisation in young female patients (Boyde *et al.,* 1998). Our findings are also in agreement with a study that showed that osteoblasts from OVX rats produced more ALP activity and mineralised bone nodules compared to cells from sham operated rats (Yokose *et al.*, 1996). However, the direct effects of oestrogen withdrawal *in vitro*, on osteoblast mineral production, as evaluated in the current study, have not before been investigated. It is revealed for the first time that silencing oestrogen receptors with fulvestrant significantly reduces mineralisation by osteoblasts compared to controls in pre-treated cells for 14 days after 7 and 14 days of culture. This highlights the role of oestrogen receptors and the importance of oestrogen pre-treatment duration, in regulating osteoblast mineralisation. Moreover, this study reveals that blocking

oestrogen receptors affects mineral production differently than simply withdrawing oestrogen from cultures.

The current study shows that oestrogen withdrawal from osteocyte-like cells induces apoptosis. This finding is in agreement with previous studies, where it was reported that oestrogen deficiency is associated with higher apoptosis rates and depleted osteocyte number, in female patients (Boyde *et al.,* 1998; Tomkinson *et al.,* 1997) and also in ovariectomized animals (Kousteni *et al.,* 2001; Zarrinkalam *et al.,* 2011). The current study showed that oestrogen treatment reduced the amount of apoptosis when cells were treated with an apoptotic inducing agent (Etoposide) compared to cells without oestrogen treatment. This is in agreement with other *in vitro* studies (Bradford *et al.,* 2010; Kousteni *et al.,* 2001). The current study is the first to investigate the direct effect of oestrogen withdrawal on apoptosis *in vitro* however.

Since empty osteocyte lacunae are known to lead to tissue hyper-mineralisation *in vivo* (Frost, 1960), it is intriguing to speculate that oestrogen deficiency induced apoptosis also leads to a local increase in mineral content. Unlike osteoblasts, which lie on the bone surface, the debris from osteocyte apoptosis is not accessible to phagocytic cells and may therefore be mineralised instead. Since osteocyte apoptosis is not ubiquitous (Zarrinkalam *et al.,* 2011), these changes might explain mineral heterogeneity during oestrogen deficiency that was seen in previous studies in Chapter 3 (Brennan *et al.*, 2011). Although it was shown in this study that higher apoptosis occurrence and mineral production both occur as a result of oestrogen withdrawal, a direct link has not been established. However, a previous study showed that in *ex vivo* cultures, higher cell apoptosis occurs with increasing mineral nodule formation (Pantschenko *et al.,* 2005). It has also been demonstrated that apoptosis occurs during osteoblast mineral production *in vitro* (Lynch *et al.*, 1998). Furthermore, IL-6 inhibits osteoblast apoptosis (Jilka *et al.,* 1998) and oestrogen inhibits IL-6 production by mice and human osteoblasts (Girasole *et al.*, 1992), therefore oestrogen may protect osteocyte cells from apoptosis by the same mechanism.

Bone adapts its mass and structure according to the mechanical loading regimes placed upon it (Lanyon *et al.*, 1982). Osteocytes are mechanosensitive cells, which can detect mechanical signals in bone and convert these into chemical signals which are conveyed to other bone cell types (Klein-Nulend *et al.,* 1995a; Klein-Nulend *et al.*, 1995b). Therefore, increased apoptosis may impair bone's ability to adaptively respond to

mechanical loading and to repair micro-damage. Osteocytes physically contact bone lining cells which in turn signal for osteoclast recruitment (Parfitt *et al.*, 1996) and an important role for osteocyte apoptosis in bone remodelling has been suggested previously (Bronckers *et al.*, 1996; Noble *et al.*, 1997). Although the withdrawal of oestrogen in the current study is much more abrupt than diminished oestrogen levels prior to the menopause in females, the oestrogen levels used are similar to normal physiological ranges for serum levels of females (Stricker *et al.*, 2006). Therefore, the changes in osteocyte apoptosis during oestrogen deficiency observed in this study might provide an insight into the increase in bone turnover characteristic of osteoporosis.

5.5. Conclusion

In conclusion, the current study reveals the important implications of oestrogen administration and oestrogen withdrawal on bone cell mineralisation. In addition, the apoptosis induction effect of oestrogen withdrawal from osteocytes and the preventative effects of oestrogen administration on osteocyte apoptosis, induced by apoptotic stimuli (Etoposide) have been shown. Dysregulated apoptosis of both osteoblasts and osteocytes may disrupt normal bone mineralisation and play a direct role in stimulating osteoclast resorption and bone loss during oestrogen deficiency. This may have important implications for osteoporotic patients as increased apoptosis may compromise the osteocyte network, leading to bone resorption and increased fragility. Higher mineral production with oestrogen withdrawal may explain the higher heterogeneity observed in Chapter 3 of this thesis. Recognizing the underlying mechanisms regulating bone cell mineral production and apoptosis during oestrogen withdrawal and their consequences is necessary to further our knowledge of bone biology and aid in the formulation of enhanced pharmaceuticals to remedy osteoporosis. In particular, the osteogenic activity and mineral production of osteoblasts in response to mechanical loading will be investigated in Chapter 6 to determine whether the mechano-responsiveness of osteoblasts is impaired during oestrogen deficiency.

6. Mechanobiology of Osteoblasts during Oestrogen Deficiency

6.1. Introduction

Bone is a dynamic tissue that adapts its structure and material properties in response to the mechanical stresses it experiences. It is believed that this adaptive nature serves to provide a structure capable of withstanding everyday loads, while also achieving a minimal weight for efficient movement, a concept commonly referred to as Wolff's Law (Wolff, 1892). It has been shown that mechanical strain imposed by physiological loading causes increased bone formation *in vivo* (Chambers *et al.*, 1993; Robling *et al.*, 2000; Turner *et al.*, 1996; Forwood *et al.,* 1996). An obvious example of this is in tennis players, where the racquet arm has significantly greater mass and size than the non-playing arm (Bass *et al.*, 2002). Conversely, unloading of bones causes significant loss of bone mass and mineral content, as observed in astronauts experiencing weightlessness in microgravity (Holick, 1998; Schneider *et al.*, 1995) and patients under strict bed rest (Baecker *et al.*, 2003; Leblanc *et al.*, 1990).

Mechano-sensitive bone cells, such as osteocytes and osteoblasts, are capable of sensing their mechanical environment and in response to changes in applied mechanical loads, they produce biochemical signals, by means of a process known as mechano-transduction (Machwate *et al.*, 1995). This mechanism activates cells to alter bone mass and architecture (Huang and Ogawa). Several biophysical forces are believed to act as the *in vivo* mechanical stimulus to bone cells in their native environment. In particular, it is thought that physiological loading results in direct tensile and compressive strains on osteoblasts at the bone surface (Carter *et al.*, 1998; Claes and Heigele, 1999). In addition, shear stress (Bakker *et al.*, 2001) and streaming potentials (Pienkowski and Pollack, 1983) have been proposed to occur as a secondary effects of applied mechanical strain, when the interstitial fluid within bone matrix is perturbed. Increased chemotransport due to mechanical loading, has been shown to modulate the effects of shear stress on bone cells (Donahue *et al.*, 2003). Shear stress is the principal biophysical stimulus that has been studied *in vitro* to understand mechano-responsiveness by bone cells. Parallel plate flow chambers of various configurations have been employed extensively to impart a constant, controlled stimulus to a homogeneous population of cells and investigate the biochemical response of osteoblasts and osteocytes to fluid shear stress (Frangos *et al.,* 1988; Jacobs *et al.,* 1998;

Reilly *et al.,* 2003; Sakai *et al.,* 1999; Sterck *et al.,* 1998).

The mechanotransduction of osteoblasts involves a cascade of signalling events. Under imposed mechanical stimuli, intracellular calcium mobilization is increased (Donahue *et al.*, 2003) by the activation of plasma membrane receptors generated by ATP release, or by direct intercellular communication through gap junctions (Jorgensen *et al.*, 2000). Other biochemical signalling molecules are produced including prostaglandin E_2 (PGE_2), nitric oxide (NO), osteopontin (OP) and extracellular signal-regulated kinase (ERK1/2) (Bakker et al., 2001; Batra et al., 2005; McGarry *et al.*, 2005a; Papachristou *et al.*, 2009; Reilly *et al.,* 2003; You *et al.*, 2001). Wnt protein expression is increased (Santos *et al.*, 2009), facilitated by down regulation of Wnt inhibitors (Sclerostin) by osteocytes (Van Bezooijen *et al.*, 2004) under applied loading (Moustafa *et al.,* 2012). PGE_2 is an important regulator of bone remodelling (Norrdin *et al.,* 1990; Suda *et al.,* 1996) and plays a pivotal role in skeletal mechano-responsiveness (Weinreb *et al.*, 1989). PGE receptors are present in MC3T3-E1 osteoblast-like cells and facilitate increased cell growth and differentiation in the presense of PGE_2 (Suda *et al.*, 1996). Shear stress has been shown *in vitro* to stimulate mechano-transduction by bone cells to a greater degree compared to direct mechanical strain, in terms of NO, PGE_2, and OP expression (Owan *et al.,* 1997; Smalt *et al.,* 1997). Fluid flow engendered shear stress also leads to increases in mineral matrix production *in vitro* (Sikavitsas *et al.*, 2003; Van den Dolder *et al.*, 2003).

Post-menopausal osteoporosis in females is associated with diminished levels of circulating serum oestrogen levels (Riggs *et al.*, 2002). It is well known that osteoporotic bone has compromised strength due to bone loss (Bourrin *et al.*, 2002; Ederveen *et al.*, 2001) and depleted micro-architecture (Aaron *et al.*, 2000). In addition, a marked increase in bone remodelling occurs at a cellular and tissue level, with bone resorption rates superseding bone formation rates (Balena *et al.*, 1993). In Chapter 3 it was observed that mineral heterogeneity is significantly altered during oestrogen deficiency (Brennan *et al.*, 2011). In addition, bone loss following ovariectomy is remarkably site specific within the distal femur of rats (Baldock *et al.*, 1998). Therefore, the synergistic effects of systemic oestrogen deficiency and local differences in mechanical forces within bones (Bergmann *et al.*, 2001; Bergmann *et al.*, 1993), might be responsible for heterogeneous alterations in tissue composition and structure during oestrogen deficiency. It has been shown that oestrogen levels affect cortical bone formation rates in response to mechanical loading *in vivo* (Devlin and Lieberman, 2007) and that blocking

oestrogen receptors substantially reduces cell proliferation stimulated by strain *in vitro* (Damien *et al.*, 1998). Osteoblasts from osteoporotic donors differ in their response to cyclic strain, as measured by cell proliferation and release of transforming growth factor-β (Neidlinger-Wilke *et al.*, 1995) and show a long-term impaired response to flow induced shear stress, measured by PGE_2 release (Sterck et al., 1998). It has been shown that combined exposure to oestrogen and shear stress increased biochemical signalling (PGE_2 and NO release) in an additive manner (Bakker *et al.*, 2005). In addition, in Chapter 4 it has been shown that mineral concentration is significantly reduced, with increasing duration of oestrogen deficiency. This study demonstrated that sheep that were oestrogen deficient for 31 months had significantly less bone tissue mineralisation, compared to sheep that were oestrogen deficient for 12 months. Normal ageing was ruled out as a cause of this diminished mineralisation. However, whether such local changes in tissue mineralisation arise due to an altered mechano-biological response by oestrogen deficient bone cells has not yet been studied.

