

Fracture healing in osteopenic bone and the influence of simvastatin

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Declaration

This thesis is submitted to the University of Edinburgh for the degree of Doctorate of Medicine. I confirm that all the experimental work was carried out by myself except where specifically acknowledged and all sources of information have been referenced. This work has not previously been submitted for any other degree.

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Abstract

Despite recent improvements in the ability to prevent osteoporotic fracture a vast number of mainly elderly patients continue to sustain fractures resulting in significant morbidity and a huge demand on health care services. Current knowledge of the process of fracture healing in osteoporotic bone is limited but there is a suggestion that it may differ from that of normal bone. An improved knowledge of the mechanical and biological properties of healing fractures in osteoporosis could ultimately lead to an enhancement of our ability to treat this problem by both medical and surgical means.

The aim of this study was firstly to use an animal model to investigate the histological and mechanical properties of fracture healing in osteopenic bone. Secondly, the same model and outcome parameters were used to study the effect on fracture healing of the systemic administration of simvastatin, which recent studies have suggested, promotes both bone formation and inhibits bone resorption.

In part one of the study 20, 3-month-old female, Wistar rats underwent ovariectomy (Ovx) while a further 20 had a sham procedure to act as controls. Seven weeks later a transverse fracture was created in the proximal tibia of each animal by three-point bending with the resulting fractures supported by an intramedullary wire. Half of the animals in each group were euthanased at two weeks and the remainder at four weeks post fracture with the tibiae removed post mortem. All tibiae were then x-rayed. The mechanical properties of half of the healing fractures were ascertained by four-point bending to failure while the remaining specimens were prepared for histological analysis and immunohistochemistry. There were no mechanical differences in the fracture calluses from the ovx animals compared with control at two weeks but by four weeks post fracture the ultimate load at failure of the fractures from the ovx animals was reduced to 71% of that from controls. Stiffness (54%) and stress at yield (74%) were also reduced while the strain at yield was increased by 40% in fractures from the ovx group. Histological differences were also seen with an increased porosity of the calluses from the ovx group at four weeks. No radiological differences were noted and there was no difference in the expression of the key

growth factors BMP 2, BMP 6 or Smad1. No difference in the replication of key cell populations was seen.

In the second part of the study the same animal model was used with the groups once again divided into ovx and sham controls. Half of each group received placebo while the other half received simvastatin 20mg/kg daily for 14 days post fracture. The same time points and outcome measures were used as in the first part of the study. In the sham groups simvastatin had no detectable effect on the radiological or mechanical features of the fractures or on the expression of the growth factors studied. The only difference was a higher proportion of immature bone in the four-week-old calluses from the simvastatin group (40%) versus placebo (34%). In the ovx animals simvastatin had a more noticeable effect with, once again, a higher proportion of immature bone in the four week old calluses in the drug group (43%) versus 28% in the placebo group. In addition the four-week-old healing fractures in the statin group had smaller calluses (16mm² compared with 28mm²) and were able to withstand only 76% of the ultimate load before failure than the fractures from the placebo fed ovx controls. An increased level of Smad1 expression was also seen in the chondrocytes of the statin fed ovx group.

The findings from the first part of this study provide evidence that an osteopenic environment has a deleterious effect on the mechanical properties of healing fractures in the rat model with an associated impairment of callus maturation. Contrary to previous work the dose and method of delivery of simvastatin in the second part of this study had no apparent effect on the fracture healing in normal bone. However simvastatin appeared to have a deleterious effect on fracture healing in the osteopenic model causing a reduction in callus size and maturity and reducing the healing fractures' ability to withstand load. This study does not support a role for simvastatin in the enhancement of fracture healing in osteopenia.

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Introduction

1 Introduction

1.1 Osteoporosis

1.1.1 Definition

The World Health Organisation (WHO) has defined osteoporosis in two ways. Firstly, as a “progressive systemic disease characterized by low bone mineral density (BMD) and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture”. Secondly, and more precisely, as a BMD of more than 2.5 standard deviations below the young adult mean ¹. Commonly the BMD is expressed relative to the young adult reference as a T-score.

1.1.2 Epidemiology

Using the second of the two WHO definitions it is estimated that as many as 30% of postmenopausal women will suffer from osteoporosis. Up to 70% of all hip fractures can be attributable to osteoporosis with population demographics predicting an exponential rise in hip fracture incidence until the middle of this century ². In 2001 over 180000 osteoporosis related fractures occurred in the United Kingdom resulting in an estimated cost of £1.7 billion ³.

Currently, the true incidence of osteoporosis in populations can only be inferred from fracture incidence, as osteoporosis sufferers who do not fracture do not usually present to health care services. There is substantial variability in the incidence of osteoporotic fracture with Caucasian women in Northern Europe having one of the highest incidences in the world. The accelerated bone loss post menopause together with the fact that women live longer than men make the life time risk for women of an osteoporotic fracture almost three times that of men in the USA ⁴. While osteoporosis has a major association with hip fracture ⁵ it is not the only factor with environment and other co-morbidities influencing fracture incidence ⁶. Inferences about osteoporosis incidence from fracture rates should therefore be made cautiously.

1.1.3 Pathogenesis

The continuous remodelling of bone throughout life maintains a healthy skeleton capable of withstanding and adapting to load. With the onset of osteoporosis the balance of bone formation and resorption fails with a net resorption resulting. Resorption on the endocortical and trabecular surfaces results in cortical and trabecular thinning with eventual loss of trabeculae and increased cortical porosity.

Loss of bone begins soon after peak bone mass has been achieved and is therefore not entirely due to gonadal failure ⁷ but may also be due to falling osteoblast numbers associated with aging ⁸. Oestrogen deficiency is however a major factor in causing the accelerated bone loss in women seen following the menopause ⁹. After the initial rapid

loss of BMD a steady state is reached where remodelling remains negatively balanced with increased numbers of remodelling sites on trabecular bone. At these remodelling sites osteoclasts predominate due to the increased osteoclastogenesis and a reduced osteoblast lifespan¹⁰ associated with oestrogen withdrawal.

A direct role for oestrogen on osteoblast and osteoclast activity was suggested by the identification of oestrogen receptors (ER) on both cell types^{11,12}. The exact response of these cells to oestrogen has not been defined but *in vitro* work has shown osteoblasts responding to oestrogen exposure by production of procollagen and expression of growth factors¹³ as well as reduced expression of osteoclastogenic cytokines interleukins (IL)-1, 6 and tumour necrosis factor^{14,15}. Oestrogen has also been shown to promote mesenchymal stem cell differentiation into osteoblastic lineage¹⁶ and promote bone morphogenic protein (BMP)-2 and transforming growth factor (TGF)- β production in osteoprogenitor cells¹⁷.

There is a clear link between oestrogen and growth factors known to promote bone formation with an apparent cross-talk between BMP and oestrogen signalling pathways¹⁸. Matched temporal expression of BMP-2 and 4 and their intracellular signalling protein, Smad-4 with ER expression has been reported^{19,20}. BMP-4 signal transduction is influenced by oestrogen²¹ and a paracrine role for BMPs in the regulation of ovarian response to the oestrous cycle has been proposed²². While there is abundant evidence of a link between oestrogen and growth factor regulation, the purpose of this and its role in the development of osteoporosis remains unclear. The promotion of pro-osteogenic

factors such as BMPs 2, 4 and 6²³ would seem to be an obvious mechanism for the osteogenic action of oestrogen²⁴ but this hypothesis is hindered by the knowledge that anti-oestrogens can have similar effects on these growth factors²⁵.

The osteocyte is known to have an important role in the regulation of bone structure in response to load²⁶. Oestrogen withdrawal has been shown to result in increased osteocyte apoptosis both *in vitro*²⁷ and *in vivo*²⁸ in cortical and cancellous bone. The loss of osteocytes in response to oestrogen withdrawal may result in a decreased capacity for bone to remodel appropriately in response to load leading to the microfracturing seen in osteoporosis.

Systemic factors such as parathyroid hormone (PTH) and vitamin D influence osteoclastogenesis via cells of osteoblastic lineage²⁹. This influence is exerted via an osteoclast differentiation factor known as RANK ligand (RANKL) which is a common factor in osteoclast promotion by any stimulus³⁰. Blockage of RANKL results in osteopetrosis due to failure of osteoclast formation and over expression of RANKL has been shown on trabecular bone from osteoporotic women³¹. The direct role for oestrogen in preventing osteoclastogenesis is seen by the down regulation of RANKL with oestrogen supplementation³².

A genetic predisposition to osteoporosis has long been suspected. Known genetic factors such as age at menarche and menopause and body mass have an influence but more specific genetic features are being identified. Polymorphisms and mutations of genes

ranging from those for ER, collagen type one, IL-6 and TGF- β have all been shown to increase the risk for osteoporosis^{33,34}. This would support the hypotheses that these factors all have a key role in the pathogenesis on osteoporosis.

The development of osteoporosis involves the disturbance of the complex regulatory factors governing the balance of bone remodelling. An increased knowledge of the mechanisms involved has facilitated the introduction of treatments aimed at maintaining and possibly even restoring bone mass in late adult life.

1.1.4 Treatment of osteoporosis

Osteoporosis is a multi-factorial disease with alterations in life style such as cessation of smoking and increased exercise known to be beneficial. However, therapies aimed at preservation of bone mass constitute the mainstay of current pharmacological treatments.

Calcium and vitamin D

Various studies have shown a reduction in the risk of fracture when elderly individuals who are calcium or vitamin D deficient receive supplementation^{35,36}. There appears to be no benefit however to those with a normal nutritional status^{37,38}. This suggests that this treatment is effective in treating co-existing osteomalacia but is not in itself an effective treatment for osteoporosis.

Oestrogen therapy and oestrogen analogues

Hormone replacement therapy (HRT) appears to be effective at inhibiting bone loss following the menopause³⁹ but following its cessation bone loss resumes at the post menopausal rate⁴⁰. The evidence that HRT reduces the risk of fracture is limited. No large trials have been conducted to look at fracture specifically but meta-analyses of available data suggest a reduction in vertebral and non-vertebral fracture by 25 to 33%⁴¹. Unfortunately withdrawal of HRT results in loss of the reduced fracture risk in a relatively short period⁴² indicating that a delay in bone loss is all that is achieved while receiving HRT. The major disadvantage of prolonged HRT is the associated increase in breast cancer. The incidence of breast cancer is increased by HRT treatment of more than 5 years although mortality from the cancer is not clearly affected⁴³. Long term oestrogen treatment for osteoporosis is therefore no longer a viable option.

The selective oestrogen receptor modulator (SERM) raloxifene acts as a competitive inhibitor of oestrogen in the breast but conversely as an oestrogen agonist in bone. A pro-osteoblastic and anti-osteoclastic action of raloxifene has been demonstrated *in vitro*⁴⁴. The MORE study (Multiple Outcomes of Raloxifene) showed that raloxifene treatment reduced the incidence of breast cancer but also reduced the incidence of vertebral fracture in osteoporotic individuals⁴⁵. Unfortunately no reduction in non-vertebral fracture has been seen as a result of raloxifene treatment.

Bisphosphonates

These pyrophosphate analogues have a strong affinity for bone apatite. This results in inhibition of osteoclast function and reduction in osteoclast number translating into an inhibition of bone resorption⁴⁶ and of bone loss following gonadal failure⁴⁷. It has been proposed that one of the mechanisms of action is by inhibition of enzymes in the latter part of the mevalonate pathway which results in osteoclast apoptosis⁴⁸. Some concern does exist however over the long-term consequences of the inhibition of bone turnover and remodelling caused by bisphosphonates. Bourrin *et al.*⁴⁹ reported no restoration of bone architecture following bisphosphonate therapy but the long-term sequelae of this are not clear.

Large clinical trials have shown prolonged treatment with the bisphosphonates alendronate and risedronate reduces the incidence of fracture by up to 50% with the protective effect occurring within 12 months of commencing treatment⁵⁰⁻⁵². Evidence for a prolonged benefit or even absence of a deleterious effect after five years of treatment is however still lacking.

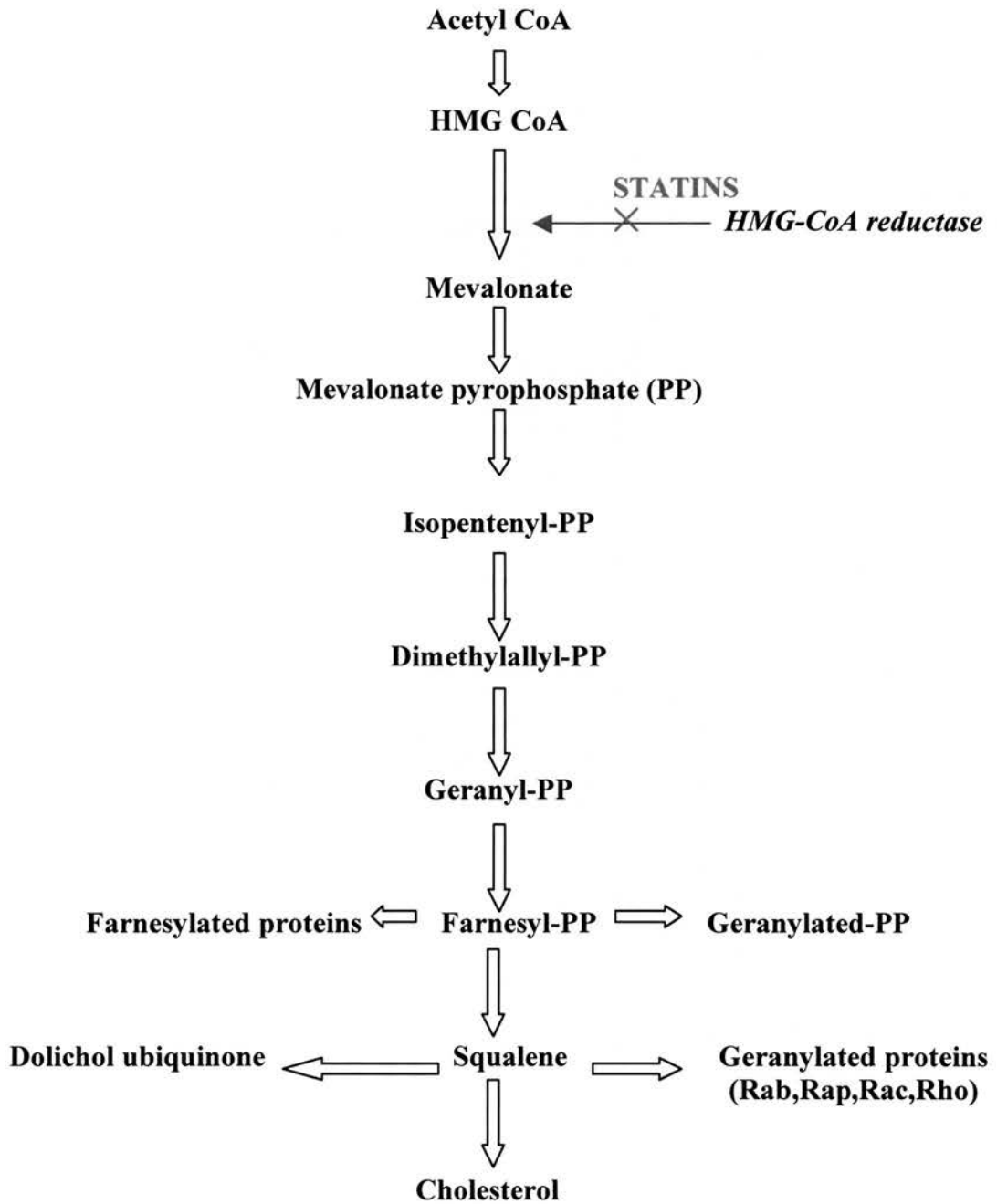
Parathyroid hormone (PTH)

PTH appears to be a truly anabolic agent when delivered appropriately. Increased bone mass has been seen in animal models after intermittent injection with anabolic effects on cortical and trabecular bone⁵³⁻⁵⁵. An initial increase in cortical porosity is seen due to stimulation of osteoclasts with this treatment but this is rapidly counteracted by osteoblast activity.

Human trials using synthetic human PTH (1-34) have all shown an increase in BMD⁵⁶⁻⁵⁸. This appears to translate into a reduction in fracture risk with Neer *et al.*⁵⁹ reporting a relative risk for vertebral fracture of 0.35 for PTH versus placebo treated women. The anabolic nature of PTH raises some concerns about possible oncogenesis with very large long-term doses of PTH resulting in the majority of animals in one study developing osteosarcoma⁶⁰. No oncogenesis has been reported with lower, more physiological doses used over the shorter term. Another disadvantage of PTH is the current necessity for it to be delivered by injection although oral preparations are being attempted.

Statins

This class of drugs is one of the most widely used in western society due to its cholesterol lowering properties. They act by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which catalyses a fundamental step in the hepatic synthesis of cholesterol via the mevalonate pathway (see Figure 1). Statins are described as pleiotropic due to a variety of differing effects on various physiological systems not all of which can be ascribed to the cholesterol lowering effect. An apparent cerebral sparing effect in cerebral ischaemia has been associated with endothelial nitric oxide enhancement resulting from reduced geranylgeranylated proteins which are an end product of the mevalonate pathway (see Figure 1)⁶¹. The extent of the pleiotropic effects appears to be due to the inhibition of formation of the non-steroidal isoprenoids which have multiple roles in cell function, signalling and replication⁶². The anti-

Figure 1: Mevalonate pathway and the action of statins ⁶³

atheromatous effect of statins is due not only to the lipid lowering function but also the effect on endothelial nitric oxide and an anti-proliferative effect on arterial myocytes⁶⁴.

Mundy *et al.*⁶⁵ first observed an apparent promotion of bone formation in a rat model after statin administration topically and systemically in normal and osteopenic animals. This finding was supported by well designed *in vitro* work which showed an increase in BMP-2 expression in osteoblastic cells which was thought to be the cause of the statins osteogenic effect. The bone forming effect of statins was inhibited by addition of the BMP-2 inhibitor, noggin, which supported this hypothesis. A dose dependent increase in BMP-2 production was also demonstrated by Sugiyama *et al.* in human osteosarcoma cells *in vitro*⁶⁶. Several other studies have produced *in vitro* evidence of osteoblast stimulation and increased BMP-2 levels as well as an apparent anti-osteoclastic effect of statin administration⁶⁷⁻⁶⁹. Prolonged administration of simvastatin has been shown to result in increased cancellous bone formation in one year old rats⁷⁰ but additional work has suggested that an initiation of the osteogenic cascade occurs after only limited administration of statins to culture and animal models⁷¹.

Contradictory results have however been reported. Maritz *et al.*⁷² in a well constructed study, found that prolonged treatment of rats with three different statins at the same doses used by Mundy *et al.* resulted in no change in bone mineral density. A stimulation of both bone formation and resorption occurred with a simvastatin dose of 20mg/kg daily leaving bone density unchanged but at lower doses resorption predominated

causing BMD to fall. Banu and Kalu⁷³ have more recently reported no beneficial effect on lumbar BMD in the rat after prolonged treatment with cerivastatin. Similarly Staal *et al.*⁷⁴ reported finding no increase in bone formation following cerivastatin treatment in a parathyroidectomy rat model but they did see an anti-resorptive effect of the drug *in vitro*.

The different results reported may be due to differing methods. The type of statin used is important as the earlier less lipophilic statins penetrate bone poorly but this would not explain the contradictory results with cerivastatin which is highly lipophilic. The dose related effects shown by Maritz *et al.*⁷² may also be important. Differing doses, methods of delivery and types of animal model make comparison between studies difficult.

Another key difference between the methods of Mundy *et al.*⁶⁵ and Maritz *et al.* was the timing of statin administration in relation to ovariectomy. Maritz *et al.* administered the drug only two weeks post ovariectomy during which time it is known that dramatic resorptive activity is occurring⁷⁵ while Mundy *et al.* waited until two months post ovariectomy. While both studies were adequately controlled suggesting the drug effects seen were genuine it may be that the simvastatin effect depends upon underlying bone resorptive activity. Comparison between species is always dangerous but it is known that rats react to HMG-CoA inhibition by increasing HMG-CoA production which is not the initial response to statins in humans⁷⁶. Comparing effective doses between species and from *in vitro* work is therefore extremely difficult.

There is some evidence for statins having an effect on bone in humans. An effect on markers of bone resorption has been seen in human trials of simvastatin and cerivastatin^{77,78}. Whether this translates into a beneficial effect on bone mineral density in humans is debatable. Observational and case controlled studies report contradictory findings. The case-controlled Chingford study⁷⁹ appeared to show a clear increase in BMD in women taking a statin for cardiovascular reasons but other population based studies failed to find any difference in BMD in similar groups⁸⁰⁻⁸². These findings have been based on analysis of secondary outcomes from other study groups and no data is available from a large, randomized controlled trial comparing the effect on human BMD of statin versus placebo.

The same is true for the evidence of a statin effect on fracture risk. Data from several retrospective, case controlled studies in large populations do appear to suggest a reduction in fracture risk associated with taking statins. Meier *et al.*⁸³ reported that data retrieved retrospectively from the general practice research database in the United Kingdom showed an odds ratio of fracture of 0.55 with statin use. A number of other similar analyses of population databases report equivalent reductions in fracture risk with statin use^{84,85}. However, the same database used by Meier *et al.* was analyzed in a different way by van Staa *et al.* who found no difference on the odds ratio for fracture in statin or non-statin users⁸⁶. This demonstrates the difficulty of drawing conclusions from retrospective data and post-hoc analyses of other studies. Multiple confounding factors such as social class, smoking, body mass or exercise are very difficult to control for and may significantly affect the results. The question of whether statins affect BMD

or fracture risk currently remains unanswered but the evidence that does exist justifies an attempt to address this question.

1.2 Fracture healing

Re-establishment of osseous integrity following fracture involves a complex cascade of cellular responses governed by autocrine, paracrine and endocrine mechanisms. These governing mechanisms are themselves influenced by the mechanical and physiological environment in which the fracture is trying to heal. This complex and incompletely understood process, results in the restoration of the ability of the bone to withstand load by regeneration of tissue rather than by scar tissue formation. This feature differentiates fracture healing from other types of tissue repair following injury.

1.2.1 Types of fracture healing

Primary cortical healing

This process is defined as an attempt to restore cortical integrity by direct healing of anatomically opposed, rigidly fixed fracture ends⁸⁷. Under such conditions of minimal interfragmentary strain, the Haversian system is reconstituted by remodelling units known as “cutting cones”. These units are comprised of osteoclasts which tunnel through cortical bone allowing penetration by mesenchymal cells accompanied by

revascularisation. The mesenchymal cells become the osteoprogenitor cells for the osteoblasts. The cutting cones are able to cross not only areas of dead cortical bone but also small gaps at the fracture site⁸⁸. The process is however slow and is intolerant of minimal mobility. It is analogous to the process of bone remodelling which occurs throughout life.

Healing of cancellous bone

Cancellous bone benefits from a rich blood supply meaning that bone turnover can occur on the surface of the trabeculae. This process is known as “creeping substitution”⁸⁷. Additionally, the ample vascularity is given as one reason why there is minimal bone necrosis at a cancellous fracture site. Charnley and Baker⁸⁹ demonstrated that cancellous bone heals rapidly at points of contact between fracture ends under compression and hypothesised that this was due to the persistent viability of the bone ends. This is to be contrasted with cortical bone which would heal in similar circumstances by the much slower process of primary cortical healing. In common with compressed, immobilised cortical bone however, cancellous bone does not heal by creeping substitution where direct contact does not exist.

1.2.2 Stages of fracture healing

Haematoma and inflammation

Common to all ultimate types of fracture healing is the initial creation of a haematoma from the damaged bone and soft tissue. This haematoma plays an important role in the

initiation of fracture healing as it appears to be a source of signalling molecules which stimulate the healing cascade⁹⁰. Inflammatory cells release chemotactic cytokines including interleukin (IL) -1 and IL-6⁹¹. Platelets within the clot have been implicated as the source of growth factors Transforming Growth Factor β (TGF- β) and Platelet Derived Growth Factor (PDGF). These factors have an important role in the regulation of cell proliferation and differentiation of stem cells⁹⁰.

Another early response is that of increased blood flow in the affected bone due to increases in both endosteal and periosteal blood flow and a hyperdynamic response in the entire region⁹². It is hypothesised that this is regulated by local and systemic pro-angiogenic substances such as vascular endothelial growth factor (VEGF) and endothelial cell growth factor (ECGF)^{93,94}. The increased vascularity is associated with invasion of the haematoma by fibrovascular tissue which promotes the laying down of a collagen network.

Following this, healing can proceed in different ways. The process of primary healing has already been described above. As mentioned this relies upon rigid stability and contact which is not a common circumstance under which fracture healing takes place in nature. More commonly varying degrees of instability and incongruence of the fracture fragments exists. These conditions lead to production of callus and the more commonly encountered process of secondary fracture healing.

The subsequent stages of secondary healing will now be described.

Callus formation

External (bridging) callus, is produced rapidly from both fracture ends in an attempt to bridge not only the fracture gap but also the dead bone at the end of the fragments and confer a degree of early stability⁹⁵. An initial, time limited, reaction termed the “primary callus response” begins rapidly after the fracture occurs. Intramembranous ossification by osteoprogenitor cells in the cambial layer of the periosteum takes place a few millimetres from the fracture ends⁸⁷. This results in advancing collars of callus from the bone ends with the apparent objective of establishing cellular contact⁹⁶. The area of callus where intramembranous ossification occurs is termed the “hard callus”. This term is also applied to the callus formed in the latter stages of endochondral ossification. Intense cellular proliferation occurs early and is relatively short lived in the hard callus formed by intramembranous ossification, occurring maximally between days three and seven following fracture⁹⁷.

