1-1-2010

Microdamage and remodelling in an ovine model.

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Microdamage and Remodelling in an Ovine Model

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A thesis submitted to the National University of Ireland for the degree of

Doctor of Medicine

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May 2010

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DECLARATION

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree (MD), is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the test such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Chapter 1

Introduction and Literature Overview

A Bit of History

Osteoporosis has haunted women since the dawn of history. Egyptian mummies from 4,000 years ago have been found with the familiar dowager's hump or curved spine. Ask anyone to portray an old woman and the first thing that most would do is stoop over. Old paintings of Greek myths and fairy tales have this classic posture associated with the elderly female.

An early medical pioneer, the eighteenth century English surgeon John Hunter (Fig 1), described himself as one who "pestered people with questions about what nobody knew or cared anything about". One of these questions was how does bone grow and develop over time? Hunter's experiments on animals, along with observations of changes in the human jaw, led him to a surprising discovery: As new bone was laid down in the body, old bone is destroyed, or resorbed. This discovery that bone is constantly being remodelled predated widespread use of the microscope and the

notion of cells as the workhorses of the body (Murray 1997).

Figure 1.1 (John Hunter)

A step towards the recognition of osteoporosis was made in the 1830s by the French pathologist Lobstein, over a hundred years after Hunter's death. Lobstein noticed that some patient's bones were riddled with larger than normal holes, and he coined the term osteoporosis (porous bone) to describe such deteriorated human bone.

Early in the last century, doctors assumed it was a natural consequence of age or immobility that caused the deterioration of bone found in osteoporosis. It was a clinical researcher, Fuller Albright (Fig 2) of Massachusett's General Hospital who was puzzled by the fact that so many of his patients with osteoporosis were older women who had gone through the menopause. A few of his patients were in their thirties or forties, but these women shared something with the older women with osteoporosis, they too were in effect postmenopausal, because their ovaries had been

removed. This is what lead to him asking the question of what it was about being postmenopausal that made women particularly susceptible to have frail bones.

Figure 1.2 (Fuller Albright)

In 1934 a pair of anatomists noticed that the bones of ovulating female pigeons were larger than those of the male pigeon. Could the hormone oestrogen, produced chiefly by the ovaries, be causing the difference in bone mass? Researchers at Yale University explored this possibility. They injected oestrogen into male pigeons, the bird's bone mass increased dramatically. Prompted by these findings, in 1940 Albright proposed his revolutionary hypothesis: Oestrogen triggers the build up of calcium reserves in bone, from which calcium can be released into the bloodstream during pregnancy and lactation to serve the needs of the foetus and newborn. The sharp reduction in oestrogen that occurs with menopause causes a loss of bone, by enabling more bone to be broken down than is subsequently built up. Women whose skeletons long outlive the functioning of their ovaries often reach the point of having

too little bone, which makes them susceptible to bone fractures. Albright named the resulting condition postmenopausal osteoporosis.

To support his hypothesis, Albright cited his laboratory findings, which showed that postmenopausal women excrete more calcium and phosphate (major components of bone mineral) in their urine, blood, and stools than they consume in their diet. He also showed that regular injections of oestrogen reserved the calcium imbalance, boosting the amount of calcium retained in the body, presumably in the bones. Not only did Albright identify postmenopausal osteoporosis, he also offered the first treatment for the condition, by means of oestrogen replacement therapy. (Patlak, 2001).

Bone Cells

Four type of cells are found in bone tissue, each having specific functions related to the formation, resorption, and remodelling of bone.

1) The osteoprogenitor cell, which ultimately differentiates into osteoblasts and osteocytes, is itself derived from mesenchymal cells, which can develop into adipocytes, myloblasts, fribroblasts, or osteoblasts (Fig 1.3). Osteoprogenitor cells are found in marrow, periosteum, and all supporting structures within the marrow cavity. They are not readily recognized by light microscopy as they are small, non-specific, stellate or spindle-shaped cells. In response to an appropriate signal, the osteoprogenitor gives rise to an osteoblast.

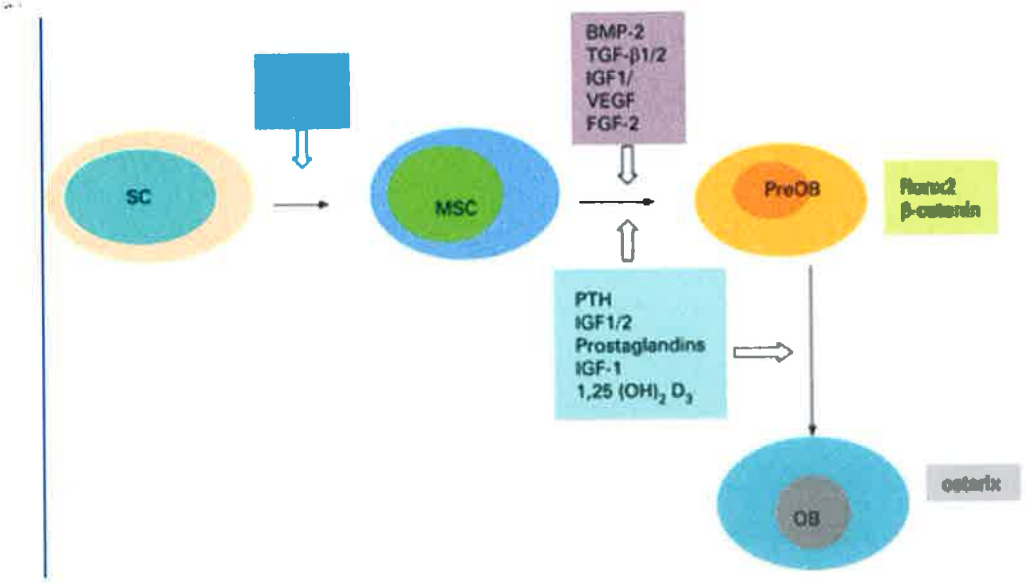


Fig 1.3 Osteoblast differentiation from stem cell involves coordinated interaction among diverse endocrine, paracrine and autocrine factors. SC, stem cell; MSC, mesenchymal stem cell; OB, osteoblast. (www.roche.com)

2) Osteoblasts are the protein-synthesizing cells that produce organic bone tissue. These large mononucleur and polygonal cells are arrayed in a line along the bone surface. Underlying the layer of osteoblasts is a thin, eosinophilic zone of organic bone matrix that has not yet been mineralized which is known as osteoid. The time from the deposition of osteoid to its mineralization is known as the mineralization lag time. Its protein synthetic capacity is reflected in its abundant endoplasmic reticulum, prominent Golgi, and mitochondria with calcium containing granules. Cytoplasmic processes that extend into the osteoid contact cells embedded in the matrix, called osteocytes. The syncytium of osteocytes and osteoblasts probably prevent bone calcium (99% of the body's calcium) from equilibrating with the general extracellular space. When an osteoblast is inactive, it flattens on the surface of bone tissue. It contains alkaline phosphatase, manufactures osteocalcin, and has parathyroid hormone (PTH) receptors. Collagenase secreted by osteoblasts may also facilitate osteoclastic activity. Finally, a number of growth factors, including transforming growth factor β (TGF-β), insulin like growth factor-I (IGF-I), IGF-2, platelet derived growth factor (PDGF), interleukin-1 (IL-1), fibroblast growth factor (FGF), and tumour necrosis factor-α (TND-α), are produced by osteoblasts and are important in regulating bone growth and differentiation.

3) The osteocyte is an osteoblast that is completely embedded in bone matrix and is isolated in a lacuna. Osteocytes deposit small quantities of bone around lacunae, but

with time they lose the capacity for protein synthesis. They have numerous cell processes that extend through bony canals, called canaliculi and communicate with those from other osteocytes. Evidence suggests that osteocytes may be the bone cell that recognize and respond to mechanical forces (Taylor et al. 2007)

4) Osteoclasts are the bone-resorption cells. They are of haematopoietic origin, being a member of the monocyte / macrophage family. Three major factors are required for osteoclastogenesis: (1) TNF-related cytokine, (2) RANK ligand (RANKL; growth factor CSF-1d), (3) the activation of RANK on the surface of haematopoietic precursor cells. Binding of RANKL to RANK activates NF-kb signalling, which leads to increased osteoclastogenesis. Osteoclasts are multinucleated and contain many lysosomes that are rich in hydrolytic enzymes. They are found in small depressions, termed Howship's lacunae, on bone surfaces (Fig 1.4). Verified using electron microscopy, they have a polarized ruffled plasmalemmal membrane when the cell is in contact with and, is actively degrading, bone. Osteoclastic resorption is a multistep process that involves attachment of the cell to bone by integrins. A tight, gasket-like seal isolates an extracellular compartment that forms between bone and the ruffled osteoclast membrane (Haltrop, 1977 & Salo, 1996). A proton pump then acidifies this compartment to a pH of 4.5, in effect creating a giant extracellular lysosome. This protein rich environment mobilizes bone mineral, thereby exposing the organic bone matrix to degradation by lysosomal enzymes. Degraded fragments

of bone are transported to the opposite side of the osteoclasts and then released to the extracellular space.

Although the machinery of an osteoclast is superbly suited for bone resorption, it functions only if the matrix is mineralized. .In fact, any bone that is lined by osteoid or unmineralized cartilage is protected from osteoclastic activity.

Constant remodelling of bone is a normal part of skeletal maintenance and is initiated by activation of the cytokine receptor RANK on osteoclasts. Soluble factors released during resorption and PTH aid in recruitment of osteoblasts to the site and their activation to form new bone. Thus, bone remodelling involves replacing old bone with newly formed bone via the functional coupling of osteoclasts and osteoblasts, termed the bone remodelling unit (BMU) (Frost et al. 1973). Bone remodelling enable bone to adapt to mechanical stress, maintain its strength and regulate calcium homeostasis.

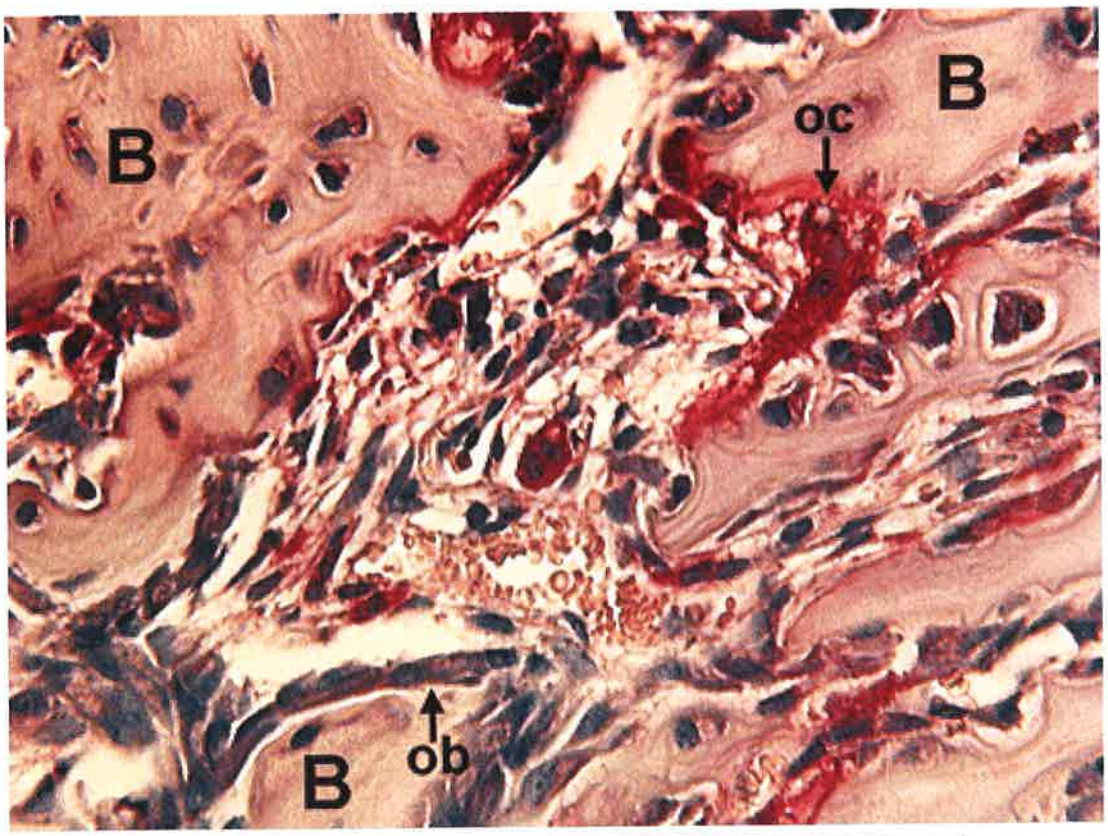


Figure 1. 4 Osteoclasts (oc), which are stained red in this microscopic section of bone (B), are responsible for the breakdown of bone. Their multiple nuclei appear as darkly stained bodies within the cell. Osteoblasts (ob) are the rectangular-shaped cells, each with a single prominent nucleus, lining the surface of the bone. These bone-building cells appear dark in color due to the presence of numerous ribosomes, which are structures in cells that synthesize protein. *Jonathan Lam, Washington University School of Medicine, St. Louis, MO.* (www.faseb.org)

Types of Bone Tissue

Compact and Trabecular –

Trabecular bone (also known as cancellous or spongy bone) has a porous structure consisting of 'rod' or 'plate-like' struts called trabeculae. Each strut and plate is composed primarily of lamellar bone and occasionally trabecular plates made up of osteonal bone can be found. Trabecular bone is found in the medullary cavity of flat and short bones, and in the epiphysis and metaphysis of long bones. The microarchitecture of trabecular bone appears random, however the connections and orientation of the trabeculae are found to have precise patterns which are thought to be related to the specific mechanical properties of the bone (Wolff, 1892).

The primary difference between the mechanical properties of trabecular and cortical bone is their effective stiffness at the macroscale level. Trabecular bone is more compliant than cortical bone and it is believed that it distributes and dissipates the energy from articular contact loads. Trabecular bone contributes approximately 20% of the total skeletal mass within the body while cortical bone contributes the remaining 80%. However, trabecular bone has a much greater surface area than cortical bone. Within the skeleton, trabecular bone has a total surface area of 7.0×10^6 mm^2 while cortical bone has a total surface area of 3.5×10^5 mm^2.

There are no blood vessels within trabeculae but the pores in the structure are filled with red marrow which provides the vital nutrients that bone requires. There are two types of bone marrow : red (consisting mainly of hemaopoietic tissue) and yellow marrow (consisting mainly of fat cells). Red blood cells, platelets and most white blood cells arise in red marrow. At birth, all bone marrow is red. With age, more and

more of it is converted to the yellow type. Red marrow is found mainly in the flat bones such as the pelvis, sternum, skull, ribs, scapula and vertebrae. Also in cancellous material at the epiphyseal ends of long bones such as the femur. Yellow marrow is found mainly in the hollow interior of the long bones. As with compact bone, a lot of research has been carried out on trabecular bone in recent decades (Carter, 1976; Gibson, 1985; Kaplan, 1985; Goldstein, 1987; Keaveny, 1994). However, there is a lot that has yet to be defined and understood about the behaviour of trabecular bone such as the reaction to tension and shear stresses, the behaviour of individual trabeculae and the interaction between compact and trabecular bone.

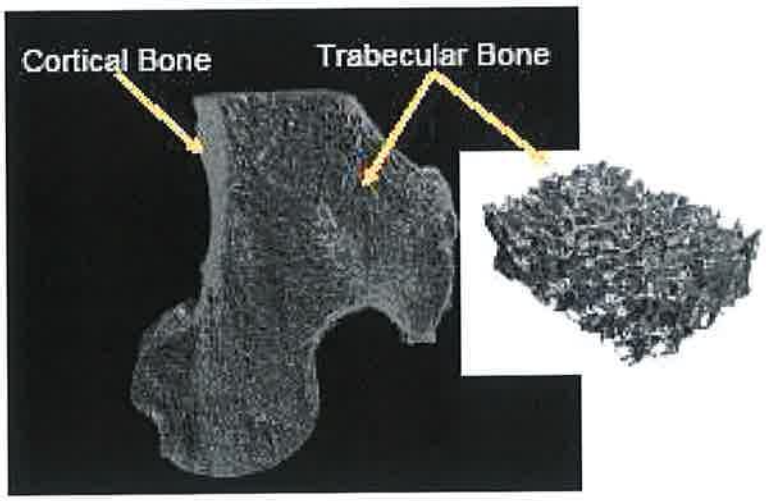


Figure 1.5

Lamellar and Woven -

1) Lamellar bone is made slowly and is highly organized. As the stronger bone tissue, it forms the adult skeleton. Anything other than lamellar bone in the adult skeleton is abnormal. Lamellar bone is defined by: (1) a parallel arrangement of type I collagen fibres, (2) few osteocytes in the matrix, and (3)

uniform arrangement of osteocytes in lacunae parallel to the long axis of the collagen fibres.

There are four types of lamellar bone:

- Cirumferential bone forms the outer periosteal and inner endosteal lamellar envelopes of the cortex.
- Concentric lamellar bone is arranged around Haversian canals. In two dimensions, concentric lamellar bone and its Haversian artery and vein constitue the osteon. In three dimensions, osteons comprise the Haversian system. These cylinders of bone around the Haversian canals run parallel to the long axis of the cortex. The osteons form only if there is appropriate stress. Thus, a paralyzed limb has a cortex composed exclusively of poorly formed Haversian systems and circumferential lamellar bone (Rubin, 2008).
- Interstitial lamellar bone represent remnants of either circumferential or concentric lamellar bone that have been remodelled and are wedged between the osteons.
- Trabecular lamellar bone forms the course cancellous bone of the medullary cavity. It exhibits plates of lamellar bone perforated by marrow spaces (Schiller, 2001)

2) Woven bone is identified by (1) an irregular arrangement of type I collagen fibers, hence the term woven; (2) numerous osteocytes in the matrix, and (3)

variation in osteocyte size and shape.

Woven bone is deposited more rapidly than lamellar bone. It is haphazardly arranged and of low tensile strength, serving as a temporary scaffolding for support. Woven bone is found in the developing foetus, in areas surrounding tumours and infections and as part of a healing fracture. Its presence in the adult skeleton is always abnormal, and indicates that reactive tissue has been produced in response to some stress in the bone.

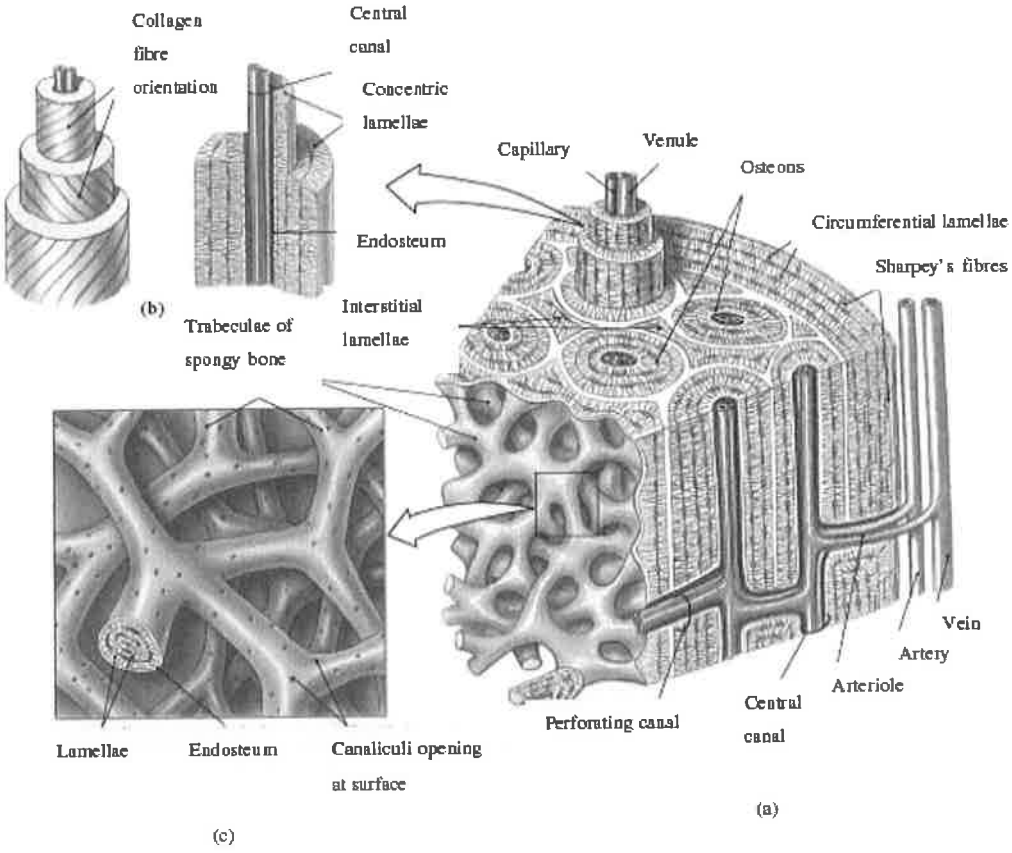


Figure 1. 6

(a) Structure of osseous tissue, (b) orientation of collagen fibres in adjacent lamellae and (c) details of organisation of trabecular bone. (Martini, 2002)

What is Osteoporosis?

Osteoporosis is a metabolic bone disease characterized by diffuse skeletal lesions in which normally mineralized bone is decreased in mass to the point where it no longer provides adequate mineral support.

Bone loss and eventually fractures are the main hallmarks of osteoporosis, regardless of underlying causes. The aetiology of bone loss is diverse but includes smoking, Vitamin D deficiency, low body mass index, hypogonadism, a sedentary lifestyle, and glucocorticoid therapy.

Regardless of the cause of osteoporosis, it always reflects enhanced bone resorption relative to formation. Thus this family of diseases should be viewed in the context of the remodelling cycle. Bone resorption and bone formation exist simultaneously. All osteoblasts and osteoclasts belong to a unique temporary structure, the basic multicellular (BMU or bone remodelling unit). The BMU is responsible for bone remodelling throughout life. Persons younger than 35 or 40 years completely replace bone resorbed during the remodelling cycle. With age, less bone is replaced in resorption bays than is removed, leading to a small deficit at each remodelling site. Given the thousands of remodelling sites in the skeleton, net bone loss, even in a short time, can be substantial.