The aim of this study is to determine if the mechano-biological response of osteoblasts in response to mechanical stimulation is impaired during oestrogen deficiency. Specifically, it was sought to understand whether changes in the production of PGE_2 and bone tissue mineral occur in response to mechanical loading in the absence of oestrogen. To address this objective, an osteoblast cell line (MC3T3-E1) was pre-treated with oestrogen to investigate the effect of subsequent oestrogen withdrawal on cell mechano-sensitivity. Fluid shear stress was applied using an orbital rotator, or a parallel plate flow chamber and cell viability (alamar blue), mineralisation (alizarin red staining), and PGE_2 production (iummunoassay) were monitored.

6.2. Materials and Methods

In this study, fluid shear stress was applied to *in vitro* cell cultures using either an orbital rotator or a parallel plate flow chamber. The governing equations to estimate the amount of mechanical stimulation imparted by each of these loading scenarios is outlined below.

6.2.1 Orbital Rotation Shear Stress

An estimate of the applied shear stress at the bottom of the culture dishes in an orbital rotator can be calculated according to equation 6.1,

$$\tau = R \sqrt{\rho \mu (2\pi f)^3} \qquad\qquad (6.1)$$

where R is the radius of orbital rotation, ρ is the density of the culture media, μ is the viscosity of the culture media, and f is the frequency of rotation (Kraiss *et al.*, 2000; Ley *et al.*, 1989; Pearce et al., 1996). This equation has been shown to be a reasonable estimation, when tested experimentally using a flow probe that uses optical velocimetry to measure shear stress within 166μm of its surface (Dardik *et al.*, 2005a; Dardik *et al.*, 2005b). The radius of orbital rotation for cells cultured in a 24 well plate is 0.8cm (calculated from the diameter of the 24 well plate wells), the viscosity of cell culture media is assumed to be 7.5 x 10^{-4} Pa.s (Pearce *et al.,* 1996), and the shaking frequency for experiments outlined below was 290 rpm (4.83 rps). As seen below, the estimated shear stress was 1.16 Pa (11.6 dynes/cm^2), which is within predicted physiological ranges of 6-30 dynes/cm^2 (Weinbaum *et al.*, 1994). Figure 6.1 presents a schematic of the orbital rotation set-up for the current study.

$$\tau = .8cm \sqrt{\frac{.1\ Pa \cdot s^2}{cm^2} \cdot 7.53 \times 10^{-4} Pa \cdot s. \left(\frac{2\pi(4.83)}{s}\right)^3} = 1.16\ Pa \approx 12\frac{dyne}{cm^2}$$

Well of a 24 well plate,

$\varnothing$=.8cm

Shear stress =1.16 Pa
on cell monolayer
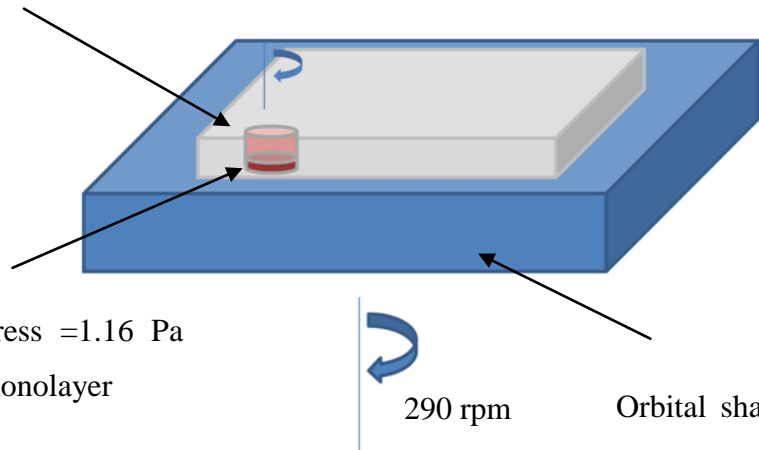
290 rpm

Orbital shaker (Stuart Microtitre SSM5)

Figure 6.1 Schematic of the orbital rotation set-up. An estimated shear stress of 1.16Pa was applied to the cell monolayer of each well within a 24 well plate using an orbital rotator at a speed of 290 rpm.

6.2.2 Design of Parallel Plate Chamber

The experimental protocol employed in this instance entails culturing cells for significant periods (up to eight days). When osteoblasts are cultured *in vitro* they proliferate and produce an extracellular matrix that they subsequently mineralise. Therefore, at high cell densities and significant culture periods, they begin to form 3D colonies and multilayer, even on flat tissue culture slides and also produce a surrounding extracellular matrix. This presents problems when using traditional parallel plate flow chambers whose height are in the range of 220-300μm (Bakker *et al.,* 2001; Frangos *et al.,* 1988; Jacobs *et al.,* 1998; Nauman *et al.,* 1999). It is necessary to have a sufficiently high chamber to ensure laminar flow conditions over the cell layer attached to the base of the chamber, because if the height of the cell layer is significant relative to the chamber height, the steady flow assumption of Poiseuille pressure driven flow through parallel plates are no longer valid (Nauman *et al.*, 2001). To overcome this problem a custom flow perfusion parallel plate configuration was designed, to confer shear stress to cell monolayers. The design of this flow chamber is outlined in detail below and detailed design drawings can be viewed in Appendix 1.

The most significant parameters in the design of a parallel plate flow chamber are, the shear stress imposed on the cells, the Reynolds number of the flow and the entrance length (Nauman *et al.*, 1999). Flow through a parallel plate configuration, known as Poiseuille flow in fluid mechanics, imparts a wall shear stress on the surface of the parallel plates of the flow chamber, i.e. on the cell monolayer attached to the surface. This can be expressed using the momentum balance for a Newtonian fluid (Equation 6.2), assuming a parallel plate geometry;

$$\tau = \frac{6\mu Q}{\omega h^2} \qquad\qquad (6.2)$$

where τ is the resultant shear stress, ω is the width of the flow field, h is the gap between the plates, Q is the volumetric flow rate and μ is the viscosity of the perfusion medium (Frangos *et al.*, 1988). Dimensions of the flow chamber in a parallel flow device are critical to ensure the cells are subjected to laminar flow conditions. A Reynolds number below 2000 is generally accepted to lie in the laminar region, however, transition from smooth or laminar flow to turbulent flow has been shown to occur at Reynolds numbers between 1000 and 8000 (Panton, 1996). Therefore, to ensure laminar flow through the

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flow chamber Re was confined <1000. The Reynolds number of the flow profile through the parallel plate chamber is expressed by equation 6.3.

$$Re = \frac{Uh\rho}{\mu} = \frac{Q\rho}{\mu\omega}$$

(6.3)

where U is the average flow velocity, h is the height of the chamber, ρ is the density of the perfusion medium, μ is the viscosity, Q is the flow rate and ω is the width of the chamber. Combining equations 6.2 and 6.3 gives equation 6.4. An estimate of the entry length for plane Poiseuille flow to develop is expressed by equation 6.5 (Schlichting, 1968) .

$$Re = \frac{\tau\rho h^2}{6\mu^2}$$

(6.4)

$$l = 0.04 \times h \times Re$$

(6.5)

The height of the constructed chamber is 0.40mm. The width of the chamber was chosen as 50mm in order to attain a height/width ratio of 0.008, which is within the ranges of literature (0.005-0.028) (Frangos *et al.,* 1988; Jacobs *et al.*, 1998; Nauman *et al.,* 1999). A minimal height/width ratio is required for the flow to adhere to the assumption of being within an infinite parallel plates, which is necessary to apply equations 6.2-6.5 (Massey and Ward-Smith, 1998). Performing a simple 'solver' analysis in Microsoft excel ensured dimensional optimisation was achieved, which conformed to constraints applied to equations 6.2-6.5 as well as those attained from computational analysis.

(A)

(B)
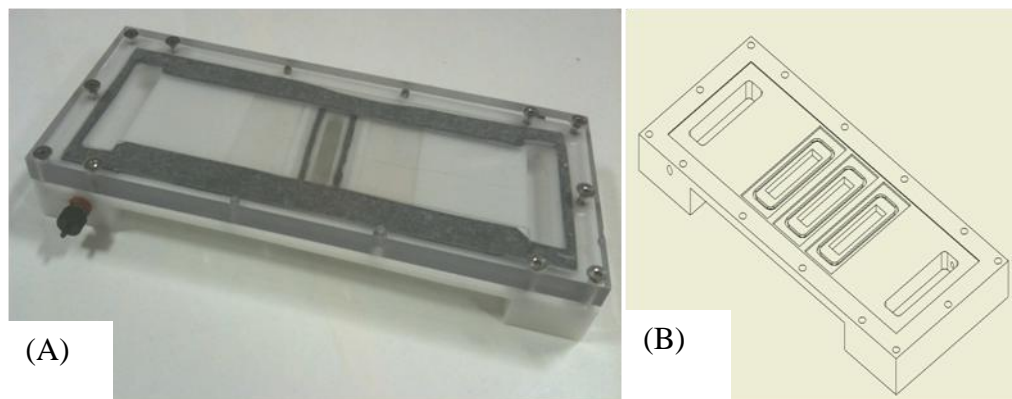
Figure 6.2 Picture (**A**) and drawing (**B**) of parallel plate flow device, designed and manufactured in house

The flow chamber consists of a machined and polished polycarbonate top plate, a polyamide machined base plate and an intermediate self adhesive backed silicone sponge sheet to form a watertight seal (RH Nuttall, Birmingham, U.K.). The assembly was held in position by threaded screws along the periphery of the plates, see Figure 6.2. Unlike most parallel plate designs, where the intermediate gaskets govern the height of the chamber, here the channel height was precision machined out of the bottom plate, ensuring an accurate and consistent height along the length of the channel. In addition to the parallel plate chamber, the flow apparatus consisted of a Masterflex multichannel peristaltic pump and Masterflex L/S 17 precision platinum cured silicone tubing (Cole Parmer Instruments Inc, London, UK), and a media reservoir, see Figure 6.3. A schematic of the flow system is also presented in Figure 6.4. The flow rate applied through cell chambers in the flow perfusion bioreactor is important, because it governs the shear stress on the cells. Flow rate and shear stress have a linear relationship in a channel of fixed dimensions (Smalt *et al.,* 1997). This relationship was confirmed for the designed chamber with height 0.4mm and width 50mm (depicted in Appendix 2).