An additional source of osteogenesis and regulation of the development of external callus is thought to be the surrounding soft tissue. Rhinelander⁹² demonstrated that the healing fracture derives a significant extraosseous blood supply from the surrounding soft tissue and the possibility of osteogenic induction in unassociated soft tissue is well established^{98,99}. The undifferentiated mesenchymal cells which infiltrate the organised haematoma are therefore potentially derived from several sources including the surrounding soft tissue, the invading endothelial cells¹⁰⁰ and the bone marrow¹⁰¹.

While external callus has a collar of bone from intramembranous ossification, bone is also formed via endochondral ossification. It is proposed that areas of low oxygen tension at the fracture site influence the undifferentiated mesenchymal cells in the callus to undergo chondrogenesis ¹⁰². Despite the attempts to establish a blood supply to the callus the mechanical forces that exist at the incompletely immobilised fracture gap result in poor perfusion and relatively low oxygen tension ¹⁰³. The relatively avascular cartilage provides the rapid contact and partial stability desired to promote vascular ingrowth of the callus and osteogenesis. This part of the callus is known as the “soft callus”. Undifferentiated mesenchymal cells show marked proliferative activity in the soft callus as early as three days post fracture with proliferation of chondrocytes occurring from around day seven to 21 ⁹⁷. The synthesis of collagen type-2 which predominates in cartilage is maximal at the end of the first week and declines dramatically by the end of week two. This has been shown to be the case in the healing tibial fractures of three month old Wistar rats with the fractures supported with an intramedullary wire ¹⁰⁴.

Medullary callus forms at a slightly slower rate than the external callus. It appears to derive its mesenchymal cells from the marrow elements of the fracture ends ¹⁰⁵. It appears to be less influenced by the mechanical environment as it has been seen to form when the fracture is rigidly fixed ⁸⁸ therefore facilitating primary bone union where small gaps are present.

The primary callus response is seen even where there is no hope of union such as in an amputation stump⁸⁷. The example of the amputation stump however demonstrated that if no contact with the other fragment has occurred with two weeks, the callus involutes. This may also occur where non-osteogenic or necrotic tissue is interposed or excessive movement prevents cellular contact and vascular bridging.

Cartilage calcification

Two weeks after fracture the rate of cell proliferation declines. Calcification of the chondroid external and medullary callus then proceeds in a very similar way to that which occurs at the physis. Hypertrophied chondrocytes release vesicles containing proteolytic enzymes to degrade the matrix and release calcium to promote calcification

¹⁰⁶.

Cartilage removal and bone formation

The ingrowth of blood vessels is accompanied by a decline in the number of chondrocytes and an increase in osteoblasts. The chondrocytes appear to undergo apoptosis as part of an ordered transition to an osteoblastic environment⁹⁷. This leads to the formation of immature, woven bone which replaces the cartilaginous callus over a period of weeks from approximately day 14 onwards.

Bone remodelling

Restoration of lamellar structure occurs in response to load as defined by Wolff's law.

Osteoclast resorption of woven bone is followed by restoration of bone architecture into lamellar and cancellous bone by the osteoblasts.

1.2.3 Regulation of fracture healing

Mechanical environment

The initial energy applied to create a fracture influences the ultimate healing process.

The greater the energy, the more the soft tissue and vascular disruption and the more complex the fracture pattern. These factors have been shown to increase the incidence of delayed and non-union¹⁰⁷.

As described above, the mechanical environment of the healing fracture exerts a significant influence on the process of healing. At every stage from proliferation of pluripotential tissue to remodelling of the healed fracture the mechanical environment is influential. Carter *et al.*¹⁰³ in a theoretical analysis, hypothesised that intramembranous ossification is permitted in areas of low to moderate stress and strain whereas increased compressive stress and strain favour chondrogenesis. It has been shown that molecular signals known to promote chondrogenesis are present very early in an unstable fracture and persist for much longer than in a stabilised fracture¹⁰⁸. Excessive tensile strain has

been shown to stimulate fibrocartilage. Claes *et al.*¹⁰⁹ showed that in a sheep model strains of less than 5% promoted intramembranous ossification while those between 5-15% promoted endochondrial ossification. Therefore, up to a point, the greater the strain applied to a healing fracture the more abundant the external, endochondrial callus formation. From the mechanical perspective this has an advantage as the larger the diameter of the callus the greater its resistance to load due to its greater moment of inertia.

The orientation of the fracture in relation to the applied load is also influential. In a well conducted study using a rabbit model, Richards *et al.*¹¹⁰ showed that increased shear due to an oblique fracture under load resulted in decreased new bone volume.

Osteoblasts are known to respond directly to load and to orientate themselves accordingly¹⁰⁹. As well as orientation, the extent of the fracture gap exerts an influence over healing. Claes *et al.*¹¹¹ postulated that a larger gap size promoted callus formation. In their ovine model however they found that a larger gap size of 6mm in a metatarsal fracture impaired the mechanical properties of the healing fracture.

The timing of the applied load is also of importance. Where cyclical strain within limits is applied early in the process healing appears to be enhanced. However if cyclical strain is inappropriately applied at a later stage healing can be impaired¹¹². Therefore the maintenance of optimal mechanical conditions throughout the healing process is desirable but the optimal timing and nature of these conditions to promote rapid healing is not yet established.

Method of fracture stabilisation

The method of stabilisation chosen for treatment of an unstable fracture will clearly have a profound effect on the process of healing. It has already been discussed how absolute stability results in primary bone healing with production of some medullary callus. “Absolute stability” would be achieved by a well reduced fracture being held by a compression plate securely applied to the bone. Intramedullary nailing does not achieve absolute stability¹¹³. Stability is improved by contact and interdigitation of the fracture fragments and by cortical contact with a large, reamed nail but bending moments still exist which prevent primary bone healing. Rotational stability is entirely dependent on the conformity of the reduced fracture fragments in the absence of interlocking of the intramedullary nail. The other biomechanical feature of the intramedullary nail is that even with interlocking, axial compression still exists at the fracture site¹¹⁴. An intramedullary nail therefore provides a stable reduction which permits secondary fracture healing.

The effect of instrumentation and in particular reaming of the medullae of long bones carried out during intramedullary nailing has been an area of concern. It has been discussed that the medulla contributes mesenchymal cells and a blood supply to a healing fracture and it would be reasonable to propose that disruption of this would interfere with the healing process. This might be particularly important where the more dominant extramedullary blood supply has been disrupted by the injury. Brinker *et al.*¹¹⁵ showed that the endosteal nutrient artery is seriously disrupted by intramedullary

reaming and does not recover for several weeks. Others have however found no difference in healing following reaming, limited reaming or no reaming ¹¹⁶. Further evidence suggests a faster time to union following reaming due to stimulation of the extramedullary blood supply and delivery of “graft” to the fracture site even in open fractures ¹¹⁷. The current evidence does not show a significant deleterious effect of intramedullary instrumentation during fracture treatment.

Physiological factors

The general health of a person with a fracture has a bearing on fracture healing.

Anaemia ¹¹⁸, diabetes ¹¹⁹ and smoking ¹²⁰ all impair healing.

A long bone fracture increases the metabolic requirement of the host by up to 25% ¹²¹ and many fracture patients are already malnourished. Vitamin D and C deficiency as well as deficiencies of proteins such as albumin and transferrin have all been linked with delayed fracture healing ¹²².

Evidence for impaired healing with advancing age is limited. It is well recognized that children's fractures heal faster than adults' which is thought to be related to the increased vascularity and cellularity of children's periosteum. Additionally, animal models have shown some impairment of fracture healing in older animals ¹²³.

The maintenance of normal levels of growth hormone are beneficial to healing ¹²⁴ but the role of oestrogen and parathyroid hormone (PTH) are receiving increasing attention as possible modifiers of the fracture healing process. Intermittent doses of PTH have

been shown to increase bone formation via stimulation of osteoblast differentiation¹²⁵. It has also been shown that PTH promotes callus formation and improves callus strength in animal fracture models^{126,127}.

The potential role of oestrogen in fracture healing is discussed in the section on fracture healing in osteoporosis.

1.2.4 Growth Factors and fracture healing

Marshall Urist¹²⁸ is credited with the discovery that extracts from demineralised bone cause bone formation when transplanted into non-osseous connective tissue. He named the responsible protein bone morphogenic protein (BMP) as, at that time, it was unclear if one or more molecules were involved. Over the years since Urist's original work it has become clear that multiple factors regulating bone growth exist of which BMPs are just one group. Table 1.1 summarises the common growth factors involved with skeletal regeneration. A clear understanding of the role of these growth factors has been sought with the hope that better knowledge might lead to therapeutic manipulation of the skeletal system in pathological conditions.

The role of the common growth factors in fracture healing will now be discussed.

Bone Morphogenic Proteins (BMPs)

The BMPs are part of a superfamily of growth factors termed the transforming growth factor β (TGF- β) superfamily. Fifteen BMPs¹²⁹ have currently been identified largely

through screening of human deoxyribonucleic acid (DNA) libraries¹³⁰ following the discovery of the genetic sequence of BMP by Wozney *et al.*⁹⁹. Not all BMPs are osteoinductive with BMPs 2, 4, 6 and 7 (also known as osteogenic protein-1 (OP-1)) generally considered the most osteogenic. As well as osteogenesis, BMPs play a vital part in the development of multiple tissue types with certain BMP deficiencies being incompatible with successful embryogenesis¹³⁰.

During the osteogenic process BMPs are secreted and act in an autocrine and paracrine fashion. They bind to serine and threonine kinase receptor complexes classed into BMPReceptor-1A and B and BMPR II, as is the case for most of the TGF- β superfamily¹³¹. Intracellular signaling is then accomplished via Smad proteins with this name derived from their homologous nature to mothers against drosophila decapentaplegic (MAD) protein. Eight Smads have been identified with Smads 1,4 and 5 known to enter the nucleus after activation of the type 1 receptors¹³². Phosphorylated Smads cross the nuclear membrane resulting in direct or indirect cell activation. It is proposed that one of the terminal mechanisms of action may be the production of osteoblast-specific factor 2 which may trigger differentiation of mesenchymal stem cells¹³³.

A role for BMPs in fracture healing is to be expected given the similarities between fracture healing and embryogenesis. Yang and Jin¹³⁴ first identified BMP in mesenchymal cells and osteoblasts at a healing fracture. Bostrom *et al.*¹³⁵ was able to identify BMPs 2 and 4 in undifferentiated cells within the maturing fracture haematoma and chondroid precursor cells during the second week following fracture. During the

third week expression in the chondrocytes decreased and more marked expression was seen in the osteoblasts. These findings were supported by Ishidou *et al.*¹³⁶ who also investigated the expression of BMP 7 and the BMP receptors. They reported that BMP 7 was present in chondroid tissue early in healing but disappeared rapidly. BMPR IA and IB up-regulation was seen by day 3 following fracture in osteogenic periosteal cells and by day 7 in fibroblasts, chondrocytes and osteoblasts. Le *et al.*¹⁰⁸ showed the presence of BMP 6 in hypertrophic chondrocytes declining prior to the end of the stage of chondrogenesis suggesting a role for this BMP in regulation of the production of cartilage. BMP 6 has however also been seen to promote osteoblast differentiation directly *in vitro* in the early stage of osteogenesis¹³⁷.

Transforming Growth Factor- β (TGF- β)

Five isoforms of this factor exist within the TGF-B superfamily. TGF- β 1 is released by platelets within the fracture haematoma and its presence has been identified in proliferating periosteal cells in early healing¹³⁸. It is present in abundance during endochondrial ossification with chondrocytes and osteoblasts expressing the receptor for TGF- β at this stage¹³⁹. It has however been shown to be present at higher than normal levels at the fracture site up to 28 days post fracture suggesting a role beyond that of early healing¹⁴⁰.

Fibroblastic Growth Factor (FGF)

Different subtypes of FGF exist with the most common in adults being acidic FGF-1 and basic FGF-2. FGF-1 has been shown to be mitogenic to chondrocytes with the more

potent FGF-2 being expressed by osteoblasts ¹⁴¹. The FGF receptors have increased expression during fracture healing suggesting a role for these factors in this process ¹⁴². Abnormalities of the FGF receptors have been implicated in abnormalities of endochondrial and intramembranous ossification resulting in skeletal dysplasias including achondroplasia ¹⁴³.

Insulin-like Growth Factor (IGF)

IGF is released following secretion of growth hormone releasing hormone. IGF-1 may have a role in promoting intramembranous ossification ¹⁴⁴ but its precise involvement in the fracture healing process has not yet been elucidated.

Platelet-derived Growth Factor (PDGF)

This factor is secreted by platelets which are involved in the formation of the fracture haematoma. It has been identified at the fracture site *in vivo* and it is postulated that it may play a role in the chemotaxis of different cell populations ^{145, 146}. Animal studies have failed to show a clear beneficial effect on fracture healing from PDGF supplementation ¹⁴⁷.

Vascular Endothelial Growth Factor (VEGF)

Increased levels of VEGF have been found in the systemic circulation following trauma and within the fracture haematoma ¹⁴⁸. It is expressed in endothelial and osteoprogenitor cells and osteoblasts at the fracture site with peak expression occurring within the first

10 days following fracture^{149,150}. In an elegant study, Street *et al.*¹⁵¹ demonstrated that inhibition of VEGF impaired both intramembranous and endochondrial ossification in a murine model and VEGF supplementation dramatically enhanced the healing of osteotomized rabbit radii. Its mechanism of action is incompletely understood but VEGF is known to effect the expression and actions of BMP-2 and other members of the TGF- β superfamily^{152,153}. As well as promoting angiogenesis at the fracture site VEGF appears to directly upregulate proosteoblast and osteoblast activity^{151,152}.

Table 1 Summary of Growth Factors involved with skeletal regeneration ¹⁴³

Growth Factor	Source	Function
Transforming Growth Factor β (TGF- β)	Platelets, cartilage and bone matrix	Stimulates undifferentiated mesenchymal cell proliferation
Bone Morphogenic Protein (BMP)	Osteoblasts, osteoprogenitor cells, bone matrix	Promotes differentiation of mesenchymal cells into osteoblasts and chondrocytes
Fibroblastic Growth Factor (FGF)	Macrophages, mesenchymal cells, chondrocytes and osteoblasts	Promotes proliferation of mesenchymal cells, chondrocytes and osteoblasts
Insulin-like Growth Factor (IGF)	Osteoblasts, chondrocytes and bone matrix	Stimulates differentiation of osteoprogenitor cells and intramembranous ossification
Platelet Derived Growth Factor (PDGF)	Platelets, osteoblasts	Chemotactic for macrophages, stimulates osteoblast and mesenchymal cell differentiation
Vascular Endothelial Growth Factor (VEGF)	Endothelial cells, osteoprogenitor cells, osteoblasts	Promotion of angiogenesis, osteoblast differentiation and callus maturation

1.2.4.1 The use of Growth Factors in the enhancement of fracture healing

Increased awareness of the key role for growth factors in the process of fracture healing has led to the expanding field of growth factor manipulation in an attempt to enhance fracture healing.

It has been known for many years that introduction of autologous bone graft at a fracture site promotes osteoinduction as well as providing osteoconduction. This is contrary to allogenic bone which provides only temporary osteoconduction¹⁵⁴. It has recently been appreciated that the osteoinductive properties of autologous bone graft relate to the presence of surviving osteogenic cells and osteoinductive growth factors¹⁵⁵. The donor site morbidity associated with autologous graft has led to a drive to deliver the osteogenic cells and growth factors in improved ways. Osteogenic mesenchymal stem cells have shown considerable promise when delivered to fracture sites in animals^{156,157} but it has been recognized that this can be enhanced by the combined delivery of stem cells with growth factors such as TGF- β , FGF and BMP¹⁵⁸.

Delivery of growth factors alone has also been evaluated with the ability to produce purified factors via recombinant DNA technology greatly enhancing this field.

BMP

Numerous animal studies using canine, rabbit and rat models, have demonstrated the ability of BMPs 2 and 7 (OP-1) to promote healing of bony defects¹⁵⁹⁻¹⁶¹. Cook *et al.*¹⁶²

has shown recombinant OP-1 (rhOP-1) to result in improved healing of bony defects in primates when compared with autologous bone graft. Sciandini *et al.* ¹⁶³ showed similar effects of rhBMP-2 in a canine model. Rh- BMP has been shown to be effective in producing spinal fusion ¹⁶⁴ and several human trials have now been carried out using BMP for the treatment of open tibial fractures, osteonecrosis and distraction osteogenesis ¹⁶⁵. RhOP-1 has now been shown to be as effective in achieving union in human tibial non-unions as autologous bone graft ¹⁶⁶.

TGF- β

Several animal studies have been conducted to evaluate the use of TGF- β in fracture healing. The evidence to date is inconclusive with studies using regular high doses of TGF- β showing enhanced bone formation and callus size ^{167,168} but others with different doses or isotypes showing little effect ¹⁶⁹. The pluripotential stimulus of TGF- β also raises concern about its use in human trials.

FGF

Introduction of exogenous FGF into fracture sites in animal models does appear to enhance fracture healing ¹⁷⁰. Improved mechanical properties and callus size have been noted after treatment with rhFGF-2 in canine and primate models ^{171,172} but method of delivery and dose again appeared influential.

1.2.4.2 Methods of Growth Factor delivery and promotion

In many studies the method of presentation of the growth factors at the fracture site proved critical. The ideal delivery vehicle needs to be biocompatible, biodegradable and osteoconductive if critical defects are being bridged and the material must not act as an impediment to bone healing as this would clearly be counterproductive. The majority of clinical trials use absorbable collagen sponges (ACS) as the delivery vehicle with the collagen often of bovine extraction. Other natural polymers such as hyaluronan, chitosan and fibrin as well as synthetic polymers and ceramics have all been tried^{173,174}. Some of these carriers allow percutaneous injection which is preferable in closed fractures.

The controlled presentation of the correct dose of factor has proved to be a major limitation in this field of research. In studies, the higher the animal the greater the dose of growth factor required. There is also an apparent need to maintain a high dose at the fracture site for a prolonged period which again provides difficulties for the choice of delivery vehicle¹⁷⁵.

The problems of direct delivery of non-physiological doses of growth factors have encouraged research into other methods of manipulating growth factor presentation at fractures sites. Promotion of growth factor expression by gene therapy techniques has been investigated in numerous animal models. Direct injection of adenoviral vectors containing BMP-2 has resulted in increased bone formation in rabbits and rats but only to a limited extent in immunocompetent animals¹⁷⁶⁻¹⁷⁹. The alternative, indirect

technique has shown that several cells of mesenchymal origin can promote bone formation if injected with adenovirus transduced with BMP-2 or 7¹⁸⁰⁻¹⁸². The advantage of genetic manipulation of growth factor expression is that more physiological doses of factor are expressed with a more appropriate temporal pattern. There is however concern about inappropriate factor expression and unanticipated sequelae such as tumour promotion as well as the currently prohibitive cost of gene therapy.

In 1999 Mundy *et al.* reported that statins appeared to stimulate bone formation both *in vitro* and *in vivo* and proposed that this effect was via an increase in BMP 2 expression⁶⁵. From this work it was hoped that systemic administration of statin might prove beneficial in conditions such as osteoporosis and further work in this area has been carried out (see section on statins in: Treatment of osteoporosis 1.1.4). The implications for manipulation of fracture healing have been less well investigated with only one study to date having been published on this subject¹⁸³. In this study it was reported that very large dietary doses of simvastatin resulted in larger calluses and improved mechanical properties of healing fractures compared with placebo in a murine model. The mechanism of action was not investigated however and it is not known whether this was related to an effect on growth factor expression. The possibility of enhancing fracture repair by oral administration of an approved pharmacological agent is however of great potential value and merits further work.

1.2.5 Models of fracture healing

Choice of animal model

A huge diversity of animals have been used in the study of fracture healing ranging from birds, rodents, lower mammals and primates¹⁸⁴. Lower animals have the advantage of reduced cost and abundant supply but the disadvantage of reduced applicability to humans. Larger animals have the advantage of a wider choice of treatment modalities but reduced availability reduces the power of much of the work. Mice and rats are commonly used but care must be exercised as these animals do not reach skeletal maturity and do not have well developed Haversian systems. Fracture healing in the rat must therefore be classed as non-osteonal. The rat skeleton does however reach a steady state following puberty at six weeks¹⁸⁵.

The rat long bone as a fracture model

Despite the disadvantages mentioned above, the rat is the most commonly used animal for the study of fracture healing. Its increased size compared with mice allows easier mechanical testing and offers a wider variety of treatment modalities for investigation. An unsupported tibial fracture in the rat can be expected to have commenced endochondrial bone formation by ten days and bony union by 21 days¹⁸⁶.

Creation of closed and open fractures of femora or tibia has been reported with treatments ranging from no stabilization¹⁸⁷ to external fixation¹⁸⁸. The commonest form

of fracture stabilization is intramedullary pinning. This is relatively easy to perform and allows good mobilization and minimal interference by the animal post operatively. It does however present the obvious disadvantages of potential introduction of infection and disruption of the medullary component of fracture healing. The rigidity of the intramedullary pin is also influential with non-union described when rigid pins have been used ¹⁸⁹. A pin diameter of no greater than 1mm is thought to be optimal ¹⁹⁰. Additionally, no rotational stability is provided by this method. Interdigitation of the fracture with axial compression is the only provider of rotational stability and may be a variable depending on fracture level and configuration. Similarly the level of the fracture in relation to the pin will dictate bending forces with tibial fractures distal to the tibiofibular junction poorly supported by an intramedullary pin due to the anatomy of the rat tibia ¹⁹⁰.

Creation of a standard long bone fracture in the rat has been achieved by various methods. Creation of a closed fracture by digital three point bending or a guillotine method has been described with the guillotine method having the advantage of reproducibility ¹⁹¹. In most studies using the guillotine method of fracture creation the femur or tibia is pre-supported by an intramedullary wire. This avoids the difficulty of wire introduction into the fractured bone but increases the energy required to create the fracture and possibly increases soft tissue damage. Despite this, this method, standardized by Bonnarens and Einhorn ¹⁹², is currently the most widely used for the study of closed mid-diaphyseal, long bone fractures.

The alternative technique of osteotomy allows consistent fracture level and pattern but changes the nature of the fracture model. Osteotomy results in more periosteal disruption and slower healing than closed fracture¹⁹³ and comparison between results using the two methods is difficult.

The ovariectomised (ovx) rat as a model of osteopenia

The ovx rat is a widely used as a model to study osteopenia. Ovariectomy results in a rapid and definite increase in bone turnover with a predominance of osteoclast activity¹⁹⁴. Increased bone resorption can be detected as early as fourteen days post ovx with increasing osteopenia up to 100 days later⁷⁵. Li *et al.*¹⁹⁵ showed that by 30 days post ovx the cancellous bone volume of the rat femoral neck was reduced to 75% of control. The changes are mainly in the trabecular bone with cortical bone volume not changing significantly until a considerable time later. It has been proposed that the loss of trabecular bone volume is related to osteoclast perforation of trabecular plates which occurs soon after ovx. Similarly, histomorphometry has shown ovx to induce endosteal bone resorption but not influence periosteal bone formation¹⁹⁶.

As with all animal models caution has to be exercised in using this model and applying the results to humans. The age at which the ovx is performed has been shown to be important with more pronounced changes occurring in younger animals¹⁹⁷. Ovx also results in weight gain. This has caused concern with experiments designed to investigate bone density or fracture healing due to the difficulties controlling for loading. Tibial

bone volume has however been shown to be only minimally affected by uncontrolled weight gain in this model ¹⁹⁸.

The appropriateness of using sham operated animals as the control is also a concern. Stress has been shown to inhibit the normal five day oestrous cycle in the rat as does any enforced dieting or fasting ¹⁹⁹⁻²⁰¹. It is therefore important to ensure as low a stress environment as possible for these animals and to ensure that sufficient time has passed for the oestrous cycle to resume following sham surgery. Ensuring that a suitable oestrous cycle has resumed can be carried out by serum oestrogen assay, vaginal or uterine histology ^{198,202}

Within these constraints, the ovx rat is considered a suitable model for the study of osteopenia secondary to oestrogen depletion ²⁰³⁻²⁰⁵.

1.2.6 Fracture healing in osteoporosis

Despite the volume of research in osteoporosis there is a relative paucity of information about the influence of osteoporosis on fracture healing. While prevention of fracture is clearly a worthwhile aim the best currently available preventive treatments only reduce the incidence of fracture by up to 50% in a high risk population⁵¹. This means that a very large number of osteoporotic fractures will continue to occur for many years to come. The osteoporotic fracture presents particular problems for treatment. Fixation is difficult to achieve as screws can pull out due to their lack of hold in osteoporotic bone. The resulting cutting-out of fixation devices limits the options for treatment. Ironically it is this very group of patients where successful internal fixation would be most beneficial. The elderly, osteoporotic patient benefits most from early mobilization with loss of independence and significant co-morbidity associated with prolonged immobilization. The ability to enhance fracture healing in osteoporosis and potentially to speed up the process or improve the mechanical properties of the healing bone would increase the treatment options for these patients. An improved understanding of the process of fracture healing in osteoporosis is therefore desirable.