Osteoporosis is classified as either primary or secondary. Primary osteoporosis, by far the more common variety, is of uncertain origin and occurs principally in post

menopausal women (type 1) (Fig 7) and elderly persons of both sexes (type 2). Secondary osteoporosis is a disorder associated with a defined cause, including a variety of endocrine and genetic abnormalities.

Figure 1. 7

Type 1 primary osteoporosis is due to an absolute increase in osteoclast activity. Given that osteoclasts initiate bone remodelling, the number of remodelling sites increases in this state of enhanced osteoclast formation, a phenomenon known as increased activation frequency. The increase in osteoclasts and remodelling in the early post menopausal skeleton is a direct result of oestrogen withdrawal. The effects of oestrogen lack are not, however, targeted directly to the osteoclast, but rather to cells derived from marrow stroma, which secrete cytokines that recruit osteoclasts. These cytokines, which are believed to be oestrogen sensitive, include IL-1 and IL-6, TNF, and macrophage colony – stimulating factor (M – CSF).

Type 2 primary osteoporosis, also called senile osteoporosis, has a more complex pathogenesis than type 1. Type 2 osteoporosis generally appears after age 70 and

reflects decreased osteoblast function. Thus, although osteoclast activity is no longer increased, the number of osteoblasts and amount of bone produced per cell are insufficient to replace bone removed in the resorptive phase of the remodelling cycle. Histologically osteoporosis is characterized by decreased thickness of the cortex and reduction in the number and size of trabeculae of the coarse cancellous bone. The loss of trabecular connectivity, which is attended by diminished biomechanical strength and ultimately leads to fracture, is due to perforation of trabeculae by resorbing osteoclasts in remodelling sites. In histological sections, the loss of connectivity results in the appearance of "isolated" islands of bone (Fig 8)

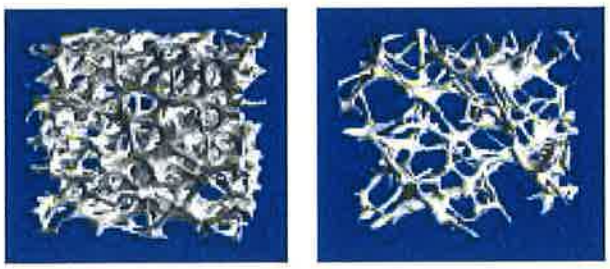


Figure 1.8

a) Normal bone and b) osteoporotic bone.

Concept of Microdamage and Remodelling

Microdamage in bone occurs in the form of microcracks as result of everyday cyclical loading activities. These small cracks are typically 100 micrometres in length, when viewed on sections cut transverse to a bone's longitudinal axis, and 500 micrometres when seen on longitudinal sections. The existence of "microdamage" in the form of these particular cracks has been known for some decades, during which time their importance, and especially their significance for the mechanical performance of bone, has increased (Taylor & Lee, 2003).

These microcracks act as a stimulus for remodelling. This microdamage is repaired by "targeted" remodelling to the site of damage (Burr et al, 1995; Prendergast & Taylor, 1994; Lee et al, 2002; Lee et al, 2006). Remodelling of bone involves an activation phase, resorption by osteoclasts and laying down of osteoid lamellae by osteoblasts, which become mineralized to form secondary osteons (Frost, 1973; Martin & Burr, 1989). Frost (1985) estimated the total time for a remodelling cycle in man – activation, resorption and formation to be 12 weeks (Fig 1.9)

As mentioned earlier, the specialized cells involved in the process of remodelling are the osteoclasts and osteoblasts. These cells combine to form a basic multicellular unit (BMU) – a cavity, about 200 micrometres in diameter, which moves along the length of the bone at a speed of about 40 micrometres per day. The result is a new portion of bone, of circular cross section, known as an osteon (Taylor et al, 2007). . It has taken

a lot of careful histological analysis (Burr et al. 1993; Lee et al. 2002) and some reasoning based on the quantitative fatigue behaviour of the material of the material to demonstrate that the remodelling process is not random and to some degree targeted towards the removal of damaged areas (Taylor et al 1998).

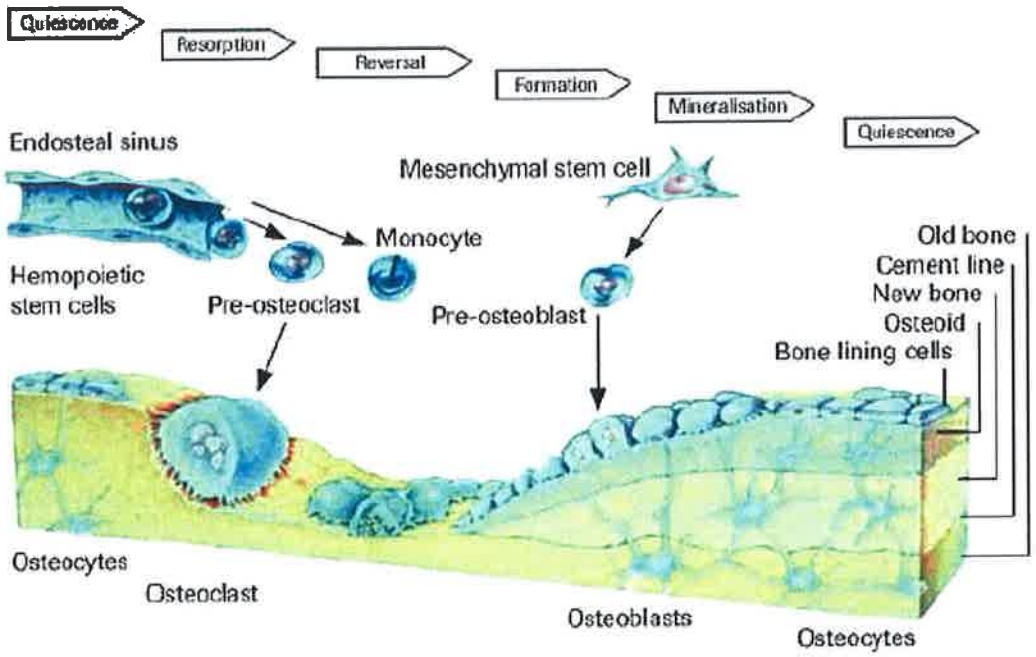


Figure 1.9

Schematic of bone remodelling in a trabecula. (www.roche.com)

Microdamage has been detected radiologically (Morris & Blickenstaff, 1967) and using reflected light photomicrography (Schaffer et al. 1989), electron microscopy (Chamay & Tschantz, 1972; Schaffer et al. 1994) and laser scanning confocal microscopy (Zioupos et al. 1994, 1996). Most widely used however, has been transmitted light microscopy following staining of specimens in basic fuschin (Frost, 1960; Burr & Stafford, 1990; Burr & Hooser, 1995). Burr (1985) viewed such

fuschin-labelled features using both transmitted light and scanning electron microscopy and confirmed that they were microcracks. Basic fuschin staining has been used to label microcracks generated in vivo and in vitro.

Stress fractures result from the contribution of damage accumulation at loading above some high threshold, and are seen in military recruits, athletes, ballet dancers and footballers (Burr, 1997; Milgrom et al. 1985, 2000, 2003). Approximately 10% of injuries seen in sports medicine centres are stress fractures and those of the lower limbs comprise about 95% of the cases (Brunet et al. 1990; Johnson et al. 1994). In contrast, if damage accumulates at "normal" rates but the bone's repair mechanism is deficient, fragility fractures may result, which occurs commonly in the ageing bone (underlying low bone density and poor microarchitecture exacerbate the effects) (Schaffler et al. 1994, 1995). Fragility fractures occur in response to minor trauma that in healthy adults would not lead to fracture (Heaney, 1993). 1.4 million such fragility fractures of the femoral neck, vertebrae and distal radius occur annually in the US at a fiscal cost of $10 billion (Riggs & Melton, 1995). Demographic trends suggest that hip fracture incidence worldwide will increase from 1.7 million in 1990 to 6.3 million by 2050 (Melton, 1996).

In Ireland, the population over 60 years of age increased 7% between 1995 and 2002 (Central Statistic's Office). This has a significant impact as it is estimated that 1 in 3 women and 1 in 5 men over 50 years of age will suffer an osteoporotic fracture. This

rises to 1 in 2 for women and 1 in 3 for men over 60 years of age (International Osteoporosis Foundation).

Between 1993 and 1997, 96,000 hospital admissions in Ireland were fall related. Of this, one third was in the over 65 age group. The main injury sustained as a result of these falls was limb fracture and this accounted for half of all admissions (Scallon, 2001). The incidence of hip fracture (Fig 10) is closely related to age and increases almost exponentially, so that about 90% of hip fractures occur in the over 70s (Melton and Riggs, 1986; Cummings et al., 1989). For women over 50 years of age, the lifetime risk of 14-18%, is greater than for men who run a 3-6% risk (Cooper et al., 1993). The Economic and Social Research Institute in Ireland reported that in the over sixties there were 1509 hip fractures in 1990, 2777 in 1998 and has risen to 3281 in 2000.

In Ireland alone, osteoporosis is estimated to cost the Exchequer 10 million euro each year. Hip fractures have an associated mortality rate of 20% within 1 year of fracture and 50% of patients never live independently again (Compston and Reeves, 2002). Hip fractures are associated with the greatest morbidity. 50% of patients are unable to walk without assistance and 25% require long term domiciliary care and within 6 months of fracture there is a 10-20% mortality rate (Riggs and Melton, 1995).

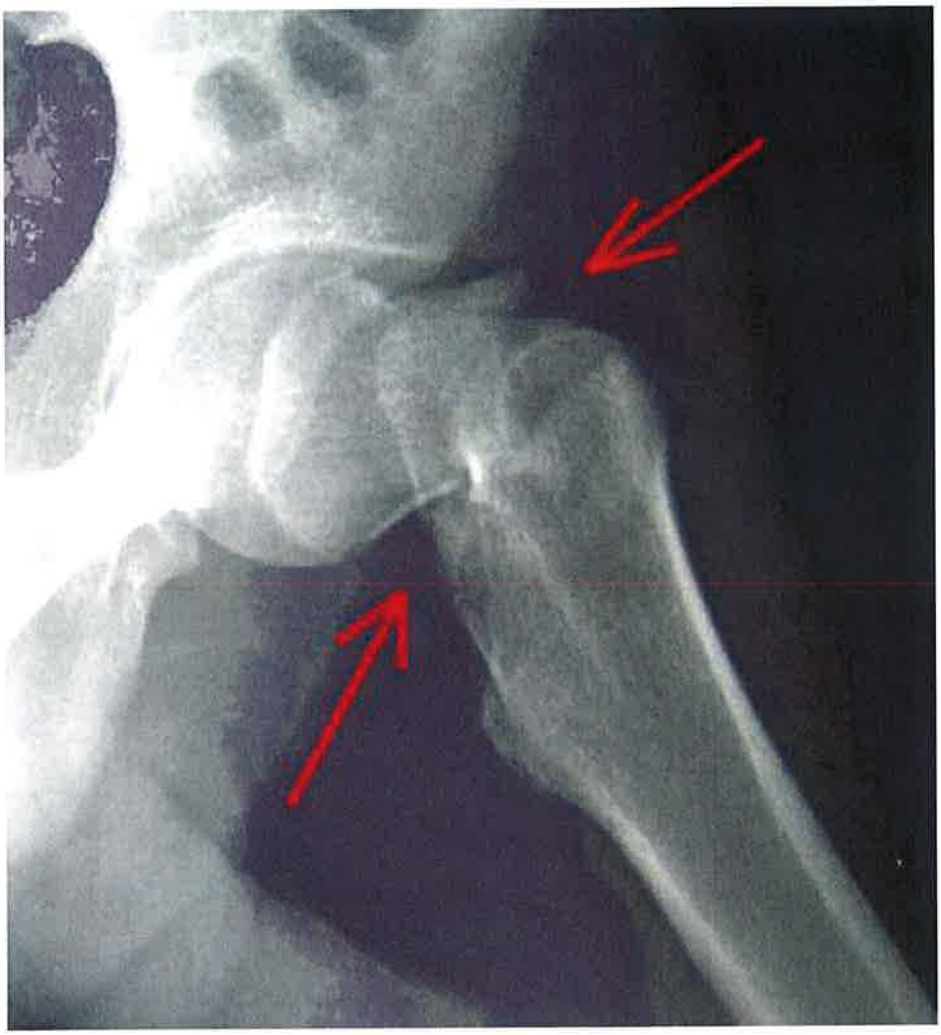


Figure 1. 10

Left Hip Fracture (Wikemedia)

Osteoporosis and Menopause

Although osteoporosis is one of the most pervasive conditions in older women, the condition is often not taken seriously enough by menopausal women. Osteoporosis is defined as a bone mineral density (BMD) equal to or greater than 2.5 standard deviations (SDs) below to peak bone mass (peak bone mass is the amount of bony tissue present at the end of the skeletal maturation and hence is deemed an important determinant of osteoporotic fracture risk – although it may vary with age and ethnicity of the subjects) or T score. Osteopenia is a BMD 1.0 – 2.49 SDs below the T score. With the onset of menopause, BMD is rapidly lost because bone resorption, uncoupled from bone formation, is accelerated, whereas formation continues at the premenopausal rate. Trabecular bone is affected more than cortical bone, and bone loss is therefore more commonly at vertebral, hip and radial sites. Bone loss in just the few years after onset of menopause may be as high as 20% of lifetime bone loss (Grady, 2001). The overall effect of menopausal bone loss is reduction of bone strength, leading to an increased risk of fracture.

Oestrogen or testosterone deficiency, regardless of age of occurrence, results in accelerated bone loss. The exact mechanisms of this bone loss potentially are numerous, but, ultimately, an increased recruitment and responsiveness of osteoclast precursors and an increase in bone resorption, which outpaces bone formation, occurs. After menopause, women experience an accelerated bone loss of 1-5% per year for

the first 5-7 years.

Evidence indicates that oestrogen deficiency causes bone to become more sensitive to the effects of parathyroid hormone (PTH), leading to an increase in calcium release from bone, a decrease in renal calcium excretion, and increased production of 1,25-dihydroxyvitamin D (1,25[OH]2 D3). Increased production of 1,25(OH)2 D3, in turn, causes increased calcium absorption from the gut, increased calcium resorption from bone, and increased renal tubular calcium resorption. PTH secretion then decreases via a negative feedback effect, causing the opposite effects. Osteoclasts are also influenced by cytokines, such as tumour necrosis factor-alpha and interleukins 1 and 6, whose production by mononuclear cells may be increased in the presence of gonadal deficiency (Hobar, 2005).

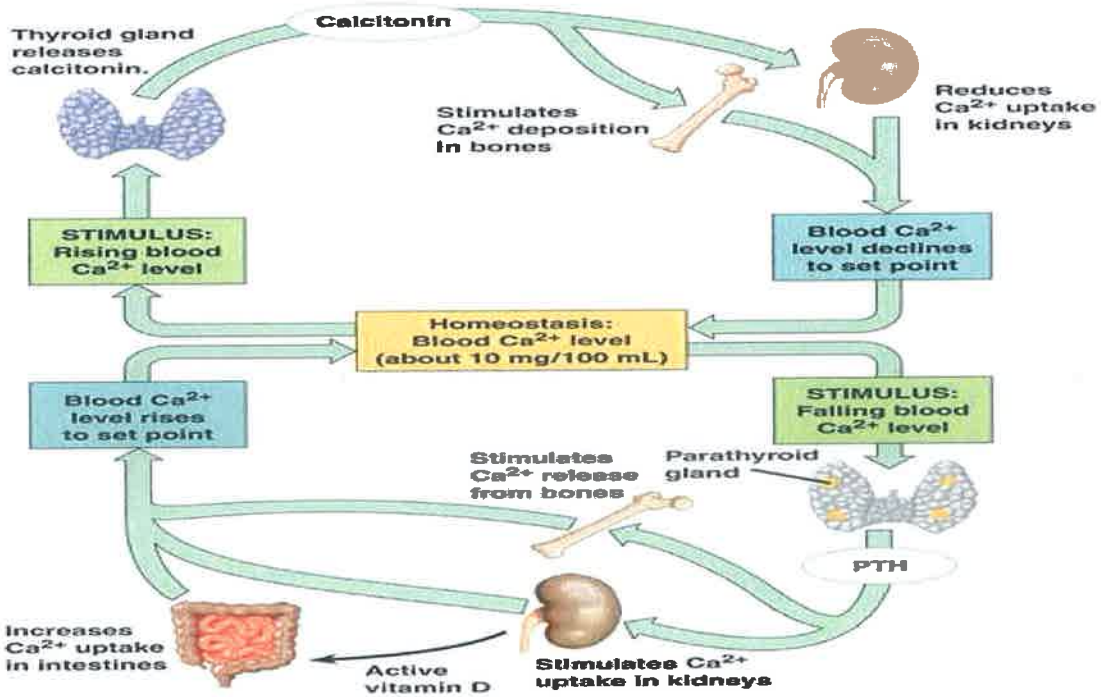


Fig 1.11 – Calcium Homeostasis (www.pbworks.com)

Treatment Options

In 2001, Grady and Cummings performed a meta-analysis of 22 trials with data on a total of 8800 women. They found a 27% reduction in risk of nonvertebral fractures in older women who received hormone replacement therapy (HRT). For hip and wrist fractures, the risk reduction was 40%, increasing to 55% in women younger than 60 years. Given that HRT is now primarily indicated for the relief of vasomotor symptoms and there is controversy regarding the use of oestrogen therapy due to the increased risk of developing breast and endometrial cancer; hormone therapy is no longer the first line treatment for osteoporosis.

Other therapies include raloxifene, calcitonin and bisphosphonates. Raloxifene is a selective oestrogen receptor modulator (SERM) and acts directly on oestrogen receptors in the bone to decrease resoprtion, resulting in reduced vertebral fracture risk and increased BMD. No effect on hip fracture risk has been documented (Ettinger, 1999).

Calcitonin is a peptide hormone that acts by inhibiting osteoclasts, which are involved in bone resorption activity. A decreased vertebral fracture rate has been demonstrated with this therapy, as has a small increase in BMD in older women. Serum calcium levels must be monitored in patients on this treatment.

A new class of inorganic compounds known as bishosphonates appear particularly useful. They work as antiresoprtives (antiosteoporotic agents), by inhibiting osteoclast resorption in a number of ways: 1) inhibition of osteoclast recruitment to the bone surface, 2) inhibition of osteoclast activity on the bone surface, 3) shortening of the osteoclast lifespan or 4) alteration of the bone or bone mineral in such a way as to reduce the rate of its dissolution (Rodan, 2002) (Fig 11). They have been shown to have a beneficial effect on vertebral and hip fracture rates and to cause a more significant increase in BMD than raloxifene and calcitonin (Harris, 1999; Black, 1996). Two widely used and effective bisphosphonates are alendronate and risedronate. The Vertebral Efficacy with Risedronate Therapy (VERT) study was conducted at 110 centres and included 2458 postmenopausal women who had vertebral fractures. Risedronate was administered at a dose of 5mg for 36 months and showed a statistically significant reduction in relative risk of new vertebral fractures. Cumulative incidence of vertebral fractures was also reduced (Harris, 1999).

Bisphosphonate given to women with postmenopausal osteoporosis reduces bone turnover into the premenopausal range, which is the aim of therapy. The decrease in bone turnover rate is associated with a reduction in fracture risk. Through their action on bone resorption and turnover, bisphosphonates increase BMD. In the first months of treatment, and before a new steady state between bone resorption and formation is achieved, there is a dissociation between the two processes, leading to a transiently

positive bone balance and increase in mineralization. With continuing treatment, bone remodelling space is decreased, leading to a further increase in BMD (Papapoulos, 2007).

All successful antiosteoporotic agents thus far developed block or slow the rate of bone resorption but do not stimulate bone formation. Thus, the drugs may prevent disease progression but cannot cure a patient who already has osteoporosis.

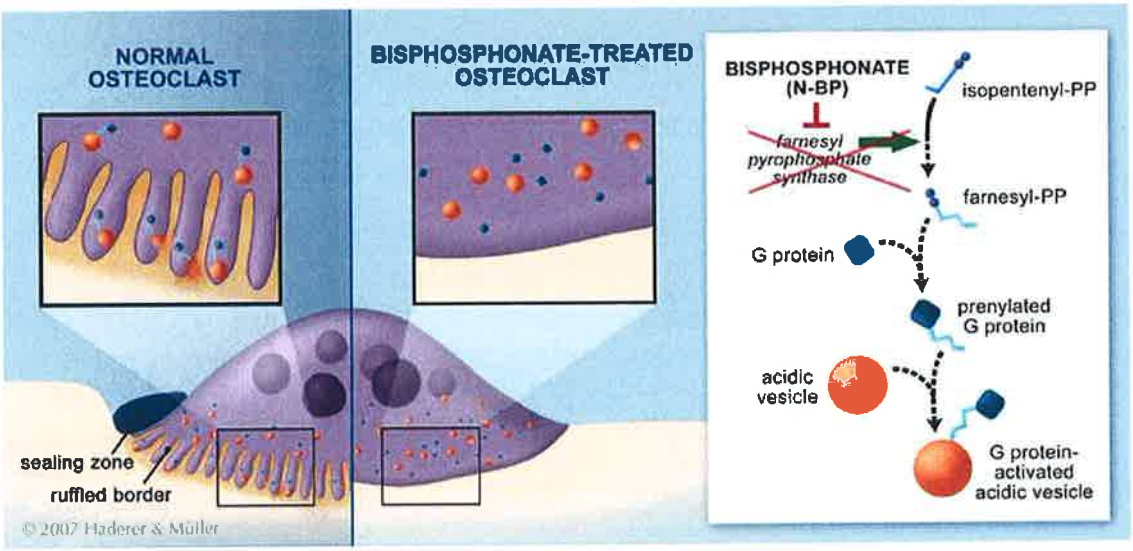


Figure 1.12

Bisphosphonates Inhibit Bone Resorption by Preventing Formation of the Ruffled Border.