Figure 6.3 Pulsatile fluid flow (PFF) pumping configuration including peristaltic pump, media reservoir, tubing, flow probe and parallel plate device operating in a CO_2 incubator at 37˚C and 5% CO_2.
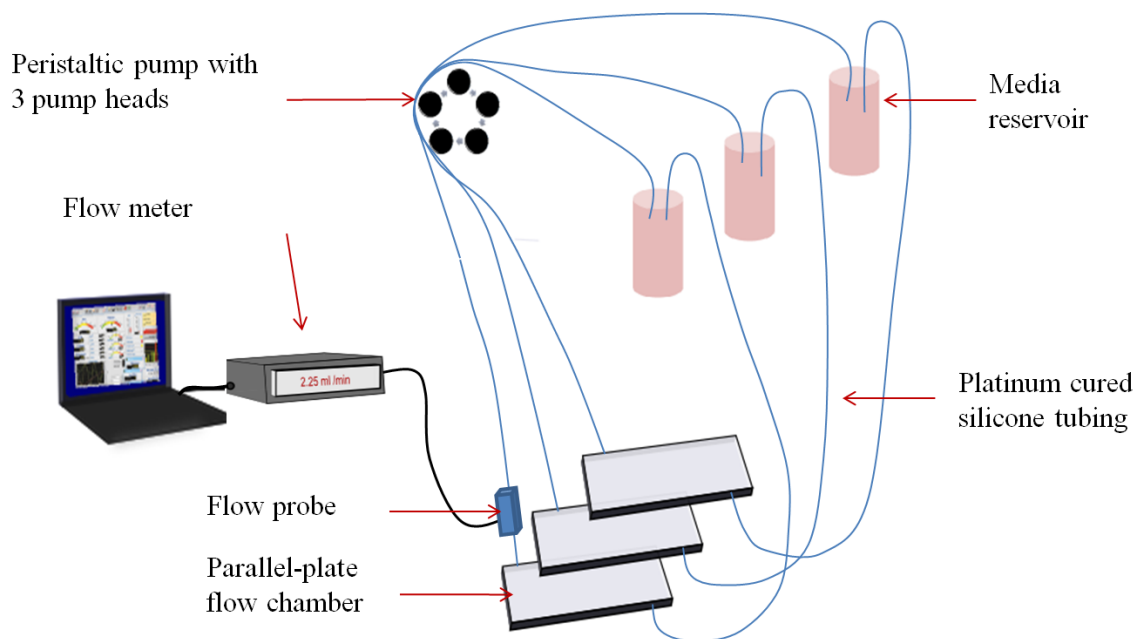
Figure 6.4 Schematic of closed loop pulsatile fluid flow system

6.2.3 Cell Culture: Oestrogen Pre-treatment

Prior to all mechanical loading experiments, MC3T3-E1 murine calvarial osteoblasts were maintained in α-modified MEM supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100U/ml penicillin and 100 µg/ml streptomycin with the addition of commercially available 17β-estradiol ($1x10^{-8}$M, Sigma Aldrich) for fourteen days. Cell culture media were replenished every 3-4 days and cells were maintained at 37 °C in a humidified 5% CO_2 environment. This allowed the cells to become accustomed to oestrogen before subsequent withdrawal.

6.2.4 Mechanical Loading Experiments

Shear stress was conferred to MC3T3-E1 osteoblast-like cells by the use of an orbital rotator as described in section 6.2.4.1 and also by using a parallel plate configuration as described in section 6.2.4.2.

6.2.4.1 Orbital Shear Stress and Experimental Conditions

Cells with or without oestrogen pre-treatment were plated at a density of 3×10^4 cells per ml in 24-well plates and cultured overnight in osteogenic culture media supplemented with 50µg/ml ascorbic acid, 10mM/L β-glycerophosphate and 10nM/L dexamethasome.

Cells were then cultured under separate osteogenic conditions for 14 days; (1) Osteogenic media with continued oestrogen treatment ($1x10^{-8}$M 17β-estradiol): **E2**, (2) osteogenic media without oestrogen: **E-**, (3) osteogenic media with oestrogen antagonist Fulvestrant ($1x10^{-7}$M, ICI 182780, Sigma Aldrich): **F**, and (4) osteogenic media with continued oestrogen treatment and the addition of the oestrogen antagonist: **E2 + F**. All cell cultures were exposed daily to 40 minutes of intermittent loading (fluid flow induced shear stress of 1.16 Pa) induced through an orbital shaker (Stuart Microtitre SSM5), similar to previous studies (Dardik *et al.*, 2005b; Haga *et al.*, 2003; Pearce *et al.*, 1996; Yun *et al.*, 2002). The rationale for one loading cycle per day was based on *in vitro* (Batra et al., 2005; Robling *et al.*, 2000) and *in vivo* studies (Umemura *et al.*, 1997) which have demonstrated that mechanical loading elicits more of an osteogenic response when applied in discrete loading bouts, compared to continuous loading without rest periods. All cell culture media were replenished every 3-4 days and cells were maintained at 37 °C in a humidified 5% CO_2 environment. Non-loaded cultures under aforementioned conditions 1-4 and under the same temperature and gaseous conditions served as static controls.

6.2.4.2 Laminar Shear Stress and Experimental Conditions

In order to evaluate the biochemical response of cells to shear stress, a parallel plate flow chamber, as described in section 6.2.2, was used to confer pulsatile fluid flow (PFF) to monolayer cultures. Although orbital shear stress provides a very useful means of evaluating applied loading to cells and has been used extensively for this purpose (Dardik *et al.,* 2005a; Dardik *et al.,* 2005b; Kraiss *et al.,* 2000; Ley *et al.,* 1989; Pearce *et al.,* 1996), a parallel plate design was used instead of orbital rotation as it confers a more controlled, constant, laminar flow throughout the test chamber, Therefore, it would be expected to give a more accurate evaluation of the effects of shear stress on oestrogen deficiency cells for the analysis of biochemical production.

Cells that were pre-treated for 14 days with oestrogen, as described in 6.2.3, were trypsinized and plated at a density of 18.48 $\times 10^{4}$/cm^{2} onto 25 x 75mm rectangular polystyrene culture slides (Nunc Brand, Biosciences, Dublin, Ireland) in 1 mL of media, confined within silicone gaskets attached to the slides using 'press to seal' technology (Biosciences). Slides were contained within slide plates, four slides per plate, and were pre-incubated overnight in osteogenic culture media supplemented with 50μg/ml ascorbic acid, 10mM/L β-glycerophosphate, 10nM/L dexamethasome and 17β-estradiol ($1x10^{-8}$M, Sigma Aldrich). Cells were then either cultured up to seven days in

osteogenic media without oestrogen: **E**-, or with continued oestrogen supplemented osteogenic media: **E2**. Media (13 mL) was replenished every 3-4 days. Half of the slides from each media pre-treatment condition were then randomly assigned to either undergo applied loading group or serve as non-loaded controls. Bioreactor components were steam sterilized prior to use and were assembled under a laminar flow hood in sterile conditions. The slides were placed into the grooves in the base of the parallel plates and the pump with six rollers was operated at 48rpm which resulted in pulsatile fluid flow with a speed of 137ml/min at 4.8 Hz. The flow apparatus was kept in a 37˚C incubator with 5% CO_2 for the duration of the fluid flow experiment, see Figure 6.3. Flow was applied for two hours, exposing cells to an estimated average shear stress of 1.25 Pa (calculated using equation 6.2). The flow rate of perfusion media through the system was measured using a Ts410 transit time tubing flow meter and flow probe (Transonic Systems Inc, Maastricht, The Netherlands). Control slides were placed beside the bioreactor and were cultured under the same temperature and gaseous conditions, but remained non-loaded in the slide plates in 13 mL of culture media, serving as non-loaded static controls. Both loaded and non-loaded cell seeded slides were subsequently post-incubated in 6 mL of culture media for one hour in the absence of PFF.

6.2.5 Cellular Assays

6.2.5.1 Cell Viability Assay

Cell metabolic activity was assessed in orbital shear stress experiments using Alamar Blue cell viability reagent (Invitrogen). This assay is designed to quantitatively measure the proliferation and viability of cells. Alamar blue is a redox indicator that yields a colorimetric and fluorometric response to metabolically active cells. It uses a blue indicator dye resazurin, which is converted to its fluorescent pink form, resorufin, by enzymes produced by metabolically active cells. Non-viable cells have lower metabolic activity and therefore produce proportionally less signal. To perform the assay, cell culture media was removed from wells and cells were washed with heated (37˚C) phosphate buffered saline (PBS). Alamar blue dye was diluted 1:10 in PBS and 350 μl was added to each well. Cells were incubated at 37 ˚C for 60 minutes, after which 100μl of cell conditioned alamar blue reagent was added in triplicate to a 96 well plate. Absorbance was measured at 550 and 595nm using a microplate reader (Wallac 1420, Victor) and viability was expressed as % reduction between the two wavelengths.

6.2.5.2 Mineralisation Assay

Mineral production was quantified in orbital shear stress experiments using Alizarin Red S staining, as described in detail in chapter 5. Briefly, cells were stained with 40mM Alizarin Red-S (Sigma Aldrich) and subsequently de-stained using 100nM cethylpyridium chloride (Sigma Aldrich). The absorbance of the extracted stain was measured at 550 nm on a microplate reader (Wallac 1420, Victor). Relative photometry units were converted to $\mu mole/cm^2$ of calcium, since one mole of alizarin red binds to two moles of calcium (Norgaard *et al.*, 2006).

6.2.5.3 PGE_2 Measurement

In parallel plate flow experiments, PGE_2 release in the conditioned medium was measured by an enzyme immunoassay (EIA) system (KO18-HX1, Arbor Assays, Ann Arbor, MI, USA). This assay is based on the competition between PGE_2 present in the test samples and a PGE_2 acetylcholinesterase (AChE) conjugate, which can both bind to a limited quantity of PGE_2 monoclonal antibody. In this way, the amount of PGE_2 AChE conjugate that binds to the PGE_2 antibody is inversely proportional to the quantity of PGE_2 present in the test samples. The PGE_2–antibody arrangement then binds to a goat polyclonal anti-mouse IgG that is attached to the bottom of the wells of the microtitre plate. After unbound PGE_2 is removed and a substrate to AChE is added, the enzymatic reaction results in a colour change in the wells, the intensity of which is directly related to the amount of PGE_2 present in the sample.

To perform the PGE_2 assay, 100µl samples from each of the experimental groups were added to the wells of the PGE_2 enzyme immunoassay. Next, 125 µl was added to the non-specific binding wells (since the PGE_2 monoclonal antibody will not be added to these wells). Then, 100 µl of assay buffer was added to wells acting as maximum binding wells. Next, 25 µl of the PGE_2 conjugate was added to each well, followed by 25µl of the PGE_2 monoclonal antibody to all wells apart from the non-specific binding wells. The plate was then placed on a rocker for 15 min at 300 rpm and incubated at 4°C for 16 hours, after which any unbound PGE_2 conjugate was removed by washing the well with wash buffer diluted in distilled water. The substrate to AChE was then added to each well and after 30 minutes incubation at room temperature and the enzymatic reaction resulted in a colour change to the well. The reaction was stopped by addition of 1M solution of hydrochloric acid (HCL) and the intensity of the colour change was read at a wavelength of 420nm on a microplate reader (Wallac Victor ,1420). A standard

curve was created from serial dilutions of a precise quantity of PGE_2 supplied and was used to determine the PGE_2 concentration of the experimental samples, after correction for the dilution factor of the sample is made.

6.2.6 Statistics

Data is expressed as a mean ± standard deviation. The general linear model ANOVA procedure was used to analyse statistical differences between groups. Pre-treatment (with/without oestrogen pre-treatment), loading status (shear stress or static), and treatment group (E2, E-, F, E2+F) were crossed, fixed factors, i.e. each level of one factor occurred in combination with each level of the other factor and their effect on the response variable was determined. % reduction was the response variable for the alamar blue experiment and μmole Ca/cm^2 for alizarin red assay. Fixed, crossed factors for the PGE_2 experiment were a) duration of oestrogen deficiency prior to loading, b) loading status (with/without PFF) and c) treatment. The response variable was pg PGE_2/mL. There was a sample size of n=3 per group and each experiment was repeated to give n=6 per group. Factor effects were determined using the ANOVA F test and group means were compared using Tukeys-Kramer pair-wise multiple comparison test (Minitab 16). Statistical significance was defined as p≤ 0.05.