A role for oestrogen in fracture healing is suggested by evidence that oestrogen receptors have increased expression in the fracture callus in the early stages of healing²⁰⁶. Additionally, cross talk between oestrogen receptors and BMPs integral to fracture healing has been identified¹⁸

Initially it was unclear if any differences existed between osteoporotic and non-osteoporotic fracture healing. A limited study by Blythe and Buchsbaum²⁰⁷ appeared to show no difference in the mechanical properties of healing fibular osteotomies in either environment. More recently however, evidence has emerged to support the hypothesis that important differences do exist. Bolander *et al.*²⁰⁸ reported increased strength of healing callus in the ovx rat following oestrogen injection. Hill *et al.*²⁰⁹ showed the mechanical properties of healing fractures to be poorer in the ovx group after 21 days compared with a sham control. Very similar results were reported by Walsh *et al.* who used healing femoral fractures supported by an intramedullary wire in the ovx rat as his model²¹⁰. Using a femoral osteotomy model in the ovx rat plus a low calcium diet, Namkung-Matthai *et al.*²¹¹ showed reduced callus formation and bone mineral density at the healing osteotomy. This was associated with a three-fold reduction in resistance to peak load in the ovx osteotomies compared with control at three weeks post osteotomy. They concluded that this demonstrated a delay in callus formation in the ovx model. This conclusion was supported by more recent work²¹² using a similar model which showed increased soft callus in the ovx group compared with sham at four weeks post fracture.

Other workers have however reported contradictory results. Cao *et al.*²¹³ reported that calluses in ovx rats were larger than sham six and 16 weeks post femoral osteotomy with histomorphological features consistent with more advanced healing. They found no significant differences in the mechanical properties of the healing osteotomies in either

group. Similarly, Kubo *et al.*²¹⁴ found no clear difference in the mechanical properties of healing femoral osteotomies at six or 12 weeks after their creation in ovx versus a non-fractured control (not sham operated). They did, however, see osteoporotic changes in the new bone formed at the osteotomy site 12 weeks after osteotomy.

The method of each of the studies mentioned differed and may be an explanation for the contradictory results. The age of animals, the time elapsed between ovx and fracture and the use of closed fractures and osteotomies were different in each study. These factors can all influence outcomes. The most comparable methods were between Walsh *et al.*²¹⁰ and Hill *et al.*²⁰⁹ who reported similar results supporting the hypothesis that fracture healing is impaired by ovx. The limited work in this field makes drawing conclusions about the process of fracture healing in osteoporosis very difficult.

Further work is clearly required to improve the understanding in this area.

1.3 Study aims

- 1 - To establish if mechanical differences exist between healing fractures of the proximal tibia in an osteopenic rat model compared with a sham operated control
- 2- To investigate if histological differences exist between the healing fracture calluses in the osteopenic rat model compared with control
- 3 - To investigate whether the expression of growth factors BMP-2, 6 and their intracellular signalling peptide Smad-1 differ in osteopenic callus versus control
- 4 - To evaluate the potential influence of oral administration of simvastatin during the early stage of fracture healing on each of the above factors

Hypothesis

That differences exist in the mechanical and histochemical properties of healing fractures in an osteoporotic model versus control and that fracture healing can be influenced by the systemic administration of simvastatin.

Materials & Methods

2 Methods

Licensing

This research was covered by personal and project licences granted under the Animals (Scientific Procedures) Act UK 1986.

2.1 Part 1

Fracture healing in the ovariectomy model versus control

2.1.1 Study design

Four month old female Wistar rats underwent ovariectomy (Ovx) or sham ovariectomy (sham) through a standard dorsal approach under general anaesthesia. This was carried out under license by the supplier Charles River (UK) prior to delivery. Opiate analgesia was used post ovariectomy with no anti-inflammatory medication given at any time. The animals were housed in groups of five and allowed to feed *ad libitum* until delivery to our research facility seven weeks following ovx or sham surgery.

One week following delivery a tibial fracture was created in every animal by the method described in Section 2.1.5.2. The animals were then divided into the following groups

each containing ten rats housed in groups of five with the following post fracture regimes:

Group A1 – Sham animals, received placebo, euthanased four weeks post fracture

Group A2 – Ovx animals, received placebo, euthanased four weeks post fracture

Group B1 – Sham animals, received placebo, euthanased two weeks post fracture

Group B2 - Ovx animals, received placebo, euthanased two weeks post fracture

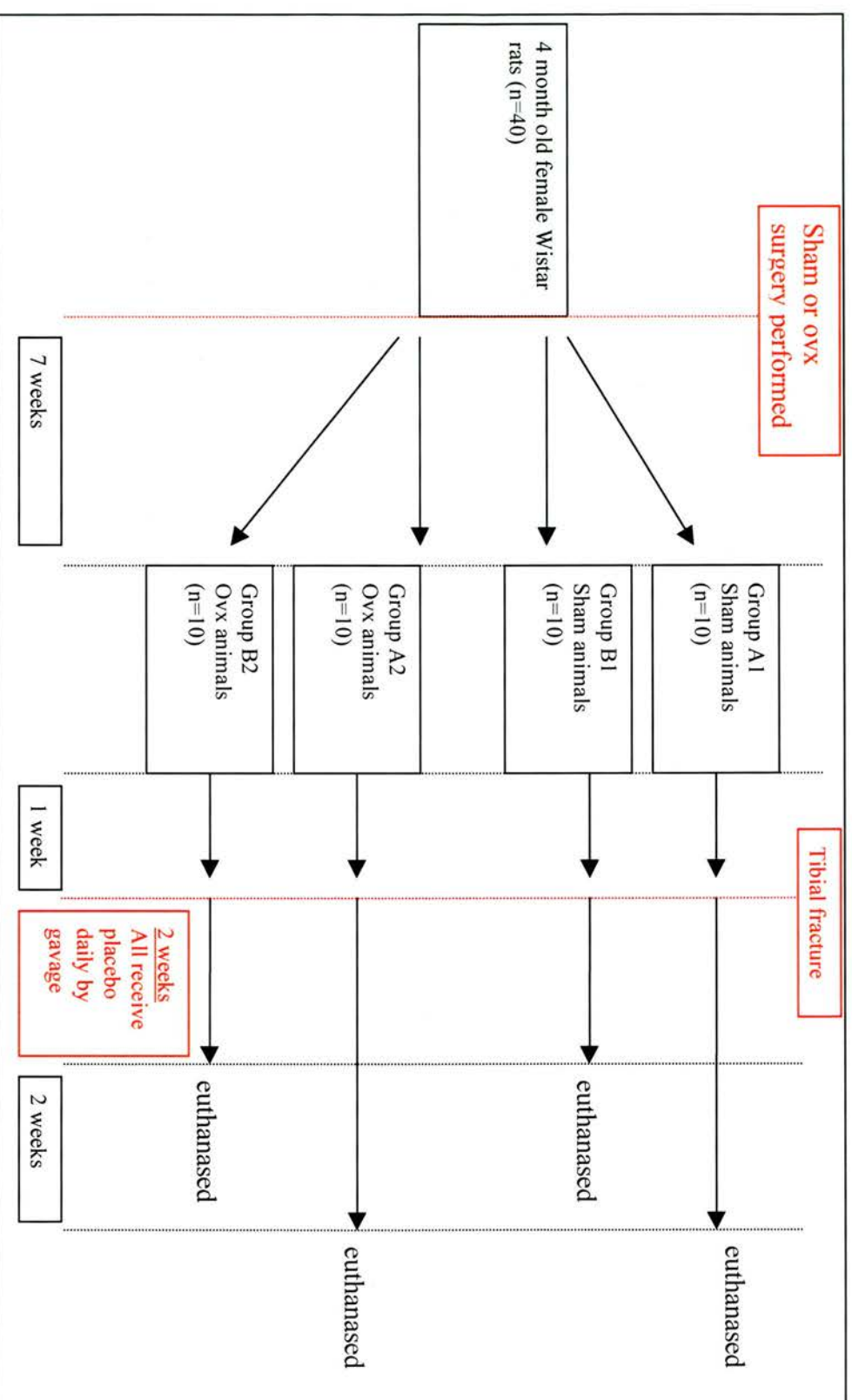
Placebo was administered to the animals in order that this arm of the trial could act as a control for part B of the study. Placebo (1ml vegetable oil) was administered by gavage daily for two weeks following fracture. Animals were allowed to eat and drink *ad libitum* throughout.

Following euthanasia the animals were weighed and the uterus and both tibiae removed for analysis. Outcome measures were:

- 1 - Histological analysis of fracture site
- 2 – Radiological analysis of the fracture
- 3 – Immunohistochemical assay for expression of bone morphogenic proteins (BMP) BMP-2 and 6, Smad 1 and bromodeoxyuridine (BRDU) as a marker of mitosis
- 4 – Mechanical testing of the healing fractures
- 5 – Assessment of the efficacy of ovariectomy by;

- Calculation of tibial bone mineral density of the intact, opposite tibia
- Body weight
- Uterine histology and weight

Figure 2.1.1 Plan of part 1 : Fracture healing in ovariectomised model versus control



2.1.2 The fracture model

2.1.2.1 Environment and diet

Animals were housed in groups of five in standard cages with wood shaving bedding. Temperature was maintained at 15 to 20 degrees Celsius with cycles of 12 hours of light and dark every 24 hours. All procedures and husbandry were carried out during hours of light. Animals were delivered one week before creation of fracture and handled daily to allow acclimatisation to handlers and environment.

2.1.2.2 Age of animals

One of the limitations of the rat fracture model is that rats do not reach skeletal maturity. Rapid growth however occurs in the first 6-8 weeks of life and by 5-6 months skeletal growth rate has reached a plateau²¹⁵. Roholl *et al.*²¹⁶ showed that at six months skeletal calcium flux had attained a steady state in female rats. An age of six months for creation of fracture was therefore chosen.

2.1.2.3 Choice of time points for euthanasia

Animals were euthanased by carbon dioxide inhalation. A two week time point was desired to allow investigation of callus formation and expression of growth factors which have been shown to be maximally expressed at this time²¹⁷.

By four weeks bony union has occurred in rat tibial fractures¹⁸⁶ and remodelling begins²¹⁸. This time point was chosen to investigate mechanical and histological features of the fractures at bony union.

2.1.2.4 Development and validation of the fracture technique

The technique for creation and stabilisation of a reliable transverse proximal tibial fracture was developed and validated using the cadavers of 3-6 month old Wistar rats kindly donated by the University of Edinburgh Small Animal Research Facility. A method was sought to create a reliable, transverse fracture of the proximal tibial metaphysis. The proximal metaphysis was chosen in order to provide a model of cancellous bone healing. This was thought to be more relevant to the study of fracture healing in osteopenia than the previously described mid-diaphyseal models. A cross section of the tibia taken at the level of the fracture in our model (Figure 2.1.2) is compared with a cross section from the mid-diaphysis of the tibia (Figure 2.1.3). These figures demonstrate the difference in the proportions of cortical to cancellous bone at the two levels. Intramedullary pinning also caused significantly less disturbance to the intramedullary, cancellous bone at the proximal metaphysis compared with the mid-diaphysis.

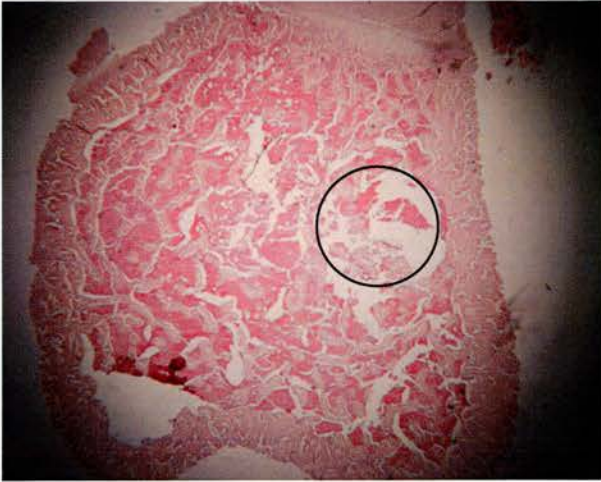


Figure 2.1.2 Cross section of proximal tibial metaphysis at the intended level of fracture. The passage created by the intramedullary pin is highlighted. (H&E x 25 light)

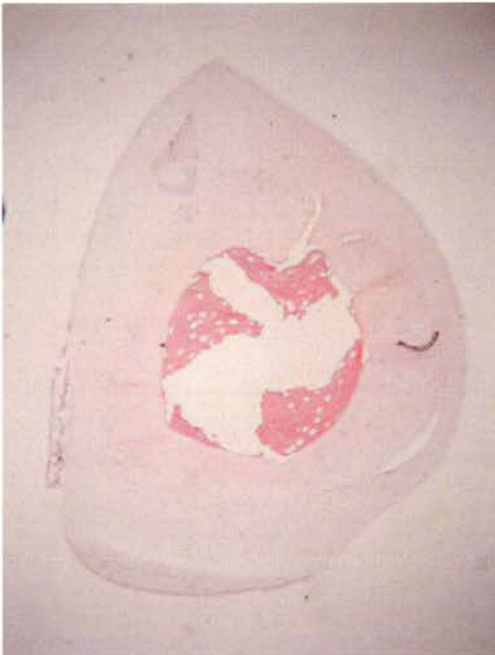


Figure 2.1.3 Cross section at the mid-diaphysis of the same tibia as figure 2.1. The disruption of the intramedullary bone caused by the intramedullary pin can be seen. (H&E x 40 light)

Reliability of the method was defined as creation of greater than or equal to 90% acceptable fractures. Fractures displaying any spiral component or comminution or those occurring out with the proximal third or involving the proximal physis of the tibia were defined as unacceptable. The following methods of fracture creation were assessed.

Digital three-point bending with the fulcrum positioned laterally, medially, posteriorly and anteriorly failed to create a reliable closed fracture of the proximal third of the tibia. Significant force was required and resulted in comminution at the fracture site (Figure 2.1.4) and in one specimen, dislocation of the distal femoral physis (Figure 2.1.5). This technique produced only 24/40 (60%) acceptable fractures.

Three-point bending with the three pressure points applied by polythene rods was also attempted. While this resulted in a more reliable fracture configuration, problems were encountered with securing the limb and preventing rotation during the fracture process due to the curved shape of the rat tibia. This resulted in an inconsistent direction of application of force and many fractures were occurring out with the proximal metaphysis. Thirty-three out of 40 fractures (82.5%) were unacceptable. Once again, the force required to fracture the metaphyseal bone resulted in an unacceptable degree of soft tissue crush injury. It was therefore decided to abandon attempts to create a closed fracture.

An open technique was then assessed. The anterior crest of the proximal tibia was exposed through a short, longitudinal incision. The same incision was used to allow

passage of the intramedullary wire for fracture stabilisation (discussed below). A 2mm notch was then made with bone cutters in the anterior crest of the tibia at the desired level. Application of digital three-point bending with the fulcrum centred over the medial side of the notch resulted in a reliable, transverse fracture with minimal soft tissue crushing. This also resulted in reliable fracture or dislocation of the fibula. This was important as it has been shown that the integrity of the fibula influences the mechanical properties of the healing tibial fracture. This technique was repeated on 40 hind limbs of 20 rat cadavers resulting in 37 (92.5%) suitable fractures. This method of fracture was therefore adopted (see Figure 2.1.6).



Figure 2.1.4 Radiograph of severely comminuted tibial fracture created by closed three-point bending



Figure 2.1.5 Radiograph of femoral physeal dislocation created by attempt at creation of closed tibial fracture



Figure 2.1.6 Radiograph of 4 cadaver limbs with tibial fractures created by open technique



2.1.2.4 *In vivo* fracture creation and stabilisation

Induction and maintenance of general anaesthesia

Anaesthesia was induced by gas inhalation of 5% halothane in air with supplemental nitrous oxide. For maintenance, halothane was reduced to 1%. A satisfactory level of anaesthesia was confirmed by loss of corneal reflex and withdrawal response.

Surgical preparation

The anterior aspect of the right lower limb was then shaved to expose the knee joint and upper third of the lower leg. The exposed area was cleaned with methylated spirit and a sterile drape applied leaving only the prepared surface of the knee and leg exposed. No intravenous antibiotics were given.

Surgical technique

Aseptic technique and disposable instruments were used throughout with the exception of a supply bone cutters which were sterilised in an autoclave between cases. A 5mm longitudinal incision was made in the midline over the lower aspect of the patella tendon and upper tibia. A 16-gauge hypodermic needle was then passed through the tendon and used to create an entry point through the anterior aspect of the proximal tibial plateau into the medullary canal of the tibia. A 0.8mm stainless steel wire was then passed down the intramedullary canal until resistance was encountered indicating it had reached the distal tibial diaphysis. The wire was then marked, withdrawn and cut to length.

Manipulation of the skin allowed exposure of the proximal tibial crest through the same incision. A pair of small bone cutters were used to create a 2mm notch in the anterior crest of the tibia 5mm distal to the tibial tuberosity. A three-point bend was then applied digitally to complete the tibial fracture. The fulcrum was placed over the medial aspect of the tibia at the level of the notch.

The wire was then re-passed down the intramedullary canal and firmly engaged in the distal tibial diaphysis. Care was taken to ensure the proximal aspect of the wire was not prominent at the knee.

The surgical wound was then closed with a subcuticular 6/0 nylon suture supplemented with acrylic glue to seal the wound. No wound dehiscence or clinically apparent infection was observed with this technique.

Subcuticular buprenorphine was then given for analgesia and the halothane discontinued. Non-steroidal anti-inflammatory analgesia was avoided due to its effects on fracture healing. Animals were recovered in isolation and returned to preoperative housing conditions on recovery. Full weight bearing through the affected limb was observed within a few hours of surgery.



Figure 2.1.7 Vertical incision exposing patellar tendon with left knee flexed



Figure 2.1.8 Creation of the entry hole in the proximal tibial plateau with 16-gauge needle



Figure 2.1.9 Insertion of pre-measured intra-medullary pin



Figure 2.1.10 Wound after closure with subcutaneous stitch and acrylic glue. The wound has retracted medially covering the patellar tendon with undamaged skin

2.2 Part 2

The effect of simvastatin on fracture healing in normal and osteopenic bone

This part of the study was run concurrently with Part 1. This allowed the placebo fed animals in part 1 to act as controls for Part 2 of the study.

2.2.1 Study design

Four month old female Wistar rats underwent ovariectomy (Ovx) or sham ovariectomy (sham) through a standard dorsal approach under general anaesthesia. This was carried out under licence by the supplier Charles River (UK) seven weeks prior to delivery. The animals were housed in groups of five and allowed to feed *ad libitum* following surgery.

One week following delivery to our research facility, a tibial fracture was created in every animal by the method described in Section 2.1.2.4. The animals were then divided into the following groups each containing ten rats housed in groups of five with the following post fracture regimes (see Figure 2.2)

Group A3 – Sham animals, received simvastatin, euthanased four weeks post fracture

Group B3 – Sham animals, received simvastatin, euthanased two weeks post fracture

Group A4 – Ovx animals, received simvastatin, euthanased four weeks post fracture

Group B4 - Ovx animals, received simvastatin, euthanased two weeks post fracture

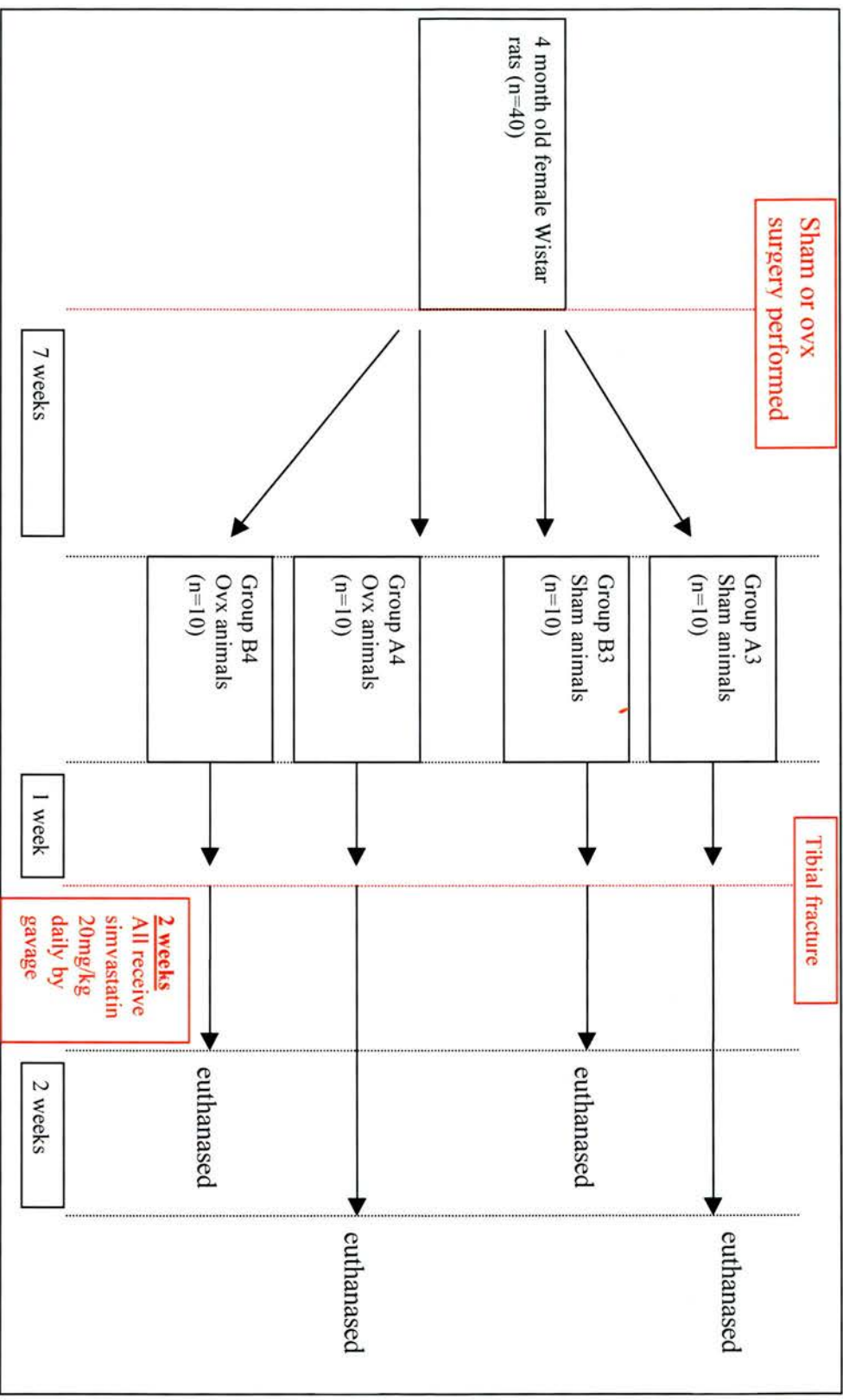


Figure 2.2 Plan of Part 2: The effect of simvastatin (20mg/kg) orally on fracture healing in normal and osteopenic bone

2.2.1.1 Administration of simvastatin

Simvastatin was administered in the form of ZOCOR (Merck & Co, USA). See Appendix A1.7 for full description of ZOCOR. This was finely ground and suspended in vegetable oil on the morning of administration. Vegetable oil has been used previously as a vehicle for oral delivery of statins⁷² as they are insoluble in water. Measured doses of statin were delivered by rigid tube gavage in the same way as delivery of placebo in Part 1 of this study.

Administration of statin or placebo was carried out at 9am each morning for 14 days starting on the day following creation of the tibial fracture.

Experimental conditions were otherwise identical to those in Part 1.

2.2.2.2 Dose of simvastatin

A dose of 20mg/kg was chosen on the basis of previous work carried out by Mundy *et al.*⁶⁵ and Maritz *et al.*⁷². Both investigators had shown effects on osteoporotic bone at this dose. The dose of 120mg/kg used by Skoglund *et al.*¹⁸³ in their study on fracture healing was considered too high with toxic effects demonstrated in mammals at 50mg/kg and above⁷⁶. The dose of 20mg/kg produces a serum level in the rat five times that in a human taking the usual dose of 80mg per day.

2.3 Histological analysis

As it was of primary interest to investigate the morphology of the healing fractures, it was decided that samples should be fixed in formalin, decalcified and embedded in paraffin to provide sections with optimum morphology. The possible effects of this method of specimen preparation will now be discussed.

2.3.1 Fixation

The tibiae and fibulae from each animal were dissected out leaving a cuff of soft tissue around the fracture site so as not to violate the callus. The samples were immediately immersed in 4% formaldehyde in phosphate buffered saline at pH 7.2-7.4 and left for 48 hours. A period of 24 to 48 hours is accepted as sufficient for fixation of most tissues after Helander²¹⁹ showed the quantity of fixed tissue plateaus after this period.

The process of formalin fixation involves development of protein cross-linking. While this process has no effect on histological appearance of the specimen it has implications for immunohistochemical techniques and will be discussed further in Sections 2.6 and 2.7.

2.3.2 Decalcification

To permit sectioning in paraffin, bone requires decalcification prior to mounting. Removal of calcium and resulting dissolution of hydroxyapatite can be achieved by strong acids or chelating agents. Acids such as nitric and hydrochloric have the advantage of achieving rapid decalcification but at the cost of hydrolysis of nucleic acids

and denaturing enzymes²²⁰. This can significantly hinder subsequent assays and immunohistochemical techniques²²¹. For this reason, specimens were decalcified in disodium ethylenediamine tetraacetic acid (EDTA) at pH 7, which acts as a chelating agent.

Complete decalcification was verified by x-ray analysis of specimens using a table-top x-ray unit (Faxitron, USA). All samples were completely decalcified after 4 weeks at 37°C with weekly changes of EDTA.

2.3.3 Processing

After complete decalcification and further trimming, the specimens were processed in an automated processor to allow embedding in paraffin. The processing protocol is shown in Appendix A1.1.

Following processing the specimens were mounted in paraffin blocks with great care taken to orientate the specimens horizontally. This was to permit longitudinal cross sections of the tibiae to be cut.

2.3.4 Sectioning

5µm sections were cut on a standard microtome (Shandon) and mounted on “Superfrost plus” slides (BDH) after floating on deionised water at 40°C to remove ridges. Slides were stood vertically to dry and then baked at 37°C for 12 hours to optimize section adherence.

Slides were then stored in a dry, dark environment at room temperature until use in histological or immunohistochemical procedures.