AIM

Main purpose of this study is to see:

1. The effect of ovariectomy on microdamage and remodelling as compared with a normal group.
2. The possibility to assess this over a given time frame, as fluorochrome dyes were administered to the sheep at set intervals. Also, there is a unique opportunity to see what changes are present in the subgroup that received zolendronic acid treatment. As osteoclastic activity will be diminished in this group, microdamage should accumulate.
3. And finally, to assess microdamage in two non weight bearing bones; the rib which is cyclically loaded and iliac crest biopsies, hopefully allowing us to draw comparisons with clinical data from humans iliac crest biopsies to determine certain extent of osteoporotic pathology.

Chapter 2

An Appropriate Animal Model for Osteoporosis

INTRODUCTION

Postmenopausal osteoporosis is a major health problem for women, the understanding of which is hindered by the difficulty of studying a disorder that is restricted to humans. Osteoporosis is a slowly progressive disease, necessitating a study of several years duration to allow for a response to therapy. As results come slowly, accumulation of data is time consuming and maintenance of a study group is made more difficult by natural attrition due to either relocation or death (Turner, 2001).

Lifestyle and health related factors such as smoking, alcoholism, and diet have been linked with an increased incidence of osteoporosis (Heaney, 1996; Seeman, 1996). This presents a difficulty when trying to create a homogenous study group, since so many elements can influence the results.

Fall related injuries pose the most serious health risks associated with the disease, causing prolonged hospital stays, greater mortality rates, and at the very least an increased dependence on people or devices for morbidity (Grisso *et al.*, 1997).

Animal models provide more uniform material and allow for extensive testing of potential therapies. They play a crucial role in osteoporosis research as they reduce high costs and long time frames of clinical testing (Hartke, 1999). Even a model with a small representation of human functions may be of use for some aspect of the human condition under examination (Hazzard *et al.*, 1992).

Selection of a suitable animal model is difficult. Each species has its strengths and weaknesses and no laboratory animal species is equally suited to model all of the risk

factors associated with osteoporosis. Many factors must be considered when discussing possible models for osteoporosis, and these are well defined by Rodes *et al.* 1993 as "convenience, relevance (comparability to the human condition) and appropriateness : a complex of other factors that make a given species the best for studying a particular phenomenon".

An appropriate animal model for any research should be based on the following considerations:

1) appropriateness as an analog

2) transferability of information

3) genetic uniformity of organisms where applicable

4) background knowledge of biological properties

5) cost and availability

6) generalizability of the results

7) ease and adaptability to experimental manipulation

8) ecological considerations

9) ethical and societal implications

(Davidson *et al.*, 1987).

Keeping the above points in mind, when considering animal models for osteoporosis research it becomes increasingly important to ask whether the disease is truly represented by the following questions:

What is the peak age of bone mass?

Is there age dependent bone loss?

Do we see oestrogen reversible bone loss (i.e. can bone loss be restored)?

Do spontaneous fractures occur?

Is the disease the same (e.g. increased fragility at relevant sites)?

Is the magnitude of the disease the same?

Are there changes in cortical bone as well as in concellous bone?

Are there confounding effects following ovariectomy (OVX) e.g. weight gain?

Is one animal model enough?

Should we consider models for different degrees of osteoporosis?

(Turner, 2001).

VARIOUS "MODELS"

Studies of the aetiology of human osteoporosis are complicated by confounding factors such as diet, smoking, alcohol usage, exercise patterns, and oestrogen replacement therapy, which are usually irrelevant in animal models. As a result, several ovariectomized (OVX) animal models have been used for osteoporosis research to minimize the effects of such factors.

PRIMATES (non-human)

As would be expected, due to a number of similarities with humans, this particular model has many advantages over other models for osteoporosis. The close resemblance of their organ systems, for example gastrointestinal tract, endocrine system and bone metabolism reinforce that.

For example, female macaque monkeys have monthly menstrual cycles and have hormonal patterns similar to those of humans. Menopause occurs in these female primates after approximately 20 years of age (Kimmel, 1996). As post menopausal osteoporosis is clearly hormone related, the similarity of the endocrine system to that of the human is a notable advantage. Unfortunately peak bone mass is not reached in macaques until 9 years of age (Jayo *et al.*, 1994), and most studies have used OVX monkeys aged 4 – 7 years (Jerome *et al.*, 1993).

Another point to note is that most studies in primates have focused on loss of trabecular bone, and cortical sites have not been examined as extensively. A significant reduction does occur in vertebral cancellous bone volume in response to ovariectomy of the female monkey (Muller *et al.*, 1986) while gonadotrophin releasing hormone agonist-treated female monkeys exhibit decreased bone mineral density at a rate of loss comparable to postmenopausal woman (Mann *et al.*, 1990).

So, there is a similarity between non-human primates and humans in the response of bone to cessation of ovarian function. But once primates are acquired for study, their handling becomes an issue of concern. Firstly, the risk of zoonotic disease transmission from the animal is relatively high. Frequently, primates used for studies are not bred for research but are caught in the wild and are therefore potential reservoirs for a host of zoonotic diseases including the primate retroviruses, which have a history of jumping host species (Newman *et al.*, 1995, Weiss, 1998), a consideration which discourages the use of these animals as an experimental model.

Unfortunately; primates are too dangerous, costly and difficult to handle, for them to be a primary model for the study of therapeutic agents for osteoporosis. They may be of most value as the final step toward clinical trials after a rodent and/or a larger animal have been used (Turner, 2001).

DOGS

Dogs are less expensive than the primate, easier to work with and like humans, they are monogastric. They have been a useful model over the years for the human skeleton because of their extensive basic multicellular unit (BMU) – based remodelling. But there have been variable results available in regards to reduction in vertebral bone density at a set time interval post ovariectomy; showing insignificant bone loss in dogs after cessation of ovarian function.

The bisphosphonate YM 175 was tested in the OVX calcium – restricted beagle. BMD, strength, structure and turnover were evaluated and it was concluded that, although calcium restriction increase the sensitivity of bone to OVX in rats and minipigs, such sensitivity was not increased in the OVX beagles. Furthermore it was stated that "contribution of OVX to the reduction in bone mass and strength at the organ level in the OVX beagle model was small" (Motoie *et al.*, 1995).

The resistance of the canine skeleton to natural oestrogen deficiency or artificial oestrogen recession may be related to the infrequent oestrous cycle (Yamaura *et al.*, 1993). Unlike humans and the primate models which are polyoestrous, dogs are dioestrous, with ovulation occurring twice a year (Fox and Laird, 1970). Despite extremely low levels of oestrogen throughout most of the year, spontaneous fractures of the appendicular or axial skeleton in dogs are almost unheard of in veterinary practice.

The remodelling changes (activation) in cancellous and cortical bone are transient and brief in nature (stabilization within 5-12 months), without sizeable bone loss (Boyce

et al., 1990, Kimmel, 1991). Furthermore, the alteration in skeletal remodelling does not appear sufficiently sustained to have a substantial impact on cancellous bone microstructure and strength.

Hence dogs are of limited use as a model for oestrogen deficient – related bone loss, yet they have been extremely useful in evaluation of general aspects of the human skeleton. In a study that compared bone composition, density and quality in bone samples derived from seven vertebrates that are commonly used in bone research: human, dog, pig, cow, sheep, chicken and rat, large interspecies differences were observed. Of all species included in the biochemical analyses, rat bone was most different whereas canine bone best resembled human bone (Aerssens *et al.*, 1998).

MINI-PIGS

Where previously the size of the pig was a limiting factor in their widespread use as a model for research, the introduction of the mini-pig had eliminated this problem. The metabolism of bone, the oestrous cycle, and gastrointestinal function of the swine are positive features as a model. The skeleton displays extensive BMU-based remodelling in cancellous and metaphyseal cortical bone (Spurrel, 1965). They are large enough to receive prosthetic implants and withstand repetitive bone biopsies. Also, bone removal and deposition of trabecular and cortical bone occurs at a rate comparable with that of a human, and swine possess lamellar bone (Mosekilde, 1987). However, the major disadvantage of using this particular animal model is the cost of the mini-pig, as well as its rarity in certain areas. If the larger farm pig is chosen , housing and handling are made more difficult and regardless of the breed, these

animals can be aggressive, making them inconvenient to use on projects which require a large amount of handling and interaction (Turner, 2001).

RATS

Rats are commonly used animal model for osteoporosis. They have numerous advantages; they are inexpensive, easy to house and the general public is accustomed to the role of rodents for use in research.

There is extensive literature studying the OVX rat including histomorphometric changes, biochemical markers, methodology for bone densitometry and evaluation of bone fragility (Wronski *et al.*, 1985, 1986, 1991, Dempster *et al.*, 1995). Genetically specific strains can be aquired, thus removing some variability in studies. Their shorter life span enables studies on the effects of ageing on bone. Cortical thinning and increased fragility are well documented in ageing rat and mouse bone, but it is unclear if this results in an increased incidence of fractures. Weight gain in OVX rats can result in an increase in bone mass with increased mechanical loading, resulting in protection of OVX animals against age related loss of bone strength (Peng *et al.*, 1997). Therefore bone changes are seen as osteopenia rather than osteoprosis.

Although aged rodents have Haversian systems and OVX results in a significant bone loss, the use of this model is hindered because young rats have a limited naturally occurring BMU – based remodeling. Nevertheless, older rats have lamellar bone, trabecular remodeling and some secondary osteonal remodeling (Wronski and Yen, 1991 ; Frost and Jee, 1992; Kalu, 1991).

As older animals more accurately effect the target population for proposed osteoporosis therapy, the very aged-rat model (30-months old) is an even better choice as a cost effective animal model (Gaumet *et al.*, 1992). The inability to restore bone following OVX in rats is similar to human bone (Abe *et al.*, 1993).

The rat is a poor model to study the effect of OVX on cortical bone because of the lack of Haversian systems, while another limitation is the absence of impaired osteoblast function during the late stages of oestrogen deficiency (Wronski and Yen, 1991). Using the rat model, there is also difficulty experienced in terms of its size. Being small they are easier to house but are not suitable in terms of evaluating implants in osteopenic bone and hence not suitable for testing of various prosthetic devices, fro example total hip arthroplasty. Finally, longer term studies which require several biopsies, or large blood samples, also are very difficult in such a small animal as the rat (Turner, 2001).

OVINE MODEL

Before 1994, there were very few studies using sheep as an animal model of osteopenia and other aspects of orthopaedic research. However, advantages in the use of sheep have emerged because societal views about use of animals for research, in general are quickly changing.

Sheep are very docile, compliant and they are flock animals suffering the least when they can be housed outdoors with minimal supervision. The husbandry of sheep greatly reduces housing costs. Finally, sheep are available in large numbers in many countries around the world so large studies are possible (Turner, 2001).

The OVX sheep model has significant advantages over other animal models for studying postmenopausal osteoporosis. The metabolic rate of sheep (based on oxygen consumption per gram of body weight) is 0.22, which is closer to that of man (0.21) than to that of the rat (0.87) or dog (0.3) (Schimdt – Nielson, 1977).

Unlike the human female, ewes have an annual anoestrous period of 1-2 months, but during the remainder of the year oestrogen production is relatively high; moreover, sheep have up to 20 oestrous cycles during this period (Frandson, 1986).

Further advantages are that there are temporal and qualitative similarities between the hormone profiles of ewes and women (Goodman, 1994). Although some breeds are seasonally polyoestrous (cycles begin in Autumn in response to shortening periods of daylight), some breeds (e.g. Merino) can continue to cycle almost year round (O'Connell, 1999). It has also been observed that the bone remodeling cycle is

approximately 3 months whish is also similar to humans (Turner and Villaneuva, 1994; Turner *et al*, 1995, Lee *et al*, 2002).

The effects of various therapeutic drugs, such as fluoride, on bone tissue were investigated by workers in France using sheep (Chavassieux, 1990). The same laboratory used a sheep model to show that OVX induced an increase in bone formation beginning at 10 weeks after surgery and persisting at the 6^{th} month (Pastoureau *et al.*, 1989).

OVX sheep are recognized as a viable model of loss of bone mineral density (BMD) (Newman *et al.*, 1995, Turner *et al.*, 1995 Johnson *et al.*, 1997, 2002). A number of studies have documented osteopenia in sheep following OVX as well as the response to various agents such as oestradiol implants (Turner et al 1995, O' Connell, 1999), salmon calcitonin (Gensen *et al.*, 1996) and the selective oestrogen receptor modulator (SERM) raloxifene (Turner *et al.*, 1999).

Seasonal changes in bone mass and biochemical markers in elderly women have been reported (Diesen et al., 1994). In a 24 month study in Northern England, significant seasonal changes in BMD, serum 25 hydroxyvitamin D and parathyroid hormone were seen; the greatest decline in measurable bone mass being in winter. Seasonal fluctuations such as this also occur in sheep and this must be addressed as a potential variable when using sheep in studies of osteopenia (Chavassieux *et al.*, 19911 Hornby *et al*, 1995, Turner *et al.*, 1995, O' Connell, 1999). For these reasons, experiments using sheep should span all four seasons to minimize seasonal changes. Seasonal

variation is also related to the periods of seasonal anoestrous, itself linked to the environmental photoperiod (Goodman, 1994).

The cortical bone of sheep is similar to other species of large domestic mammals. The bone of young sheep (less than 3-4 years of age) is plexiform and is a combination of woven and lamellar bone with functional similarities but with distinctly different patterns of deposition and organization (Newman *et al.*, 1995). Like woven bone, plexiform bone is deposited rapidly but achieves better mechanical properties for large, rapidly growing animals. Plexiform bone is also found in humans in the medial side of the mandibular ramus and in growing children around the time of growth spurts (Martin and Burr, 1989). As the ovine skeleton ages, one of the first places to see Haversian remodeling is the caudal aspect of the femur, while other place of such remodelling are the diaphyses of the radius and humerus (Newman *et al.*, 1995).

Like dogs and primates, the size of the sheep allows researchers to meet other criteria for a model of osteoporosis. Specifically they are large enough to accommodate prosthesis implantation, substantial blood and urine sampling, and ample iliac crest biopsies (histomorphometry). This facilitates research to correlate events at the clinical, tissue, cellular and biochemical levels.

OSTEOPOROSIS IN SHEEP?

Metabolic bone diseases or osteodystrophies are caused by deficiencies or imbalance in nutrients, such as Vitamin D, calcium and phosphorus, essential for normal bone growth and remodeling. Nutrition at early age has long term – steady effects on the skeletal development and properties of sheep (Tartura *et al.*, 2007).

Although the ovine model is being used in this case as a model for osteoporosis itself, it is important to appreciate that osteoporosis is the most common metabolic bone disease in grazing sheep, especially lambs where the skeleton is still growing. Many mild cases probably remain undetected as the shape of the bone is not altered and unless there have been pathological fractures, lameness is not likely to have been a presenting sign (Thomson, 2008).

Most cases of osteoporosis in sheep are either nutritional or parasitic in origin. Osteoporosis can often be detected during post – mortem examination of lambs with severe or prolonged gastrointestinal parasitism, but diagnosis requires sectioning of long bones so its prevalence is therefore grossly underestimated (Fig 2.1).

Although malabsorption may be a factor, the mechanism is likely to be more complex and probably involves the generation of pro-inflammatory cytokines, such as interleukin-1 and interleukin-6 as part of the immune response to infection. Both cytokines are known to induce osteoclastic bone resorption and are considered to be important in the pathogenesis of osteoporosis associated with inflammatory bowel disease in humans (Andreassen *et al.*, 1997).

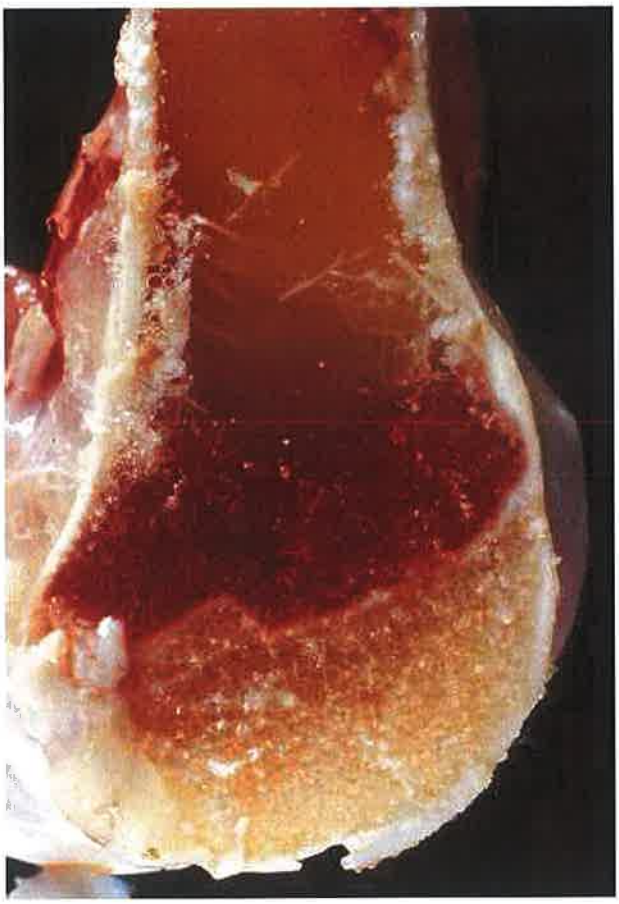


Figure 2. 1

Sagittal section through the distal femur of a lamb with osteoporosis. Presence of thin cortices and reduced density of trabecular bone in the metaphysis and epiphysis; the gelatinous nature of medullary fat (serous atrophy) indicates either starvation or malabsorption. (Thompson, 2008).

"BONE FOR LIFE" PROJECT

This is an ongoing multi-disciplinary project involving several teams and researchers with a primary goal of being able to better understand, the pathogenesis of osteoporosis by studying various aspects of the ovine model.

For this particular part of the project, two specific skeletal regions of the ovine model are assessed. 1) Cortical bone within rib segments and 2) Trabecular bone from iliac crest biopsies.

To facilitate the animal experiment, an animal license was granted by the Department of Health under the Cruelty to Animals Act, 1876 (license number: B100/2443) and also approved by the Ethics Committee in the School of Veterinary Science in University College Dublin. All animals were housed on Lyons Estate, Newcastle, Co. Kildare where they were maintained at pasture. Animals underwent routine health checks by a veterinarian and were vaccinated, dipped and treated to prevent infectious foot rot and other diseases.

The study groups were obtained from a flock of skeletally mature aged female mixed breed sheep, age range of 5-9 years (Kennedy and Brennan, 2006). A 12 month group and a 31 month group were devised (sacrificed at 12 and 31 months post OVX). Within these two distinct categories there was a further subdivision in the form of a control (CON) batch and an ovariectomized (OVX) batch. Within the 31 month group a small proportion received "treatment" in the form of bisphosphonate, Zolendronic acid (OVX + ZOL) at a supraclinical dose.

In terms of numbers, a total of 44 sheep, divided as follows:

1) 12 Month Group a) 10 control (CON)

b) 10 ovariectomized (OVX)

2) 31 Month Group a) 10 CON

b) 10 OVX

c) 4 (OVX + ZOL)

Fluorochrome Administration

Bone turnover can be measured experimentally using fluorochrome markers. These were injected intravenously into the sheep at specific time intervals (Table 1). These flurorochromes, are chelating agents which bind to exposed calcium phosphate through stable covalent bonds (Parkesh *et al.*, 2007). Rahn classified and optimized a series of five of these chelating agents, which could be used to label bone (Rahn, 1997). The fluorochromes that were used were calcein, calcein blue, oxytetracycline, xylenol orange, alizarin complexone (Table 2). Previous work had shown this technique to be a very useful method of labeling new bone formation (Canyon *et al.*, 1984, Lee *et al.*, 2002).

	12 Month Group	31 Month Group
Week 0 17/11/03	Oxytetracycline	Oxytetracycline
Week 12 5/02/04	Alizarin Complexone	-
Week 24 29/04/04	Calcein	-
Week 36 19/07/04	Xylenol Orange	-
Week 48 18/10/04	Calcein Blue	Alizarin Complexone
Week 52 22/11/04	Sacrifice	-
Week 72 29/03/05	-	Calcein
Week 84 13/07/06	-	Xylenol Orange
Week 108 15/12/05	-	Calcein Blue
Week 138 19/06/06	-	Sacrifice

Table 2. 1

Table 2. 1. Schedule of flourochrome administration and point of sacrifice.

Flurochrome	Sol. Conc. (g/L)	Dosage (mg/kg)	Excitation Wavelength (nm)	Emission Wavelength (nm)	Colour
Oxytetracycline[1] $C_{22}H_{24}N_2O_9HCl$	200	50^	390	520	Yellow
Alizarin Complexone[2] $C_{19}H_{15}NO_8$ $(2H_2O)$	30	25^	580	625	Red
Calcein[3] $C_{30}H_{26}N_2O_{13}$	5	10^*	495	540	Green
Xylenol Orange[3] $C_{31}H_{30}N_2Na_2O_{13}S$	90	90^*	377	615	Orange
Calcein Blue[3] $C_{15}H_{15}NO_7$	30	30^*	375	435	Blue

^ From (Lanyon et al., 1982) * from (Rahn, 1977)
1 Pfizer Animal Health, Dublin
2 Lennox Laboratory Supplies, Dublin
3 Sigma Aldrich, Dublin

Table 2. 2

Concentrations and dosages for in vivo injections, along with excitation and emission wavelengths for the 5 fluorochromes. (Kennedy and Brennan, 2006).

ZOLENDRONIC ACID

Zoledronic acid is a third-generation aminobisphosphonate showing the greatest inhibition of farnesyl disphosphate synthase and highest affinity for hydroxyapatite. In human studies, it is a potent inhibitor of bone resorption that can be administered intravenously annually.(2) In postmenopausal women, its use is associated with increases in spine and hip BMD, reduced bone turnover, and a significant reduction in the risk of vertebral, hip, and nonvertebral fractures (Reid *et al.*, 2002).