6.3. Results

6.3.1 Cell Viability

Cells without oestrogen pre-treatment: At day 7, when data from all treatment groups was pooled, there was significantly lower cell viability in cultures with applied loading (orbital shear stress), compared to static cultures (10.49 ± 1.01 vs. 12.44 ± 3.81, p<0.03). Following pair-wise analysis between treatment groups, it was found that cell viability was significantly lower with **F** compared to **E2** (6.85 ± 3.44 vs. 13.88 ± 3.62, p<0.01), see Figure 6.5. In the **E2** and **E2+F** groups at day 7, cell viability was lower with orbital shear stress compared to static conditions (9.07 ± 3.12 vs. 3.12 vs. 13.88 ± 3.62, p<0.05) and (10.47 ± 1.37 vs. 15.41 ± 3.01, p<0.05) respectively.

At day 14, when all data was pooled from all treatment groups, there was no significant evidence of a loading status affecting cell viability. Similarly, when data was compared between treatment groups, no significant difference between group means was found at day   14.

Cells *without* Oestrogen Pre-treatment
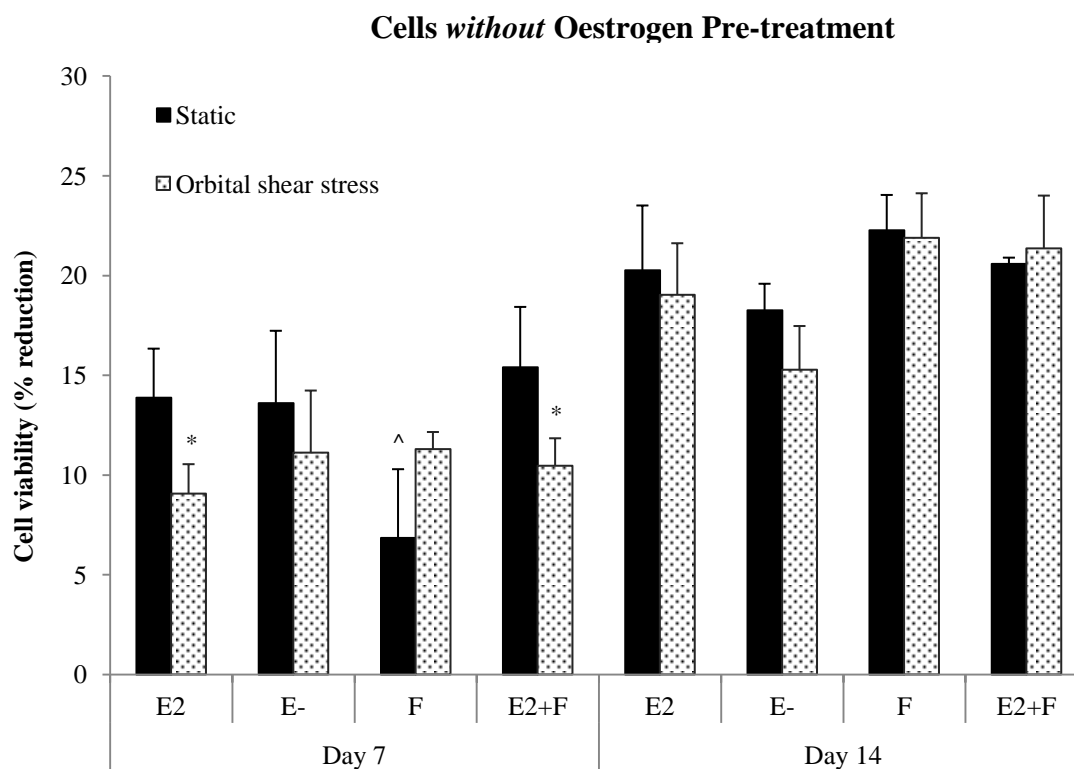
Figure 6.5 Cell viability was quantified using alamar blue viability assay in cell cultures without oestrogen pre-treatment. ^ indicates significantly lower compared to **E2**, n=6 per group, per loading status, at each time point. * indicates significantly lower compared to static conditions.

Oestrogen pre-treated cells: At day 7, exposure to fluid shear stress (orbital) had no effect on cell viability compared to non-loaded cultures, either when data from all treatments was pooled, or within each treatment group, as depicted in Figure 6.6. Likewise, at day 14, cell viability from cultures with applied orbital shear stress did not differ from static cultures, either when data from all treatments was pooled, or when means were compared between groups. Furthermore, no significant difference was found when treatments were compared to continued oestrogen controls (**E2**), either at day 7 or day 14. Finally, cell viability was not altered in cultures pre-treated with oestrogen, compared previously untreated cultures.

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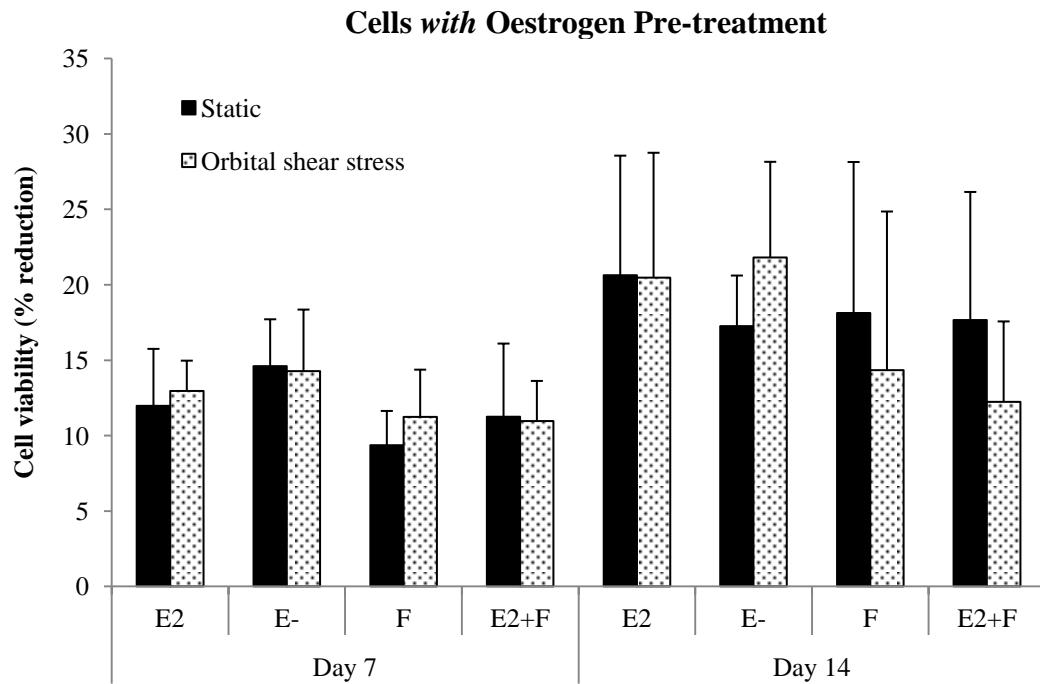
Figure 6.6 Cell viability in cell cultures with oestrogen pre-treatment. There was no significant difference between treatment groups compared to controls (**E2**), or between non-loaded and loaded conditions. n=6 per group, per loading status, at each time point.

6.3.2 Mineral production

Cells without oestrogen pre-treatment: At day 7, when data from all treatments was pooled, significantly higher mineralisation was found in cultures with exposure to fluid shear stress compared to static conditions (0.23 ± 0.16 vs. 0.09 ± 0.08, $p<0.01$). As depicted in Figure 6.7, by day 7, mineral production was significantly higher with orbital shear stress compared to static conditions in the **E-** group (0.39 ± 0.31 vs. 0.09 ± 0.04, $p<0.01$). With applied shear stress, mineral production was higher in oestrogen deficient controls (**E-**) compared to **E2** controls (0.39 ± 0.31 vs. 0.06 ± 0.03, $p<0.03$).

After 14 days of culture, when data from all treatments was pooled, similarly to day 7, mineralisation was higher with applied shear stress compared to static conditions (0.81 ± 0.23 vs. 0.45 ± 0.05, $p=0.001$). Within treatment groups, it was observed that mineralisation was higher with applied shear stress compared to static conditions in the **E-** group (1.12 ± 0.19 vs. 0.47 ± 0.24, $p<0.05$). Similar to day 7, with applied shear stress, **E-** was significantly more mineralised compared to the **E2** control group (1.12 ± 0.19 vs. 0.55 ± 0.19, $p<0.03$).

Cells *without* Oestrogen Pre-treatment
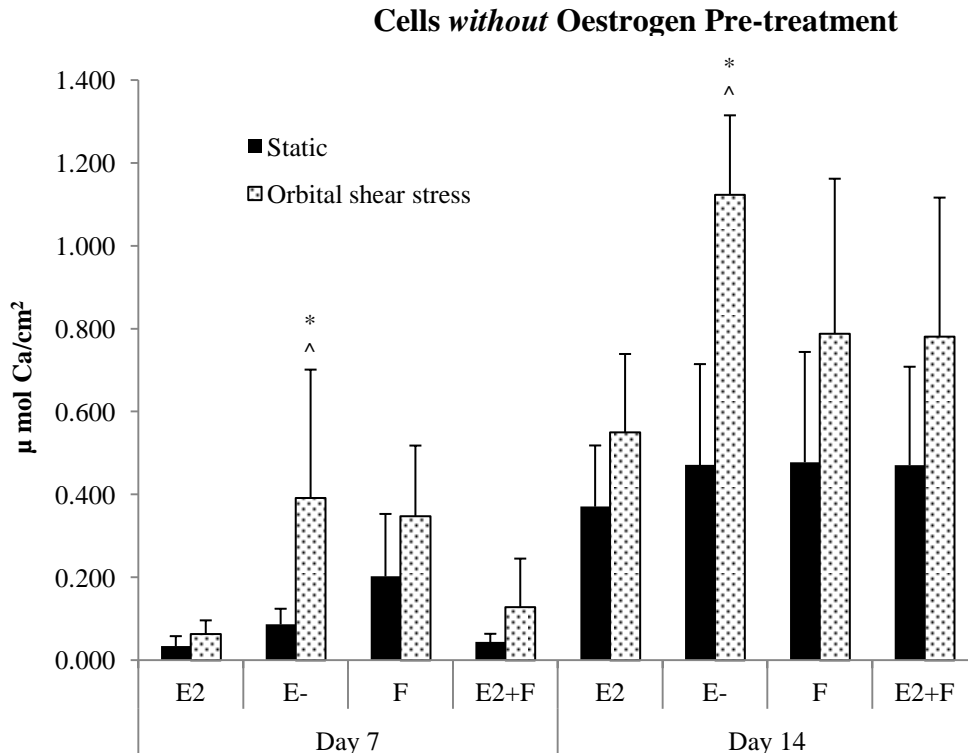
Figure 6.7 Mineralisation after 7 and 14 days under static or orbital shear stress conditions, in cultures without oestrogen pre-treatment, n=6 per group, per loading status, at each time point. ∗ indicates significantly higher compared to static conditions of the same treatment group. ^ signifies statistically higher compared to **E2** controls, with applied shear stress, at the same time point.

Oestrogen pre-treated cells: At day 7, when data from all treatments was pooled, higher mineralisation was found with exposure to fluid shear stress compared to static conditions (0.50 ± 0.10 vs. 0.20 ± 0.06, $p<0.01$). As depicted in Figure 6.8, at day 7, mineral production was higher in cultures with exposure to intermittent shear stress compared to static controls in the **E2** and **E-** groups (0.56 ± 0.14 vs. 0.27 ± 0.15, $p<0.05$) and (0.63 ± 0.17 vs. 0.26 ± 0.04, $p<0.01$).