2.3.5 Analysis of sections

In order to study the general morphology of the healing fractures three slides from each specimen were stained with haematoxylin and eosin (H&E) and viewed under light microscopy at magnifications from x25 to x400. See Appendix A1.2 for H&E method. Five sections were taken 250 μm apart to control for variability through the specimen. Formal quantification of tissue types present at the fracture site was performed using a 36-point graticule. The fracture site was defined as beginning where the periosteum became elevated to where it returned to cortical contact beyond the fracture and all tissue within this area was quantified using the graticule. Tissue types were recorded as:

Lamellar or trabecular bone

Woven bone

Cartilage

Marrow or trabecular space

Undifferentiated tissue

Each section was mapped twice at different times and a third time by an independent observer to control for inter and intra-observer error (see Appendix 2).

Results were expressed as number of points for each tissue type at the fracture site over the number of total points mapped and then expressed as a percentage. The results from the three sections were averaged to provide a mean score for each specimen.

2.4 Radiological analysis

After fixation but prior to decalcification, all tibiae were x-rayed. Tibiae were placed in a right lateral position after removal of the fibula to facilitate consistent positioning. All samples were exposed at 50 KvP for 45 seconds with the image captured on Polaroid film (Polaroid Inc, USA).

Negative images were analysed for presence of fracture line and scored as:

Fracture line complete	= 1
>2/3 diameter	= 2
1/3 to 2/3 diameter	= 3
<1/3 diameter	= 4
Fracture line absent	= 5

The median, range and 95% confidence intervals for each group were then calculated.

2.5 Immunohistochemistry for detection of Bone Morphogenic Proteins (BMP) 2 & 6

2.5.1 Basis of technique

Immunolocalization of BMP in frozen and paraffin sections of rat tissue by indirect techniques has been successfully demonstrated previously²²². The primary antibody to BMP-2 used in the following technique was successfully used by Yu and co-workers¹⁴⁰

2.5.2 Specimen preparation

Sections prepared in the standard way, as described in Section 2.3, were dewaxed and rehydrated to distilled water.

2.5.3 Effect of prolonged storage

To control for possible effects on the sections of storage at room temperature for an average of eight weeks, a selection of fresh sections were cut and processed in the same experimental run. As no difference in staining intensity between the fresh and stored sections was demonstrated, the stored sections were accepted as representative.

2.5.4 Antigen retrieval

Most antigen retrieval is carried out using a combination of heat and pressure.

Unfortunately this led to unacceptable damage to sections and was abandoned as a technique in this study.

Antigen retrieval was performed with trypsin solution as described in Appendix A1.4. Slides were immersed in trypsin solution at 37°C for 20 minutes. This time was derived from trials of 5, 10, 15, 20 and 30 minutes with 20 minutes appearing to result in optimal staining. EDTA antigen retrieval (see Appendix A1.5) resulted in patchy, sub-optimal staining with this technique.

2.5.5 Primary antibody concentration and incubation time

Both BMP 2 & 6 goat polyclonal antibodies were purchased from Santa Cruz Biotechnology (BMP-2 sc-6895, BMP-6 sc-7406) and were supplied at 200µg/ml. The assay was performed for both antibodies with concentrations of primary antibody of 1:80, 1:40 and 1:20. A final working dilution of 1:40 proved optimal for antiBMP-2 which was in-keeping with the findings of Yu *et al.*¹⁴⁰. Staining was best for anti-BMP-6 at a concentration of 20:1.

Sections were incubated with primary antibody at 4°C overnight in a humid environment. Staining proved inadequate with shorter durations of incubation.

2.5.6 Assay technique

The indirect avidin-biotin complex (ABC) technique was used with the exact method described in Appendix A1.3. This technique relies upon detection of the biotinylated secondary antibody by an avidin-biotin-horse radish peroxidase complex. This, in turn, is identified by utilisation of the reaction between diaminobenzidine (DAB) and peroxidase which results in production of a visible, insoluble brown product at the site of

the reaction. This improved sensitivity of this technique compared with other IHC methods was shown by Hsu *et al.*²²³.

One negative (primary antibody omitted) and one positive slide (section of rat foetus) were included with every experimental run.

To ensure positive staining was representative of the presence of BMP 2 or 6, blocking peptides were used. The antigens against which the antibodies had been raised were obtained from Santa-Cruz Biotechnology (BMP-2 blocking peptide sc-6895p, BMP-6 blocking peptide sc-7406p). In accordance with the supplier's instructions, the antibodies were incubated with their blocking peptide overnight at 4°C with a ratio of blocking peptide to antibody of 5:1. This solution was then used in place of primary antibody in the technique described in Appendix A1.3. Suppression of staining was demonstrated indicating that positive staining was representative of the presence of BMP 2 or 6 (Figure 2.5.1).

2.5.7 Analysis

Three random high power fields (x400) of the mid-fracture specimen were viewed at the fracture. All positive stained osteoblasts and chondrocytes were expressed as a ratio of total number of the same cell type visible within the field. Results were expressed using the Positive Stained Index²²⁴ (expressed as a percentage) which was derived for each cell type as follows:

$$\text{Positive Stained Index (PSI)} = \frac{\text{Positively Staining Cells}}{\text{Total number of same cell type visible}}$$

The PSI for each field was then scored as follows:

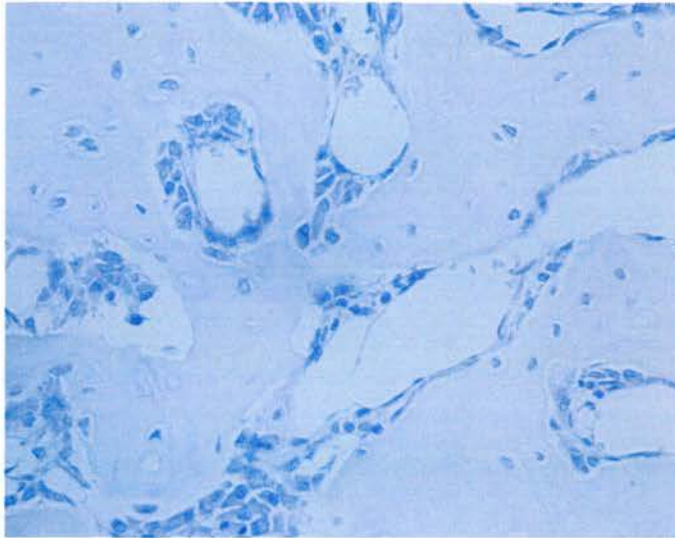
0 = no staining, 1= <25%, 2= 25-50%, 3= 51-75%

4= >75%

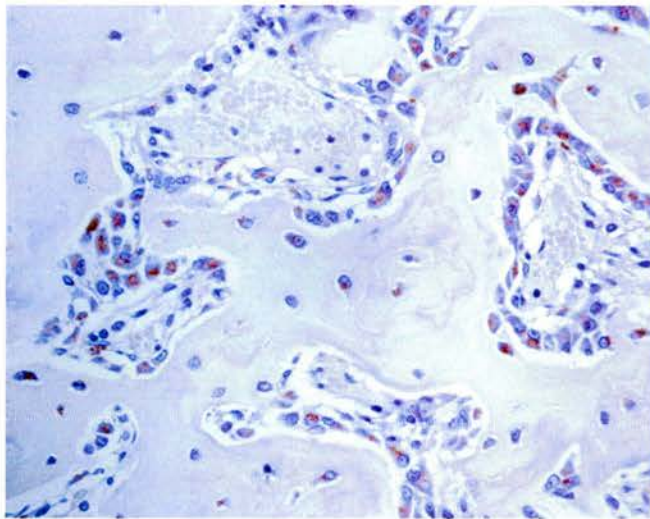
The scores for each field were then added for a total score ranging from 0 to 12 for each specimen.

It has been shown that the reliability of quantitative immunohistochemical analysis can be improved by using computerised image analysis²²⁵. Unfortunately this facility was not available during this study.

Figure 2.5.1 Efficacy of BMP-2 antibody (sc-6895)



BMP-2 assay after antibody incubation with blocking peptide (sc-6895p)



BMP-2 assay without blocking peptide

Sections of cancellous bone at fracture site of sham & placebo (group A1)

specimen. (light x 400)

2.6 Immunohistochemistry for detection of Smad 1

2.6.1 Specimen preparation

Sections prepared in the standard way, as described in Section 2.3, were dewaxed and rehydrated to distilled water.

2.6.2 Effect of prolonged storage

As in Section 2.5, fresh sections were cut and used in parallel with sections stored for eight weeks at room temperature. No difference in antigen expression was identified and stored sections were therefore used.

2.6.3 Antigen retrieval

Antigen retrieval was performed with trypsin solution as described in Appendix A1.4. Slides were immersed in trypsin solution at 37°C for 20 minutes. This time was derived from trials of 5, 10, 15, 20 and 30 minutes with 20 minutes appearing to result in optimal staining.

2.6.4 Primary antibody concentration and incubation time

The primary antibody used was murine polyclonal anti-Smad 1 (sc-7965, Santa Cruz Biotechnology) supplied at 200 µg/ml. Dilutions of 1:20, 1:40 and 1:80 were tried with 1:20 representing 10µg/ml final working concentration providing optimal staining. Incubation overnight at 4°C proved optimal.

2.6.5 Assay technique

The assay was run using the Animal Research Kit (ARK) (DAKO) according to the protocol described in Appendix 1.6. This was used to minimize background staining which was problematic using the avidin-biotin technique described above.

2.6.6 Analysis

Three random high power fields (x400) were viewed at the fracture. All positive stained osteoblasts and chondrocytes were expressed as a ratio of total number of the same cell type visible within the field. Results were expressed using the Positive Stained Index (expressed as a percentage) which was derived for each cell type as follows:

$$\text{Positive Stained Index (PSI)} = \frac{\text{Positively Staining Cells}}{\text{Total number of same cell type visible}}$$

The PSI for each field was then scored as follows:

$$0 = \text{no staining}, \quad 1 = <25\%, \quad 2 = 25-50\%, \quad 3 = 51-75\% \\ 4 = >75\%$$

The scores for each field were then added for a total score ranging from 0 to 12 for each specimen.

2.7 5-Bromo-2-deoxyuridine (BRDU) Immunohistochemistry (IHC)

2.7.1 Basis of technique

BRDU is a pyrimidine analogue of thymidine which is incorporated into the deoxyribosenucleic acid (DNA) of cells in the S-phase of mitosis. Development of monoclonal antibodies to BRDU has permitted the identification of cells in S-phase of mitosis using immunohistochemical techniques.

This technique has been shown to be effective in identifying both replicating bone and cartilage cells *in vivo*. Farquharson and Loveridge²²⁶ showed that BRDU immunohistochemistry can be used on both fresh, frozen and fixed and decalcified sections. Apte²²⁷ validated this assay against thymidine autoradiography for decalcified tissues and found it to be a useful and reliable technique.

2.7.2 Sample preparation

Animals were labelled by intraperitoneal injection of 25mg BRDU/kg body weight one hour prior to death. Animals were euthanased and the tibiae were collected, processed, decalcified and sectioned as described earlier.

Sections were dewaxed and rehydrated through alcohols to distilled water as described in Section 2.3.

2.7.3 Effect of prolonged storage of sections

Sections were stored in the dark at room temperature for eight weeks prior to carrying out the BRDU immunohistochemistry. As before, freshly cut sections were stained with

the experimental sections to establish if storage had effected experimental staining.

There was no difference between stored and freshly cut sections with this technique.

2.7.4 Antigen retrieval

In order to allow exposure of the BRDU antigen the DNA firstly had to be denatured.

This was performed by placing the sections in 2M hydrochloric acid (HCL) at 37°C for 30 minutes. After washing in phosphate buffered saline (PBS) further antigen retrieval was carried out by placing the sections in trypsin solution at 37°C for 20 minutes (see Appendix A1.4).

This method of antigen retrieval was selected as optimum after trials with the following combinations:

2M HCL for 30 minutes at 37°C followed by trypsin solution for 10, 20 and 30 minutes

5M HCL for 45 minutes at room temperature followed by trypsin solution for 10, 20 and 30 minutes

2M HCL followed by EDTA antigen retrieval technique described in Appendix A1.5.

The selected technique provided the best staining with minimum background.

2.7.5 Primary antibody concentration and incubation time

The primary antibody used was mouse monoclonal anti-BRDU (IgG, clone BU 33, SIGMA UK).

The experiment was run using the following dilutions of primary anti-BRDU mouse monoclonal antibody: 1:125, 1:250, 1:500, and 1:1000. A concentration of 1:250 was

found to be optimal. This represented a final concentration of 16ug/ml as stock was supplied at 4000ug/ml.

Sections were incubated with primary antibody in the following conditions: 30, 60 and 120 minutes at 37⁰C and overnight at 4⁰C. 120 minutes at 37⁰C was selected as optimal.

2.7.6 Technique

After specimen preparation and antigen retrieval, the assay was conducted according to the Animal Research Kit (ARK) (DAKO) protocol shown in Appendix A1.7.

This commercially available kit was used due to problems with excessive background staining encountered using the indirect staining technique described earlier for the BMP assays. The ARK is designed to minimise background which may arise due to reactivity of secondary anti-mouse antibody with endogenous antigen in rodent tissue.

For this assay the diluent used was DAKO antibody diluent.

2.7.7 Analysis

Three random high power fields (x400) were viewed at the fracture site. All positive stained cells were expressed as a ratio of total number of the same cell type visible within the field. Results were expressed using the Positive Stained Index which was derived for each cell type as follows:

$$\text{Positive Stained Index (PSI)} = \frac{\text{Positively Staining Cells}}{\text{Total number of same cell type visible}}$$

Scoring was applied as described in Section 2.5.7

2.8 Verification of the osteopenia model

2.8.1 Confirmation of ovariectomy

After euthanasia and removal of the tibiae the cadavers were dissected to confirm the absence of ovaries in the ovariectomy animals. Sham models were also dissected to ensure the ovaries were present.

2.8.2 Uterine weight

All uteri were removed from the cadavers and fixed in formalin. All uteri were then weighed three times. The mean weight of each uterus was expressed in grammes.

2.8.3 Calculation of bone mineral density

Volume

Volume was calculated using Archimedes' principle²²⁸. Tibiae were stripped of all soft tissue and the fibulae removed. The tibiae were then weighed (weight A).

A bijou was filled with water and sealed underwater to ensure the absence of air bubbles. The filled bijou was then dried externally and weighed (weight B).

The tibia was then placed in the filled bijou which was once again sealed underwater to ensure the absence of air. The water filled bijou containing the tibia was then externally dried and weighed again (weight C).

From the principle that 1ml of water weighs 1g, the volume of the tibia was calculated from the following formula:

$$\text{Weight of water in bijou displaced by bone} = B - (C - A)$$

$$\text{Weight of displaced water (g)} = \text{volume of bone (ml)}$$

Ash weight

Tibiae were placed in individual, pre-weighed crucibles and placed in a muffle furnace at a temperature of 600°C for 24 hours. After cooling, the crucibles plus the ash were weighed and after subtraction of the crucible weight the ash weight of each bone was derived. The mean of three repeat measurements was taken as representative.

Bone mineral density (BMD)

BMD was calculated as follows:

$$\text{BMD (g/ml)} = \text{Ash weight (g)} / \text{Volume (ml)}$$

2.9 Mechanical testing

2.9.1 Testing method

The healing fractures were tested by four-point bending as described below. Bending is a practical and well accepted technique for obtaining biomechanical data about small animal bones or healing fractures²²⁹. The alternative of tensile testing does provide accurate assessment of biomechanical properties of both cortical and cancellous bone. However, the technicalities of mounting specimens for tensile testing mean that this requires relatively large or well machined specimens and it is therefore rarely used for testing rodent bone.

Testing in compression can provide useful information on the mechanical properties of cancellous bone in particular. It has the additional advantage of reproducing a more physiological model with for example vertebra normally being under compression *in vivo*. Accuracy relies upon uniform compression being applied and this could not be achieved to test the proximal tibia in the axial plane due to the geometry of the rat tibia.

Previous workers have tested healing fractures in torsion^{230,231}. This provides information about the behaviour of the sample in shear. However, the specimen requires to be circular in cross section so that a constant centre of rotation exists at which shear is zero. With a non-circular specimen the polar moment of inertia changes during the test rendering the results inaccurate²²⁹. Once again the shape of the proximal tibia was thought to be unsuitable for testing in torsion for this reason.

Bending tests are applicable to small specimens and reproduce an element of the normal physiological environment under which long bone fractures heal. Bending a bone results in tension on one side and compression on the other with failure usually occurring on the tension side as bone is weaker in tension than compression²³². Bending can be achieved using three or four contact points. Three point bending is simple to perform but results in significant shear at the mid-loading point²³³. This problem is compounded by the fact that small specimens result in a low span to depth ratio as biological specimens are of a predetermined length and depth. A span to depth ratio of greater than 16 is recommended to exclude shear but this cannot be achieved with rodent tibia or femur.

Four point loading results in minimal shear between the two mid-loading points. It does however rely on equal contact by all four loading points which is elementary on standardized samples but can be more difficult with bone. For this reason a pivot is usually incorporated into the design of the loading contact to ensure constant contact throughout. On this basis, it was decided to design apparatus to test specimens in four-point bending to failure.

2.9.2 Retrieval and storage of samples

In order to reduce error, all samples were stored and tested in one experimental run. It has previously been shown that bone can be frozen in saline at -20°C and thawed out without affecting material or mechanical properties²³⁴.

Immediately after euthanasia both tibiae were dissected free of all soft tissue with disarticulation of the tibiofemoral and tibiotalar joints. The intramedullary pins were removed and the samples immersed in 0.9% normal saline (NaCl). Samples were then frozen to -20°C in a standard freezer. Storage time under these conditions varied from 1 to 3 weeks to accommodate the two experimental time points.

2.9.3 Preparation of samples for testing

All samples were removed from the freezer and placed within their saline filled containers into a water bath set at 37°C . Adequate thawing of the samples was ensured by verifying saline temperature had reached 37°C for 30 minutes prior to mechanical testing. During the thawing period the fracture calluses were measured in the antero-posterior and medio-lateral planes using a precision calliper measuring to 0.1mm. The mean of three readings was taken for each orientation for each sample. Samples were re-immersed in the warmed saline for a further five minutes then removed and immediately tested to failure. On average the total time from removal from saline to end of the experiment was 90 seconds.

2.9.4 Testing apparatus and specification

All tibiae were tested to failure by four-point bending using a Instron materials testing machine (Series 4500, Instron Corp. Canton, Mass, USA).

The four point loading jig shown in Figure (2.9.1) was designed and produced specifically for this work by University of Edinburgh Engineering Workshop, Kings Buildings, Edinburgh.

The upper loading assembly was designed to incorporate a pivot to ensure symmetrical loading by the upper two contacts. The upper loading span was 5mm and the lower, static span was 20mm. Contact points were rounded to minimise notching of the bone after application of load.

All samples were tested in the same orientation with the tibiae resting on their lateral surface and the force being applied from the medial aspect. The tibiae were found to be stable in the testing apparatus in this orientation after a trial or various different orientations.

Testing specifications were as follows:

Load cell 500N

Rate of deformation 2mm/minute

Temperature of specimen 37°C

Room temperature 22°C

Room Humidity 50%

2.9.5 Standardisation

Thirty tibiae from the cadavers of rats weighing between 300g and 400g were collected, frozen, thawed then tested using the above criteria. This allowed assessment of the reliability of the testing method. The results are shown in Appendix 3.7. Standard environmental conditions have been shown to be important with changes in humidity and room temperature known to influence material testing results²²⁹.

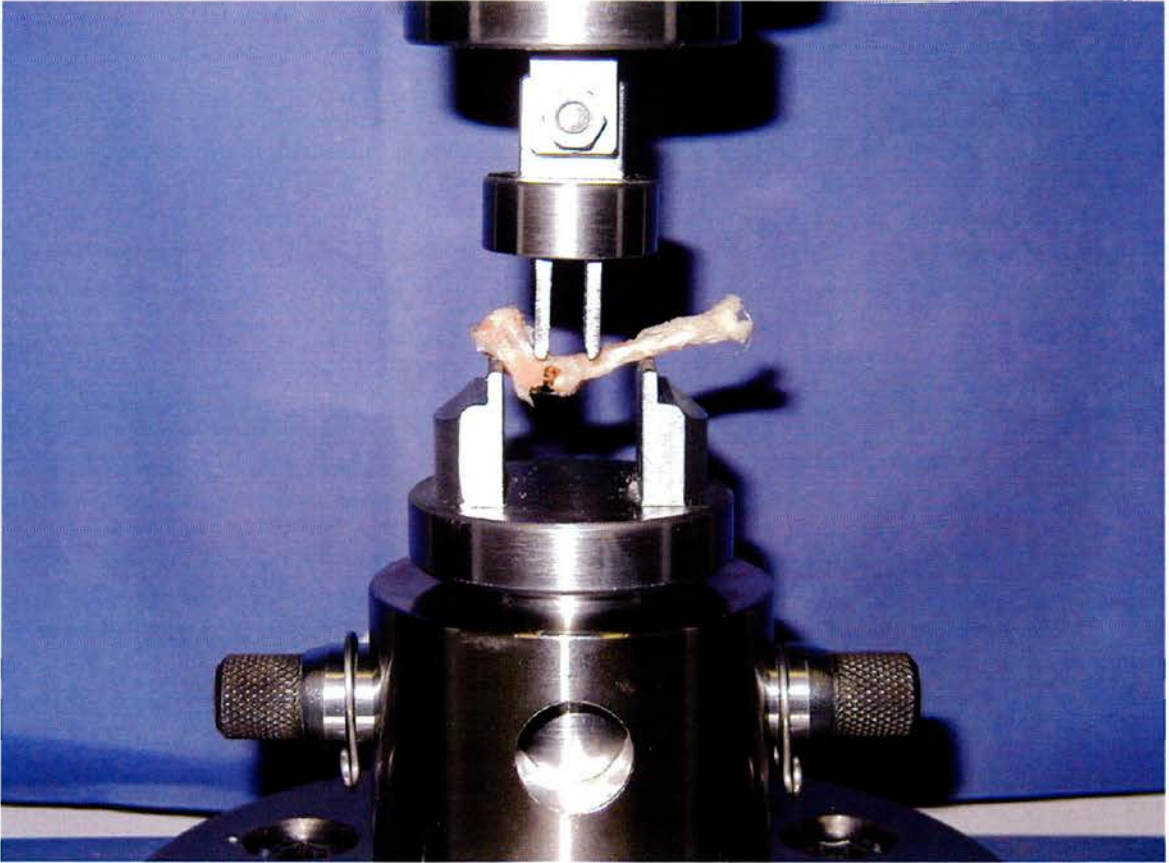


Figure 2.9.1 Four point bending apparatus with failure of tibia through fracture callus

2.9.5 Expression of Results

Data derived from the materials testing machine was expressed as load (N) / deformation (mm). Load at yield and failure were taken directly from the load / deformation graph with yield defined as the point where the load /deformation curve became non-linear.

Load at failure was defined as the maximum load applied. Extrinsic stiffness was calculated from load / deformation at the point of yield.

Other workers have directly measured the cross sectional area of the fracture plane using image analysis and pixilation²¹³. This highly accurate technique was not available and the cross sectional area was therefore estimated from biplanar measurements taking the fracture plain as an ellipse.

The area of an ellipse is:

$$\text{Width} \times \pi \times (\text{Height}/4)$$

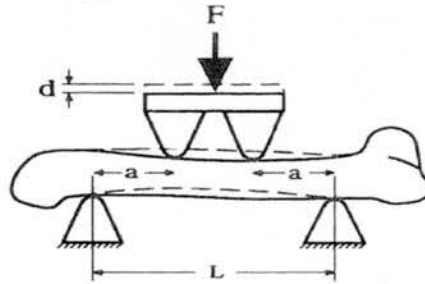
The cross sectional moment of inertia (I) is a measure of material distribution around a neutral axis. In order to calculate stress, I must be known.

Cross sectional moment of inertia for an ellipse:

$$I_y = \frac{1}{4} \times \pi \times \text{vertical radius} \times \text{horizontal radius}^3$$

Figure 2.9.2 allows interpretation of the following formulae

Figure 2.9.2 Diagram of four point bending model



(F=force, d=displacement, L=span, a=distance from outer to inner contact)

Stress can therefore be calculated from:

$$\text{Stress } (\sigma) = (\text{force} \times a \times c) / 2I$$

(c = distance from the centre of the mass or half the height of the callus)

Strain describes the deformation of an object under load and is expressed as new length / original length. For bone which undergoes relatively small degrees of strain, this is usually expressed as μ strain. Strain can be measured directly using a strain gauge or derived from load/deformation data for four point bending as follows ²²⁹:

$$\text{Strain } (\epsilon) = \frac{(6 \times \text{vertical radius} \times \text{displacement})}{a(3L - 4a)}$$

Young's Modulus (E) was calculated from the data as follows:

$$E = (\text{force/displacement}) \times (a^2/12I) \times (3L-4a)$$

Energy to the point of yield was calculated from area under the graph to the yield point

Units

Load	Newton (N)	
Stiffness	N/mm	
(I) Moment of inertia	(mm ⁴)	
(σ) Stress	megapascals (Mpa)	(1 Pascal = 1 Newton/m ²)
(ϵ) Strain	μ strain	
(E) Young's Modulus	gigapascals (GPa)	

Energy to yield Megajoules MJ

All measurements of distance expressed in mm

2.10 Statistical analysis

Data were compared using the statistical package Graphpad (Graphpad Software Inc. El Camino Real, San Diego, USA).

The individual tests used in each analysis are given with the results in Section 3.