The purpose of the subgroup that received Zolendronic acid (within 31 month group) was to see if there was any predictable effect on the OVX group, after administering them with supraclinical doses of intravenous zolendronic acid. As sheep are ruminants, not monogastric like humans; oral bisphophonate supplements would not have been adequate. In humans, oral bisphosphonates have also been shown to cause gastrointestinal problems.

DISCUSSION

Laboratory animals have played a major role in the recent improvements in the management of osteoporosis. They have contributed to enhanced knowledge of the aetiology of osteoporosis. Additionally, animals have been essential for preclinical evaluation of the efficacy and safety of interventions intended to prevent and / or reverse bone fragility (Turner, 2001).

The choice of an appropriate species is highly important in regards to achieve meaningful results and ultimately to allow for data interpretation and eventual comparison with clinical studies. Each species has its strength and weakness and no laboratory animal species is equally suited to model all of the risk factors associated with osteoporosis.

The ovine model, had been thoroughly researched and prepared to undergo a number of varying experiments. It is the optimal candidate for the purpose of this particular study.

Chapter 3

Microdamage and Remodelling in Cortical Segment of Ovine Ribs

INTRODUCTION

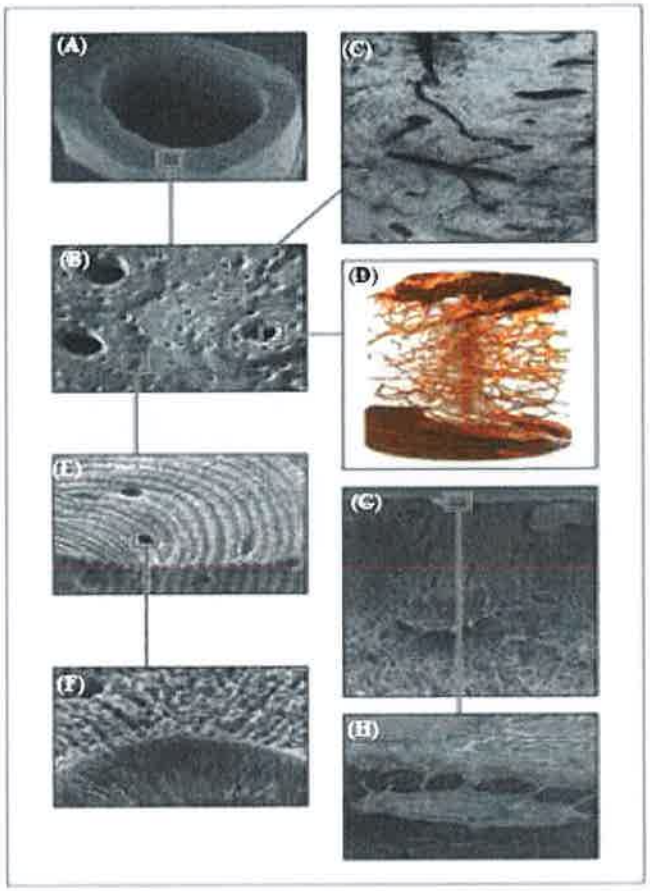
The concept of microdamage and remodeling has been covered in Chapter 1. It is important though to reemphasize the sequence of events that occur and the "major players" in it.

Simplified version of chain of events:

1) Microcrack → 2) Resorption Cavity → 3) Secondary Osteon

Microdamage initiates a remodeling cycle in which osteoclasts resorb damaged bone which is then replaced by new bone laid down by osteoblasts.

The cyclical loading of bone creates microdamage which contributes to stress fractures (Burr et al. 1990) and acts as a stimulus for bone remodeling. Cortical bone itself contributes rigidity and forms the dense outer shell. The basic units of cortical bone are Haversian systems (osteons) arranged in vertical columns. Accumulation of microdamage in this region can ultimately result in excess stressed loads and eventual fracture may ensue (Fig 3.1)



An illustration of the cortical bone hierarchical structure.

Figure 3. 1

Within a cortical bone shaft, shown in cross-section (A) are osteons surrounded by interstitial bone and osteocytic lacunae distributed around the central Haversian canal (B). Panel C shows a microcrack that is largely confined to interstitial bone. Panel D shows the Haversian canal network in cortical bone. In Panel E, alternating high-density and low-density concentric lamellae of an osteon produce a composite structure. Panel F depicts an osteocyte lacuna at a high resolution showing collagen fibres. In Panel G, osteocytes connect with lining cells and with one another through a network of canaliculi. Panel H shows the detail of a bone lining cell connected to an osteocyte (Seeman and Delmas, 2006)

Remodeling occurs lifelong; during growth it contributes to the maturation of bone, while in the "adult" it provides metabolically active bone tissue for calcium homeostasis and also helps in eliminating avascular necrotic bone compartments, and prevents fatigue fracture by local repair of microcracks (one of the stimuli for remodelling itself).

Bone apposition by the cortical envelopes results in the formation of circumferential lamellae and primary osteons. Both are at least partially replaced by secondary osteons. Replacement means substitution, and is initiated by resorption followed by formation. In human bone, secondary osteons reach an outer diameter of about 200-250 μm and inner diameter of 50-80 μm. The wall thickness amounts to 70-100 μm and varies somewhat with age. Secondary osteons can run parallel to the long axis of the bones, but measurements of their length are impeded by frequent branching or ramifications. As a uniform cylindrical structure, they are rarely more than 2-3mm long. In addition, they are interconnected by transverse vascular channels (Volkmann's canals) at intervals of 0.5-1 mm.

In contrast to primary osteons, secondary osteons are always delineated from the surrounding bone matrix by a cement line. Although cement lines are easily identified by their refractive properties and by various staining methods, their exact composition is not clear. Cement lines appear at sites where the bone surface stays quiescent (undergoing neither resorption nor formation) for some time. Microscopically, two types are distinguished: 1) Resting (or arresting) lines appear where bone formation is temporarily arrested and then resumes again. 2) Reversal

lines, which indicates the bone formation was preceded by oteoclastic resorption.

Cement lines surrounding secondary osteons belong to this latter type.

Below is a schematic of the 6 major steps in the bone remodeling cycle (Fig 3.2):

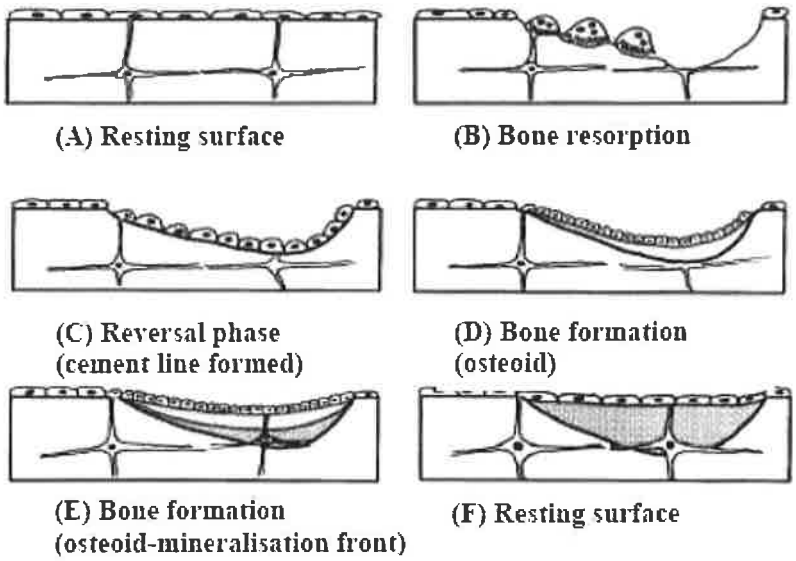


Figure 3. 2

Each remodelling process is initiated by activation at a resting surface (A) by which osteoclastic lineage cells start to secrete collagenase which removes the thin layer of unmineralised bone typical of a resting bone surface. This exposes the mineralised bone underneath to the multinucleated mobile, osteoclasts. During osteoclastic bone resorption (B), Howship's lacunae are excavated to a maximum depth of 50-60 μm. A short reversal phase follows, when the cement line is formed (C). Then bone formation begins (D).
Osteoblasts produce osteoid at a rate of 0.5-1.0 μm per day. When the osteoid thickness has reached approximately 12-15 μm, mineralisation begins from the bottom (mineralisation front) (E). At the termination of each remodeling process, the bone surface is again covered by an extremely thin layer of non-mineralised bone and a layer of flat lining cells. The bone is again converted into a resting surface (F) (Kennedy, 2007).

In order for bone remodeling to occur, a coupled sequence of bone resorption and formation occurs in space and time via discrete remodeling sites or "bone multicellular units" (BMUs). The formation of resorption cavities by osteoclasts is the starting process. The resorption cavities may also act as points of "raised stress" (Hernandez, 2006) and hence a strain enhancing effect locally. Thus, remodeling could be described as being governed by strain pertubations, be they generated externally by load or internally by resorption cavities (Huiskes, 2000).

Within the tip of a resorption cavity, a group of osteoclasts is assembled in a cutter cone (Fig 3). This lengthens the resorption cavity and at the same time widens it to the final diameter of the future osteon. The resorption "canal" is well vascularised. The vessels are accompanied by perivascular cells, possibly including osteoclast and osteoblast precursors. After osteoclasts have passed by, the wall is lined by ill defined mononuclear cells. This portion, however, is only 100-200 μm long and represents a reversal phase of 1 or 2 days. During this phase the cement line may be formed. Then osteoblasts appear and start depositing lamellar osteoid that will undergo mineralization 8-10 days later. Because the system advances longitudinally, the canal assumes a conical shape. Lamellar formation continues until, several weeks or months later and the evolving osteon is complete (Schenk, 1986).

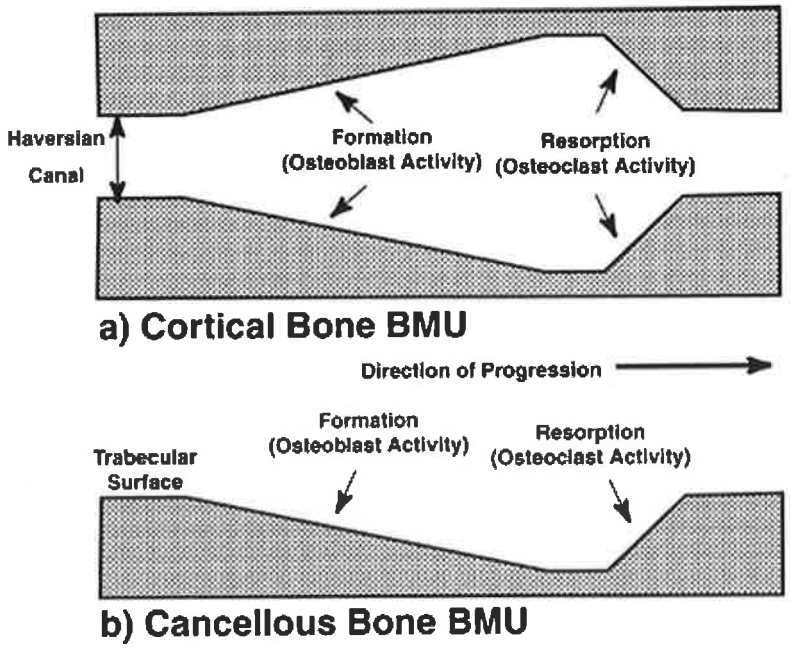


Figure 3. 3

The basic multicellular unit (BMU) in a) cortical and b) cancellous bone. In cortical bone, (a), remodeling takes place in Haversian canals; in cancellous bone, (b), remodeling takes place on the surfaces of the trabeculae (Parfitt, 1994).

TARGETED AND NON-TARGETED REMODELLING

Bone remodelling achieves three goals. First, it provides a way for the body to alter the balance of essential minerals by increasing or decreasing the concentration of these in serum. Second, it provides a mechanism for the skeleton to adapt to its mechanical environment, reducing the risk for fracture and increasing the organism's chances for passing its genes to the next generation. Third, it provides a mechanism to repair the damage created in bone by repetitive cycles of mechanical loading. The first of these goals can be easily accomplished without site-dependent remodeling. To re-establish mineral balance, it matters little where the bone is removed or replaced, as long as mechanical integrity is not compromised, and the mineral balance is restored. The other two goals require site-dependent remodeling (Burr, 2002)

Frost, originally proposed that remodeling would occur to repair microdamage in bone. He suggested that disruption of canalicular connections that occurred when cracks crossed them could provide the stimulus to initiate remodeling. Several lines of evidence lend support to this view (Frost, 1960 & 1985, Taylor et al., 2007).

There is recent evidence that osteocyte apoptosis is an important factor in initiating new remodelling sites. The prevailing view, without much supporting evidence, is that an intact osteocyte-canalicular system inhibits the recruitment or activation of osteoclasts, and that disruption of the network, by microcracks for instance, releases the normal inhibition to resorption (Burger, 1999). A positive correlation has been shown between osteocyte apoptosis and bone resorption by osteoclasts in growing bone (Bronkers, 1996), and between osteocyte apoptosis and increased activation frequency in oestrogen-deficiency osteoporosis. The concept has recently received additional support with the identification of an osteocyte derived protein that has been shown to inhibit osteoclastic resorption (Ikeda, 1997).

There is increasing interest in the degree to which bone remodeling, particularly in cortical bone, is "targeted" at fatigue microdamage. The theory that microdamage initiates remodeling in close proximity to microcracks, thereby removing them, and that this accounts for a significant fraction of the overall remodeling activity, has been gaining acceptance. However, the association between the initial, resorptive stage of remodeling and microcracks in histologic sections of cortical bone is far from complete; indeed, the great majority of resorption cavities are not spatially associated with microcracks (Martin, 2002).

For example, in one experiment, 59% of microcracks were not associated with resorption spaces, and 90% of resorption spaces were not associated with microcracks (Li et al., 2001). The lack of complete spatial association between microcracks and resorption cavities has maintained support for the older concept that most remodeling serves functions other than microdamage removal, for example calcium homeostasis. Thus, some may be "targeted" at (i.e., initiated by, and in proximity to) microdamage, whereas other BMUs serve metabolic or other purposes through "stochastic" or non-targeted remodeling.

Research Question - Does any significant difference exist in microdamage and remodelling, between the control and OVX groups? And does the introduction of bisohosphonate treatment in the form of zolendronic acid have any effect on the outcome of either of the two phases?

MATERIALS AND METHODS

As discussed in Chapter 2, sheep were divided into 2 groups; 12 month and a 31 month. They were further divided into a Control (CON) and an Ovariectomized (OVX) subgroup in each and a further subdivision was where 4 OVX specimens underwent intravenous zolendronic acid treatment (OVX + ZOL) in the 31 month group.

Ribs were chosen to assess microcracks and remodelling due to cyclical loading they undergo during the respiratory cycle (Frost, 1960; Lee et al., 1998).

"ANATOMICAL DESCRIPTION"

Sheep usually have either 13 or 14 thoracic vertebrae, and a corresponding number of paired ribs. The thoracic cage is formed by thoracic vertebrae, ribs, sternum and is an essential component of the respiratory system (Fig 3. 4).

Figure 3.4

Sheep Ribcage (lateral view) (www.aps.uoguelph.ca)

The ribs are joined to the vertebral column dorsally so that the head of each rib articulates with the bodies of two adjacent vertebrae. Each rib has a tubercle that articulates with the transverse process of the more caudal of its two vertebrae (Fig 5). Ventrally, the anterior ribs articulate with the sternum and are termed sternal ribs. The more caudal ribs are called asternal ribs and they connect to the sternum via costal cartilages. The structure of the ribcage is rather variable and at as few as 12 ribs on one side and more on the opposite side (left and right differences). There are approximately 8 pairs of sternal ribs and 5 to 6 asternal ribs.

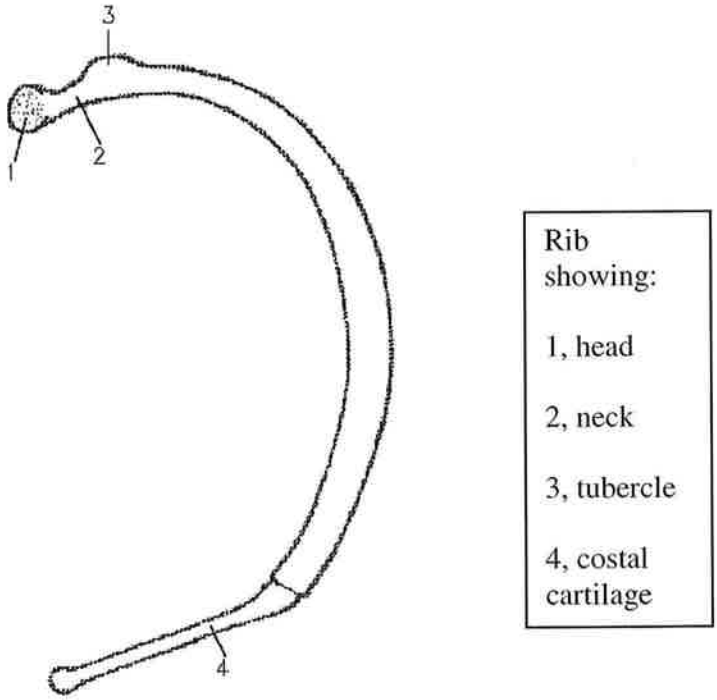


Figure 3.5 – Schematic diagram of rib (www.aps.uoguelph.ca)

As mentioned earlier, ribs undergo cyclical loading during respiration. The intercostal muscles attach to the main body of the rib in between the tubercle and costal cartilage and would hence cause varying strain patterns at their attachment sites, external and internal thoracic surface.

SAMPLE SELECTION

For the purpose of the study, the 2 groups were separated out as follows:

12 Month Group -

Control (CON)	Ovariectomized (OVX)
12 samples	12 samples

31 Month Group -

Control (CON)	OVX	OVX + Zolendronic Acid)
10 samples	10 samples	4 samples

The right 7th rib (sternal) from each thoracic cage was chosen for preparation. The entire rib was removed from the thoracic vertebrae, length measured and subsequently the midpoint identified. 1 cm cross – sectional samples were removed from the mid point of each rib using a slow speed diamond saw (Struers, Acutom 50). Eventual thickness of specimen prior to mounting would be ~100 μ

STAINING OF SECTIONS IN BASIC FUCHSIN

The following protocol was used in the making of sample slides followed by microscopic evaluation:

1% solutions of basic fuchsin are prepared in 70%, 80%, 90% and 100% ethanol and mixed using a magnetic stirrer. After rinsing under running water to remove fatty marrow, each 1 cm thick specimen is fixed over night in 70% ethanol and bulk stained in 4mls of the following solutions in individual vials in a desiccator at -20psi vacuum:

1. 1% basic fuchsin in 70% ethanol (ETOH) for 2 hours – Change solution
2. 1% basic fuchsin in 80% ethanol (ETOH) for 2 hours – Change solution
3. 1% basic fuchsin in 90% ethanol (ETOH) for 2 hours – Change solution
4. 1% basic fuchsin in 100% ethanol (ETOH) for 2 hours – Change solution
5. 1% basic fuchsin in 100% ethanol (ETOH) for 2 hours – Change solution
6. 1% basic fuchsin in 100% ethanol (ETOH) for 2 hours – Change solution
7. Rinse in 100% ETOH for 1 hour to remove excess stain.

If there is a break in protocol, specimens are held in their current alcohol concentration without stain or vacuum.

The specimens are then rehydrated in 100mls of distilled water, with two changes, for 48 hours prior to sectioning and mounting on glass slides for histological examination.

PREPARATION OF GROUND SECTIONS OF BONE

The technique of grinding is used to obtain slides of bone that are of high quality and facilitate detailed histological examinations (Frost, 1958). The big advantage of this method is that it is simple, quick and can be used to obtain either longitudinal or transverse sections of compact bone.

The sections are obtained as follows:

1. Specimen is placed in a small clamp and approximately 250μm is cut using a diamond saw (Streuers Minitom).
2. A sheet of No. 400 silicon carbide paper is placed on a flat surface under running water and the section is placed upon it. Another piece of paper is wrapped around a glass slide and the section is manually ground down between the two pieces of paper in a circular fashion under light pressure.
3. This grinding is continued until the required thickness (100-150 μm) has been obtained.
4. Specimens are then agitated in 0.01% washing-up liquid in a beaker, placed in a Coors porcelain funnel with fixed perforated plate and washed in distilled water.

Specimens are then air dried and mounted using a mounting medium (DPX) and coverslip.

MICROCRACK IDENTIFICATION CRITERIA

1. They are intermediate in size, being larger than canaliculi but smaller than vascular canals.
   Method: Fluorescence, green light incident (546nm), x125 magnification.
2. They have sharp borders with a halo of basic fuchsin staining around them.
   Method: Fluorescence, green light incident (546nm), x125 magnification.
3. They are stained through the depth of section.
   Method: Fluorescence, UV incident light (365nm), x125 magnification.
4. When the depth of focus is changed, the edge of the crack can be observed to be more deeply stained than the intervening space.
   Method: Transmitted light microscopy, x250 magnification.

Table 3. 3

(Lee at al, 1998)

Each slide was examined "blind", using a combination of ultra violet (UV) (λ = 365nm), blue (λ = 470nm) and green (λ = 546nm) epifluorescence microscopy at X10 magnification.