After 14 days, when data from all treatments was pooled, higher mineralisation was found in cultures with exposure to fluid shear stress compared to static conditions (0.75 ± 0.30 vs. 0.38 ± 0.16, $p<0.01$). Higher mineral production was found with applied shear stress compared to static conditions in the **E2** (1.18 ± 0.11 vs. 0.29 ± 0.11, $p<0.01$) group. With applied shear stress, the mineral in each of the oestrogen deficiency groups was significantly less; **E-** (0.76 ± 0.08, $p<0.04$), **F** (0.53 ± 0.16, $p<0.01$) and **E2+F** (0.52 ± 0.10, $p<0.01$) compared to the mineral concentration with continued oestrogen treatment controls, **E2** (1.18 ± 0.11). Under static conditions, **E-** resulted in more mineral production compared to **E2** (0.62 ± 0.08 vs. 0.29 ± 0.11, $p<0.04$).

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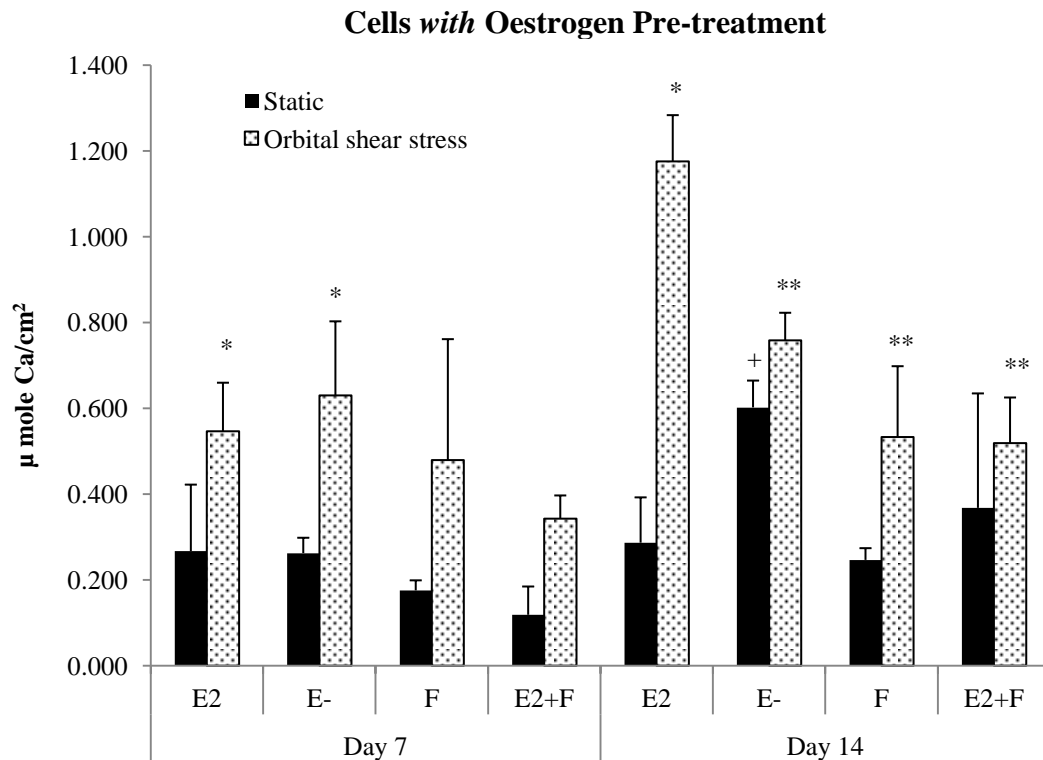
Figure 6.8 Mineralisation after 7 and 14 days under static or orbital shear stress conditions, in cultures that were pre-treated with oestrogen. n=6 per group, per loading status, at each time point. * indicates significantly higher compared to static conditions of the same treatment group. ** indicates significantly lower than **E2**, with applied shear stress, at the same time point, + indicates significantly higher than **E2** under static conditions, at the same time point.

Effect of oestrogen pre-treatment on cell mineralisation: When the effect of pre-treatment on subsequent mineral production was evaluated, at day 7, higher mineralisation was observed with oestrogen pre-treatment, compared to previously untreated cells in the **E2** group, under applied shear stress (0.55 ± 0.11 vs. 0.06 ± 0.03, $p<0.02$) and in static conditions (0.27 ± 0.16 vs. 0.03 ± 0.02, $p<0.02$). Finally, after 14 days it was observed that pre-treatment with oestrogen caused significantly increased mineralisation compared to untreated cultures in the **E2** group with applied shear stress (1.18 ± 0.11 vs. 0.55 ± 0.19, $p<0.03$).

6.3.3 PGE$_2$ release

Effect of Pulsatile Fluid Flow on PGE$_2$ Release

As depicted in Figure 6.9, when pulsatile fluid flow (+PFF) was compared with static cultures (-PFF) at day 2, +PFF caused significantly higher PGE$_2$ release compared to static cultures. This was found both with continued oestrogen controls, **E2** (679.03 ± 212.62 vs. 50.94 ± 25.65, $p<0.01$) and with oestrogen deficiency, **E-** (547.05 ± 223.45 vs. 96.02 ± 32.68, $p<0.01$). Similarly, at day 7, there was significantly more PGE$_2$

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release in +PFF compared to static cultures with continued oestrogen treatment, **E2** (738.50 ± 158.42 vs. 23.17 ± 13.15, p<0.01) and in the oestrogen deficient group, **E-** (367.19 ± 306.27 vs. 37.84 ± 27.03, p<0.04).

Effect of Oestrogen Withdrawal on PGE$_2$ Release

Comparing oestrogen deficient cultures with continued oestrogen treatment, it was observed that in static cultures, there was no statistical difference between groups after either two or seven days. When cultures were subjected to PFF, there was no significant difference after two days of oestrogen withdrawal compared to continued oestrogen treatment, however, by seven days of oestrogen depletion there was significantly lower PGE$_2$ release in the oestrogen deficient group compared to continued oestrogen treatment, **E2** (367.19 ± 306.27 vs. 738.50 ± 1.58.42, p<0.01), indicating that the release of PGE$_2$ is significantly reduced with oestrogen withdrawal. There was no significant difference between time points (day 2 versus day 7) in cultures with applied PFF. However, it was found that under static conditions, PGE$_2$ release in oestrogen deficient cells at day 7 was significantly lower compared to day 2 (37.84 ± 27.03 vs. 96.02 ± 32.68, p<0.01), indicating that the release of PGE$_2$ is significantly reduced with increasing duration of oestrogen deficiency.
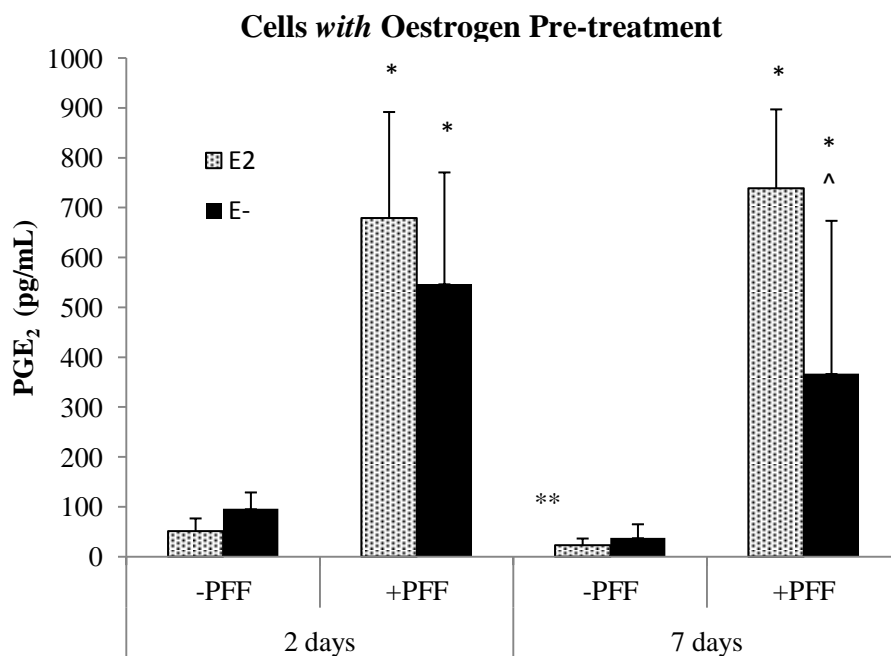
Figure 6.9 PGE$_2$ release in MC3T3-E1's undergoing oestrogen deficiency (**E-**) compared with continued oestrogen treatment (**E2**), with/without pulsatile fluid flow (PFF). n=6 per group, per loading status, at each time point. * denotes significantly higher compared to static conditions, ^ indicates significantly lower compared to

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continued oestrogen controls (**E2**). ** indicates significantly lower compared to the same group at two days.

6.4. Discussion

Oestrogen withdrawal has long been associated with rendering bones weak and more susceptible to fractures; however, the cellular mechanisms controlling such changes are still being delineated. This study demonstrates that fluid shear stress significantly enhances mineral matrix production by osteoblasts *in vitro*, particularly in cultures pre-treated with oestrogen. This study has shown for the first time that under applied loading, oestrogen deficiency imposed either by withdrawal of oestrogen or by blocking oestrogen receptors, causes significantly lower mineral production in cultures that had become accustomed to oestrogen compared to continued oestrogen exposure. Conversely, in cultures without oestrogen pre-treatment, higher mineral production was found when cells were prevented from oestrogen exposure, compared to those treated with oestrogen. Fulvestrant reduced cell viability of cultures and orbital shear stress reduced viability in cultures without oestrogen pre-treatment, but it had no affect on the viability of those with oestrogen pre-treatment. Most interestingly, significant reductions in PGE_2 release in response to mechanical loading were observed in oestrogen deficient cultures compared to those with continued oestrogen treatment. Furthermore, the reductions in PGE_2 release as a result of oestrogen withdrawal were amplified with increasing duration of oestrogen deficiency. Together, these findings support the hypothesis that the anabolic bone formation response of bone cells to mechanical loading is impaired during oestrogen deficiency.

There are a number of limitations associated with the current study that should be noted. Firstly, for orbital shear stress experiments all cell cultures underwent shear stress stimulation on rocker platforms, in laminar flow hoods at ambient temperature and gaseous conditions. Therefore, for the duration of the loading bouts (40 minutes daily) the cells were not cultured under conditions of 37˚C and 5% CO_2. However, static cultures were exposed to the same temperature and gaseous conditions, for the same durations as mechanically stimulated cultures, therefore, this limitation should affect both groups equally and direct comparisons between groups is still valid. A further limiting factor of this study design is the different means of shear stress application employed for the separate studies i.e. the orbital shear stress using the rotational rocker for mineralisation and viability studies, and the parallel plate flow system for PGE_2 studies. The rocker technique does not apply a uniform laminar stress across the entire

monolayer (Dardik *et al.,* 2005b) and although important differences were indeed detected in mineral production, this method was deemed unsuitable for the more sensitive biochemical studies. In addition, cells were plated at different densities in both set-ups, with a lower starting cell density in the longer duration rocker study, whereas, a higher cell density was employed for parallel plate experiments to ensure adequate cell coverage by two days of oestrogen deficiency. The advantages of employing the rocker apparatus initially, was the quick and easy method of attaining important information, regarding the mineral production of oestrogen deficient cells under applied loading. It permitted investigation of a range of treatment groups and the mechanisms behind the results obtained were then investigated using the parallel plate device. A limitation to note regarding the laminar flow study was that during the two hour loading sessions using the parallel plate device, a circulating volume of 150 mL of media was used, while the non-loaded static control cells were kept in 13 mL media. However, different quantities of media between loaded and non loaded cells could not have affected the results obtained since both groups were replenished with the same quantity of fresh media (6 mL) following each loading session and incubated for one hour, after which the biochemical release into media was measured.