Consideration was given to the application of Bonferroni's correction to various multiple analyses carried out in this work. While the use of the correction to avoid inadvertent Type 1 errors is appropriate in certain circumstances it was considered that the interdependence of the data made the use of the correction inappropriate. The use of Bonferroni's correction in the multiple analyses of interdependent data has been shown to excessively reduce the power of studies where multiply analysed data are interdependent. It remains the case however that where p values are close to an alpha of 0.05 the significance of the result should be interpreted with caution due to the theoretical risk of type one errors in such analyses.

2.11 Power calculation

The sample size of five per outcome group (five for histology and five for mechanical testing equalling ten per group) was chosen based on a power analysis of the following parameters:

Evaluation of a 30% difference

Estimated from previous work by Walsh *et al.* ²¹⁰ and Namkung-Matthai *et al.* ²¹¹

Alpha = 0.05

Beta = 0.8

Samples of unequal variance, $\text{Sigma}_1=12$, $\text{Sigma}_2 = 7$

Gives $n_1 = 4.487$, $n_2 = 2.513$

(Performed using Powercalc, UCLA, Los Angeles, California, USA)

Results

3 Results

3.1 Confirmation of the osteopenic model

3.1.1 Uterine weight and histology

The mean uterine weight from the ovx animals was 57% of the mean of the sham animals (Figure 3.1.1.1). This difference was significant when tested with an unpaired, two-tailed t-test ($p < 0.0001$).

H & E sections of randomly selected uteri from sham and ovx animals showed marked endometrial and myometrial atrophy in the specimens from the ovx group (Figure 3.1.1.2 & 3.1.1.3).

The significant reduction in uterine weight and tissue atrophy confirmed the efficacy of ovariectomy in these animals.

Figure 3.1.1.1

Comparison of mean uterine weights from sham versus ovx animals

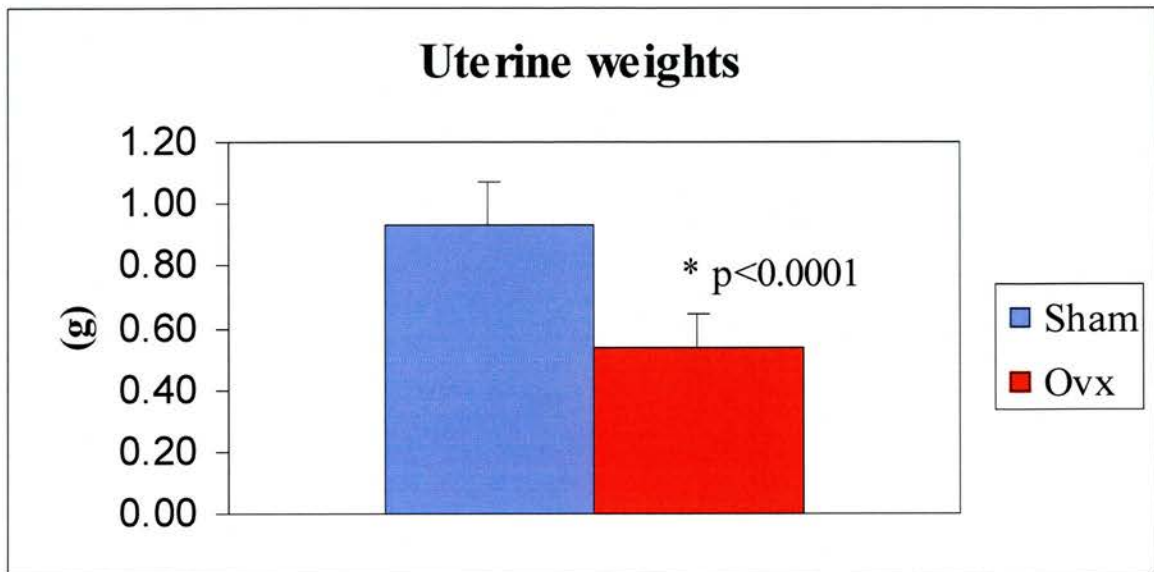


Figure 3.1.1.2 Transverse section of uterus from sham animal (H&E, x40)



Figure 3.1.1.3 Transverse section of uterus from ovx animal (H&E, x40)



3.1.2 Body weight

Creation of fracture occurred eight weeks after ovariectomy or sham procedure. As expected, by this time the ovx animals had gained weight and had a significantly higher mean body weight than sham animals. The mean weight of all ovx animals at the time of fracture was 365.07g compared with 299.75g for sham animals ($p < 0.001$, un-paired two tailed, t-test). Mean body weight was unchanged in all groups during the first two weeks after fracture as shown in Table 3.1.2.1.

The mean body weights of the groups euthanased at four weeks had however increased significantly. Once again, the ovx animals had gained the most weight since the time of fracture (mean gain 41.6g for ovx versus 20.07g for sham, $p < 0.001$, unpaired, two tailed t-test). This would suggest that weight gain occurs during weeks 2 to 4 following fracture.

There was no difference in weight change between groups given simvastatin versus placebo.

Table 3.1.2.1 Body weights for animals euthanased at two weeks post fracture

	Time of fracture		Two weeks post fracture		P value
	Weight (g)	SD	Weight (g)	SD	
Sham & placebo B1	307.38	13.1	300	17.4	0.105
Ovx & placebo B2	371.11	25.2	359.44	29.9	0.340
Sham & statin B3	293.01	15.7	286.2	13.69	0.445
Ovx & statin B4	363.2	22.7	364.8	27.6	0.762

Table 3.1.2.2 Body weights for animals euthanased at four weeks post fracture

	Time of fracture		Four weeks post fracture		P value
	Weight (g)	SD	Weight (g)	SD	
Sham & placebo A1	298.75	27.0	322.38	27.4	0.0207
Ovx & placebo A2	367	29.1	410.4	27.5	0.0021
Sham & statin A3	288.89	11.7	315.4	18.8	0.0192
Ovx & statin A4	359	15.2	398.8	18.3	0.0001

Data tested with unpaired, two tailed t-test. Normal distribution of data confirmed using Kolmogorov Smirnov method.

3.1.3 Tibial ash weight, volume and mineral density

The data shown in Table 3.1.3 were derived using the techniques described in section 2.8.3. While mean ash weight of the tibiae was the same in the ovx group and sham controls, the mean volume was higher in the ovx group. This resulted in a significantly lower mean tibial bone mineral density in the ovx group compared with sham control. This confirmed that eight weeks after ovariectomy the rat tibiae had become osteopenic. This is illustrated in Figure 3.1.3.

Table 3.1.3 Ash weight, volume and bone mineral density of tibiae

	All sham animals			All ovx animals			P value
	Mean	SEM	95% CI	Mean	SEM	95% CI	
Ash Weight (g)	0.318	0.004	0.310,0.326	0.312	0.004	0.303,0.320	0.766
Volume (cm³)	0.436	0.007	0.421,0.451	0.476	0.011	0.452,0.500	0.0226
BMD (g/cm³)	0.729	0.006	0.719,0.744	0.662	0.013	0.635,0.689	0.0137

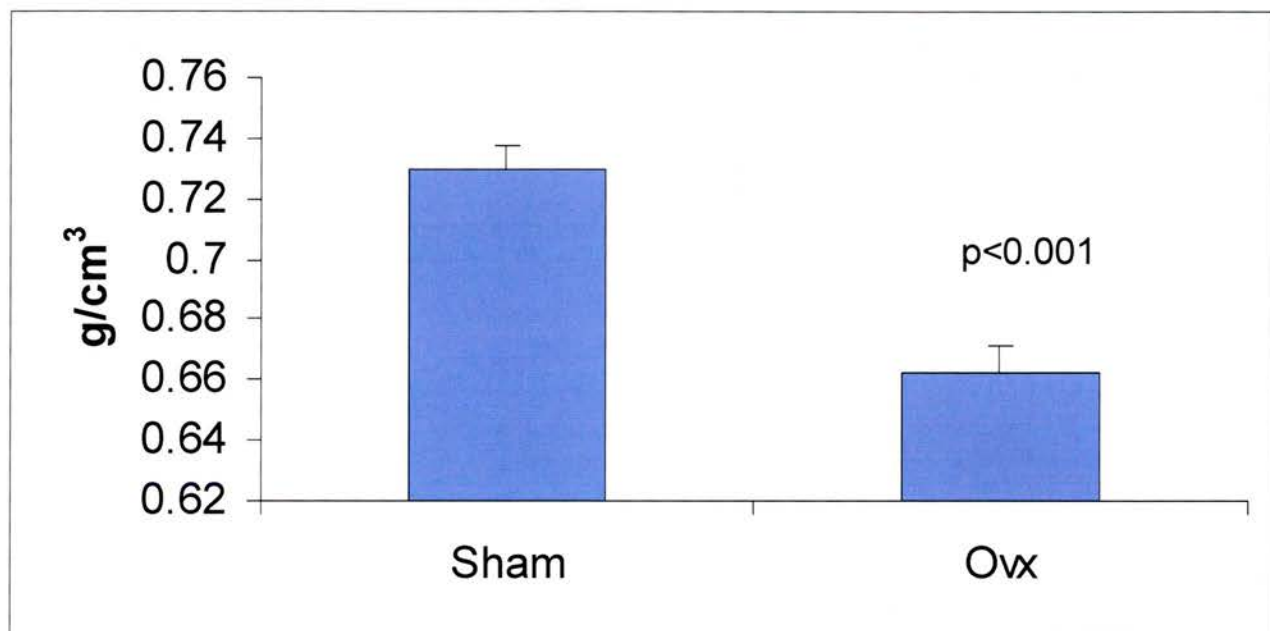
BMD = bone mineral density, SEM = standard error of the mean,

CI = confidence interval

Tested by unpaired, two tailed t-test. Normal distribution of data confirmed using

Kolmogorov Smirnov method.

Figure 3.1.3 Tibial bone mineral density of animals 8-10 weeks post ovariectomy or sham procedure



3.2 Results of Part 1

Fracture healing in the ovariectomy model versus sham control

3.2.1 Exclusions (sham v ovx)

A total of five out of the 40 (12.5%) animals in this arm of the study were excluded. A summary of exclusions per group is shown in Table 3.2.1. The most frequent problem was creation of an unsatisfactory fracture. In one case this was identified at the time of fracture creation by palpation of obvious comminution and failure to obtain stabilisation with the intramedullary wire. This animal was euthanased while under anaesthesia. In the two other cases unsatisfactory comminution was identified on radiographs following euthanasia and the specimens were discarded.

In two cases, stabilisation had been lost following creation of satisfactory fractures. This was identified post mortem by loss of fracture reduction and insecure intramedullary wires.

Table 3.2.1 Exclusions in the sham & placebo and ovx & placebo groups

Group	Description	Exclusions	Reason for Exclusion
B1	Sham & placebo, Two weeks	2/10 (20%)	1 – unsatisfactory fracture 2 – loss of stabilisation
B2	Ovx & placebo, two weeks	1/10 (10%)	1 – unsatisfactory fracture
A1	Sham & placebo, Four weeks	2/10 (20%)	1 – unsatisfactory fracture 2 – loss of stabilisation
A2	Ovx & placebo, Four weeks	0	n/a

3.2.2 Histological Results

A lower proportion of trabecular and lamellar bone was present at the fracture site in the ovx model compared with sham control two weeks following fracture. Although no statistically significant differences were seen between proportions of other tissue types a trend towards an increased amount of woven bone and marrow in the ovx model was noted at two weeks.

By four weeks post fracture the trend towards an increased proportion of marrow at the fracture site in the ovx model had become statistically significant. This was compensated for by a continued, significant reduction in the proportion of trabecular or lamellar bone present. The four week old callus in the ovx model therefore appeared more porous with a lower proportion of calcified material present overall.

Table 3.2.2.1 Summary of histological tissue types at the fracture site at two weeks post fracture (sham versus ovx). Results expressed as percentage of total number of graticule points counted (averaged from 15 repetitions)

	Group B1 SHAM Mean (%)	SEM	Group B2 OVX Mean (%)	SEM	P value
Trabecular or lamellar bone	39.27	1.76	26.93	5.07	0.022
Woven bone	40.14	3.20	51.90	5.83	0.106
Cartilage	8.50	2.29	5.07	1.27	0.170
Marrow	8.76	2.98	12.99	4.37	0.713
Undifferentiated	4.36	2.05	3.11	0.99	0.967

Statistical comparison made using unpaired two tailed t-test. Data tested for normality using Kolmogorov-Smirnoff test.

Table 3.2.2.2 Summary of histological tissue types at the fracture site at four weeks post fracture (sham versus ovx). Results expressed as percentage of total number of graticule points counted (averaged from 15 repetitions)

	Group A1 SHAM Mean (%)	SEM	Group A2 OVX Mean (%)	SEM	P value
Trabecular or lamellar bone	30.51	3.64	23.13	1.64	0.068
Woven bone	34.29	6.05	28.35	1.72	0.31
Cartilage	0.73	0.48	0.81	0.41	0.914
Marrow	33.50	4.08	47.06	1.82	0.0054
Undifferentiated	0.97	0.47	0.65	0.37	0.767

- Statistical comparison made using unpaired two tailed t-test. Data tested for normality using Kolmogorov-Smirnoff test.

3.2.3 Radiological Results (sham v ovx)

Radiographs of the healing tibial fractures from the groups euthanased at 2 weeks (B1 & B2) showed calcifying external callus but the persistence of complete fracture lines in all specimens (Figure 3.2.3.1).

At the four week time point the presence of a fracture line on the radiographs was more variable. This variability however appeared to be similar in both groups (A1 & A2). The presence of fracture line was scored according to the method described in Section 2.4 but no significant difference in scores existed between groups A1 and A2. Results for scoring of these two groups are shown in Table 3.2.3 with an example of the radiological appearance of the healing fractures at four weeks shown in Figure 3.2.3.2.

Table 3.2.3 Results of fracture line scoring in groups euthanased at four weeks

Group	Description	Median score	Range	P value
A1	Sham & placebo	2.5	1-4	0.1505
A2	Ovx & placebo	3.0	2-4	

Groups compared using Mann Whitney U Test

Figure 3.2.3.1 Typical radiographic appearance of healing fractures in all groups two weeks post fracture

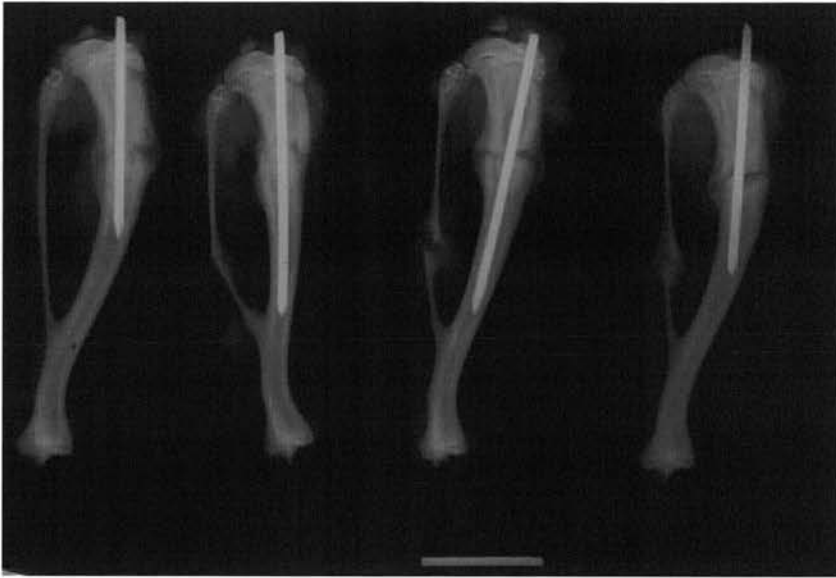
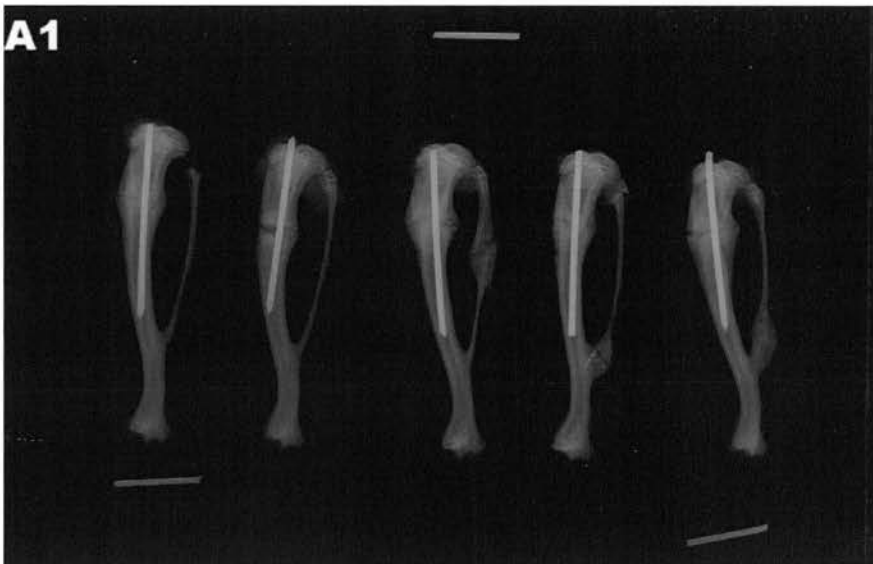


Figure 3.2.3.2 Typical radiographic appearance of healing fractures in all groups four weeks post fracture



3.2.4 Immunohistochemistry results (sham v ovx)

The results of staining for BMP 2 and 6, Smad 1 and the marker of proliferation BRDU, are summarised in Tables 3.2.3.1 and 3.2.3.2. Staining for each of the antigens was seen mainly in osteoblasts and chondrocytes two weeks following fracture. By four weeks following fracture chondrocytes were sparse and as a result only osteoblasts were scored for antigen expression at this time point.

3.2.4.1 BMP - 2 expression

Cytoplasmic staining for the BMP-2 antigen was seen in osteoblasts and chondrocytes at the healing fractures from both sham and ovx groups at two weeks. The number of positively staining cells varied within each group. Typical examples are shown in Figure 3.2.4.1.

No statistically significant difference was seen between the scores for BMP-2 expression at two or four weeks in osteoblasts when comparing the sham versus the ovx groups.

Similarly, no difference existed in the expression of BMP-2 in fracture site chondrocytes from either group two weeks following fracture.

No staining for BMP-2 was seen in the periosteum in either group.

Table 3.2.4.1 Summary of immunohistochemistry results in fracture osteoblasts of sham versus ovx animals
(results expressed as median score for positive staining index, described in Section 2.5.7)

OSTEOBLASTS	Assay	Sham & Placebo Median (95%CI)	Ovx & Placebo Median (95%CI)	P Value (Mann Whitney U Test)
2 weeks Post Fracture	BMP 2	1 (-2.462, 5.128)	3 (0.744, 6.256)	0.096
	BMP 6	3 (-2.303, 6.303)	4 (1.095, 6.905)	0.373
	Smad 1	3 (-2.303, 6.303)	2.5 (-0.25, 4.25)	0.990
	BRDU	4 (1.516, 6.484)	4 (1.864, 6.637)	0.990
4 weeks Post Fracture	BMP 2	0 (-3.303, 5.303)	2 (-2.828, 3.483)	0.760
	BMP 6	3 (-0.535, 5.202)	3 (1.16, 5.24)	0.762
	Smad 1	3 (-2.303, 6.303)	3 (-0.239, 3.84)	0.990
	BRDU	3 (0.535, 5.202)	0 (-0.839, 3.24)	0.363

Table 3.2.4.2 Summary of immunohistochemistry results in fracture chondrocytes of sham versus ovx (results expressed as median score for positive staining index, described in section 2.5.7)

CHONDROCYTES	Assay	Sham & Placebo Median (95%CI)	Ovx & Placebo Median (95%CI)	P Value (Mann Whitney U Test)
2 weeks Post Fracture	BMP 2	3 (0.798, 6.535)	4.5 (1.744, 7.255)	0.587
	BMP 6	3 (1.232, 4.101)	2 (0.701, 3.299)	0.366
	Smad 1	0 (-3.03, 5.303)	2 (0.44, 3.16)	0.652
	BRDU	3 (1.232, 4.101)	2.5 (1.581, 3.419)	0.855

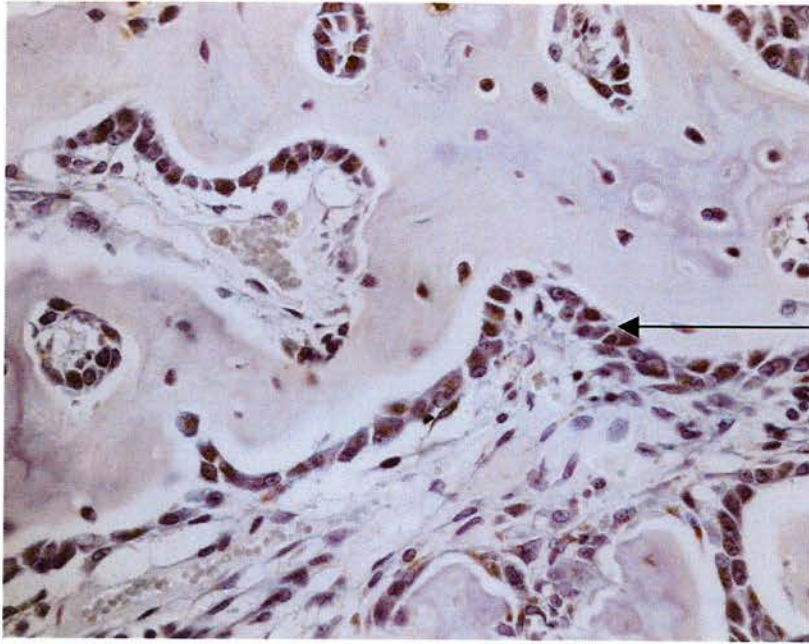
3.2.4.2 BMP – 6 expression

Cytoplasmic staining was seen for BMP-6 antigen at both two and four weeks in the osteoblasts from both groups with no significant difference found in the number of cells staining positively in each group. Staining for BMP-6 was also seen in the cytoplasm of chondrocytes within the fracture callus two weeks following fracture but no difference existed between ovx or sham groups for the extent of positive staining in this cell population.

3.2.4.3 Smad – 1 expression

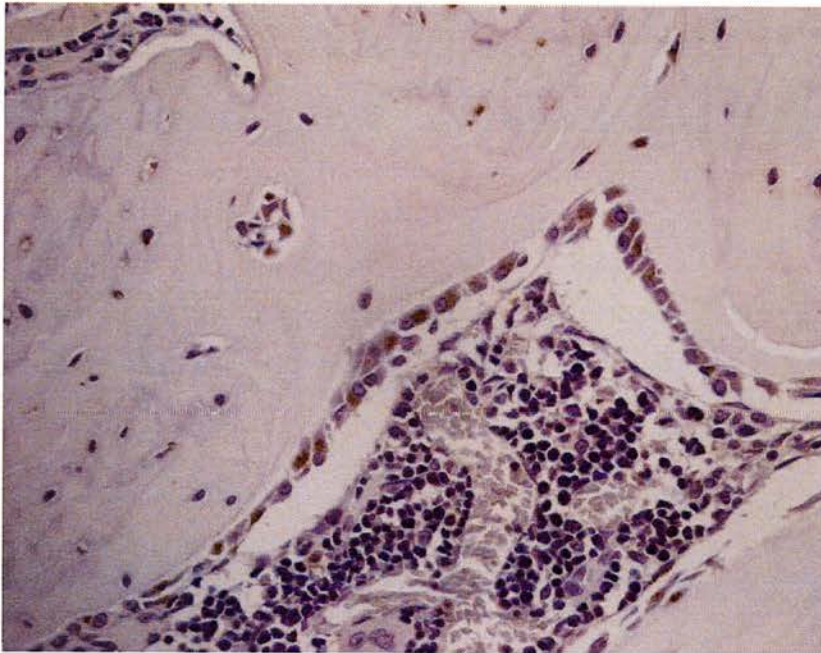
As with BMP 2 and 6, strong staining was seen in the cytoplasm of both osteoblasts and chondrocytes in the fracture callus of specimens from both ovx and sham groups two weeks following fracture. Fracture site osteoblasts continued to express Smad-1 four weeks following fracture but again no difference was seen in the degree of expression between either sham or ovx groups.

Figure 3.2.4.1 BMP-6 staining in osteoblasts four weeks post fracture



Cytoplasmic staining (brown) in osteoblast

Group A1 (sham & placebo), light x400



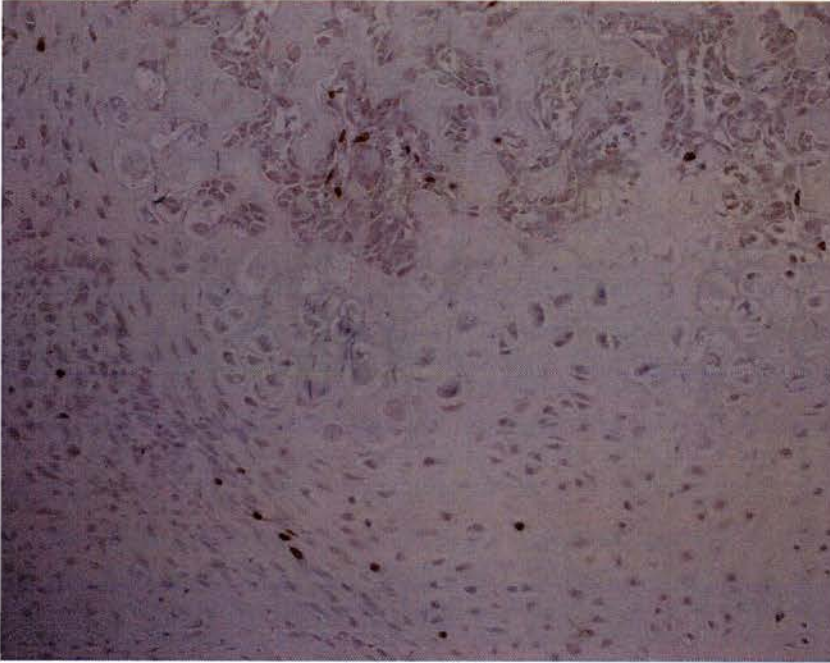
Group A2 (ovx & placebo), light x400

3.2.4.4 Bromodeoxyuridine (BRDU) expression

Strong nuclear staining was seen in chondrocyte and osteoblast populations in the fracture callus from both sham and ovx groups. This is shown in Figures 3.2.3.2-4.