The following measurements were made for each slide:

1. Surface area (mm^2)
2. Crack number (n)
3. Crack length (μm)
4. Number of resorption cavities
5. (Labelled) Secondary osteon count

The following calculations followed:

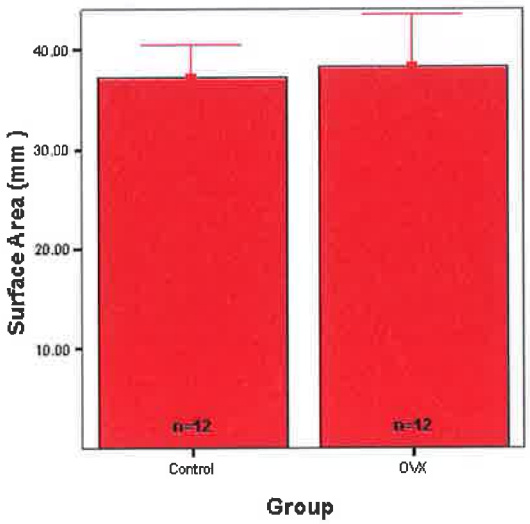
1. Numerical density (n / mm^2) – total number of cracks per area
2. Surface density ($\mu m / mm^2$) – total length of cracks per area
3. Average length (μm)
4. Resorption cavity density (n / mm^2) – total number of resorption cavities per area
5. Osteon density – total number of osteons per area

STATISTICAL ANALYSIS

Groups were assessed for normal distribution (not normally distributed data) and then compared using Mann- Whitney U test for the 12 month group. 31 month group (3 subgroups – Control, OVX and OVX + ZOL) underwent Kruskal and Wallis assessment prior to Mann – Whitney U tests comparing each subgroup to one another. SPSS statistical package, version 15.0 was used for statistical analysis. A p value of < 0.05 was considered to be significant.

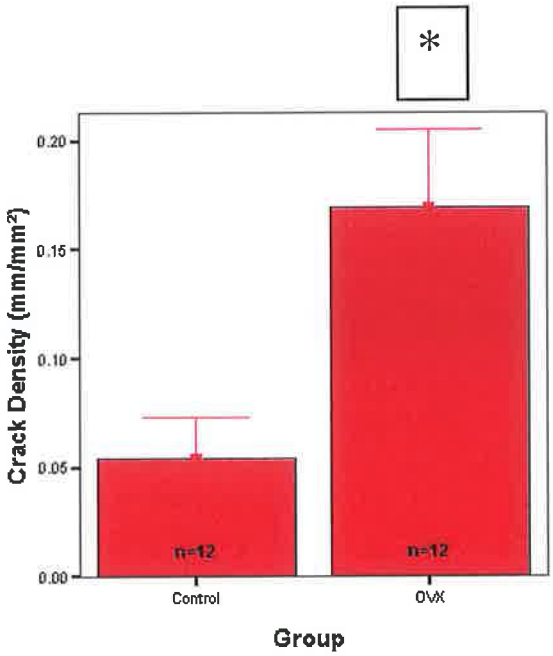
RESULTS – 12 Month Group

Control and OVX subgroups, graphical representation:



Error Bars show 95.0% CI of Mean

Bars show Means

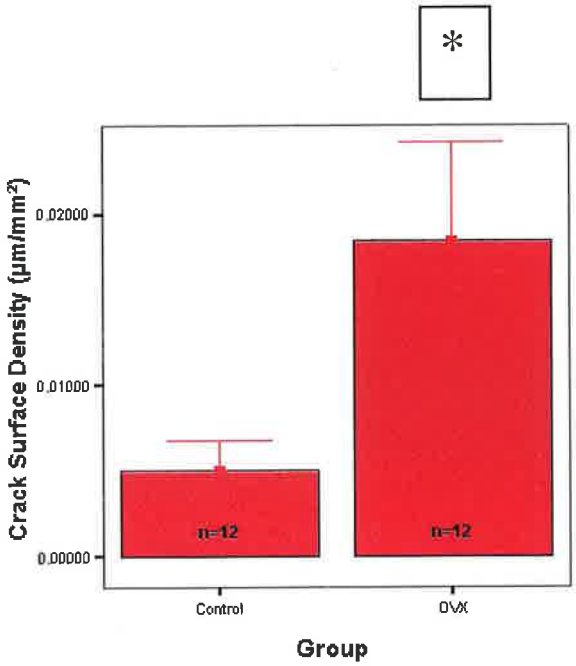


Error Bars show 95.0% CI of Mean

Bars show Means

Crack density highest in OVX group, p value < 0.001)

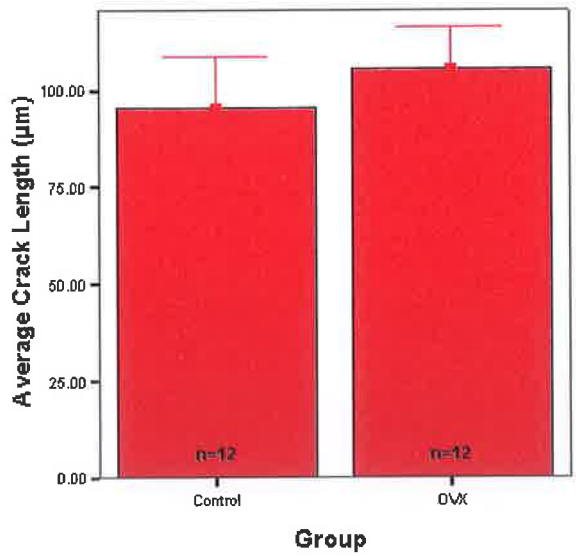
Figure 3. 6



Error Bars show 95.0% CI of Mean

Bars show Means

Crack Surface Density highest in OVX group, P value <0.0001



Error Bars show 95.0% CI of Mean

Bars show Means

Figure 3. 7

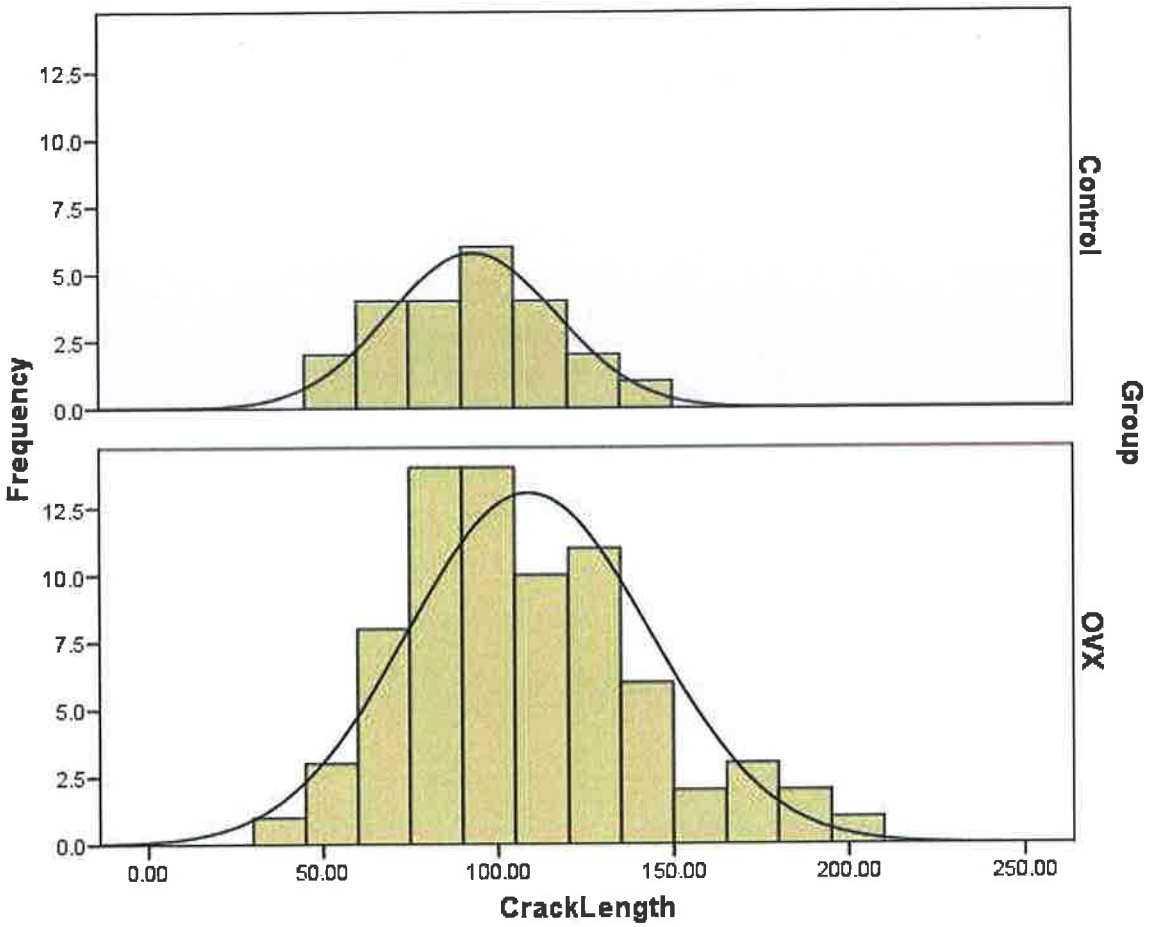


Figure 3. 8

Histogram showing crack lengths in μm, the range for both groups is similar, except a larger number / frequency of cracks in OVX subgroup and on average, longer cracks, but no statistical significant difference.

CONTROL – Mean crack length = 93.30 ± 23.86 μm

OVX – Mean crack length = 108.83 ± 34.41 μm

12 month group – (Remodelling graphs)

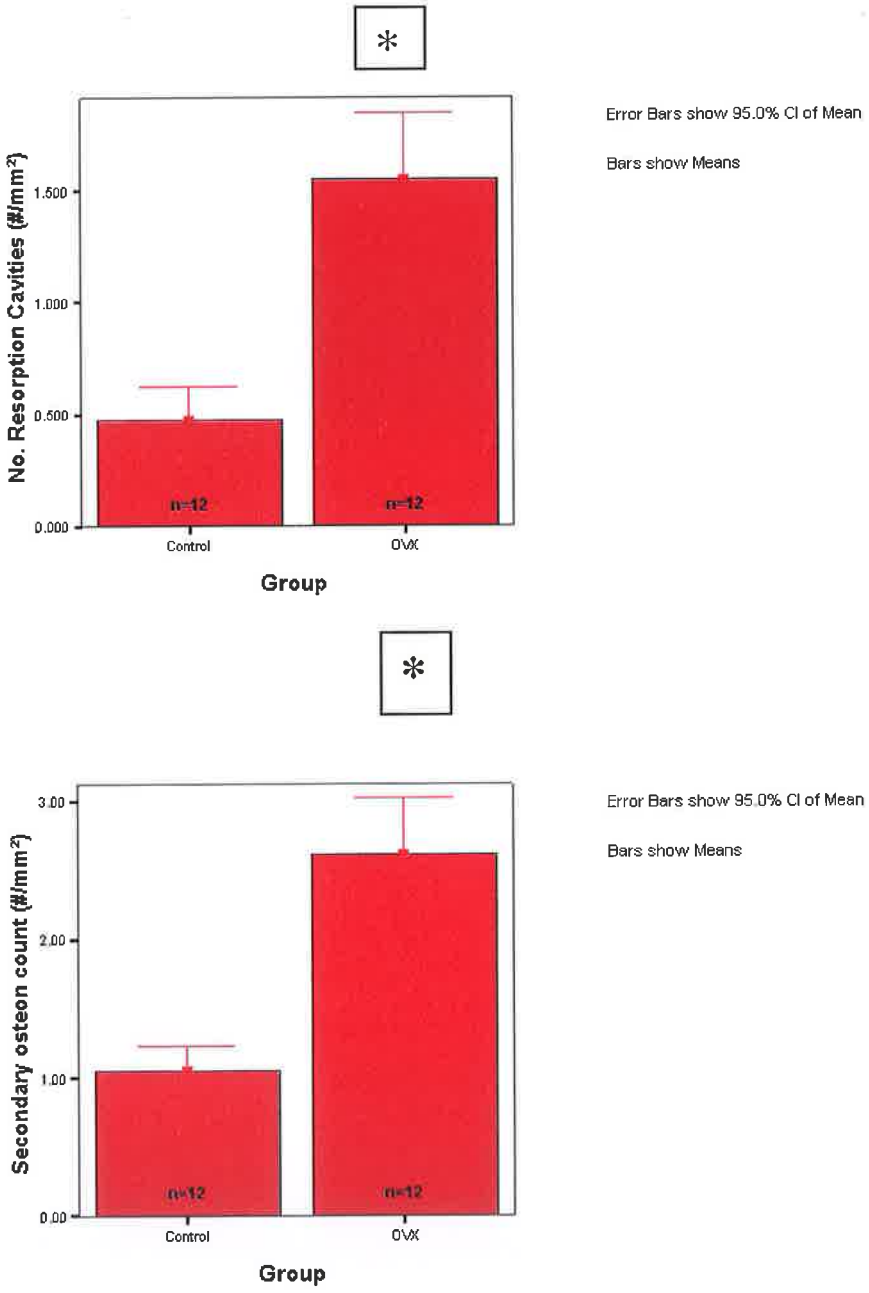


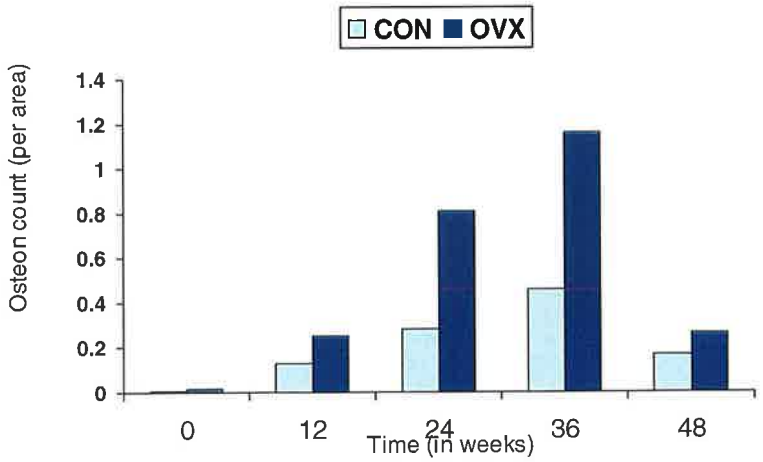
Figure 3. 9

In the OVX group both resorption cavities and labeled secondary osteon counts were increased, significant difference in both, p value < 0.001

Figure 3.10

Intracortical labelled osteons over a period of time post – OVX

(12 Month Group)



In terms of time of year:

0 weeks – November
12 weeks – February
24 weeks – May
36 weeks – August
48 weeks – November

Labelled osteon densities in control and OVX group at 0, 12, 24, 36 and 48 weeks post – OVX, represented in the graph above (example of labeled osteon microscopy images to follow in chapter). Different fluorochrome dyes were administered at those time intervals and the remodelling rate was highest in the OVX group, with statistical significant differences in all time intervals except at 0 weeks.

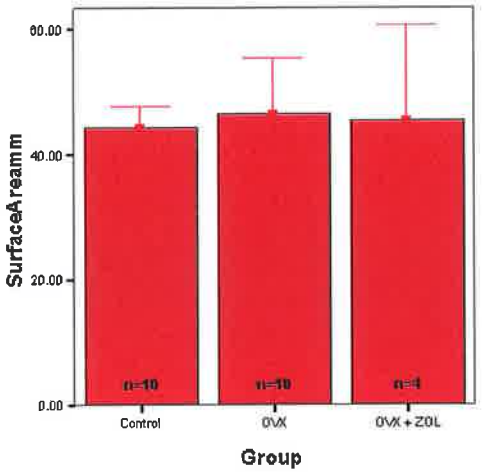
P value < 0.05 at 12 and 48 weeks

P value < 0.001 at 24 and 36 weeks

The labelled osteon densities in control and OVX group varied over the 12 month period, in keeping with the seasonal effect on bone turnover in sheep. After turnover increased from 0 – 36 weeks (0 – 9 months), and then decreased at 48 weeks (12 months).

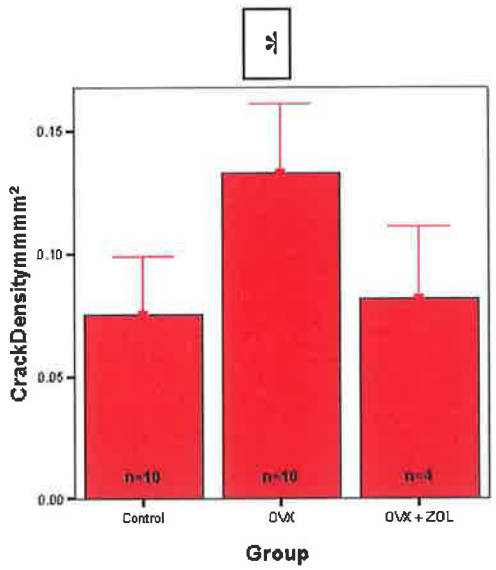
RESULTS – 31 Month Group

CON, OVX and OVX + ZOL subgroups, graphical representation:



Error Bars show 95.0% CI of Mean

Bars show Means

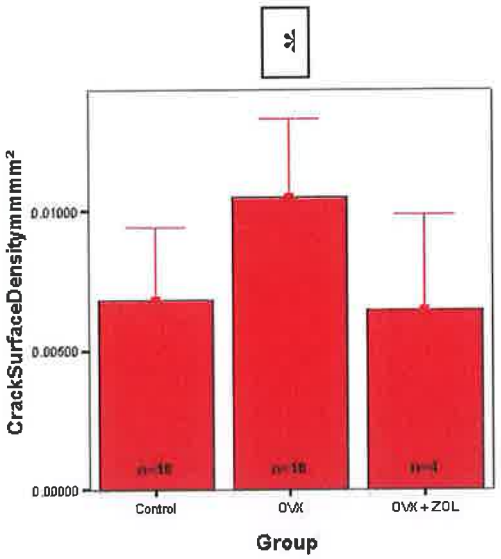


Error Bars show 95.0% CI of Mean

Bars show Means

Crack density highest in OXV, p value < 0.05 when compared to CON and OVX + ZOL

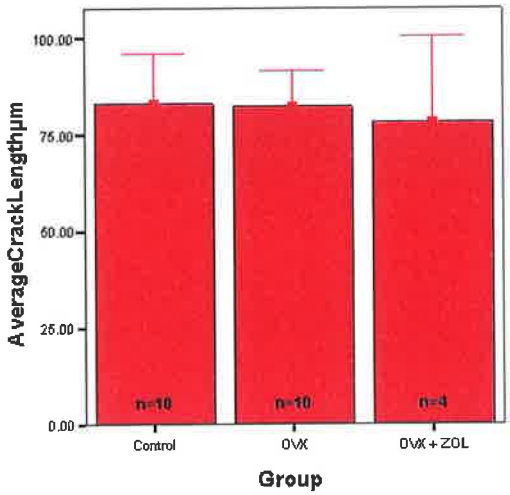
Figure 3. 11



Error Bars show 95.0% CI of Mean

Bars show Means

Crack surface density highest in OXV, p value < 0.05 when compared to CON and OVX + ZOL



Error Bars show 95.0% CI of Mean

Bars show Means

Figure 3.12

Similar lengths, P value > 0.05. OVX + ZOL shortest microcracks (see histogram)

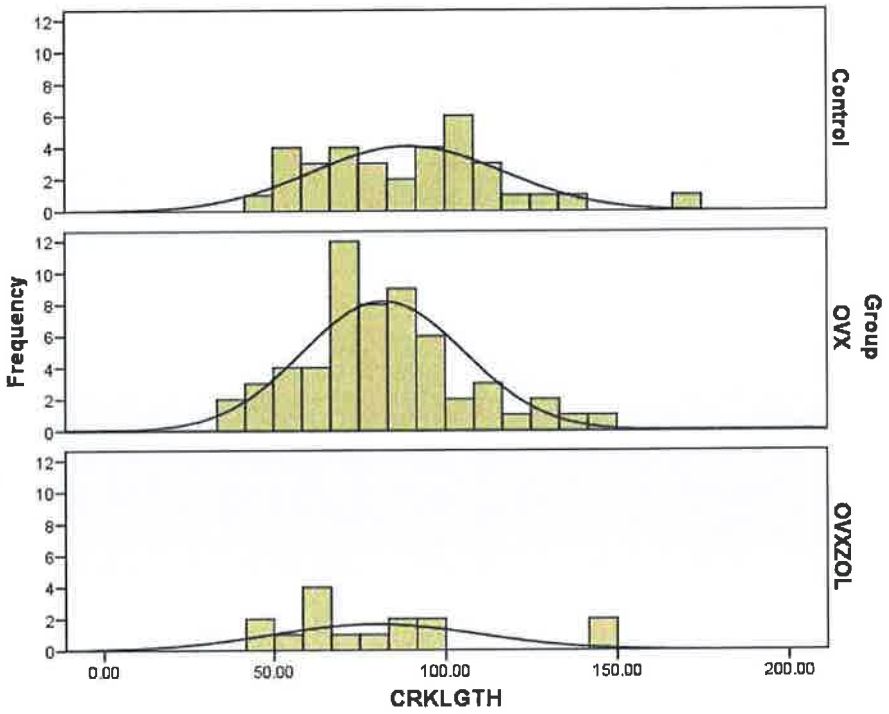


Figure 3. 13

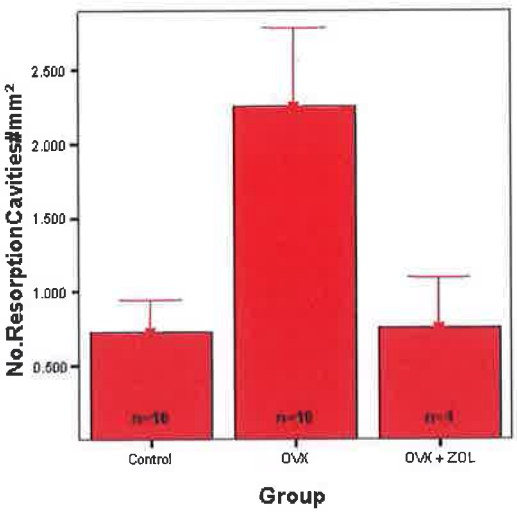
Histogram showing crack lengths in μm. Similar range distribution; longer cracks in Control group, no significant differences.