In traditional parallel plate configurations the microscope slide with cell monolayer attached, forms the base of the channel, therefore, any flow entrance effects would be applied to a portion of the cell monolayer under investigation. The estimated entrance length of the flow through the flow chamber was 1.07mm, as calculated using equation 6.5. However, the entrance length calculated using ANSYS CFX was 20mm (Vaughan, 2011). Therefore, to ensure laminar flow was achieved prior to reaching the cell monolayer, slides were positioned greater than 20 mm beyond the entrance of the chamber. The estimated Reynold's number was calculated as 66.74 (Equation 6.4), ensuring laminar flow over the cell monolayer. In the parallel plate design presented in this chapter, three grooves were machined out of the base plate to accommodate three microscope slides in series, which lay flush with the base of the chamber. The advantages of this feature were twofold. Firstly, the entrance and exit flow effects in the chamber were eliminated and the flow was fully developed before reaching the cell monolayer on each of the microscope slides, thereby, ensuring that Poiseuille flow assumptions were valid over 100% of the length of the slides. Secondly, three slides allowed for optimal analysis, which overcame, to a degree, the inherent sample size constraints imposed by most parallel plate designs, thereby, reducing the requirement for

serial repetitive, experimental runs. The base plate has two entrance channels, which led to reservoirs through which media enters and exits the chamber. The reservoirs allowed the flow to steady after a change in direction, prior to entering the flow chamber.

The results of the current study emphasize the importance of mechanical stimulation on osteoblastic cell activity. They show that osteoblasts exhibited significantly higher PGE_2 release in response to applied shear stress. This finding is in agreement with other studies which have demonstrated the enhancing effect of mechanical stimulation on PGE_2 release by osteoblasts (Bakker *et al.,* 2001; Nauman *et al.,* 2001; Reich and Frangos, 1991) and osteocytes (Klein-Nulend *et al.*, 1995b; Reilly *et al.,* 2003). The enhancing effect of fluid shear stress on mineralised matrix deposition in this study is evident, in particular in cultures that were pre-treated with oestrogen. A previous study demonstrated no distinguishable effects of shear stress on mineral production (Nauman *et al.*, 2001) however. In that study, cells were cultured statically for 14 days, prior to shear stress initiation, therefore, mineral matrix production was already well advanced and subsequent differences in mineral production as a consequence of shear stress may have been undetectable (Nauman *et al.*, 2001). Furthermore, the height of the cell colonies formed after 21 days may have been significant in relation to the chamber height (220μm), in which case the assumptions of Poiseuille flow may no longer have been valid (Nauman *et al.*, 2001). The finding of higher mineral production presented in this study, is in agreement with studies that demonstrated enhanced mineral matrix production in 3D scaffolds with increasing fluid shear stress *in vitro* (Sikavitsas *et al.*, 2005; Sittichockechaiwut *et al.,* 2009; Van den Dolder *et al.,* 2003). In addition, the enhancing effect of mechanical stimulation on *in vivo* bone formation has been well established (Chambers *et al.*, 1993; Forwood *et al.,* 1996; Robling *et al.*, 2000; Turner *et al.*, 1996).

In this study, it has been shown for the first time, that when cells are subjected to mechanical stimulation, mineral production is significantly lower with oestrogen withdrawal compared to cultures with continued oestrogen exposure. The enhancing effect of oestrogen treatment in unison with physiological loading on bone formation has been well documented: oestrogen increases bone mineral density, trabecular bone volume, bone mass and the degree of bone mineralisation in postmenopausal females, (Boivin *et al.*, 2005; Khastgir *et al.*, 2001; Wahab *et al.*, 1997) and also enhances bone formation in animal studies (Chow *et al.*, 1992; Edwards *et al.*, 1992). Together, these studies demonstrate that *oestrogen treatment* is capable of exerting an anabolic effect on

bone and indicate that oestrogen stimulates osteoblast activity, which is in agreement with the findings of the current study. However, altered mineral production as a direct consequence of *oestrogen withdrawal in vitro* has not been demonstrated previously. Furthermore, this study has demonstrated that with applied loading, blocking oestrogen receptors with fulvestrant, an oestrogen antagonist, also significantly reduces mineralisation, in comparison to continued oestrogen treatment. However, lower viability and mineral production with fulvestrant compared to oestrogen withdrawal was also observed, indicating that a down-regulation of oestrogen receptors impairs cell activity to a greater degree than simply withholding oestrogen. Similar to Chapter 5, it was found that pre-treatment with oestrogen significantly altered cell activity compared to untreated cultures. For example, in oestrogen pre-treated cultures under loading, lower mineral production was found when oestrogen was withdrawn, compared to continued oestrogen treatment, whereas, in previously untreated cells during loading, lower mineral production was observed with oestrogen deficiency, compared to oestrogen exposure.

Interestingly, this study highlights the necessity for *both* mechanical stimulation and hormone balance in unison to maintain bone formation. This is evidenced from our studies on pre-treated cultures where oestrogen treatment under applied loading caused significantly higher mineralisation compared to both 1) oestrogen withdrawal under mechanical loading and 2) oestrogen treatment of non-loaded static cultures. The requirement for both oestrogen and mechanical stimulation may explain why female amenorrheic (lack of menstrual cycle) runners who place ample mechanical loading on their bones, but who lack oestrogen circulation, suffer from bone fragility (Cobb *et al.*, 2003). On the other hand, this may also shed light on why astronauts, with normal hormone circulation and lack of mechanical forces on their bones, suffer from weak bones characteristic of osteoporosis (Schneider *et al.*, 1995).

Most interestingly, this study has shown that the mechano-responsiveness of osteoblasts, in terms of PGE_2 release, is impaired during oestrogen deficiency. A previous study showed that bone cells from osteoporotic donors have reduced PGE_2 release, compared to those from healthy donors (Sterck *et al.*, 1998). However, as noted by the authors, it was 'unclear whether the abnormal response of the osteoporotic cells was the cause of the osteoporotic status, or whether it resulted from the osteoporotic status'. The results of the current study have explained, in part, these particular findings by revealing that the mechano-sensitivity of osteoblast-like cells became impaired as a direct consequence

of oestrogen withdrawal. Furthermore, by showing that increasing duration of oestrogen withdrawal exacerbated the impaired response to mechanical loading, this study suggests that the abnormal response of osteoporotic cells found by Sterck et al may occur as a direct result of being exposed to prolonged oestrogen deficiency and not that the abnormal response resulted from the osteoporotic status. It is interesting to speculate on the mechanisms behind such altered biochemical responses. Mechano-sensing by primary cilia, which are solitary organelles that project from the cell surface, is mediated by cyclic adenosine monophosphate (cAMP) (Kwon *et al.*, 2010) and they are required for flow induced PGE_2 release (Malone *et al.*, 2007).

In addition, PGE_2 induces the production of AMP in bone cells (Scutt *et al.*, 1995); therefore, the up-regulation of AMP with shear stress could occur indirectly via a mechanism involving increased prostaglandin production with shear stress. A reduction in PGE_2 release by oestrogen deficient cells may therefore represent an impairment of this signalling mechanism, or decreased primary cilia numbers. Furthermore, shear stress imposed on the cell membrane by fluid flow would confer drag forces to the glycocalyx, or pericellular matrix, the composition of which varies according to cell function (Rambourg and Leblond, 1967). Therefore, it could increase or decrease cell sensitivity to shear stress. Indeed, when the glycocalyx of osteocytes was degraded with hyaluronidase treatment, it was found that the shear stress induced PGE_2 release was eliminated (Reilly *et al.,* 2003). Is it possible therefore, that oestrogen acts to maintain mechano-sensitivity by acting on the glycocalyx? Further studies are required to delineate this question.

The lower PGE_2 release levels under loading found in oestrogen deficient cell cultures in the current study is in agreement with the lower mineral production observed during mechanical loading in oestrogen deficient cultures. In fact, the lower PGE_2 release may contribute to the impaired mineralisation accompanying oestrogen withdrawal since PGE_2 has been shown to be a potent stimulator of mineral production *in vitro* (Kaneki *et al.,* 1999; Nagata *et al.,* 1994; Sakamoto *et al.,* 2003; Keila *et al.,* 2001) and *in vivo* (Mori *et al.,* 1992; Weinreb *et al.,* 1997; Keila *et al.,* 2001). Exposure of fetal and adult rat calvarial cells, as well as human dental pulp cells to PGE_2, evoked a significant increase in ALP and mineral production *in vitro* (Kaneki *et al.,* 1999; Nagata *et al.,* 1994; Sakamoto *et al.,* 2003; Keila *et al.,* 2001). Similarly, administration of PGE_2 increases bone mass, density and mechanical strength in young rats (Weinreb *et al.,* 1997) as well as bone formation and bone area in aged rats (Keila *et al.,* 2001). Most

interesting in the context of the current study, previous research showed that PGE_2 administration to ovariectomized rats restored bone area to levels of age matched sham operated animals as well as increasing mineral apposition and bone formation rates (Mori et al.,1992).

In conclusion, this study has shown that oestrogen withdrawal inhibits the mechano-responsiveness of osteoblasts by reducing biochemical signalling, which may be responsible for the reduced mineralisation found in oestrogen deficient cells under applied loading. This study has also highlighted the synergistic effects of oestrogen and mechanical loading on bone cell activity. Impaired capacity of bone cells to respond to mechanical stimulation may be the underlying cause of abnormal bone metabolism and consequential increased fragility associated with osteoporosis. Therefore, understanding the cellular mechanisms involved in impaired mechanically driven bone adaptation, by either de-synthesizing bone cells to mechanical loading or inhibiting biochemical signal responses are vital for the development of adequate therapeutic interventions.

7. Conclusions

7.1. Main Finding of the Thesis

The research outlined in this thesis has focused on investigating bone tissue mineral alterations during osteoporosis by analyzing animal tissue from an ovariectomised model and a drug treated cohort over the course of ageing. Static and dynamic *in vitro* cell culture experiments during oestrogen withdrawal were also performed to investigate the role of oestrogen deficiency on bone mineral production, apoptosis, proliferation, and mechano-biological responsiveness. The primary findings of the thesis are presented in this chapter, together with recommendations for future studies.

1) The first study of this thesis entailed investigating trabecular bone mineral distribution during oestrogen deficiency in an animal model of osteoporosis. Site specific changes in mineral heterogeneity occurred within trabeculae as a consequence of oestrogen withdrawal. Furthermore, alterations in tissue mineral distribution occurred along the inter-trochanteric fracture line, the most common osteoporotic fracture site. This finding is of immense importance since it demonstrates the fact that local factors have a significant impact on bone tissue during oestrogen depletion. Therefore, it highlights that those analytical methods, which only evaluate the mean mineral content of bone tissue, cannot detect important tissue level mineral alterations. Furthermore, this study indicates that in addition to important changes in bone mass and micro-architecture during osteoporosis, altered mineral distribution may be a contributing factor for weakened bone at an increased risk of fracture.