Positive staining in the periosteum was sparse in all specimens. Quantification of positive staining revealed no difference between sham and ovx groups for expression of BRDU in osteoblasts at either time point, or chondrocytes at two weeks post fracture.

Figure 3.2.4.2 Specimen from group B1 (sham & placebo) showing positive staining for BRDU in osteoblasts and chondrocytes at the healing fracture site two weeks post fracture



(light x 200)

Figure 3.2.4.3 Specimen from group A1 (sham & placebo) showing positive staining for BRDU in osteoblasts at the healing fracture site four weeks post fracture (light x200)

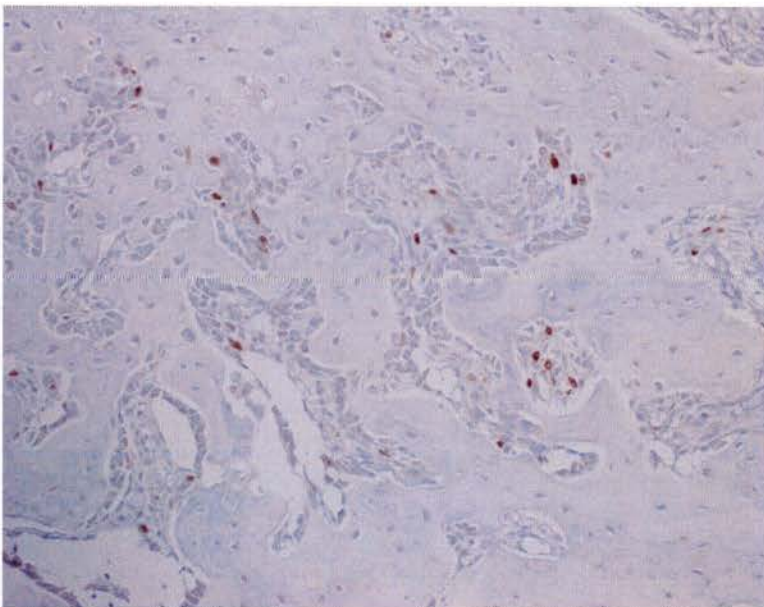
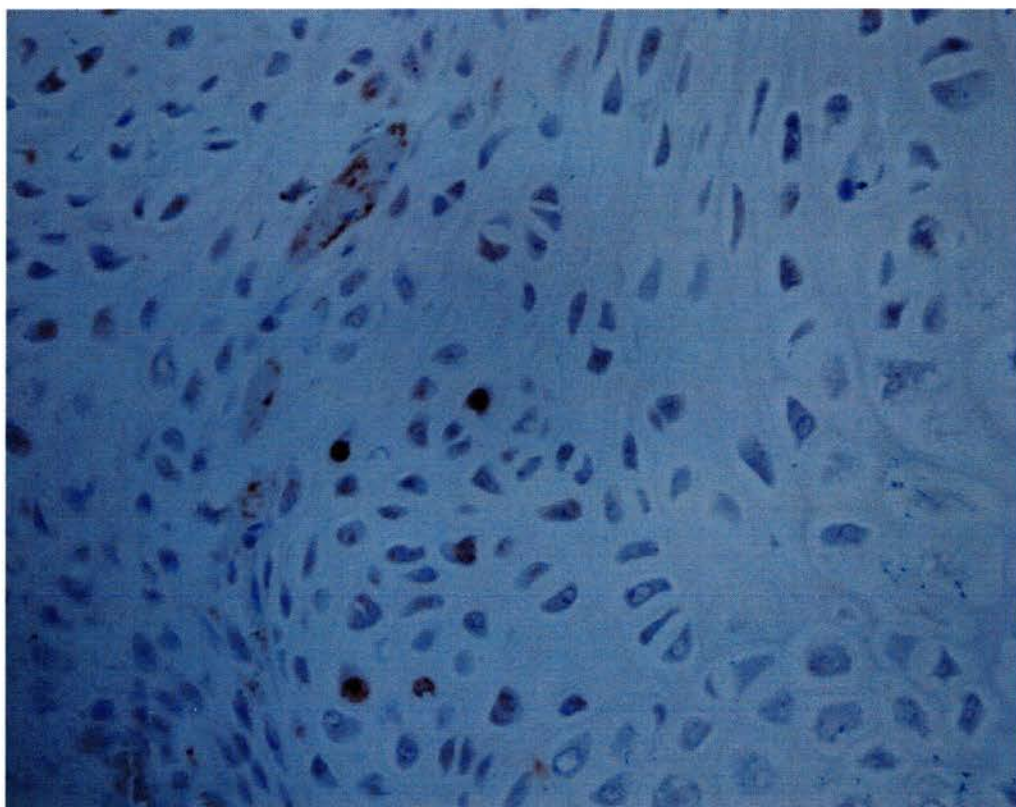


Figure 3.2.4.4 Specimen from group B1 (sham & placebo) showing positive staining for BRDU in chondrocytes at the fracture site two weeks post fracture



(light x 400)

3.2.5 Results of mechanical testing (sham v ovx)

The following data has been derived from the load / displacement curves generated by testing the healing fractures in four-point bending to failure. The method is described in Section 2.9. The intrinsic properties of stress, strain, Young's modulus and energy to yield have all been calculated from the formulae described in Section 2.9.5.

The data is summarised in Table 3.2.5.1 for the healing fractures two weeks post creation of fracture (groups B1 and B2) and Table 3.2.5.2 for those at four weeks post creation of fracture (groups A1 and A2).

Unfortunately two specimens from both two week time point groups were excluded due to loss of position on the testing apparatus during initial loading. Data are presented for reference in Table 3.2.5.1 expressed as mean with 95% confidence intervals.

3.2.5.1 Load at yield and failure

At two weeks post fracture there was no difference between the sham or ovx groups in the mean load applied at the points of yield or failure. The load at the point of yield had increased in both groups by four weeks post fracture. By this time the load at yield in the ovx group was only 71% that of the load at yield of the sham group. The same result was found for the load at failure with the healing fractures in the ovx animals withstanding only 71% of the load at failure of the sham group four weeks after fracture creation.

Both of these differences were statistically significant and are illustrated in Figures 3.2.5.1 and 3.2.5.2.

3.2.5.2 Stiffness

The mean stiffness of the healing fractures at two weeks was similar in both sham and ovx groups. By four weeks however, a statistically significant difference in the stiffness was seen with the mean stiffness of the healing fractures in the ovx group being approximately 54% that of the sham group. These findings are illustrated in Figure 3.2.5.3.

3.2.5.3 Callus area and moment of inertia

Callus area was estimated by the method described in Section 2.9.5. The calluses decreased slightly in size between two and four weeks post fracture but no significant difference was seen between callus areas in the sham or ovx groups at either time point.

No difference was seen in the moments of inertia in either group or time point reflecting the similar callus areas and distribution of material.

3.2.5.4 Intrinsic mechanical properties of the healing fractures

At two weeks post fracture the stress and strain at the point of yield were the same in both sham and ovx groups. As Young's modulus and energy to yield are calculated directly from these data they did not differ either.

By four weeks post fracture, the stress at yield was significantly greater in the sham group compared with the ovx group. The inverse was true however of the strain at yield with the ovx fractures withstanding 140% of the strain of the sham fractures before yielding. This resulted in the four week old healing fractures in both groups absorbing approximately the same mean energy before yield. This is illustrated in Figure 3.2.5.4 which plots the mean stress and strain for both groups to show a model of the linear portion of the groups' stress / strain curves.

The moduli of the healing fractures were low in both groups at both time points. The moduli did increase between two and four weeks but the increases were not significant in either group. Although the mean modulus of the four week old healing fractures in the sham group was 146% of the mean modulus in the ovx group this difference was also not significant.

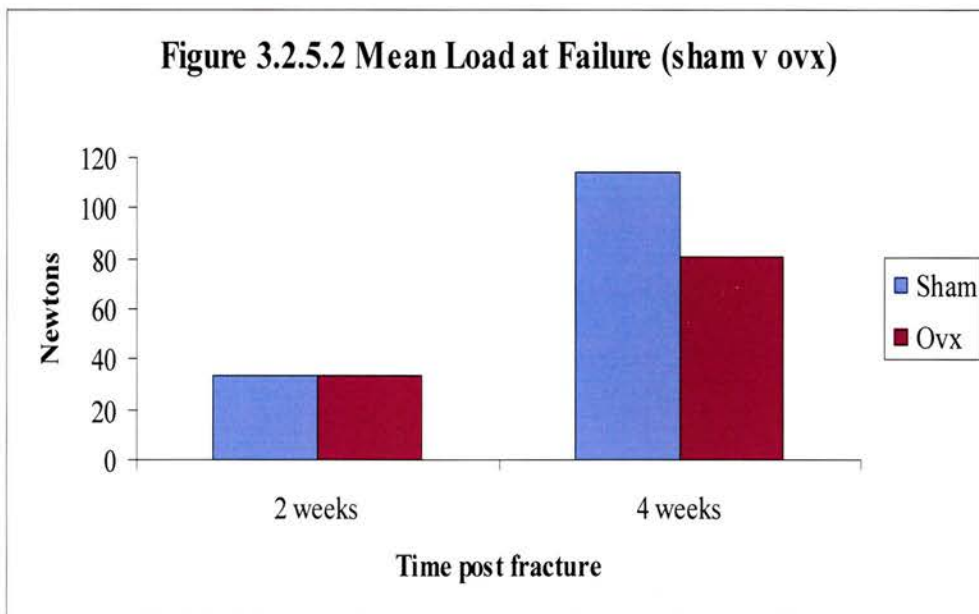
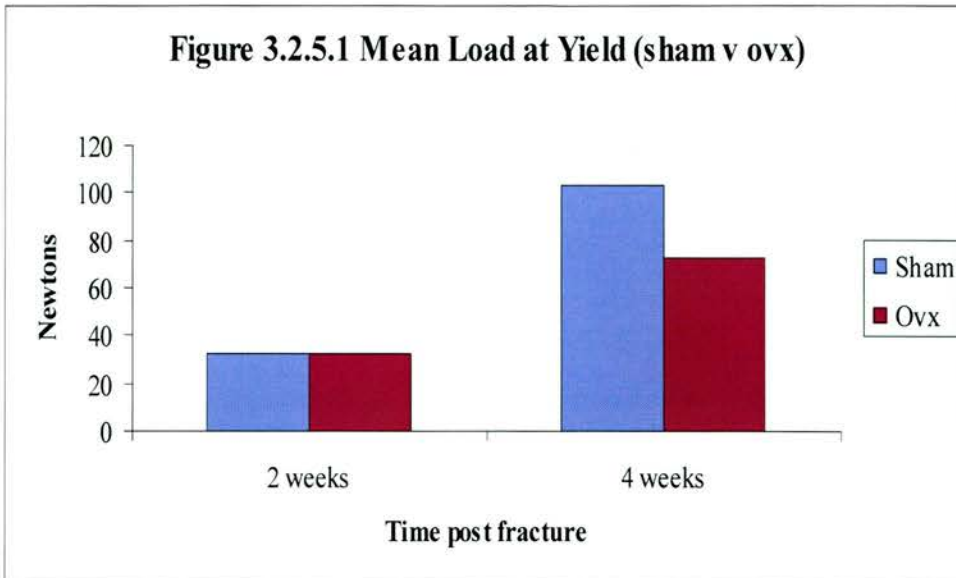
Table 3.2.5.1 Summary of mechanical testing results two weeks post fracture (sham v ovx)

	Sham & placebo (B1) n=3			Ovx & placebo (B2) n=3			P value
	mean	SEM	95% CI	Mean	SEM	95% CI	
Callus area (mm ²)	30.06	2.03	24.93, 35.19	30.49	5.65	19.42, 41.56	ns
Stiffness (N/mm)	60.81	24.6	12.64, 108.9	76.82	39.64	0, 154.52	ns
Load at yield (N)	32.0	3.51	25.12, 38.88	32.0	8.26	11.1, 52.9	ns
Load at failure (N)	33.22	4.86	23.63, 42.81	33.4	11.37	11.11, 55.69	ns
Moment of Inertia (mm ⁴)	87.57	14.91	58.34, 116.8	98.09	36.08	27.36, 168.82	ns
Stress at yield (MPa)	16.75	1.184	14.43, 19.07	16.34	5.37	5.84, 26.84	ns
Strain at yield	31.12	5.12	21.09, 41.15	23.22	9.47	4.72, 41.72	ns
Young's Modulus (GPa)	0.12	0.01	0.096, 0.144	0.12	0.035	0.051, 0.189	ns
Energy to yield (MJ/m ³)	2.57	0.356	1.698, 3.445	2.377	0.344	1.497, 3.243	ns

Table 3.2.5.2 Summary of mechanical testing results four weeks post fracture (sham v ovx)

	Sham & placebo (A1) n=5			Ovx & placebo (A2) n=5			P value
	mean	SEM	95% CI	Mean	SEM	95% CI	
Callus area (mm ²)	27.38	2.15	22.68, 32.08	27.95	2.46	23.13, 32.77	0.866
Stiffness (N/mm)	172.17	11.66	146.61, 197.73	93.25	6.38	80.75, 105.75	0.016
Load at yield (N)	103.6	7.35	87.41, 119.79	73.4	3.90	67.75, 81.05	0.014
Load at failure (N)	114.75	3.25	108.31, 121.12	81.4	2.67	76.16, 86.64	0.016
Moment of Inertia (mm ⁴)	78.48	11.58	55.1, 102.22	81.87	16.41	49.7, 114.03	0.878
Stress at yield (MPa)	50.46	4.32	41.94, 58.86	37.26	3.48	30.43, 44.09	0.045
Strain at yield	40.83	4.76	31.49, 50.17	56.74	2.91	51.03, 62.45	0.020
Young's Modulus (GPa)	0.22	0.045	0.124, 0.316	0.15	0.036	0.076, 0.224	0.189
Energy to yield (MJ/m ³)	10.056	1.859	5.98, 14.13	10.599	1.1830	8.28, 12.917	0.818

- Statistical comparison made using two tailed, unpaired t-test. Data tested for normality using Kolmogorov-Smirnoff test



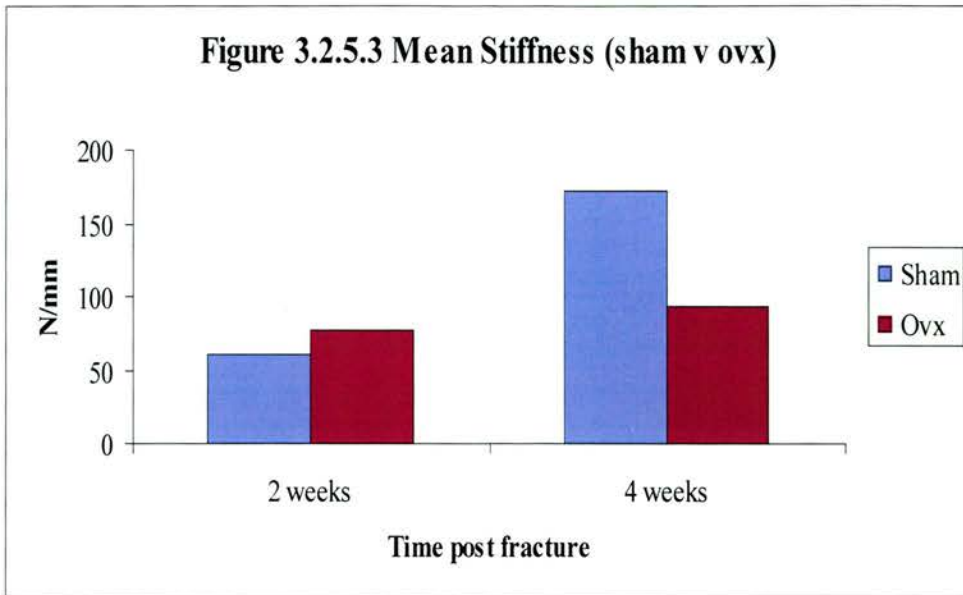
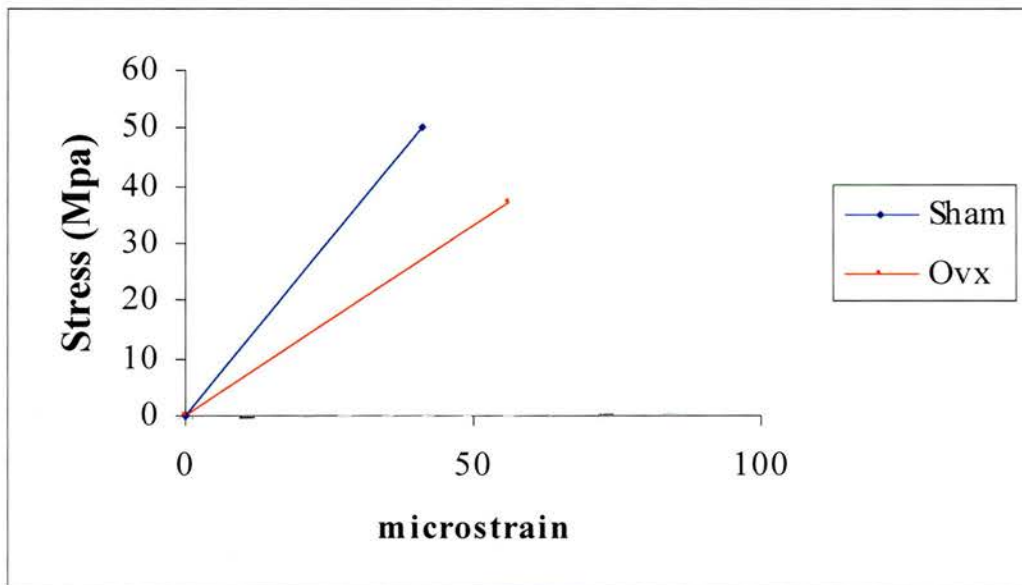


Figure 3.2.5.4 Linear portion of stress / strain curve of healing fractures at four weeks post fracture (sham v ovx)



3.3 Results of Part 2

The effect of simvastatin on fracture healing in normal and osteopenic bone

3.3.1 Exclusions

Only one specimen was excluded from this section of the study. This specimen was from group A3 and was excluded due to unsatisfactory fracture pattern as defined pre-test but seen post mortem.

3.3.2 Histological results

The data from histological analysis of the fracture calluses in both groups given simvastatin versus placebo are summarised in Tables 3.3.2.1 to 3.3.2.4.

In the sham model, no difference was seen in the proportion of tissue types present two weeks following fracture. However by four weeks post fracture a difference was seen in the amount of woven bone still present at the fracture sites of sham animals given simvastatin compared with placebo. The statistical significance of this finding with a p value of 0.029 is questionable in a multiple analysis of this kind but simvastatin did appear to result in decreased maturity of the callus in the sham animals.

In the ovx group a similar picture was seen. At two weeks post fracture simvastatin treatment the histological appearance of the fracture callus was not significantly

different compared with the control. By four weeks however a more immature callus was once again seen in the simvastatin treated group. An even greater proportion of woven bone compared to trabecular or lamellar bone was seen than had been noted in the sham model. Simvastatin treatment had again resulted in a less mature callus but this effect appeared to be exaggerated by ovariectomy. As mentioned previously, the statistical significance of these findings is not certain due to the risk of a type one error in such analyses but a trend towards reduced callus maturity was certainly apparent.

Table 3.3.2.1 Summary of histological tissue types at the fracture site at two weeks post fracture (simvastatin v placebo) in sham model.

Results expressed as percentage of total number of graticule points counted (averaged from 15 repetitions)

	Group B1 PLACEBO Mean (%)	SEM	Group B3 Simvastatin Mean (%)	SEM	P value
Trabecular or lamellar bone	39.27	1.76	36.93	5.78	0.659
Woven bone	40.14	32.0	38.24	1.65	0.600
Cartilage	8.50	2.29	6.41	1.46	0.450
Marrow	8.76	2.98	9.97	3.1	0.772
Undifferentiated	4.36	2.05	8.44	2.15	0.134

Table 3.3.2.2 Summary of histological tissue types at the fracture site at four weeks post fracture (simvastatin v placebo) in sham model.

Results expressed as percentage of total number of graticule points counted (averaged from 15 repetitions)

	Group A1 Mean (%)	SEM	Group A3 Mean (%)	SEM	P value
Trabecular or lamellar bone	30.51	3.64	26.06	4.11	0.451
Woven bone	34.29	6.05	40.07	1.46	0.029
Cartilage	0.73	0.48	0	0	0.149
Marrow	33.50	4.08	33.87	3.13	0.943
Undifferentiated	0.97	0.47	0	0	0.058

Tested with unpaired, two tailed t-test. Data checked for normality using Kolmogorov-Smirnoff test.

Table 3.3.2.3 Summary of histological tissue types at the fracture site at two weeks post fracture (simvastatin v placebo) in ovariectomised model.

Results expressed as percentage of total number of graticule points counted (averaged from 15 repetitions)

	Group B2 Mean (%)	SEM	Group B4 Mean (%)	SEM	P value
Trabecular or lamellar bone	26.93	5.07	33.06	3.22	0.428
Woven bone	51.9	5.83	44.77	3.67	0.302
Cartilage	5.07	1.27	3.22	0.91	0.329
Marrow	12.99	4.37	13.48	2.45	0.598
Undifferentiated	3.11	0.99	5.47	1.42	0.176

Table 3.3.2.4 Summary of histological tissue types at the fracture site at four weeks post fracture (simvastatin v placebo) in ovariectomised model.

Results expressed as percentage of total number of graticule points counted (averaged from 15 repetitions)

	Group A2 Mean (%)	SEM	Group A4 Mean (%)	SEM	P value
Trabecular or lamellar bone	23.13	1.64	15.61	3.28	0.041
Woven bone	28.35	1.72	43.49	6.02	0.013
Cartilage	0.81	0.41	0	0	0.067
Marrow	47.06	1.82	40.83	6.48	0.289
Undifferentiated	0.65	0.37	0.07	0.07	0.503

Tested with unpaired, two tailed t-test. Data checked for normality using Kolmogorov-Smirnoff test.

3.3.3 Radiological results

Radiographs of the tibiae two weeks post fracture showed persistence of complete fracture lines throughout all the groups.

Variability did exist within all groups with regard to the degree of presence of fracture line in the tibiae four weeks post fracture. In both sham (A3) and ovx (A4), simvastatin supplementation resulted in no significant radiological difference in presence of fracture line when compared with placebo using the Mann Whitney U Test. Results are summarised in Table 3.3.3.1.

Table 3.3.3.1 Summary of scores for presence of fracture line seen on radiographs four weeks post fracture

	Placebo		Simvastatin		P value
	Group	median	Group	Median	
Sham	A1	2.5	A3	3.0	0.085
Ovx	A2	3.0	A4	3.0	0.591

3.3.4 Immunohistochemistry results

This section compared the expression of BMP 2, 6, Smad 1 and the marker of proliferation BRDU in sections from the fracture calluses of sham and ovx animals fed

either simvastatin or placebo. For the factors BMP 2, 6 and Smad 1 sporadic cytoplasmic staining was seen in osteoblasts and chondrocytes. Strong BRDU staining was seen in the nuclei of osteoblasts and chondrocytes indicating these cells were undergoing replication.

A summary of the results from this section is shown in Tables 3.3.4.1 to 3.3.4.4.

3.3.4.1 BMP-2 expression

Supplementation with simvastatin made no difference to BMP-2 expression in the osteoblasts or chondrocytes at the fracture sites in sham animals. Cytoplasmic staining was present in both groups to an equal degree at two and four weeks post fracture.

The same was true of BMP-2 expression in specimens from ovx animals with simvastatin supplementation appearing to make no difference to the amount of positive staining seen at either time point compared with placebo.

3.3.4.2 BMP - 6 expression

Cytoplasmic staining was seen in approximately 25 to 50% of osteoblasts and chondrocytes at two and four weeks post fracture in both the sham and ovx animals fed placebo or simvastatin (see Figure 3.3.4.1). There was no statistical difference between any of the groups for the amount of positive staining for BMP-6.

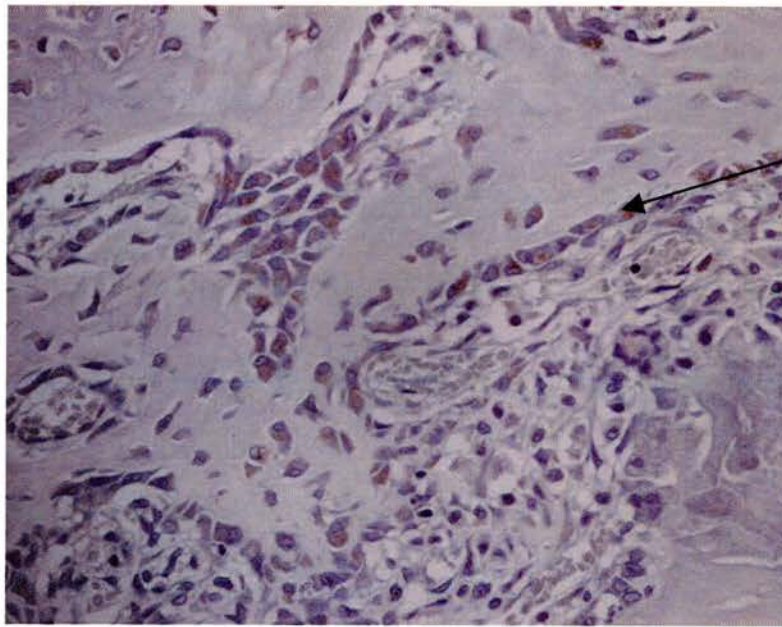
3.3.4.3 Smad-1 expression

The expression of Smad-1 in osteoblasts at the fracture site was similar to the levels of expression of BMP-2 with between 25 to 50% of cells staining positively. No

differences between any of the groups existed in the proportion of osteoblasts expressing Smad-1 at two or four weeks (Figure 3.3.4.2).