CONTROL – Mean crack length = 89.24 ± 27.76 μm

OVX – Mean crack length = 82.03 ± 23.54 μm

OVX + ZOL – Mean crack length = 79.43 ± 30.45 μm

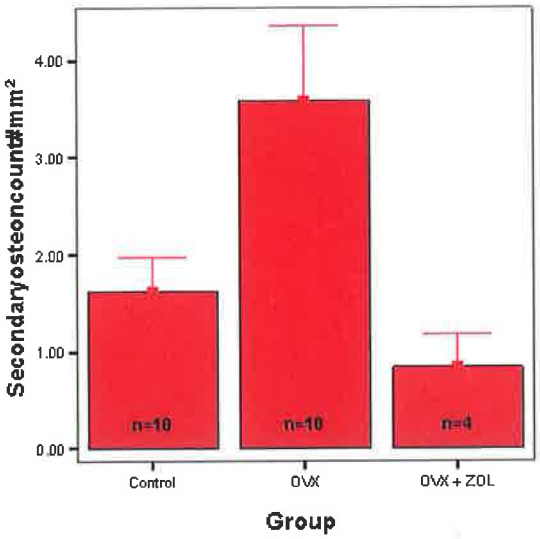
31 Month Group – (Remodelling Graphs)



Error Bars show 95.0% CI of Mean

Bars show Means

Resoprtion cavities, highest in OVX group, P<0.0001 when comparing OVX to CON. P value <0.001, comparing OVX to OVX + ZOL



Error Bars show 95.0% CI of Mean

Bars show Means

P value < 0.001 OVX v CON
P value <0.001 OVX v OVX + ZOL
P value < 0.05 CON v OVX + ZOL

Figure 3. 14

Bone turnover is at its lowest level in the OVX + ZOL group, highest in the OVX group.

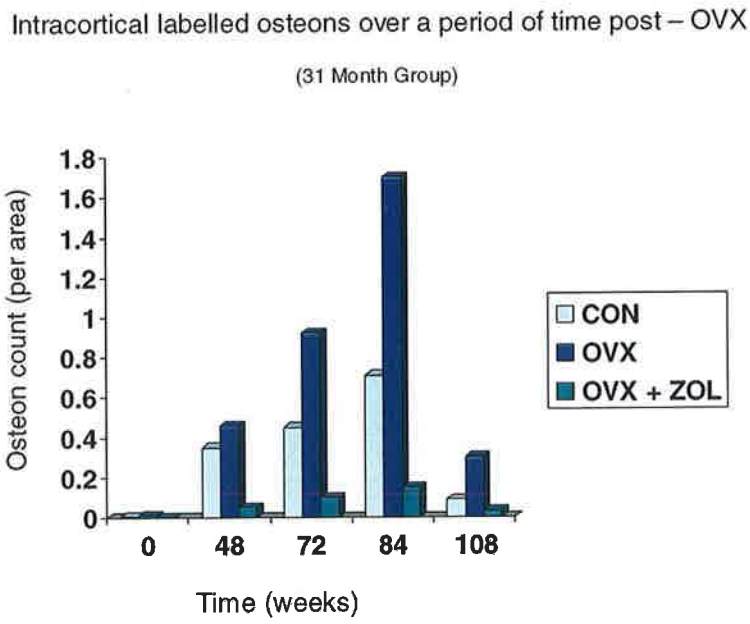


Figure 3. 15

Labelled osteon densities in the 3 subgroups, showing similar pattern to that found in the 12 month group; gradual increase in bone turnover, reaching maximum levels in all three groups at 84 weeks post OVX.

In weeks, 48, 72, 84 and 108 there is a significant difference in secondary labelled osteon count numbers, P value < 0.001 comparing OVX to OVX + ZOL.

P value < 0.01 in weeks 72, 84, 108 when comparing OVX to Control.

P value < 0.01 comparing CON to OVX + ZOL at weeks 48, 72, 84.

SUMMARY of RESULTS

12 Month Group (Table 3. 2)

	Control	OVX
Surface Area	-	↑
Crack Density	-	↑ *
Crack Surface Density	-	↑ *
Average Crack Length	-	↑
No. Resorption Cavities	-	↑ *
Seondary Osteon Counts	-	↑ *

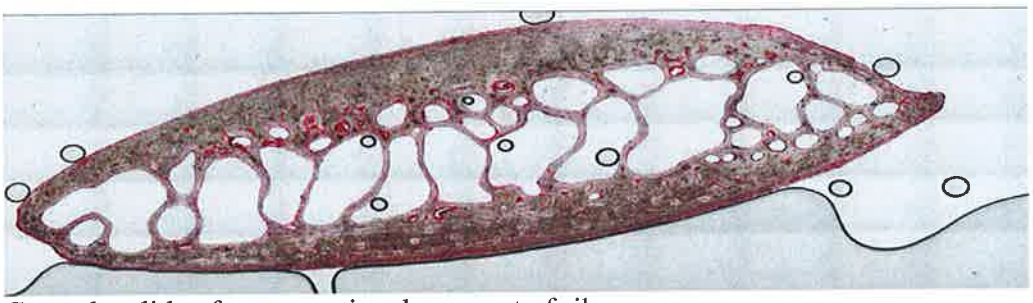
* denotes a statistically significant p value. (OVX v CON)

31 Month Group (Table 3. 3)

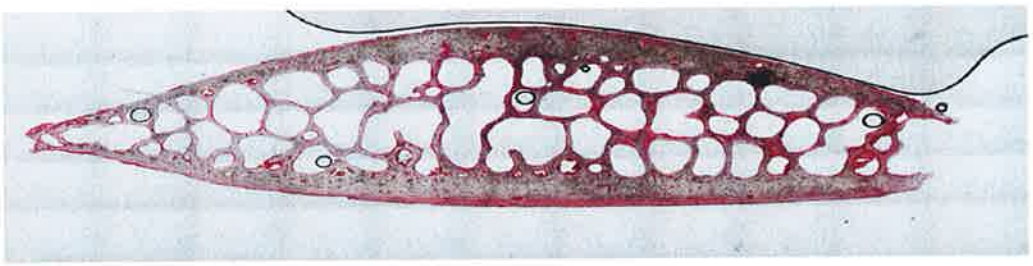
	Control	OVX	OVX + ZOL
Surface Area	-	↑	↑
Crack Density	-	↑ *	↑
Crack Surface Density	-	↑ *	↓
Average Crack Length	-	↓	↓
No. Resorption Cavities	-	↑ *	-
Seondary Osteon Counts	-	↑ *	↓

* denotes a statistically significant p – value (OVX v CON and OVX + ZOL v CON)

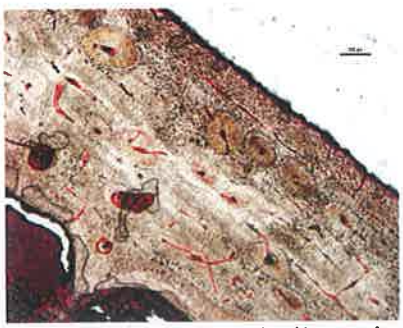
MICROSCOPY IMAGES



Control – slide of cross sectional segment of rib.

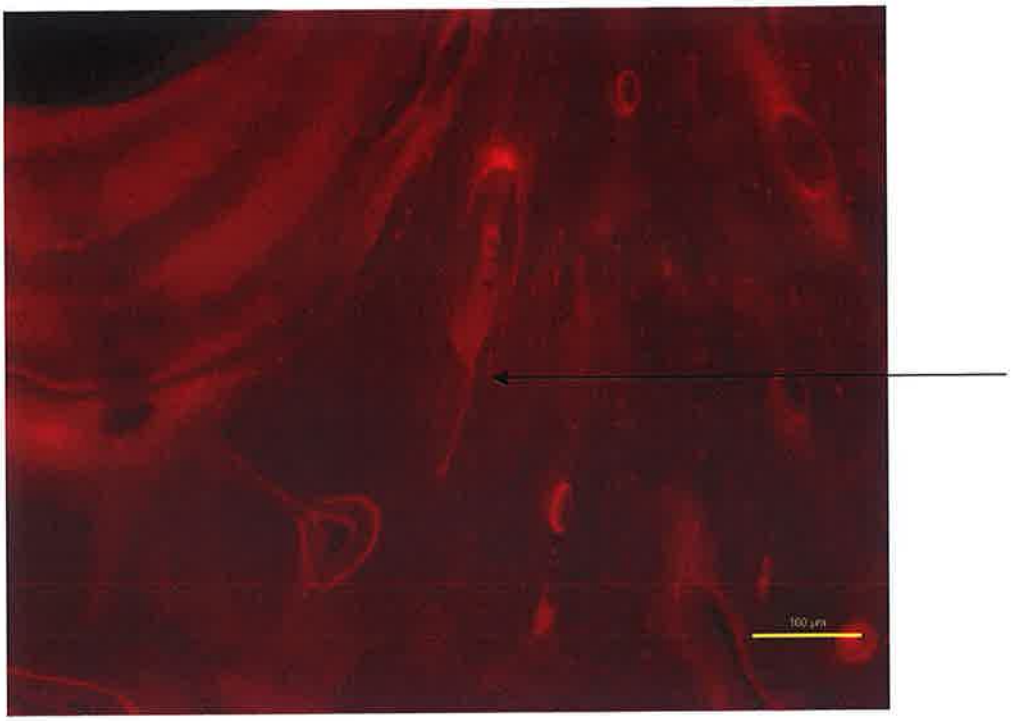


OVX – sample slide

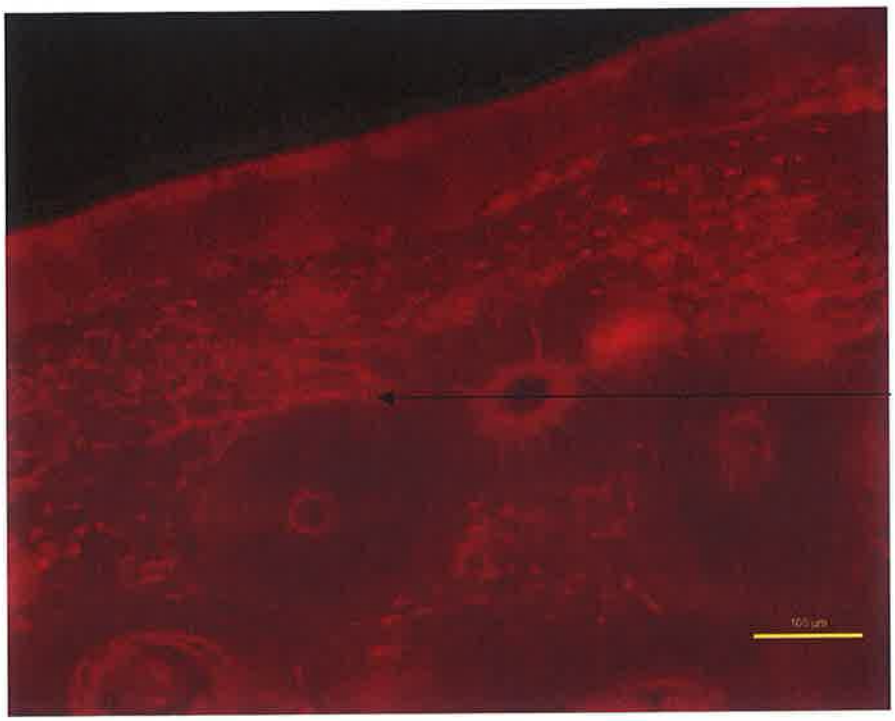


Cross section through rib cortical section viewed under light microscopy and epifluorescence with green incident light.

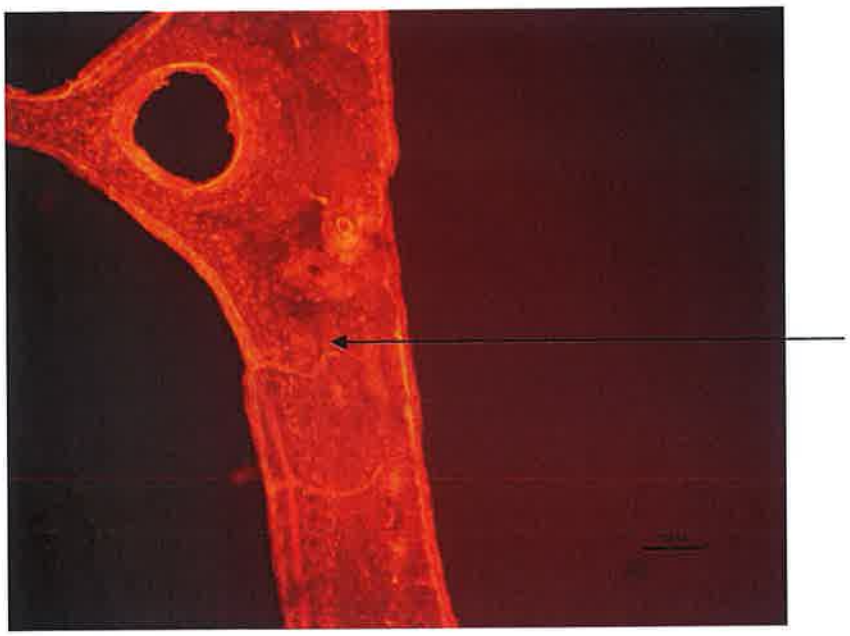
(Scale bar = 100μm)



Epifluorescence with green incident light; microcrack visible.

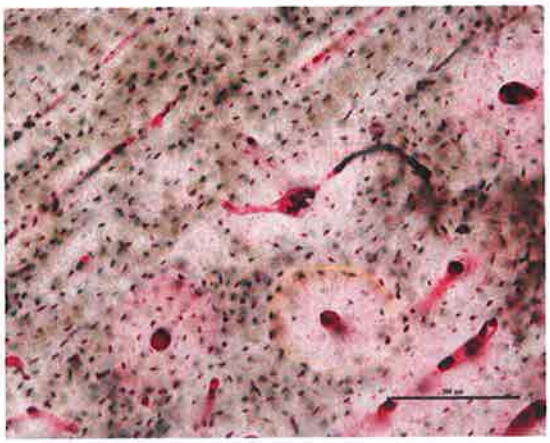


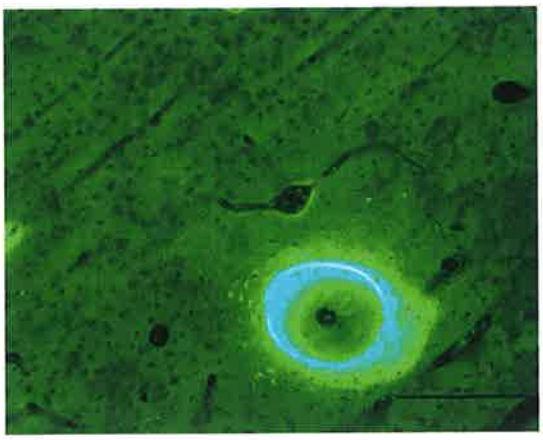
Epifluorescence with green incident light, microcrack running in horizontal place.
(Scale bar = 100 µm)

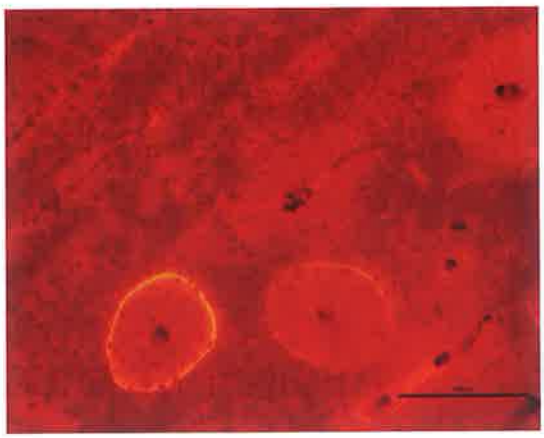


Epiflurorescence with green incident light, notching and crack propagation from trabecular region, luminal side of cross section (to the left).

Calcein fluorochrome labelled secondary osteons; viewed under blue incident light
(Scale bar = 100 µm)







Intracortical bone turnover sites labelled with differing fluorochrome dyes, viewd under light microscopy initially, can observe the pink and yellow colouration around the margins of the secondary osteon. Followed then by viewing under blue and green incident light via epifluorescence microscopy.

(Scale bar = 200μm)

DISCUSSION

Fatigue damage in bone occurs in the form of microcracks. This microdamage contributes to the formation of stress fractures and fragility fractures and contribution to the loss of bone quality in osteoporosis. Microdamage accumulation also increases with age in humans (Schaffler et. al., 1995). Therefore ovariectomy, which is an accepted means of inducing postmenopausal changes to bone, would be expected to result in increased levels of microdamage. In ovariectomised rats the crack density and crack surface density have both been shown to increase relative to sham operated rats (Dai et al., 2004).

In this study, in both the 12 month group and 31 month group crack numerical density and crack surface density were increased in the OVX samples and there was a statistical significance in the figures p value < 0.001. There also appears to be an overall increase in microdamage accumulation when comparing the 12 month and 31 month group, in keeping with the increased time lapse, although this was not statistically significant.

There was no statistical difference in the average crack length or surface area of cortical bone under examination.

Bisphosphonates have previously been reported to increase crack density and surface density at both clinical and supraclinical doses (Mahiba et al., 2000; Allen et al., 2006). Their effects on damage have also shown to peak during the early

period (1 year) of bisphosphonate treatment with no subsequent increase in damage levels after 3 years (Allen and Burr, 2007).

Bisphosphonates suppress osteoclastic activity and hence, microdamage accumulation is what is to be expected as there is no resorption possibly due to diminished osteoclasts. Along with having an effect on crack density, bisphosphonates have also been shown to increase mineralization levels. It has previously been demonstated that microdamage initiates more readily in highly mineralized areas of bone (Wasserman et. al., 2005).

In the subgroup within the 31 month group, the four sheep rib samples that were examined, which had received intravenous zolendronic acid at a supraclinical dose, the findings in terms of microdamage were nearly equal to the control specimens. There had been no accumulation on microdamage, as it may have been expected.

In both the 12 month and 31 month group number of resorption spaces were high along with number of labelled secondary oseteons per unit area. Overall there was significantly increased remodeling taking place in the OVX sheep. Multiple fluorochrome labeling techniques have been used to assess various aspects of bone physiology in a number of animal models (Frost, 1969; Lee et al., 2002). For this study the sheep had been administered at three monthly intervals during the year beginning on the day of surgery and finishing four weeks prior to sacrifice. The three month interval coincides approximately with ovine and human remodeling cycles (Turner et al, 1995; Lee et al, 2002). The data showed

that over the given time period there were more labelled osteons in the OVX than in the control group. Numbers reached a significant level after 3 months and a maximum was attained in the first 9 months in the OVX group with a drop and then again a similar pattern was seen in the 31 month cycle. This information suggest that ovariectomy begins to increase bone turnover in ovine cortical bone. These findings are in support of work carried out by Turner et al, 1995, who used biochemical markers to show accelerated remodeling in sheep 3 months post OVX. Kennedy et al, 2007 also showed that there was a similar pattern in remodeling rates with the similar sampled sheep where the vertebrae was being investigated.

The interesting and differing results in this study as compared to some others are:

In the OVX group both microdamage and remodeling is significantly increased as compared to the control group. It may be expected that if there is increased bone turnover, microdamage levels should not be up significantly.

The other interesting result that has been mentioned earlier, were the findings in the group that had undergone zolendronic acid treatment. In terms of microdamage there was no accumulation and the numbers were very similar to the control groups. The remodeling aspect was in keeping with expected findings, that they were significantly lower than OVX samples, lower than control or at par with them. That could be explained due to osteoclastic suppression.

The reasons for these possibly boil down to a number of facts and mainly, it may be due to the structural and mineral properties of the rib. It is a non weight bearing, cyclically loaded bone that undergoes cylical levels of strains. In the OVX group increased bone turnover gave numerous secondary osteons within the cortical segment, and resorption cavities prior to that or during a different phase of remodeling, these would allow for increased "stress" levels across the plane of the rib itself and hence can cause initiation and encouragement of microcrack formation and accumulation.

Apart from secondary osteons causing areas of potential weakness and altered strain and hence provoking microdamage accumulation, resorption cavities that initate BMU work and formation of the osteons also act as potential regions of increased stress levels in bone (Hernandez et al., 2006).

A study by Skedros et al, 2003, looked at regional differences in cortical bone organization and microdamage, where ribs had an increased microcrack accumulation along with new remodeling events, both were up. They hypothesized that due to different loading conditions, e.g weight bearing bones as compared to non weight bearing bone, and due to their mineral content, some are more susceptible to microdamage than others.

In the zolendronic acid group there appears to be an uncoupling effect as osteoclast activity is suppressed, microcrack numbers are low. Resorption cavities are also significantly lower than the OVX group, and if we were to extrapolate these findings it may be possible to hypothesise that due to the fact of the

decreased remodeling sites in this non weight bearing cyclically loaded cortical segment of rib, there are lower potential "spots" of weakness, lower strain levels, and hence microcrack is not initiated as it may have done if they were present as seen in the OVX group.

Potential areas of concern in terms of errors related to this study, could be possibly due to the sheep used in the study were of a mixed breed variety and their age ranged from across 3-5 years. Plus only a small number were treated with zolendronic acid, as compared to a much larger cohort of control and ovariectomized sheep. So accurate comparison would be difficult to make, a larger treatment group would be necessary. Overall though significant differences were obtained within the subgroups and findings were in keeping with changes expected and there was clarity between the ovariectomized samples, control and treatment group despite small number in the latter.

CONCLUSION

- Ovariectomy significantly increased both microdamge and remodeling in both 12 and 31 month groups.

- Resorption cavity numbers were also significantly increased in the OVX group along with bone turnover over a period of time as measured by labelled osteon densities. Reinforced the seasonal variation that is to be expected in sheep as they undergo regular oestrous cycles along with anoestrous periods.

- Zolendronic acid treated group caused an uncoupling reaction, where remodeling was decreased due to inhibition of osteoclasts and at the same time there was no accumulation of microdamage, possibly due to lack of resoprtion cavities and mechanical changes as a result in the levels of potential stresses in cortical bone.