2) The second study of this thesis focused on evaluating the effects of ageing, prolonged oestrogen withdrawal, and bisphosphonate drug treatment on tissue level mineral distribution. This study found that mineralisation is higher with increasing age in healthy sheep (12 month CON versus 31 month CON), and that long term oestrogen deficiency reduces the calcium concentration of ovine bone tissue. It was observed that Zoledronic acid treatment during oestrogen deficiency led to a more homogeneous calcium distribution both within trabeculae and between femoral regions. It is proposed that these changes in mineral distribution may contribute to the ability of Zoledronic acid treatment to prevent fracture occurrence during oestrogen deficiency. Together, these findings

are of potential importance in understanding the underlying mechanisms of altered mechanics during osteoporosis and the long-term efficacy of drug treatment.

3) The third study of the thesis sought to delineate the mechanisms responsible for the altered mineral distribution *in vivo* during oestrogen deficiency detected in Chapters 3 and 4. It was observed that oestrogen pre-treatment promoted bone cell mineral production, and that bone cells became accustomed to their oestrogen environment and responded to oestrogen withdrawal by increasing mineral production. Furthermore, the apoptosis induction effects of oestrogen withdrawal from osteocytes have been highlighted, which might play a role in altered tissue mineral heterogeneity. The preventative effect of oestrogen on caspase 3/7 activation by apoptotic stimuli was also reported. Increased apoptosis during oestrogen deficiency may have important implications for osteoporotic patients, as it may compromise the osteocyte network, leading to impaired mechano-responsiveness and increased fragility.

4) The final study in this thesis investigated whether bone cells ability to adaptively respond to mechanical stimulation is impaired during oestrogen deficiency. This entailed the development of a custom parallel plate configuration to confer mechanical loading to bone cells *in vitro*. Under applied shear stress, significantly less mineralisation was observed when oestrogen was withdrawn from cells, compared to continued oestrogen exposure. Furthermore, this study showed that the mechano-responsiveness of osteoblasts, in terms of PGE_2 production, is impaired during oestrogen deficiency. This finding may explain the observed reduction in mineral production since PGE_2 is an important regulator of bone formation. Together, these studies have shown altered osteoblast activity during oestrogen deficiency, which may contribute to the lower mineralization seen in the *in vivo* sheep study, and increase our understanding of the etiology of osteoporosis.

7.2. Future Work

Based on the findings of this thesis the following recommendations are made for future research;

7.2.1 SEM and CT Scanning of Trabecular Bone Tissue at Early Time Points Following Oestrogen Withdrawal

It was interesting to find that although increased heterogeneity of bone tissue occurred 12 months post-ovariectomy, no change in mean tissue mineral concentration was found. In contrast, after 31 months of oestrogen deficiency, significantly lower mean mineralisation was found. This highlights that duration of oestrogen deficiency has important consequences for bone fragility. A study that would investigate this observation further is advised. An SEM study, which could track the material level mineral changes, paralleled with an investigation into the structural changes in trabecular bone tissue using CT scanning, at earlier time points following oestrogen withdrawal is advised. This could help to delineate whether altered tissue composition leads to bone tissue depletion, or whether altered mineralisation is a compensatory mechanism for the compromised trabecular architecture, which is characteristic of osteoporosis.

7.2.2 Mechanoreceptor Expression during Oestrogen Deficiency: Primary Cilia

It has been shown that oestrogen withdrawal altered mineralisation *in vivo* and impaired cell responses to mechanical loading; however, the mechanisms by which oestrogen deficiency imposes these changes are unknown. Several means of bone cell mechano-sensing have been explored in previous studies, and as discussed in section 2.4.1.1 and 2.4.3.1, it is believed that cytoskeleton/integrin/cell processes interactions, in addition to the functions of the glcocalyx, are responsible (Pavalko *et al.,*1998; Reilly *et al.,* 2003; Toma *et al.,* 1998;). In addition, it has long been recognised that primary cilia act as sensory organelle in kidney cells and sense fluid flow (Liu *et al.*, 2005; Praetorius and Spring, 2001), however, it was only recently demonstrated that mechano-responsiveness of both osteoblast and osteocyte cells, in terms of PGE_2 production, osteopontin mRNA levels, and cyclooxygenase 2 (COX2) gene expression, is dependent on the presence of primary cilia (Malone *et al.,* 2007). A further study identified a molecular mechanism responsible for primary cilium-dependent mechano-sensing involving adenylyl cyclase 6 (AC6) and cAMP (Kwon *et al.*, 2010). However, the functions and physiology of primary cilia in bone diseases have not yet been investigated. There is evidence that endometrial cells alter the quantity and length of microvilli and primary cilia according to oestrogen concentration or availability (Anderson *et al.*, 1975; Craig and Jollie, 1984; Rambo and Szego, 1983). Therefore, oestrogen deficiency might also alter microvilli and primary cilia on bone cells and since primary cilia are required for mechano-sensation, their down-regulation during oestrogen deficiency might be responsible for

the impaired mechano-responsiveness in terms of PGE$_2$ releases and mineral formation that was found in Chapter 6. In this way, during osteoporosis bone's ability to adaptively respond to biophysical loads may be impaired, in a manner similar to unloading or disuse. It is therefore recommended that a thorough investigation of the effects of oestrogen deficiency on primary cilia expression in bone cells be performed and compared to the alterations in biochemical responses, as a result of oestrogen deficiency.

7.2.3 Co-culture of Bone Cells with Loading and Oestrogen Deficiency

Although important findings of oestrogen deficiency on the apoptosis of osteocytes and the bone formation capabilities of osteoblasts *in vitro* have been demonstrated, bone physiology *in vivo* involves the interaction of various cell types. Yet few studies investigate mechanisms of bone loss using a co-culture system, most opt for monolayer cultures of one cell type. Functional gap junctions between neighbouring osteocytes were discovered, in addition to gap junctions between osteoblasts and osteocyte cells (Yellowley *et al.*, 2000). Furthermore, the ability of osteocytes to communicate a mechanically induced calcium response to osteoblasts was demonstrated. Therefore, although the effects of oestrogen deficiency on osteoblast cells under static conditions and with applied loading *in vitro* have been determined, the vital interplay of biochemical signalling between cell types is lost in this environment. Therefore, it is recommended to co-culture oestrogen deficient osteocyte and osteoblast cells with applied mechanical loading in order to further understand the underlying cellular mechanisms of altered tissue mineral distribution during oestrogen deficiency, presented in Chapters 3 and 4.

7.3. Conclusion

In conclusion, this thesis has presented experimental work carried out throughout the course of the author's PhD studies in the field of tissue mineralisation and cell mechano-biology during oestrogen deficiency. A comprehensive literature review of the current state of research in this area was firstly presented, together with the rationale for the studies performed. The findings of each study and their implications were discussed in detail in each chapter.

These studies sought to determine whether bone tissue mineral was altered as a consequence of oestrogen deficiency and found firstly that changes in tissue mineral heterogeneity were site specific. This coincided with important osteoporotic fracture lines, indicating that altered mineral distribution may contribute to increased fracture

risk during osteoporosis. Secondly, it was found that mineral concentration is higher in more aged healthy sheep than in younger healthy sheep, but conversely is reduced with prolonged oestrogen deficiency which may lead to diminished strength and fracture resistance. Zoledronic acid treatment of ovariectomised animals significantly homogenized bone tissue, both within individual trabeculae and between proximal femora sites, suggesting that altered mineral distribution during bisphosphonate treatment may contribute to reduced risk of fracture associated with its administration.

In order to understand the tissue level alterations in mineralisation found in Chapters 3 and 4 of this thesis, cell culture experiments were carried out and presented in Chapters 5 and 6, in which oestrogen was withdrawn from osteoblast and osteocytes and bone formation, proliferation, apoptosis, and mechano-sensitive was assessed. In static cultures, oestrogen withdrawal from cells causes an elevation in mineral production, however under applied loading conditions, oestrogen withdrawal reduced mineral production, most likely due to the reduced mechano-responsiveness of bone cells found in Chapter 6 of this thesis. Together, these studies have contributed significantly to a greater understanding of the etiology of osteoporosis, and have proposed many further questions, therefore paving the way for future studies aimed at delineating the cellular mechanisms responsible for osteoporosis.

8. Appendices

8.1. Appendix 1: Design Drawings of Parallel Plate Chamber

8.2. Appendix 2: Estradiol and Progesterone Levels in Sheep

The average, minimum and maximum estradiol and progesterone levels measured in the serum of control and ovariectomized animals from the 12 month group are presented in Table 8.1. Figures 8.1 and 8.2 show typical estradiol and progesterone cycles for one control and one ovariectomised sheep. These measurements were not gathered in the course of this PhD and are from the PhD thesis of Dr. Orlaith Brennan, Department of Anatomy, Royal College of Surgeons in Ireland (RCSI), Dublin 2, Ireland, who was directly involved in the attainment of this data. They are included in this thesis as requested, to demonstrate the success of the ovariectomy procedure in reducing the level of circulating hormones in these sheep.

Table 8.1 Mean, min and max estradiol and progesterone levels in 12 month control and OVX animals.

12 Month Post-OVX	Estradiol (pg/ml)			Progesterone (mg/ml)		
	Mean	**Min**	**Max**	**Mean**	**Min**	**Max**
Control	0.848	0.588	1.351	3.781	1.183	11.429
OVX	0.351	0.0314	0.760	0.596	0.159	1.114
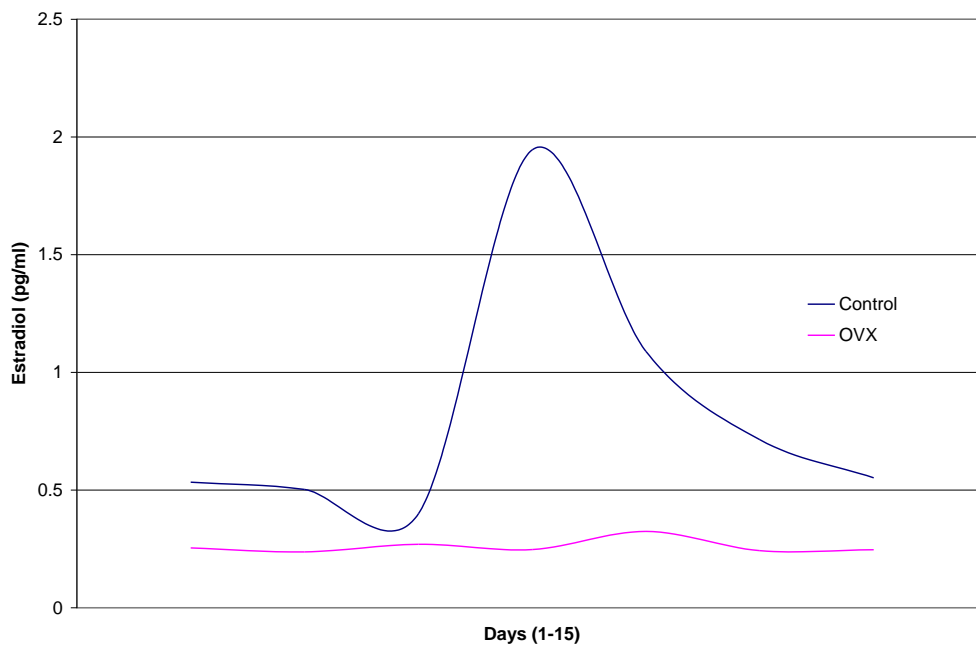
Figure 8.1: Average estradiol cycles in a 12 month control and ovariectomised animal.
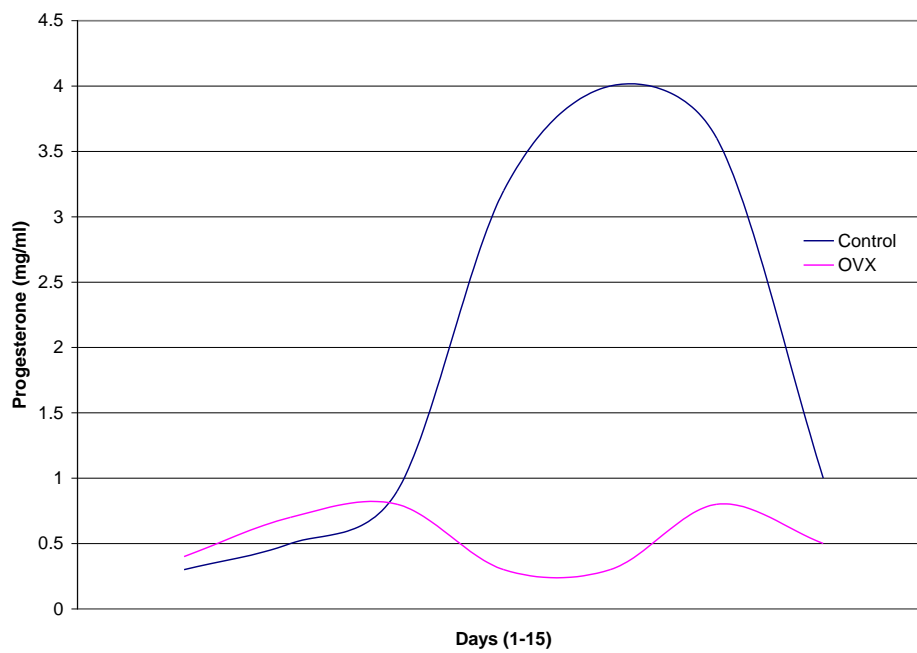
Figure 8.2: Average progesterone cycles in a 12 month control and ovariectomised animal.