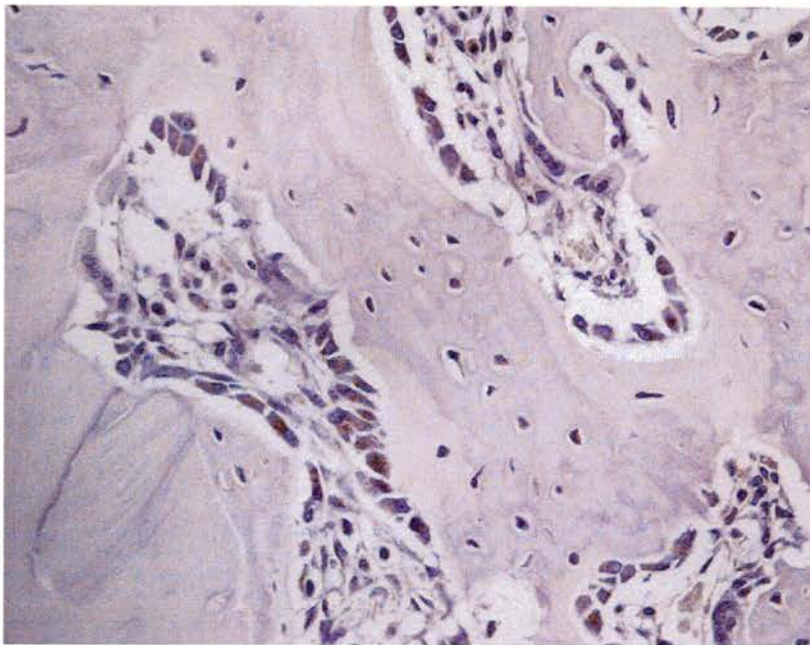
However in the chondrocytes present in the fracture callus two weeks following fracture the level of expression of Smad-1 varied. In ovx animals an increased proportion of chondrocytes stained positively for Smad-1 in the simvastatin group compared with placebo (see Table 3.3.4.4 and Figure 3.3.4.3). A trend towards a similar result was noted in the sham animals when comparing those fed simvastatin with placebo but this did not achieve statistical significance ($P=0.0758$).

Figure 3.3.4.1 BMP-2 staining in osteoblasts 4 weeks post fracture



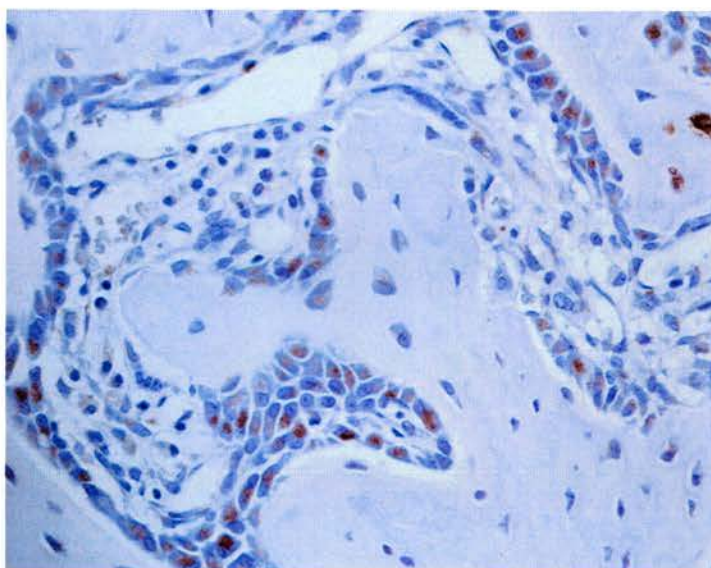
Positive brown cytoplasmic staining

Group A1 (sham & placebo), light x 400

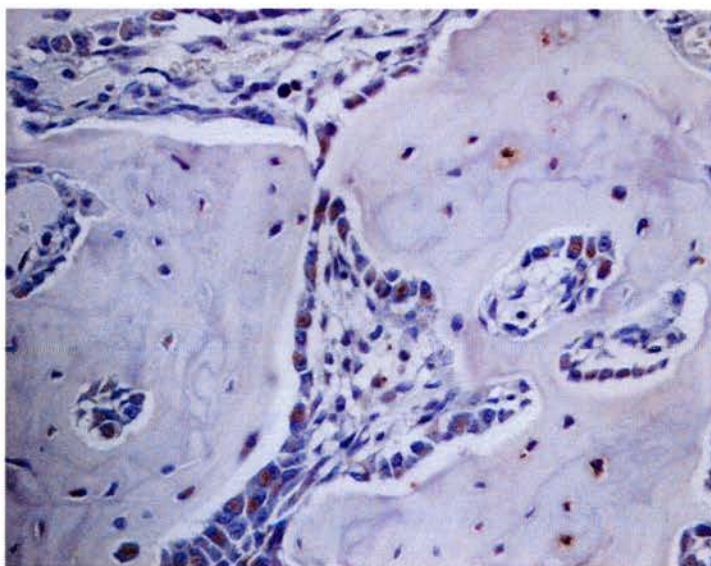


Group A3 (sham & simvastatin), light x 400

Figure 3.3.4.2 Smad1 staining in osteoblasts four weeks post fracture

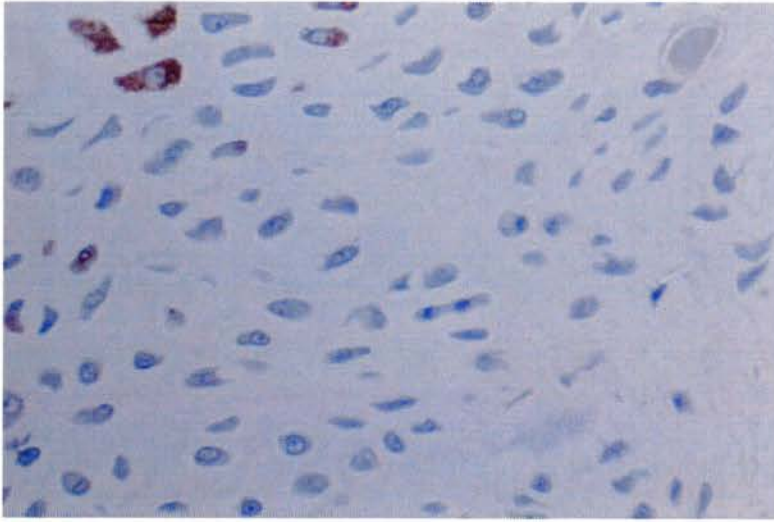


Group A2 (ovx & placebo), light x 400

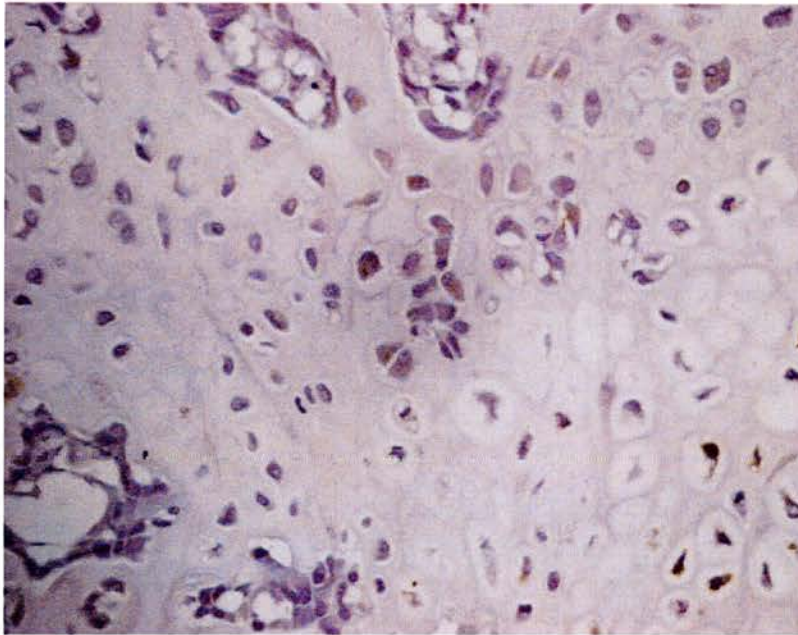


Group A4 (ovx & simvastatin), light x 400

Figure 3.3.4.3 Smad1 staining in chondrocytes two weeks post fracture



Fracture site chondrocytes from group B2 (ovx & placebo), Light x 400



Fracture site chondrocytes from group B4 (ovx & Simvastatin), light x 400

Table 3.3.4.1 Summary of immunohistochemistry results in fracture osteoblasts of sham animals (simvastatin versus placebo)

(results expressed as median score for positive staining index, described in section 2.5.7)

OSTEOBLASTS	Assay	<u>Sham & Placebo</u> Median (95%CI)	<u>Sham & Simvastatin</u> Median (95%CI)	P value (Mann Whitney U Test)
2 weeks Post Fracture	BMP 2	1 (-2.462, 5.128)	3 (2.509, 7.891)	0.173
	BMP 6	3 (-2.303, 6.303)	6 (2.980, 7.82)	0.143
	Smad 1	3 (-2.303, 6.303)	3 (1.76, 5.84)	0.363
	BRDU	4 (1.516, 6.484)	3 (1.840, 4.560)	0.364
4 weeks Post Fracture	BMP 2	0 (-3.303, 5.303)	4 (-0.1753, 9.675)	0.156
	BMP 6	3 (-0.535, 5.202)	4.5 (0.970, 7.530)	0.373
	Smad 1	3 (-2.303, 6.303)	3 (1.76, 5.84)	0.990
	BRDU	3 (0.535, 5.202)	1 (-0.837, 2.837)	0.229

Table 3.3.4.2 Summary of immunohistochemistry results in fracture chondrocytes of sham animals (simvastatin versus placebo)

(results expressed as median score for positive staining index, described in section 2.5.7)

CHONDROCYTES	Assay	Sham & Placebo Median (95%CI)	Sham & Simvastatin Median (95%CI)	P Value (Mann Whitney U Test)
2 weeks Post Fracture	BMP 2	3 (0.798, 6.535)	3 (1.517, 5.283)	0.759
	BMP 6	3 (1.232, 4.101)	3 (0.086, 5.491)	0.879
	Smad 1	0 (-2.202, 3.535)	4 (1.364, 6.137)	0.076
	BRDU	3 (1.232, 4.101)	3 (3, 3)	NS

Table 3.3.4.3 Summary of immunohistochemistry results in fracture osteoblasts of ovx animals (simvastatin versus placebo)

(results expressed as median score for positive staining index, described in section 2.5.7)

OSTEOBLASTS	Assay	Ovx & Placebo Median (95%CI)	Ovx & Simvastatin Median (95%CI)	P Value (Mann Whitney U Test)
2 weeks Post Fracture	BMP 2	3 (0.744, 6.256)	5 (1.836, 7.764)	0.524
	BMP 6	4 (1.095, 6.905)	6 (2.484, 8.716)	0.387
	Smad 1	2.5 (-0.25, 4.25)	5 (2.717, 6.483)	0.063
	BRDU	4 (1.864, 6.637)	3 (1.761, 3.839)	0.173
4 weeks Post Fracture	BMP 2	2 (-2.828, 3.483)	5 (1.836, 7.764)	0.344
	BMP 6	3 (1.16, 5.24)	5 (1.836, 7.764)	0.344
	Smad 1	3 (-0.239, 3.84)	3 (0.583, 3.819)	0.915
	BRDU	0 (-0.839, 3.24)	0 (-0.56, 2.16)	0.748

Table 3.3.4.4 Summary of immunohistochemistry results in fracture chondrocytes of ovx animals given simvastatin versus placebo.

(results expressed as median score for positive staining index, described in section 2.5.7)

CHONDROCYTES	Assay	Ovx & Placebo Median (95%CI)	Ovx & Simvastatin Median (95%CI)	P Value (Mann Whitney U Test)
2 weeks Post Fracture	BMP 2	4.5 (1.744, 7.255)	3 (1.934, 5.266)	0.524
	BMP 6	2 (0.701, 3.299)	3 (2.29, 4.51)	0.062
	Smad 1	2 (0.44, 3.16)	6 (4.517, 8.283)	0.008
	BRDU	2.5 (1.581, 3.419)	3 (3, 3)	NS

3.3.5 Results of mechanical testing

3.3.5.1 Simvastatin versus placebo in sham animals

The following data have been derived from the load / displacement curves generated by testing the healing fractures in four-point bending to failure. The method is described in Section 2.9. The intrinsic properties of stress, strain, Young's modulus and energy to yield have all been calculated from the formulae described in Section 2.9.5.

The data is summarised in Table 3.3.5.1.1 for the healing fractures two weeks post creation of fracture (groups B1 and 3) and Table 3.3.5.1.2 for those at four weeks post creation of fracture (groups A1 and 3).

Two specimens from group B1 were excluded due to loss of position during initial loading. Unfortunately one specimen from group A3 had to be excluded for the same reason making a total of 15% exclusion of samples in this section.

3.3.5.1.1 Load at yield and failure

The loads applied at both the points of yield and failure were lower in the simvastatin group compared with placebo. However the differences were small and did not achieve statistical significance. At four weeks post fracture the loads applied at the points of yield and failure were the same. These results show that simvastatin had no effect on

ability of the healing fractures in sham animals to withstand load at two or four weeks post fracture.

3.3.5.1.2 Stiffness

While the mean stiffness of the healing fractures appeared slightly greater in the simvastatin group the difference was not statistically significant at either two or four weeks post fracture.

3.3.5.1.3 Callus area and moment of inertia

The cross sectional area of the calluses did not differ between simvastatin or placebo groups at either two or four weeks. The calculated cross sectional moment of inertia was also the same in each group and time point.

3.3.5.1.4 Intrinsic mechanical properties of the healing fractures

Although the stress and strain at yield two weeks following fracture appeared to be lower in the simvastatin group, no statistical difference was demonstrated. Analysis was however limited by the small sample size in group B1. The sample size necessitated comparison of the groups using non-parametric testing which increases the likelihood of a type two error. Table 3.3.5.1.1 shows that the 95% confidence intervals of the mean stress at yield do not overlap. This suggests a possible effect of simvastatin reducing the mean stress at the point of yield of healing fractures in the sham operated groups.

There was no difference between simvastatin or placebo groups in mean stress or strain at yield by four weeks after fracture.

The moduli of the healing fractures were equal by four weeks post fracture.

The simvastatin group absorbed 43% of the mean energy before yield of the placebo group. This difference had however disappeared by the time the healing fractures were four weeks old.

Table 3.3.5.1.1 Summary of mechanical testing results for sham groups fed simvastatin 20mg/kg versus placebo. Data at two weeks post fracture

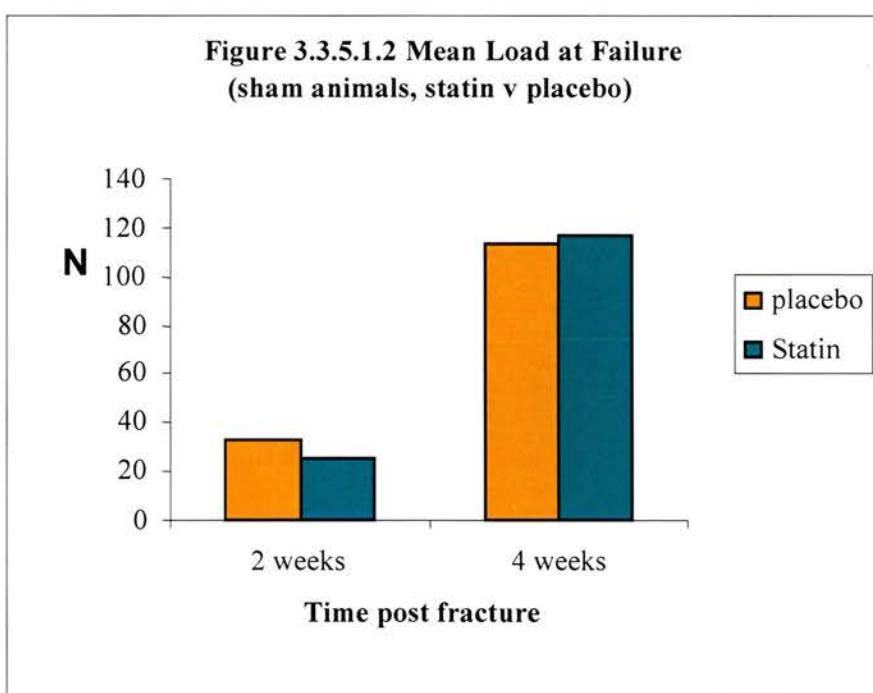
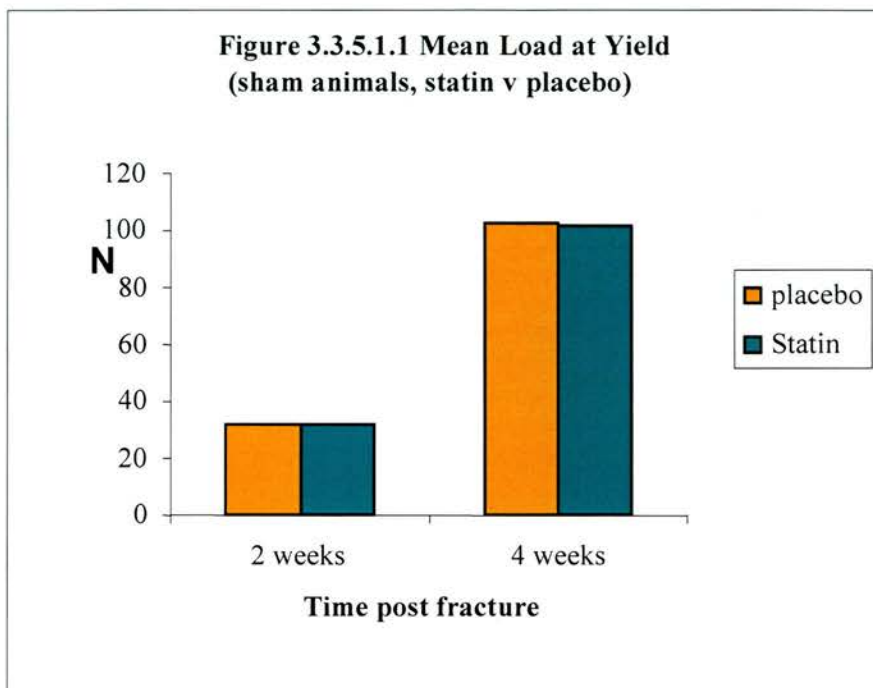
- Statistical comparison made using Mann Whitney Test

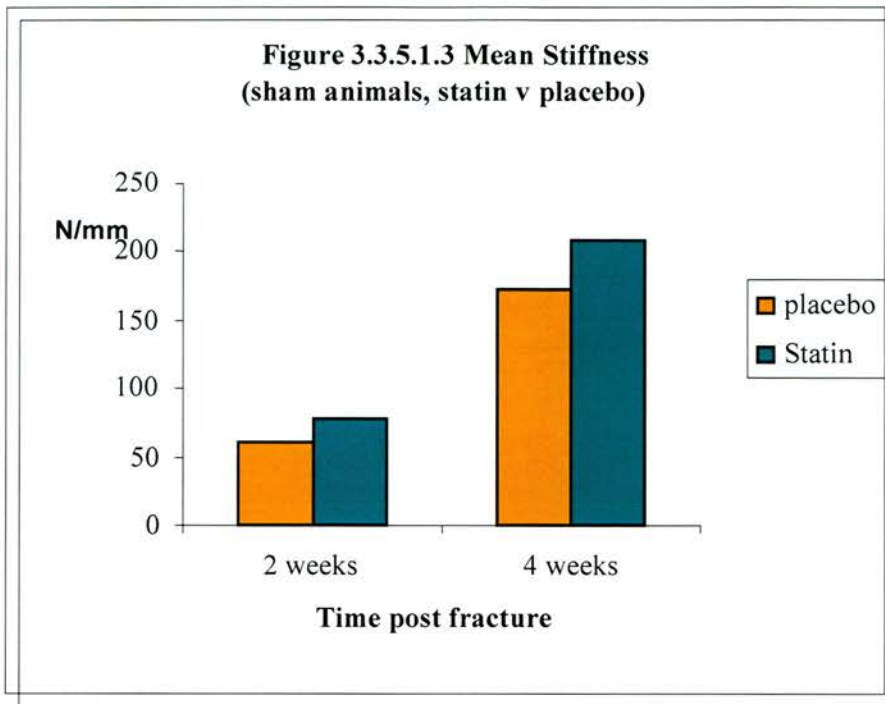
	Sham & placebo (B1) n=3			Sham & simvastatin (B3) n=5			P value
	mean	SEM	95% CI	mean	SEM	95% CI	
Callus area (mm ²)	30.06	2.03	24.93, 35.19	24.25	1.40	21.5, 27.0	0.143
Stiffness (N/mm)	60.81	24.6	12.64, 108.9	76.67	9.62	57.82, 95.52	0.905
Load at yield (N)	32.0	3.51	25.12, 38.88	25.6	3.93	17.87, 33.33	0.571
Load at failure (N)	33.22	4.86	23.63, 42.81	26.64	4.045	18.72, 34.56	0.071
Moment of Inertia (mm ⁴)	87.57	14.91	58.34, 116.8	72.86	14.54	44.37, 101.35	0.571
Stress at yield (MPa)	16.75	1.184	14.43, 19.07	10.88	1.659	7.63, 14.13	0.393
Strain at yield	31.12	5.12	21.09, 41.15	19.15	3.081	13.11, 25.19	0.071
Young's Modulus (GPa)	0.12	0.01	0.096, 0.144	0.11	0.02	0.066, 0.154	0.786
Energy to yield (MJ/m ³)	2.572	0.356	1.698, 3.446	1.107	0.283	0.551, 1.663	0.036

Table 3.3.5.1.2 Summary of mechanical testing results for sham groups fed simvastatin 20mg/kg versus placebo. Data at four weeks post fracture

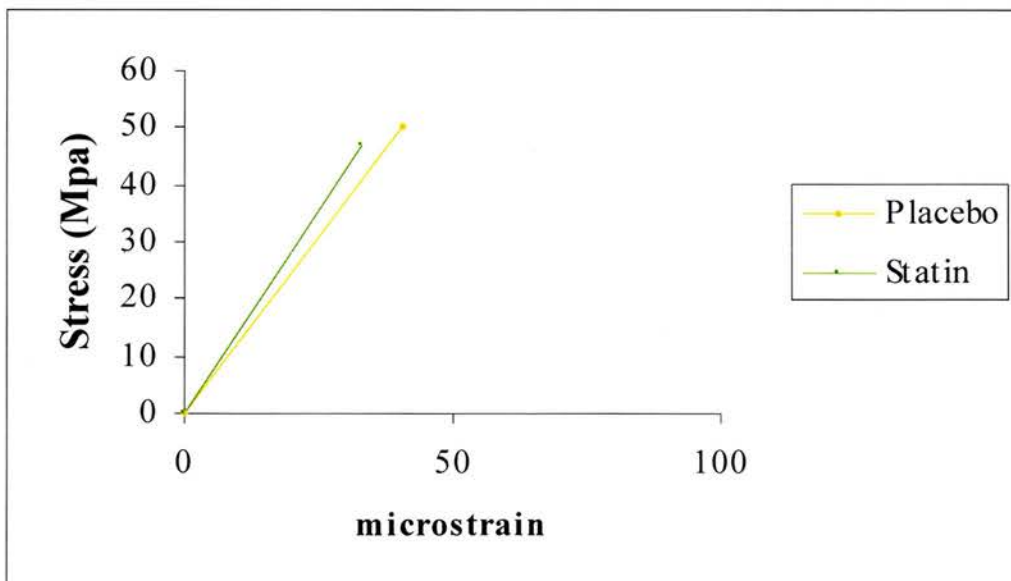
	Sham & placebo (A1) n=5			Sham & simvastatin (A3) n=4			P value*
	mean	SEM	95% CI	mean	SEM	95% CI	
Callus area (mm ²)	27.38	2.15	22.68, 32.08	23.55	3.06	17.85, 29.25	0.413
Stiffness (N/mm)	172.17	11.66	146.61, 197.7	208.64	30.96	147.97, 269.37	0.459
Load at yield (N)	103.6	7.35	87.41, 119.79	102	11.49	79.47, 124.53	0.730
Load at failure (N)	114.75	3.25	108.38, 121.12	116.75	14.63	88.06, 145.44	0.990
Moment of Inertia (mm ⁴)	78.48	11.58	55.1, 102.22	58.07	11.5	35.29, 80.85	0.556
Stress at yield (MPa)	50.46	4.32	41.94, 58.86	47.66	9.13	29.25, 65.57	0.556
Strain at yield	40.83	4.76	31.49, 50.17	33.01	3.29	26.56, 39.46	0.343
Young's Modulus (GPa)	0.22	0.045	0.124, 0.316	0.23	0.065	0.1, 0.36	0.990
Energy to yield (MJ/m ³)	10.056	1.8594	5.981, 14.13	8.248	2.357	3.629, 12.86	0.343

• * Statistical comparison made using Mann Whitney Test. Too few values in A3 to ensure Gaussian distribution.