Chapter 4

Trabecular Analysis of Ovine Iliac Crest Biopsies

INTRODUCTION

Microarchitecture can be understood in terms of the trabecular microstructure, which encompasses the orientation, thickness and spacing of trabeculae as well as the extent to which they are interconnected (Fig 4.1). Majority of the studies that investigate osteoporotic bone quality tend to focus on trabecular bone tissue. This is because of the prevailing belief that the rate of bone turnover is higher in areas of trabecular bone; thus most of the deterioration in bone quantity and quality, including micoarchitecture will be found in these areas. Neither of these factors is necessarily true. Parfitt (2002) noted that "it has often been asserted, without qualification that cancellous bone has higher turnover than cortical bone". He went on to comment that there are circumstances in which this is indeed true, but there are also circumstances in which it is not.

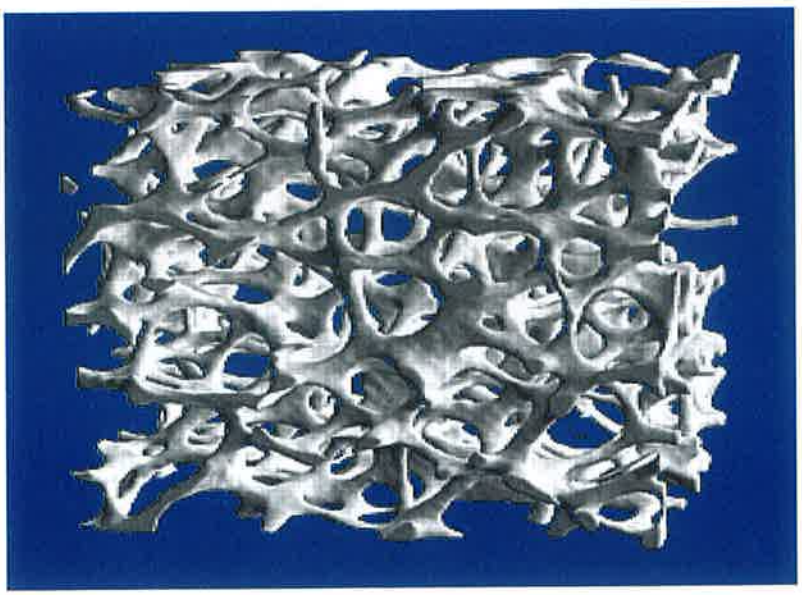


Fig 4.1 Trabecular Bone www.sciencecases.org (MicroCT image)

As previously mentioned, bone remodelling is a continuous process, a coupled action of bone resorption and deposition, carried out by osteoclasts and osteoblasts. In trabecular bone, osteoclasts travel along the surface of trabeculae, resorbing the tissue and forming a resorption pit, and osteoblasts follow along behind filling in the cavity with osteoid, which subsequently mineralizes (Parfitt, 1984). It is generally agreed that this process tends to adapt the architecture of cancellous bone so that there is minimum stress in the bone tissue relative to its weight (Hart, 2001). However, it has been observed that microdamage is generated in both trabecular (Vashishth et al., 2000) and cortical bone (Lee et al., 2002) under everyday physiological loading conditions and these observations lead to the suggestion that trabecular bone remodelling may also prevent accumulation of microdamage, as has been hypothesised for compact bone (Carter et al., 1987; Frost, 1986; Martin and Burr, 1989; Prendergast and Taylor, 1994; Martin, 2002). Because resorption cavities occurring during trabecular remodelling can be large relative to the size of the trabeculae, it is possible that significant elevations of stress occur around them (Van der Linden et al., 2001). Similar reasoning can be attributed to the findings in cortical rib specimens (Chapter 3).

The current clinical standard of diagnosing osteoporosis and assessing the risk sustaining an osteoporotic bone fracture is Dual Energy Xray Absormetry (DXA) for the measurement of BMD (Bone Mineral Density) at the spine and hip, the two skeletal locations most prone to fracture. The structure and spatial arrangements of

bone at the macroscopic and microscopic levels are thought to provide additional, independent information and may help to better predict fracture risk and assess response to drug intervention (Genant et al., 2008)

While many parameters that were developed to capture macro- and microstructural properties can be easily assessed *in vitro*, non-destructive and non-invasive techniques for *in vivo* use are at the forefront of research in the radiology of osteoporosis. The imaging of specimens, bone biopsies and small animals for the investigation of bone structure currently is almost exclusively done with microCT (μCT) scanners.

BONE BIOPSY

This particular part of the study concentrates on trabecular analysis, using a μCT scanner to study iliac crest specimens. The reasoning behind choosing this skeletal site for investigation, is to be able to correlate the data, with various animal studies of a similar nature and to allow a comparison with data from clinical studies of human iliac crest samples.

Bone biopsy is a simple technique which harvests bone cylinders through the iliac bone in a clinical setting. The technique appeared in the 1960s and was considerably popularized and developed after 1970 with the development of bone histomorphometry as a useful tool in the diagnosis of metabolic bone disease (Durchschlag et al., 2006). In this particular case "architectural" elements are of concern hence, frozen sections were adequate as no marrow cytological evaluation was to be carried out.

An intact full thickness transiliac biopsy can be regarded as representative of the entire bone (Malluche et al. 1982) since the length of the cylindrical biopsy core perpendicular to the external surface depends mainly on the width of the iliac bone at the site of sampling (Malluche et al., 1986). The same principle can be applied to rib biopsies, by using the whole area enclosed by periosteum as the referent (Parfitt et al., 1987).

The aim of this study was to investigate the effects of ovariectomy and treatment with zolendronic acid on trabecular architecture in iliac crest specimens in an ovine model.

MATERIALS AND METHODS

Sample Selection

For the purpose of the study, the 2 groups were separated as follows:

12 Month Group

Control (CON)	Ovariectomized (OVX)
10 samples	10 samples

31 Month Group

CON	OVX	OVX + Zolendronic Acid
8 samples	8 samples	4 samples

Each of the 12 and 31 month groups had 20 core biopsies in total.

Obtaining Core Biopsies

Iliac crest core biopsies were obtained with the aid of a milling machine, using a drill piece with a diameter of 10mm. A minimum of 7mm is required for a sufficient

diameter in order to correctly preserve bone microarchitecture and to provide enough marrow for cytological analysis if required (Durchschlag et al., 2006).

The core biopsies were taken from the right ilium, 2cm medial from the anterior superior iliac spine and 2cm inferior from that point on each specimen (Fig 4.2, Fig 4.3). Each pelvis was fixed in a manner to allow the drill piece to cut through at a perpendicular level, using a vice grip on the milling machine itself.

Once the cylindrical core samples were obtained, they were wrapped in moist guaze and stored in a freezer, until scanned.

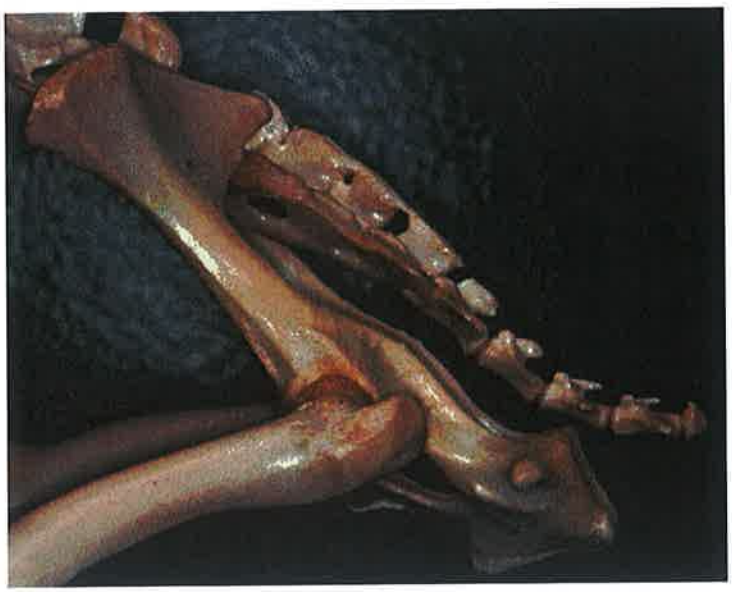


Fig. 4.2.Left lateral view of the sheep pelvis (www.aps.uoguelph.ca)

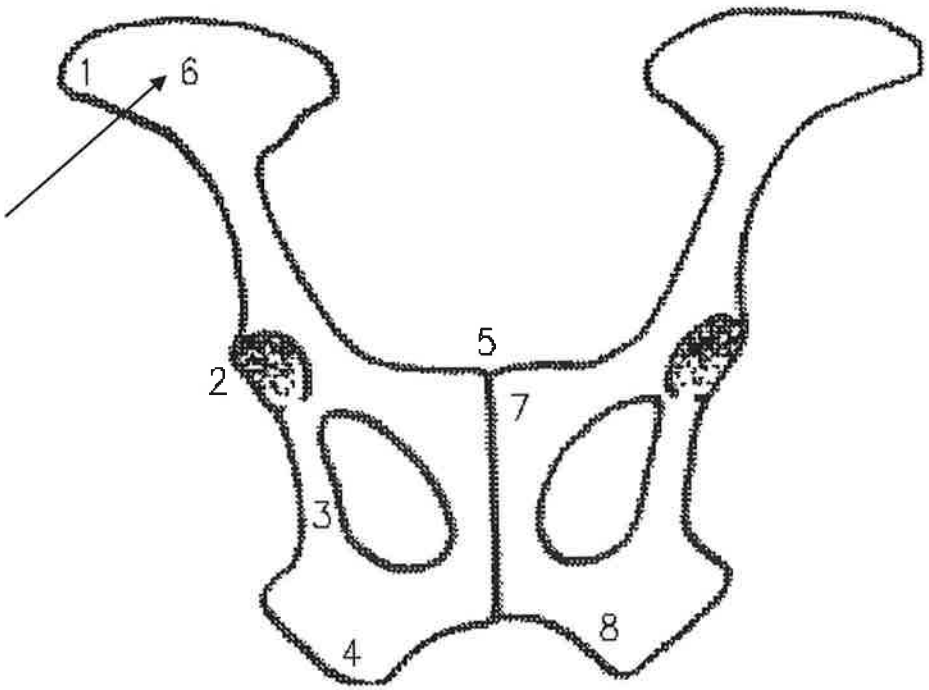


Fig 4.3 Anterior view - Pelvic Anatomy (www.aps.uoguelph.ca)

1, tiber coxae (anterior superior iliac spine); 2, acetabulum; 3, acetabular ramus of ischium; 4, tuber ischii; 5, symphysis pubis; 6, ilium; 7, pibis; and 8, ischium. The arrow indicates the biopsy site.

Micro CT Analysis

Each of the iliac crest core biopsies were thawed at room temperature for ~ 6 hours prior μCT scanning (Scanco, μCT – 40, Bassessdorf, Switzerland). Samples were placed in a cylindrical specimen holder and fixed into the scanner. Samples were wrapped in moist gauze for the duration of scanning.

During the scanning process, xrays are directed towards the sample and, after passing through the sample, they are detected by a 2048 × 256 element CCD array which is controlled by a dedicated workstation (Kennedy et al., 2008). A specific measurement protocol (control file) was created before scanning began so that all parameters, such as source energy, scan time and resolution, were identical for every sample in the study. Source energy was 70 kV, scan time for each sample was less than 30 minutes and the scan resolution was 8 μm (Laib, 2000).

Fig 4.4 Micro CT (Scanco Medical). Model used for this study was no.40

After 3-D reconstruction via software on the workstation connected to the μCT scanner, calculations were performed and the following measurements were obtained and used to compare the different subgroups:

TV = total volume (mm^3)

BV = bone volume (mm^3)

BV / TV = relative bone volume present

BS = surface area (mm^2)

TbN = trabecular number (#/mm)

TbTh = trabecular thickness (μm)

TbSp = trabecular separation (μm)

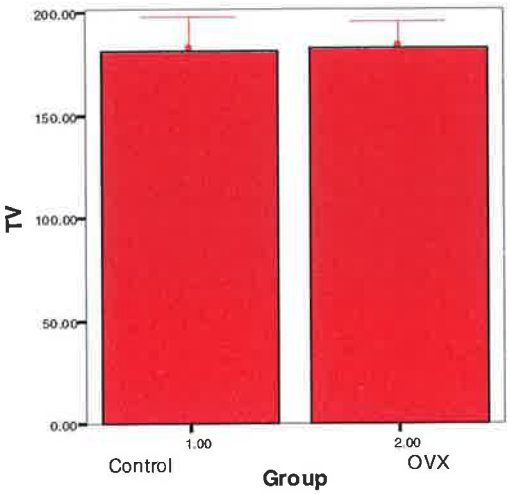
STATISTICAL ANALYSIS

Groups were assessed for normal distribution and then compared using a Mann - Whitney U test for the 12 month group. The 31 month group (3 subgroups) underwent Kruskal and Wallis assessment prior to Mann – Whitney U tests comparing each subgroup to one another. SPSS statistical package, version 15.0 was used for statistical analysis. A p value of < 0.05 was considered to be significant.

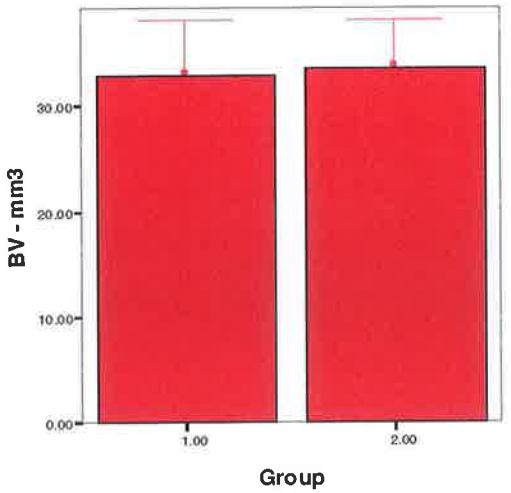
RESULTS – 12 Month Group

Control and OVX subgroups, graphical representation:

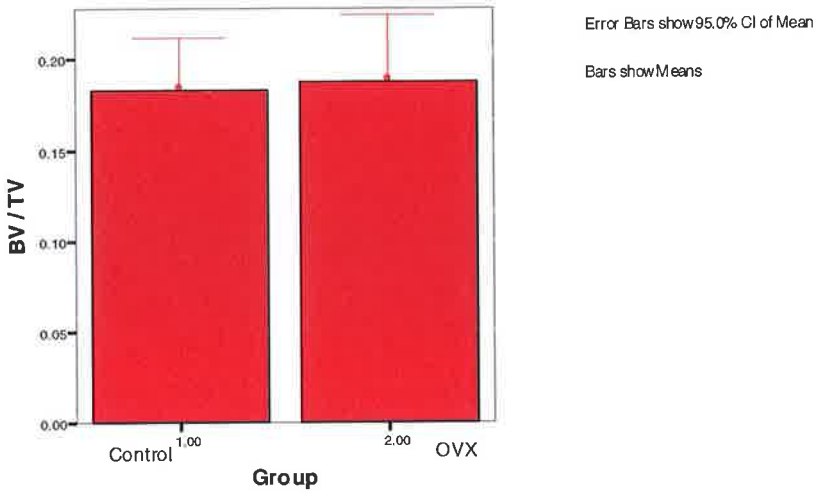
a) Fig 4.5 – Total volume



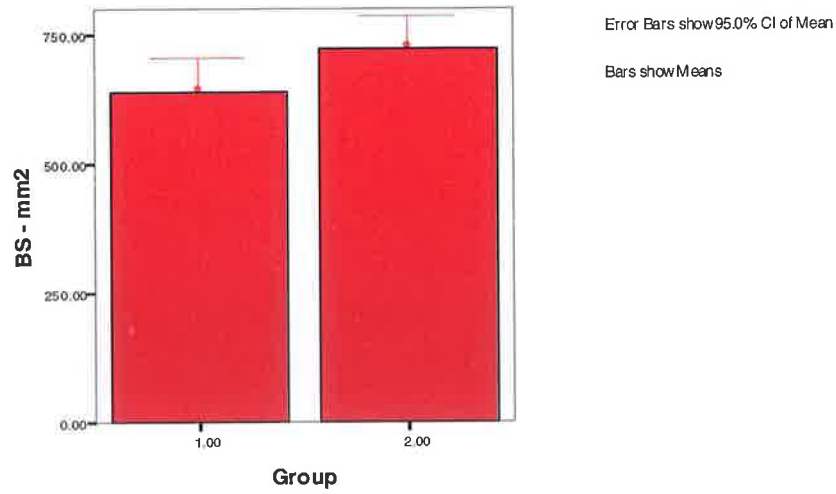
b) Fig 4.6 – Bone volume



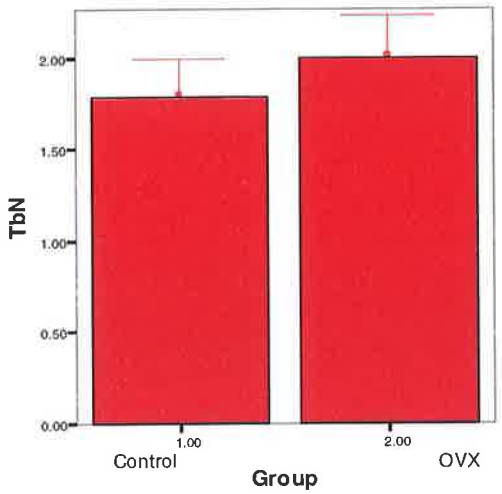
c) Fig 4.7 – Relative bone volume



d) Fig 4.8 – Surface area



e) Fig 4.9 – Trabecular number



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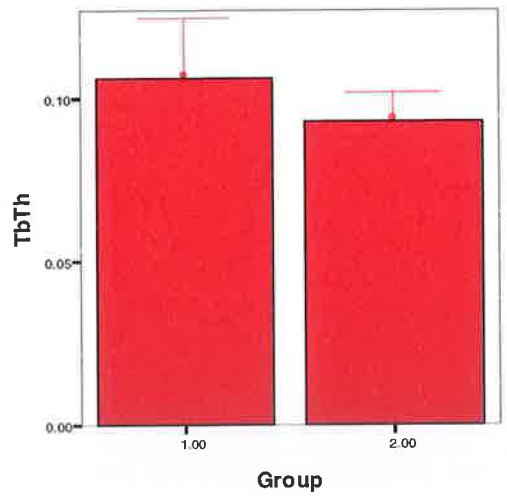
f) Fig 4.10 – Trabecular thickness



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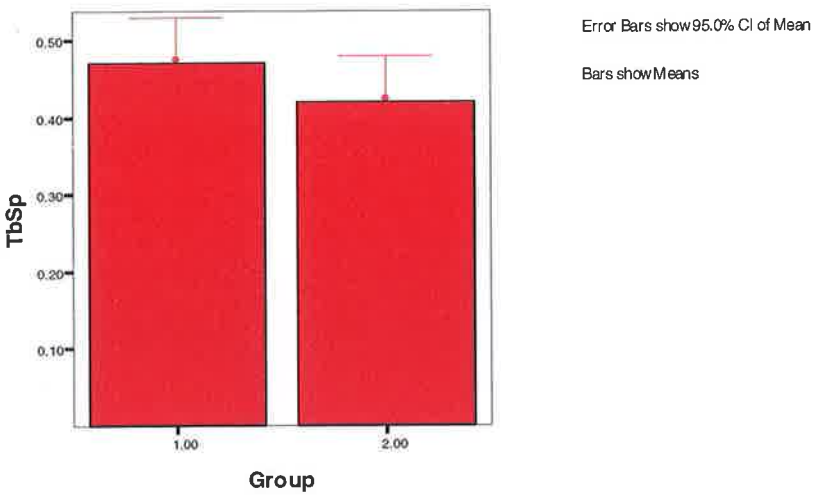
g) Fig 4.11 – Trabecular separation

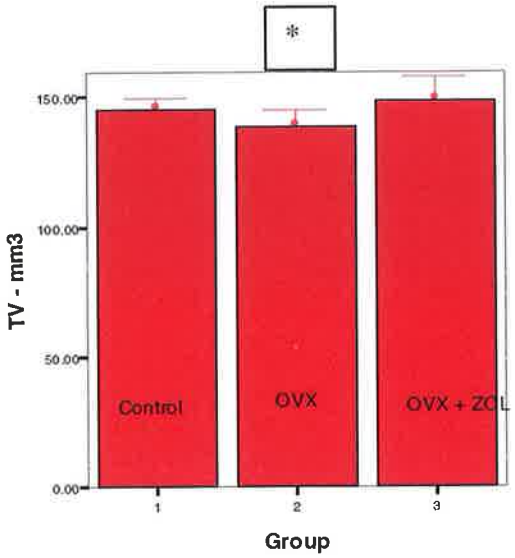


There was no statistical difference in any of the parameters measured within this group.