8.3. Appendix 3: Standard Curves
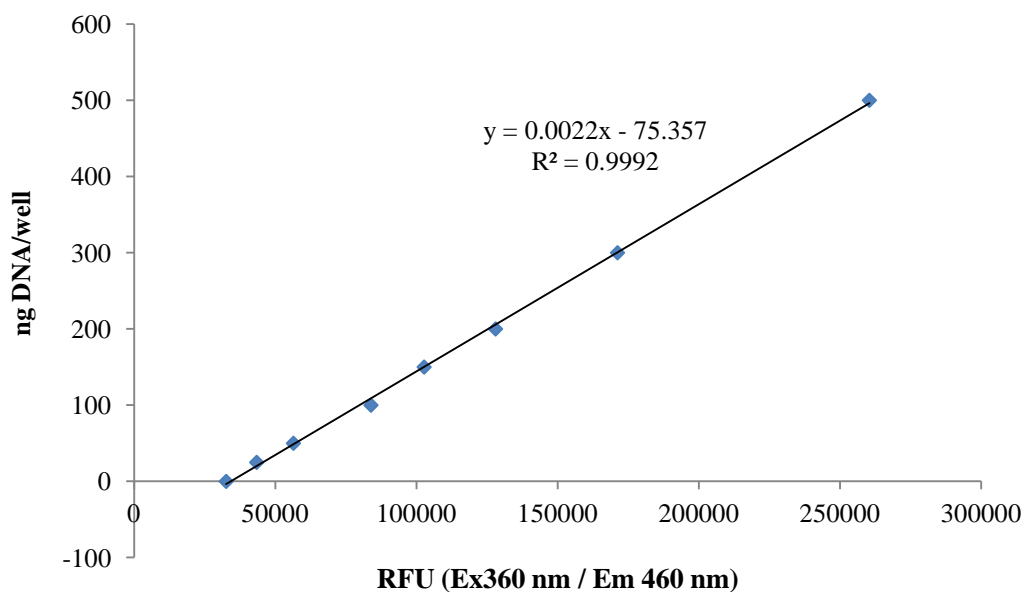
Figure 8.3 Standard curve of serial dilution of known quantities of DNA content (calf thymus) measured on a microplate reader at exitaton 360nm and emission 460nm.
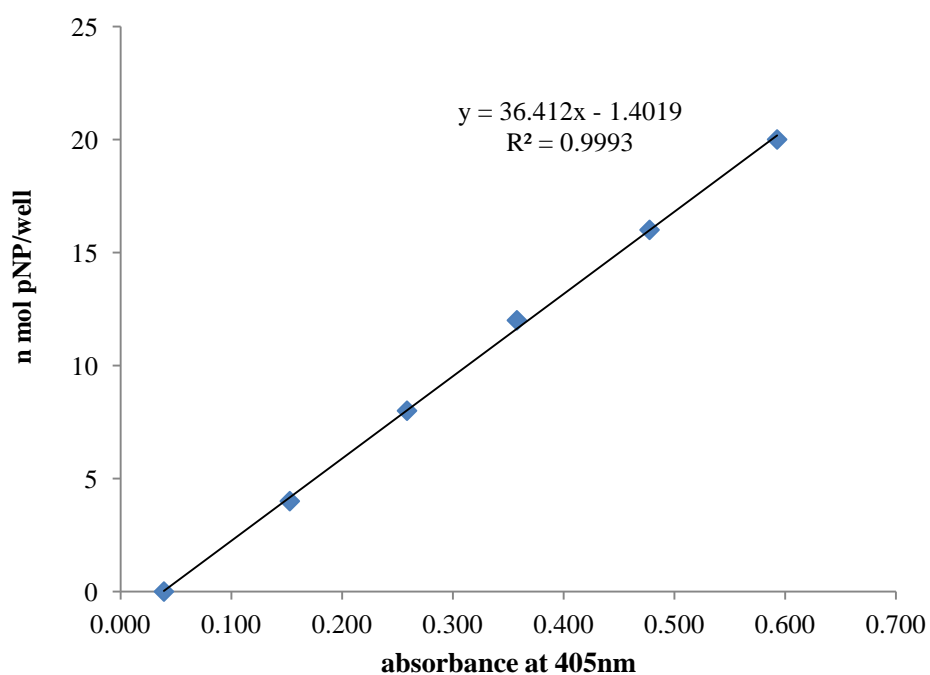
Figure 8.4 Standard curve of known values of pNP per well measured on a microplate reader at an absorbance of 405nm. ALP activity (U/ml) is determined as; A/V/T, where A is amount of *p*NP generated by samples (in μmol), V is volume of sample added in the assay well (in ml) and T is reaction time (in minutes).
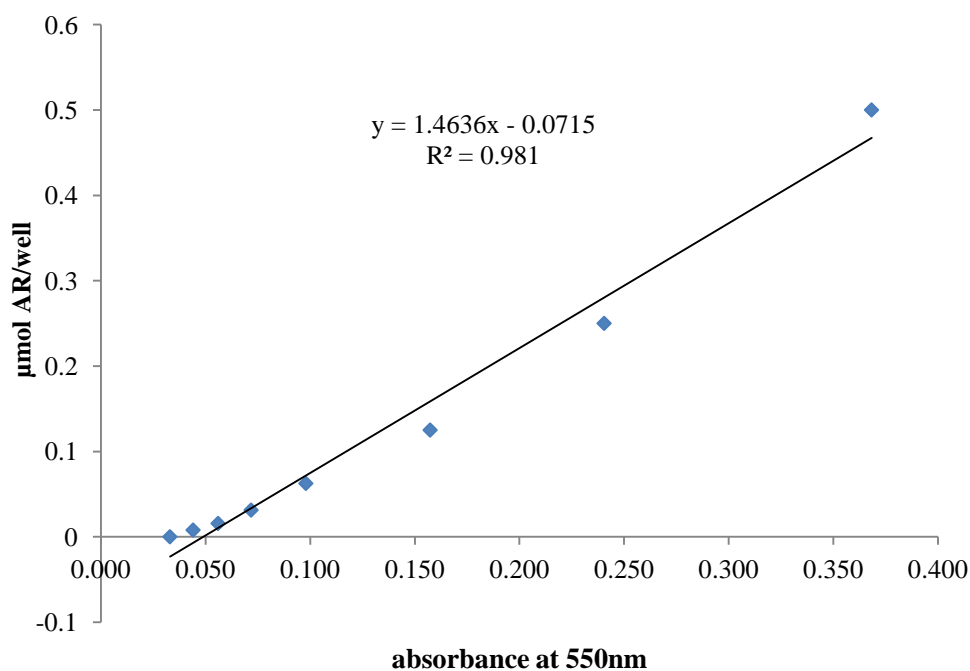
Figure 8.5 Alizarin red standard curve created by serial dilutions of a known quantity of alizarin red, which were measured on a microplate reader at 550nm. Moles of alizarin red were subsequently converted to moles of calcium; one moles of alizarin red binds to two moles of calcium (Norgaard et al., 2006). Mineral production was then normalized to DNA content and expressed as n moles Ca/pg DNA.
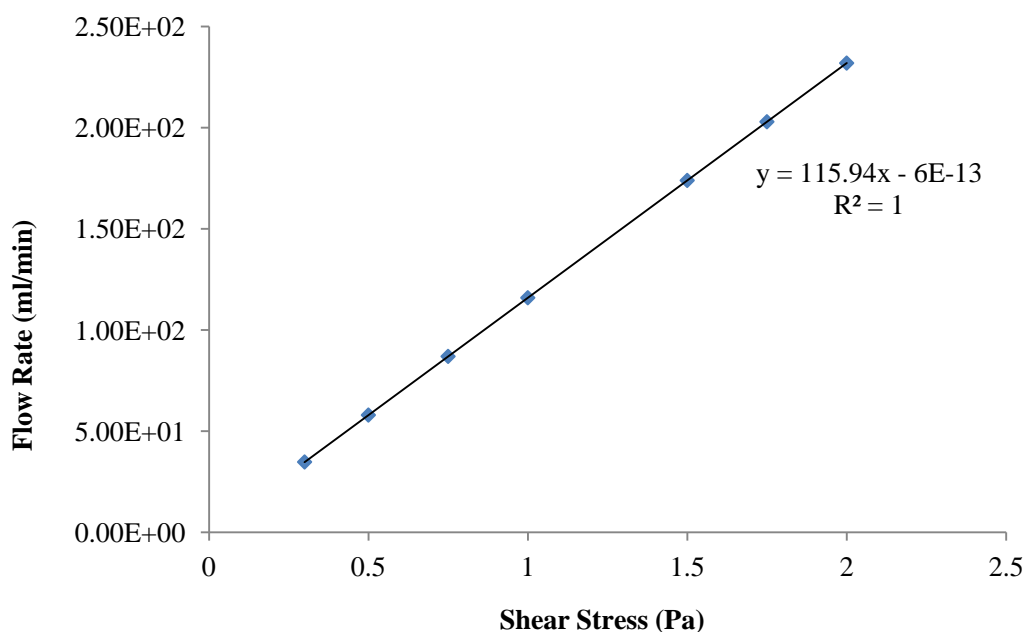
Figure 8.6 Relationship between shear stress and flow rate within the parallel plate flow system configuration. Flow rate was measured using a Ts410 transit time tubing flow meter and flow probe (Transonic Systems). Shear stress was calculated using equation 6.2.

9. References

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