RESULTS – 31 Month Group

CON, OVX and OVX + ZOL subgroups, graphical representation:

a) Fig 4.12 – Total volume



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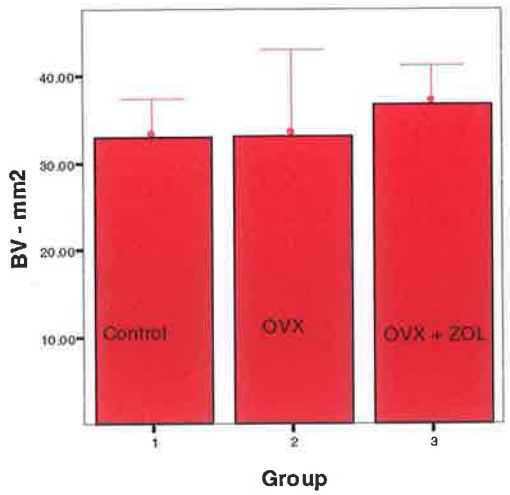
Total volume $p < 0.05$ in control v OVX

b) Fig 4.13 – Bone volume



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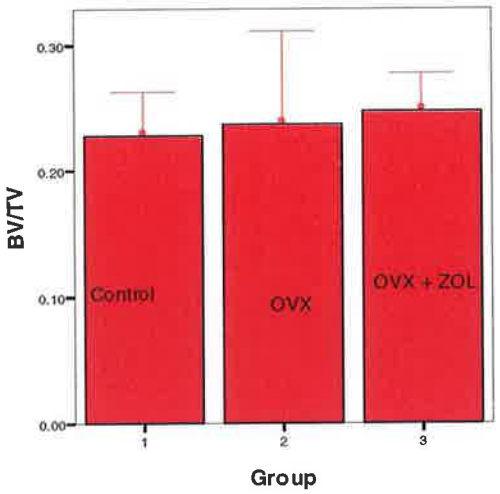
c) Fig 4.14 – Relative bone volume



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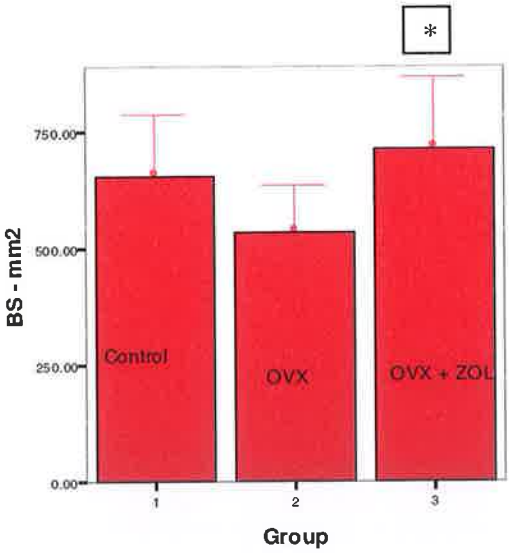
d) Fig 4.15 – Surface area

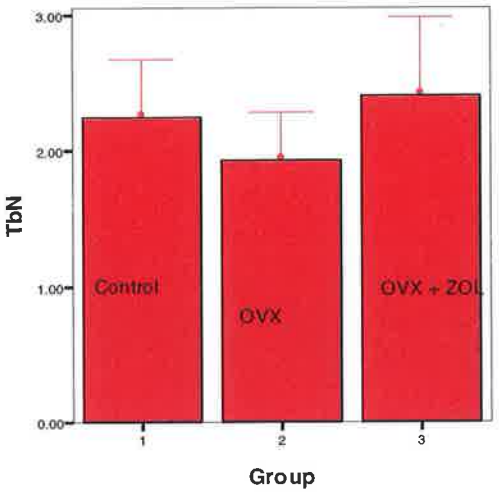


Error Bars show 95.0% CI of Mean

Bars show Means

BS highest in OVX + ZOL, $p < 0.05$ when compared to OVX

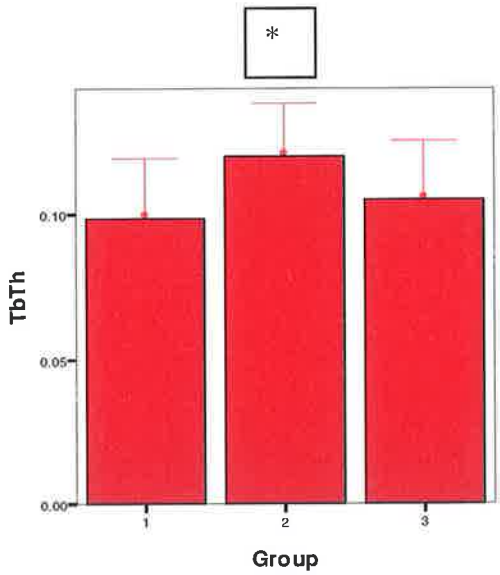
e) Fig 4.16 – Trabecular number



Error Bars show 95.0% CI of Mean

Bars show Means

f) Fig 4.17 – Trabecular thickness



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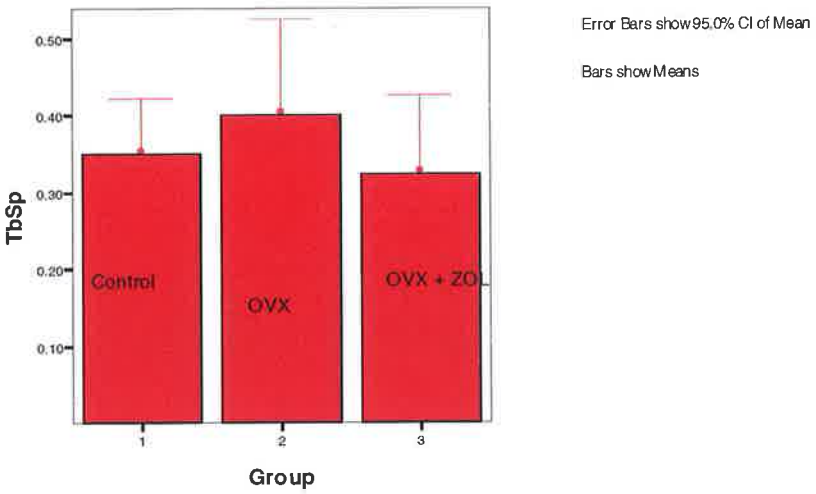
TbTh highest in OVX $p < 0.05$, when compared to Control

g) Fig 4.18 – Trabceular separation



Summary for 31 month group: (Table 4.1)

	Control	OVX	OVX+ZOL
TV	-	↓ *	↑
BV	-	-	↑
BV/TV	-	↑	↑
BS	-	↓	↑ *
TbN	-	↓	↑
TbTh	-	↑*	↑
TbSp	-	↑	↓

* denotes a statistically significant p value

↑ - increase in the measured parameter as compared to the control

↓ - decrease in the measures parameter as compared to the control

Figure 4.19 – MicroCT image of iliac crest core biopsy.

DISCUSSION

Studies of the aetiology of human osteoporosis are complicated by confounding factors such as diet, smoking, alcohol usage, exercise patterns, and oestrogen replacement therapy, which are usually irrelevant in animal models. As a result several OVX models have been used for osteoporosis research to minimise the effects of such factors. The advantages of the OVX sheep over other animal models have been discussed in length earlier (Chapter 2). An additional point to note is that the large bones of OVX sheep present an opportunity to examine the mechanical behaviour of osteoporotic bone. Since the mechanical properties of bone are also dependent on its trabecular structure, this study was designed to determine the effects of ovariectomy on the trabecular portion of the pelvis by looking at the 12 month and 31 month groups and a treatment subgroup was also looked at in the 31 month group, the OVX + Zolendronic acid group.

A point to note here was that no statistical significance was found between control and OVX animals in the 12 month group, which is not what might have been expected. A study by Newton et al., 2004, which looked at the ovariectomized sheep as a model for human bone loss, used a similar set up. They had a 12 month period, and two groupings similar to this study: Control and OVX. The results however were in the form of time 0 months and 12 months for iliac crest biopsies. The results showed that there was a significantly different result in the 12 month group as compared to 0 months in BV measurements, BV was reduced in the OVX group as compared to time 0 and TbSp was reduced in the OVX group as well.

In our study here there is no iliac crest biopsy at time 0 months, only at 12 months. However, the results are comparable with the study by Newton et al, in terms of the 12 month period. This point may lead us to believe, that although there are minute changes within the trabecular structure in the ovariectomized and control groups there are minimal differences, and the non weight bearing aspect of the iliac bone may not be an ideal sample in detection for osteoporotic changes; none were found in two studies looking at femoral neck and vertebral changes in the ovine model as well.

Results from the 31 month group showed 3 distinct areas of significantly differing results ($p < 0.05$). Before those are discussed, the treatment group with zolendronic acid, showed that its total volume, bone volume, trabecular number thickness and surface area all increased as compared to the control group, but only surface area was statistically different than the OVX group. This maybe due to a remodelling effect as a result of inhibiting osteoclastic activity, leading to an increase in surface area.

The total volume in the OVX group is significantly lower than the control group and the TbTh is increased ($P < 0.05$). Studies in the ilium revealed an increase in trabecular thickness during bone development (Parfitt et al., 2000) and no change was found in trabecular number. A similarity with osteoporotic changes in terms of thickness increase; that the increase in trabecular thickness was due to remodelling with a positive balance. This means that during each remodelling cycle osteoblasts add more bone than was previously resorbed by osteoclasts.

Trabecular bone transforms from plate to a rod like structure in osteoporotic changes and some of those findings are evident in clinical stuidies. These changes are associated with decreased BV / TV and an increase in TbSp in osteoporotic women

(Akhtar et al., 2007), similar findings were obtained as far as TbSp is concerned with this present study but no significant difference recorded.

A study investigating application of micro-CT assessment of 3-D bone microstructure in preclinical and clinical studies, via iliac crest biopsies, showed a change in trabecular morphology with a shift towards a more platelike structure. Paired bone biopsy specimens from the iliac crest in postmenopausal women with osteoporosis before and an average of 2 years after beginning of oestrogen replacement therapy demonstrated that post treatment biopsies showed change in the ratio of plates to rods and statistically insignificant changes in other 3-D trabecular parameters (Jiang et al, 2005).

CONCLUSION

- Minimal changes between the two groups noted in the 12 month period. Trabecular architecture is altered in the control and OVX group, more so in the 31 month group as compared to the 12 month group.
- OVX + Zolendronic acid treatment, do cause an overall rise in TV, BV, BV/TV, TbN, TbTh and a decrease in TbSP as would be expected but not a statistically significant difference noted.
- Main limitation from this study and the one assessing cortical bone in ribs; may well be due to the small sample size of the treatment group and hence it is not as straightforward to extrapolate and draw comparisons with actual clinical trials, although some similarities in terms of subtle changes have been found and importantly, the ovine model does offer the opportunity to study this disease process further.

REFERENCES

Akhter, M. P, Lappe J. M, Davis K. M, Recker R. R (2007). Transmenopausal changes in the trabecular bone structure. *Bone* **41**(1):111-6.

Allen, M. R. and D. B. Burr (2007). Mineralization, Microdamage and Matrix: How Bisphosphonates Influence Material Properties of Bone. *BoneKEY Osteovision* **4**(2): 49-60.

Allen, M. R. and D. B. Burr (2007). Three Years of Alendronate Treatment Results in Similar Levels of Vertebral Microdamage as After One Year of Treatment. *Journal of Bone and Mineral Research.*

Allen, M. R., K. Iwata, R. Phipps and D. B. Burr (2006). Alterations in canine vertebral bone turnover, microdamage accumulation, and biomechanical properties following 1-year treatment with clinical treatment doses of risedronate or alendronate. *Bone* **39**(4): 872-9.

Brennan, O (2008). Bone quality and its relationship with bone fragility and osteoporosis. (PhD).

Burr, D. B., M. R. Forwood, D. P. Fyhrie, R. B. Martin, M. B. Schaffler and C. H. Turner (1997). Bone microdamage and skeletal fragility in osteoporotic and stress fractures. *Journal of Bone and Mineral Research* **12**(1): 6-15.

Burr, D. B., T. Hirano, C. H. Turner, C. Hotchkiss, R. Brommage and J. M. Hock (2001). Intermittently administered human parathyroid hormone(1-34) treatment increases intracortical bone turnover and porosity without reducing bone strength in the humerus of ovariectomized cynomolgus monkeys. *Journal of Bone and Mineral Research* **16**(1): 157-65.

Burr, D. B. and R. B. Martin (1993). Calculating the probability that microcracks initiate resorption spaces. *Journal of Biomechanics* **26**(4-5): 613-6.

Burr, D. B., R. B. Martin, M. B. Schaffler and E. L. Radin (1985). Bone remodeling in response to in vivo fatigue microdamage. *Journal of Biomechanics* **18**(3): 189-200.

Burr, D. B., L. Miller, M. Grynpas, J. Li, A. Boyde, T. Mashiba, T. Hirano and C. C. Johnston (2003). Tissue mineralization is increased following 1-year treatment with high doses of bisphosphonates in dogs. *Bone* **33**(6): 960-9.

Burr, D. B., A. G. Robling and C. H. Turner (2002). Effects of biomechanical stress on bones in animals. *Bone* **30**(5): 781-6.

Burr, D. B., C. H. Turner, P. Naick, M. R. Forwood, W. Ambrosius, M. S. Hasan and R. Pidaparti (1998). Does microdamage accumulation affect the mechanical properties of bone? *Journal of Biomechanics* **31**(4): 337-45.

Chappard, C., B. Brunet-Imbault, G. Lemineur, B. Giraudeau, A. Basillais, R. Harba and C. L. Benhamou (2005). Anisotropy changes in post-menopausal osteoporosis: characterization by a new index applied to trabecular bone radiographic images. *Osteoporos Int* **16**(10): 1193-202.

Chapurlat, R. D. and P. D. Delmas (2006). Drug insight: Bisphosphonates for postmenopausal osteoporosis. *Nat Clin Pract Endocrinol Metab* **2**(4): 211-9; quiz following 238.

Ciarelli, T. E., D. P. Fyhrie, M. B. Schaffler and S. A. Goldstein (2000). Variations in three-dimensional cancellous bone architecture of the proximal femur in female hip fractures and in controls. *Journal of Bone and Mineral Research* **15**(1): 32-40.

Cooper, C., E. J. Atkinson, S. J. Jacobsen, W. M. O'Fallon and L. J. Melton (1993). Population-based study of survival after osteoporotic fractures. *Am J Epidemiol* **137**(9): 1001-5.

Dai, R. C., E. Y. Liao, C. Yang, X. P. Wu and Y. Jiang (2004). Microcracks: an alternative index for evaluating bone biomechanical quality. *J Bone Miner Metab* **22**(3): 215-23.

Dawson-Hughes, B. (1996). Calcium and vitamin D nutritional needs of elderly women. *J Nutr* **126**(4 Suppl): 1165S-7S.

Day, J. S., M. Ding, P. Bednarz, J. C. van der Linden, T. Mashiba, T. Hirano, C. C. Johnston, D. B. Burr, I. Hvid, D. R. Sumner and H. Weinans (2004). Bisphosphonate treatment affects trabecular bone apparent modulus through micro-architecture rather than matrix properties. *J Orthop Res* **22**(3): 465-71.

Follet, H., J. Li, R. J. Phipps, S. Hui, K. Condon and D. B. Burr (2007). Risedronate and alendronate suppress osteocyte apoptosis following cyclic fatigue loading. *Bone* **40**(4): 1172-7.

Forwood, M. R., D. B. Burr, Y. Takano, D. F. Eastman, P. N. Smith and J. D. Schwardt (1995). Risedronate treatment does not increase microdamage in the canine femoral neck. *Bone* **16**(6): 643-50

Frost, H. M. (1960). Presence of microscopic cracks in vivo in bone. *Bull Henry Ford Hospital* **8**: 27-35.

Frost, H. M. (1973). Metabolism of bone. *N Engl J Med* **289**(16): 864-5.

Holtrop, M. E and King, G. J (1997). The ultrastructure of osteoclasts and its functional implications. *Clin. Orthop* **123**, 177-196.

Kennedy, O. D. (2007). Bone Quality in Osteoporosis. Mechanical and Manufacturing Engineering. Dublin, Trinity College Dublin. PhD.

Komatsubara, S., S. Mori, T. Mashiba, M. Ito, J. Li, Y. Kaji, T. Akiyama, K. Miyamoto, Y. Cao, J. Kawanishi and H. Norimatsu (2003). Long-term treatment of incadronate disodium accumulates microdamage but improves the trabecular bone microarchitecture in dog vertebra. *Journal of Bone and Mineral Research* **18**(3): 512-20.

Lee, T. C. (1997). Detection and Accumulation of Microdamage in Bone. Dublin, University of Dublin. M.D.

Lee, T. C., E. R. Myers and W. C. Hayes (1998). Fluorescence-aided detection of microdamage in compact bone. *J Anat* **193** (Pt 2): 179-84.

Lee, T. C., A. Staines and D. Taylor (2002). Bone adaptation to load: microdamage as a stimulus for bone remodelling. *J Anat* **201**(6): 437-46.

Li, J., T. Mashiba and D. B. Burr (2001). Bisphosphonate treatment suppresses not only stochastic remodeling but also the targeted repair of microdamage. Calcif Tissue *Int* **69**(5): 281-6.

Lill, C. A., A. K. Fluegel and E. Schneider (2002). Effect of ovariectomy, malnutrition and glucocorticoid application on bone properties in sheep: a pilot study. *Osteoporos Int* **13**(6): 480-6.

Martin, R. B. (2000). Toward a unifying theory of bone remodeling. *Bone* **26**(1): 1-6.

Martin, R. B. and D. B. Burr (1982). A hypothetical mechanism for the stimulation of osteonal remodelling by fatigue damage. *Journal of Biomechanics* **15**(3): 137-9.

Martin, R. B., O. C. Yeh and D. P. Fyhrie (2007). On sampling bones for microcracks. *Bone* **40**(4): 1159-65.

Mashiba, T., T. Hirano, C. H. Turner, M. R. Forwood, C. C. Johnston and D. B. Burr (2000). Suppressed bone turnover by bisphosphonates increases microdamage accumulation and reduces some biomechanical properties in dog rib. *Journal of Bone and Mineral Research* **15**(4): 613-20.

Mashiba, T., C. H. Turner, T. Hirano, M. R. Forwood, D. S. Jacob, C. C. Johnston and D. B. Burr (2001). Effects of high-dose etidronate treatment on microdamage

accumulation and biomechanical properties in beagle bone before occurrence of spontaneous fractures. *Bone* **29**(3): 271-8.

Mohsin, S., F. J. O'Brien and T. C. Lee (2006). Microcracks in compact bone: a three dimensional view. *J Anat* **209**(1): 119-24.

Mosekilde, L., E. N. Ebbesen, L. Tornvig and J. S. Thomsen (2000). Trabecular bone structure and strength - remodelling and repair. *J Musculoskelet Neuronal Interact* **1**(1): 25-30.

Newman, E., A. S. Turner and J. D. Wark (1995). The potential of sheep for the study of osteopenia: current status and comparison with other animal models. **Bone** *16*(4 Suppl): 277S-284S.

Newton, B. I., R. C. Cooper, J. A. Gilbert, R. B. Johnson and L. D. Zardiackas (2004). The ovariectomized sheep as a model for human bone loss. *J Comp Pathol* **130**(4): 323-6.

Nyman, J. S., O. C. Yeh, S. J. Hazelwood and R. B. Martin (2004). A theoretical analysis of long-term bisphosphonate effects on trabecular bone volume and microdamage. *Bone* **35**(1): 296-305.

O'Brien, F. J., O. Brennan, O. D. Kennedy and T. C. Lee (2005). Microcracks in cortical bone: how do they affect bone biology? *Curr Osteoporos Rep* **3**(2): 39-45.

O'Brien, F. J., D. Taylor and T. Clive Lee (2005). The effect of bone microstructure on the initiation and growth of microcracks. *J Orthop Res* **23**(2): 475-80.

O'Brien, F. J., D. Taylor, G. R. Dickson and T. C. Lee (2000). Visualisation of three dimensional microcracks in compact bone. *J Anat* **197** Pt 3: 413-20.

O'Brien, F. J., D. Taylor and T. C. Lee (2003). Microcrack accumulation at different intervals during fatigue testing of compact bone. *J Biomech* **36**(7): 973-80.

Parfitt, A. M. (2002). High bone turnover is intrinsically harmful: two paths to a similar conclusion. The Parfitt view. *Journal of Bone and Mineral Research* **17**(8): 1558-9; author reply 1560.

Parfitt, A. M. (2002). Misconceptions (2): turnover is always higher in cancellous than in cortical bone. *Bone* **30**(6): 807-9.

Parfitt, A. M., C. H. Mathews, A. R. Villanueva, M. Kleerekoper, B. Frame and D. S. Rao (1983). Relationships between surface, volume, and thickness of iliac trabecular bone in aging and in osteoporosis. Implications for the microanatomic and cellular mechanisms of bone loss. *J Clin Invest* **72**(4): 1396-409.

Prendergast, P. J. and D. Taylor (1994). Prediction of bone adaptation using damage accumulation. *Journal of Biomechanics* **27**(8): 1067-76

Rubin. R. (2008) Rubin's Pathology : Clinicopathologic Foundations. Edition 5. Lippincott, Williams & Wilkins.

Salo J, Melsiko K, Palokangus. H, Lehenkan. P (1996). Bone resorbing osteoclasts reveal a dynamic division of basal plasma membrane into two different domains. *Journal of Cell Science* **109**, 301-307.

Schaffler, M. B., D. B. Burr and R. G. Frederickson (1987). Morphology of the osteonal cement line in human bone. *Anat Rec* **217**(3): 223-8.

Schaffler, M. B., K. Choi and C. Milgrom (1995). Aging and matrix microdamage accumulation in human compact bone. *Bone* **17**(6): 521-25.

Schenk . R, Eggli. P, Fleisch. H and Rossini. S (1986). Quantitative morphometric evaluation of the inhibitory activity of new aminobisphosphonates on bone resorption in the rat. *Calcif. Tissue Int* **38**, 342-349.

Schiller. P, G. Balkan, W. Roos (2001). Gap junctional communication is required for the maturation process of osteoblastic cells in culture. *Bone* **28**, 362-369.

Turner, A. S. and A. Villanueva, R (1994). Static and dynamic histomorphometric data in 9- to 11-year old ewes. *Veterinary Comparative Orthopaedics and Traumatology* **7**: 101-109.

Turner, A. S., A. R. Villanueva, M. R. Alvis and H. M. Aberman (1995). Unusual histomorphometric changes in the iliac crest in ovariectomizes and sham-operated ewes. *Veterinary Comparative Orthopaedics and Traumatology* **(8):** 184-190.

Vashishth, D., O. Verborgt, G. Divine, M. B. Schaffler and D. P. Fyhrie (2000). Decline in osteocyte lacunar density in human cortical bone is associated with accumulation of microcracks with age. *Bone* **26**(4): 375-80.

Wolff, J. (1892). Das Gesetz der Transformation der Knochen. Berlin, Hirschwald.

Wronski, T. J., L. M. Dann, K. S. Scott and M. Cintron (1989). Long-term effects of ovariectomy and aging on the rat skeleton. *Calcif Tissue Int* **45**(6): 360-6.

World Health Organisation.

Yao, W., G. Balooch, M. Balooch, Y. Jiang, R. K. Nalla, J. Kinney, T. J. Wronski and N. E. Lane (2006). Sequential treatment of ovariectomized mice with bFGF and risedronate restored trabecular bone microarchitecture and mineralization. *Bone* **39**(3): 460-9.