**Figure 3.3.5.1.4**

Linear portion of stress / strain curve of healing fractures at four weeks post fracture in sham animals (simvastatin v placebo)



3.3.5.2 Results of simvastatin versus placebo in ovariectomised animals

The following data has been derived from the load / displacement curves generated by testing the healing fractures in four-point bending to failure. The method is described in Section 2.9. The intrinsic properties of stress, strain, Young's modulus and energy to yield have all been calculated from the formulae described in Section 2.9.5.

The data is summarised in Table 3.3.5.2.1 for the healing fractures two weeks post creation of fracture (groups B2 and B4) and Table 3.3.5.2.1 for those at four weeks post creation of fracture (groups A2 and A4).

As mentioned previously, interpretation of results in this section was hindered by the necessary exclusion of specimens due to loss of position on the mechanical testing apparatus. For this reason, two specimens were excluded from group B2 and one from group B4.

3.3.5.2.1 Load at yield and failure

The values for mean load at yield and failure were lower at the two week time point in the ovx group fed simvastatin compared with placebo. Wide confidence intervals however reflect large standard deviations for these mean values and no statistical difference was seen between the groups at two weeks following creation of fracture. By four weeks post fracture creation the mean load at failure was significantly lower in the simvastatin group compared with placebo (Figure 3.3.5.2.2). The mean load at the point

of yield was also lower in the simvastatin group at this time point but the difference did not achieve statistical significance.

3.3.5.2.2 Stiffness

No significant difference was seen in the mean stiffness of the healing fractures at either two or four weeks following fracture. Mean values were however, again reduced in the simvastatin groups compared with placebo at four weeks post fracture (Figure 3.3.5.2.2).

3.3.5.2.3 Callus area and cross sectional moment of inertia (CSMI)

At two weeks following fracture there were no differences in the mean callus area or CSMI between the simvastatin or placebo groups. By four weeks however, the callus size and CSMI were both significantly smaller in the simvastatin fed group. The mean callus size in the simvastatin group was only 57% that of the placebo fed animals.

3.3.5.2.4 Intrinsic mechanical properties of the healing fractures

Interpretation of the intrinsic mechanical data from the two week time point is hindered by the small sample sizes in groups B2 and B4. No clear differences were seen between the groups for mean stress, strain, energy to yield or Young's modulus.

By four weeks following fracture significant differences between the groups were seen. Both stress and strain at the point of yield were reduced by 43% and 30% respectively in the simvastatin group. This was reflected in a 60% reduction in the energy absorbed prior to yield in the simvastatin fed group compared with placebo. This can be seen by

the different areas under the lines in Figure 3.3.5.2.4 where the mean stress and strain at yield have been plotted for values four weeks post fracture. Figure 3.3.5.2.4 does not however show a clear difference in the slope of the lines yet Young's modulus calculated by the method described in section 2.9 differed significantly between the simvastatin and placebo groups. The mean modulus of the healing fractures prior to yield in the simvastatin group was only 26% of the mean modulus in the placebo group.

Feeding with simvastatin at 20mg/kg for two weeks following fracture appeared to impair significantly the mechanical properties of the healing fractures in the ovx groups.

Table 3.3.5.2.1 Summary of mechanical testing results for ovx groups fed simvastatin 20mg/kg versus placebo. Data at two weeks post fracture

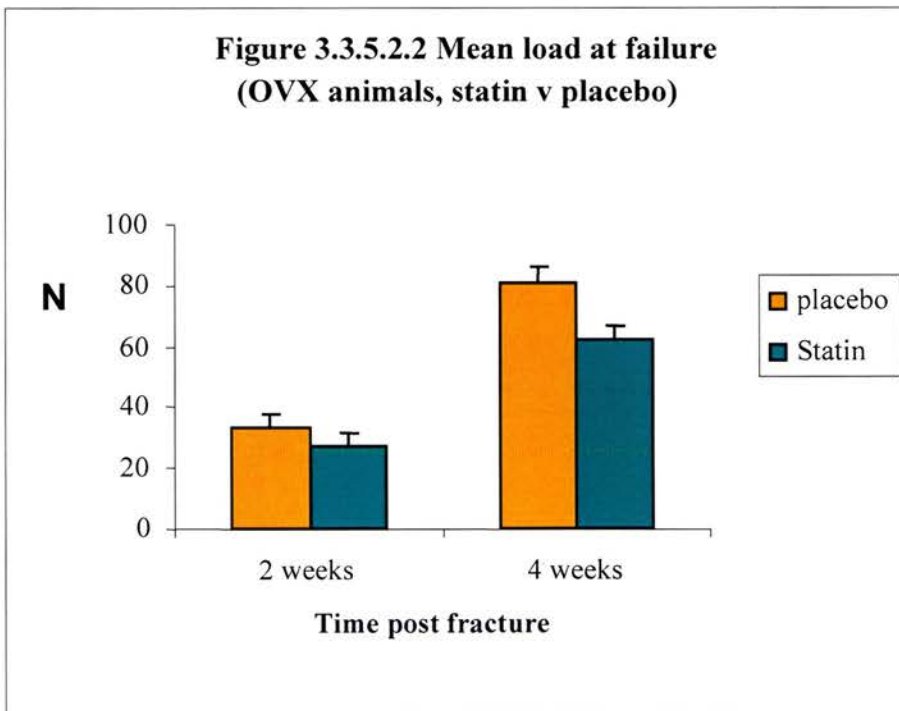
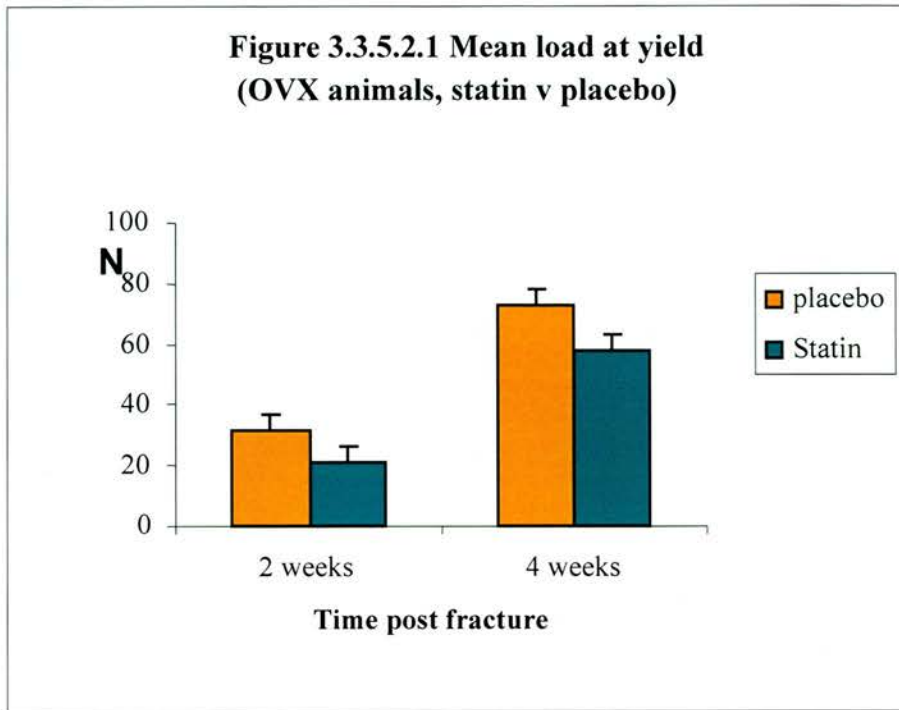
	Ovx & placebo (B2) n=3			Ovx & Simvastatin (B4) n=4			P value *
	mean	SEM	95% CI	mean	SEM	95% CI	
Callus area (mm ²)	30.49	5.65	19.42, 41.56	27.97	2.93	22.23, 33.71	ns
Stiffness (N/mm)	76.82	39.64	0, 154.52	36.89	4.68	27.72, 46.06	ns
Load at yield (N)	32.0	8.26	11.1, 52.9	21.5	2.595	16.41, 26.59	ns
Load at failure (N)	33.4	11.37	11.11, 55.69	26.79	1.723	23.42, 30.16	ns
Moment of Inertia (mm ⁴)	98.09	36.08	27.36, 168.82	84.27	23.06	39.07, 129.46	ns
Stress at yield (MPa)	16.34	5.37	5.84, 26.84	10.72	1.58	7.62, 13.82	ns
Strain at yield	23.22	9.47	4.72, 41.72	47.07	3.89	39.46, 54.68	ns
Young's Modulus (GPa)	0.12	0.035	0.051, 0.189	0.05	0.02	0.006, 0.09	ns
Energy to yield (MJ/m ³)	2.377	0.344	1.497, 3.243	2.482	0.3062	1.882, 3.082	ns

• * insufficient numbers to allow statistical comparison

Table 3.3.5.2.2 Summary of mechanical testing results for ovx groups fed simvastatin 20mg/kg versus placebo. Data at four weeks post fracture

	Ovx & placebo (A2) n=5			Ovx & Simvastatin (A4) n=5			P value
	mean	SEM	95% CI	mean	SEM	95% CI	
Callus area (mm ²)	27.95	2.46	23.13, 32.77	15.9	1.09	13.76, 18.04	0.002
Stiffness (N/mm)	93.25	6.38	80.75, 105.75	77.74	6.15	65.68, 89.8	0.119
Load at yield (N)	73.4	3.90	65.75, 81.05	58.8	6.56	45.94, 71.66	0.092
Load at failure (N)	81.4	2.67	76.16, 86.64	62.0	5.91	50.42, 73.58	0.017
Moment of Inertia (mm ⁴)	81.87	16.41	49.7, 114.03	25.76	3.88	18.16, 33.4	0.010
Stress at yield (MPa)	37.26	3.48	30.43, 44.09	21.82	1.89	18.12, 25.52	0.005
Strain at yield	56.74	2.91	51.03, 62.45	39.3	4.59	30.31, 48.29	0.012
Young's Modulus (GPa)	0.15	0.036	0.076, 0.224	0.04	0.004	0.033, 0.047	0.020
Energy to yield (MJ/m ³)	10.599	1.183	8.28, 12.917	4.224	0.566	3.114, 5.335	0.002

- Statistical comparison made using two tailed, unpaired t-test. Data tested for normality using Kolmogorov-Smirnoff test



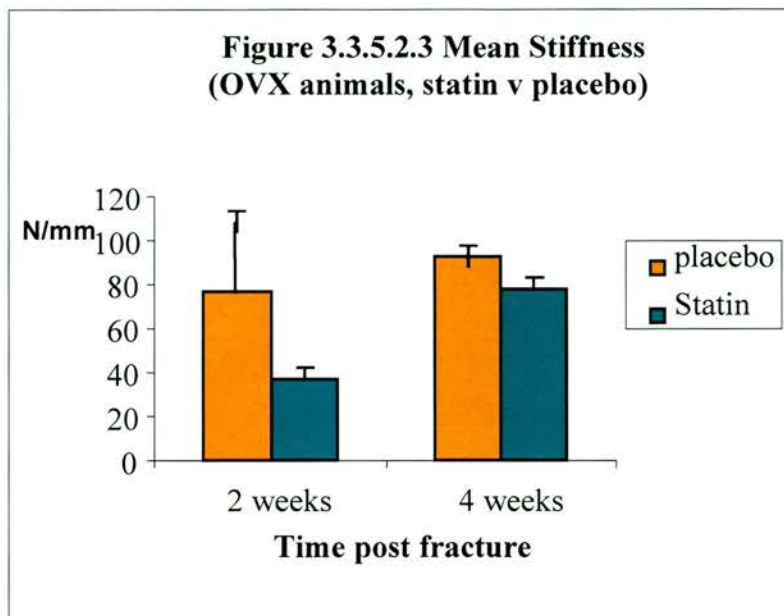
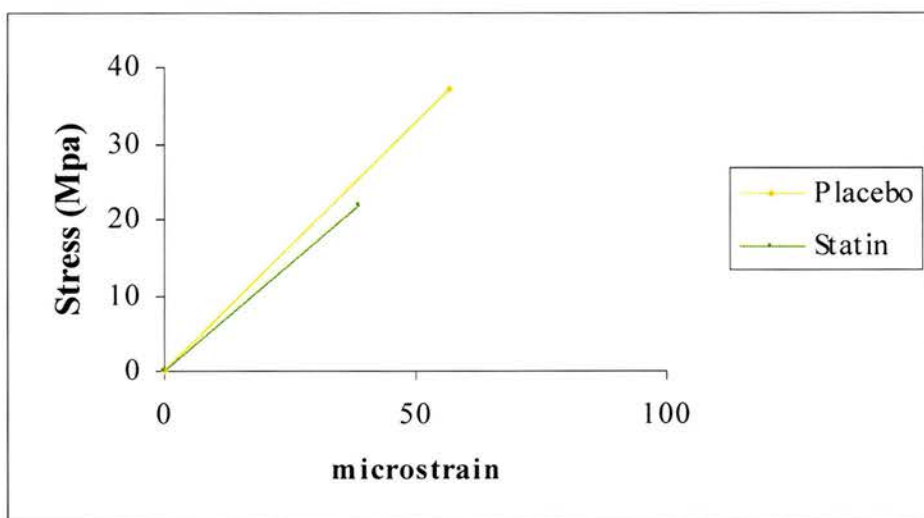


Figure 3.3.5.2.4 Linear portion of stress / strain curve of healing fractures four weeks post fracture in OVX animals (statin v placebo)



Discussion

4 Discussion

Fragility fractures in humans are becoming increasingly common and will present an ever increasing burden on health care for the foreseeable future. Advances in the current limited understanding of the process of fracture healing in osteoporosis could have significant benefits and translate into improved management of this large patient group. There is no doubt that prevention is better than cure and attention is deservedly directed towards pharmaceutical and lifestyle interventions to reduce the incidence of fragility fractures. The process of bone healing is however integral to the development of fragility fractures as it is the altered bone turnover and resulting development of microfracture that ultimately facilitates clinical fracture in these individuals. Additionally, even with the best currently available preventative measures, the incidence of fragility fracture remains high and advances in treatment should continue to be sought.

The primary objective of this study was to compare the early stage of fracture healing in osteoporotic bone versus non-osteoporotic bone and establish if clinically relevant differences existed. Processes believed integral to fracture healing were also investigated in an attempt to elicit possible mechanisms for any differences demonstrated.

The exciting early results of Mundy *et al.*⁶⁵ and Skoglund *et al.*¹⁸³ showing a potentially beneficial effect of simvastatin supplementation on bone formation created

the prospect of a direct role for this simple pharmacological intervention in fracture healing. These results were however controversial and had not been applied to the setting of fracture healing in osteoporosis. The second part of this study was therefore designed to investigate further the role of simvastatin in fracture healing and its application in osteoporotic fracture.

4.1 The fracture model

The most appropriate model to study is the human but as with many studies impracticalities existed in attempting to use humans for this investigation. Obtaining tissue from healing human fractures which required no operative intervention was ethically questionable. Controlling for factors such as comorbidity, age, sex and fracture configuration would have required recruitment of very large numbers and mechanical outcome measures are not yet well defined in the field of human fracture repair. Attempts should continue to be made however to study the human wherever possible and certainly analysis of human systemic markers, bone and callus will provide us with the most relevant information in the future.

The rat model overcomes many of the problems mentioned above which is why it is a commonly used and accepted model for the study of fracture repair. As with all animal models however, some limitations had to be accepted. Perhaps the most important consideration was the difference in skeletal maturity between the three-month old rat and the mainly elderly human population to whom these data would be applied. Rats do

not reach skeletal maturity and their growth plates remain open. Longitudinal bone growth therefore continues and the additional impact of senescence occurring in osteoporotic humans²³⁵ was not reproduced by the rat model used in this study. An attempt to minimise the impact of this was made by using rats beyond adolescence but an effect from the continued growth stimulus on fracture healing in the model must be considered when extrapolating from this data. Similarly, the local fracture environment in the model differed from that of the common osteoporotic fractures in humans. In elderly humans, reduced vascularity and poor soft tissues are often a feature which was not the case in the model. Additionally the creation of an acceptable, standard fracture in the model required an open technique while most human osteoporotic fractures are closed.

A strength of this study was the use of a cancellous fracture model. Most fragility fractures in humans occur in bone metaphyses and the applicability of the more commonly studied mid-diaphyseal models is questionable. Creation of a standard, transverse fracture in the proximal tibia proved more difficult than creating a mid-diaphyseal fracture but careful development of the technique resulted in few exclusions. This fracture model appears to be entirely novel in the investigation of fracture healing in osteopenia, yet it showed similar degrees of impairment of fracture healing in the osteopenic animals when compared with diaphyseal models. One of the major drawbacks of the use of the rat for studying fracture healing is that osteonal healing does not occur. This would certainly influence cortical healing but may be of lesser importance in a cancellous bone fracture such as the one used here.

The use of intramedullary fixation did not confer absolute stability to the fracture but as rigid fixation is not commonly used in the operative treatment of osteoporotic fracture, this was felt to be a suitable and practical technique for maintaining the consistent fracture alignment required for meaningful mechanical testing. While a closed fracture with no operative fixation would be a purer model for the study of fracture repair the inconsistency of fracture configuration and position at union meant that this model could not be used.

4.2 The ovariectomised rat as a model for osteoporosis

Many investigators have used the castrated female rat for studying the effects of the anoestrous environment. It has been widely accepted as an appropriate model for the study of “osteoporotic” bone with pronounced decreases in bone mineral density seen a few weeks after ovariectomy. Caution must be exercised however, when using this model. The timing of the index intervention following ovariectomy is crucial and adequate time must be allowed to elapse to ensure a steady state of bone turnover has occurred. Intervention too early would introduce the potentially confounding effects of inadequate loss of bone mineral density against the background of the rapid bone resorption which occurs in the first four weeks post ovariectomy⁷⁵. Allowing seven weeks to elapse prior to fracture creation in this study was considered adequate.

The degree of osteopenia induced by ovariectomy is difficult to compare with the human situation and a definition for osteoporosis in the rat does not exist. Measurement of bone mineral density varies depending on the method used and no population data is available for the rat making it impossible to derive a T-score in this species. It is therefore more accurate to refer to the model as osteopenic rather than osteoporotic. It is also important to note that spontaneous fractures do not occur in the ovariectomised rat as they do in the osteoporotic human ²⁰⁴. There is however no doubt that the ovariectomised rat does provide a useful model to study fracture repair in the anoestrous environment with the effects of oestrogen withdrawal on bone and the reproductive tract being confirmed in this study.

The use of the sham operated control is of great importance. The effect of stress on oestrogen levels can be pronounced and unpredictable and the stress of surgery, transport and environment must be controlled for. Difficulty exists in controlling for the weight gain which results following ovariectomy due to accumulation of adipose tissue ²¹³. In this study the decision not to pair feed resulted in ovariectomised animals being approximately 20% heavier than control. This will have resulted in differential loading of the healing fractures which was not controlled. Pair feeding however results in animals being in a constant state of hunger which translates into stress and a resulting hormonal disturbance. This would have had unpredictable results and pair feeding was therefore not employed as it has not been in many other similar studies.

4.3 Fracture healing in the ovariectomy model versus control

This study provided clear evidence for a deleterious effect on early fracture healing of the osteopenic / anoestrous environment. The substantial reduction in ability to withstand load to the point of yield and failure in the fractures from osteopenic bone compared with control complies with the previous findings of Walsh *et al.*²¹⁰ and Namkung *et al.*²¹¹. These investigators used very similar methods with the exception that their models were of femoral, mid-diaphyseal fractures. This implies that the cause of the poorer mechanical properties of osteopenic fracture callus is a mechanism common to diaphyseal and metaphyseal healing. Deficiencies therefore seem less likely to be primarily related to altered vascularity or angiogenesis as cancellous bone is known to benefit from a significantly more abundant blood supply than cortical bone.

The fact that both cortical and cancellous bone healing are affected implicates the process of osteogenesis as the main factor influenced by an osteopenic environment as it is the major process common to the healing of both types of bone. In the diaphyseal fracture model of Bonnerans and Einhorn¹⁹² endochondrial ossification predominates initially whereas in the metaphyseal model a greater proportion of intramembranous ossification should be expected where fracture fragment contact occurs. It is possible that osteopenia affects both processes but the step common to both is the production and maturation of calcified material and this should be the focus of further work in this field.

Histologically there was a higher proportion of woven bone to trabecular bone in the early stages of healing with a subsequent increased porosity in the osteopenic fracture callus at four weeks. This suggests a decreased maturity which might translate into decreased ability to withstand load, particularly in compression. The hypothesis that the poorer mechanical properties are related to reduced ability of osteopenic bone to withstand compression would not explain the results of Walsh *et al.*²¹⁰ who tested fractures to failure in tension and still found differences. Additionally, while bending creates compressive forces, the common mechanism of beam failure in bending is firstly through failure on the tension side. Tension is primarily resisted by collagen and collagen production is certainly influenced by the presence of oestrogen²³⁶. Whether the absence of oestrogen results in poorer collagen production is not clear and would benefit from further investigation.

The effect of ovariectomy on callus size is controversial. This study showed no difference in callus size between control and ovariectomy animals at either time point. Some investigators have reported similar findings^{210,214}. Others have seen a decrease in callus size in ovariectomy models^{211,212} and Cao *et al.*²¹³ reported an increase in callus size in their ovariectomy model six weeks following fracture compared with control. This study did show histological evidence of reduced callus maturity with higher levels of immature bone and porosity compared with control. Taken together these findings raise the interesting possibility of an initial delay in callus formation in osteopenia with a subsequent increase in size to exceed that of control later in fracture healing. This may be related to the poorer intrinsic mechanical properties of the osteopenic callus during

the early phase in healing leading to continued mechanical stimulus for callus formation. The increased callus size would then appear to compensate for mechanical deficiencies by increasing the moment of inertia. Work by Walsh *et al.*²¹⁰ and Cao *et al.*²¹³ supports this hypothesis and shows that mechanical differences between osteopenic and normal bone have been abolished by six weeks post fracture.

Overall a picture of decreased callus maturity emerges in the osteopenic model. As mentioned earlier this appears to reflect a failure of the normal processes of tissue formation with evidence suggesting that the effect is independent of the better known pro-resorptive effect of osteopenia. Flick *et al.*²³⁷ showed that complete blockade of RANK, a key activator of osteoclasts, had no effect on callus formation or early fracture healing²³⁷. This means that attempts to influence fracture healing in osteopenia with anti-osteoclast cytokines such as osteoprotegerin would be unsuccessful. In other words, more benefit is likely to be derived from efforts to promote anabolic processes in early osteopenic fracture repair and this is borne out by the exciting results reported with recombinant PTH (teriparatide) when it is given in the early stages after fracture²³⁸. Komatsubara *et al.*²³⁹ showed that teriparatide promoted the early replacement of woven bone to lamellar bone which would appear to address one of the main deficiencies seen in the maturation of the calluses in this study. The absence of any effect of RANK blockade on fracture healing would also make it unlikely that the deficient bone healing in osteopenia is due to the upregulation of RANK seen in the anoestrous state. This hypothesis is supported by the findings of Ulrich-Vinther *et al.*²⁴⁰ who found that gene therapy with adeno-associated virus transformed to induce production of osteoprotegerin

actually hindered fracture repair in a rat model. A reduction in the number of osteoclasts was seen but it was associated with a reduction in the production of woven bone and a reduced elastic modulus of the callus. This work implies that functioning osteoclasts are required for normal maturation of the callus.

The growth factors investigated in this study did not appear to have a clear role in the differential behaviour of the osteopenic callus versus control. Differences in osteoblast or chondrocyte proliferation were not detected at either time point studied. An important role for these factors cannot be excluded by this work and it can only be stated that no difference was seen at the time points studied. Given the evidence for early callus differences, the study of the very early stages of fracture healing might prove fruitful. In particular, the early expression of growth factors and angiogenesis would be of interest. Links between vascular endothelial growth factor (VEGF), oestrogen and bone mineral density have been established^{241,242} and as angiogenesis is integral to early fracture healing, this area merits further work. There is, of course, a vast array of growth factors now identified and in some way implicated in the process of fracture repair but in addition to vegf, IL1 and 6 and TGF- β are likely to be important. It was impossible to study all of the potentially relevant factors in this study and those studied were chosen on the currently best available evidence of a likely key role in osteoporotic fracture healing.

4.4 The influence of simvastatin on fracture healing

The effect of statins on bone mineral density remains controversial. Their inhibitory effect on protein prenylation should result in an anti-osteoclastic action as this is also one of the mechanisms of action of the anti-resorptive nitrogen containing bisphosphonates. Mundy *et al.*⁶⁵ report of a bone formation effect via BMP-2 promotion together with the anti-resorptive effect invited investigation of the role of statin supplementation in fracture healing. Other than this study, only Skoglund *et al.*¹⁸³ has conducted a study on statin supplementation in fracture healing using a murine model with normal bone mineral density. They showed a dramatic and apparently beneficial effect on fracture healing using a very high dose of simvastatin. Unfortunately the dose used would not be applicable in human practice due to toxicity but their findings were nevertheless encouraging.

In this study, the dose of simvastatin used was the same as that used by Mundy *et al.*⁶⁵ and Maritz *et al.*⁷² in their work reporting contradictory, but definite, effects on bone turnover. This dose was the equivalent of 400mg per day for an adult human which represents five times the usual dose for treatment hypercholesterolaemia but would still be tolerable. It was hypothesised that two weeks of 20mg/kg orally administered simvastatin during the initial stage of fracture healing would be the most likely regime to demonstrate any effect. It is during this stage that BMP-2 production is maximal and promotion of BMP-2 production has been proposed as the anabolic mechanism of action for simvastatin.

In the sham operated animals no effect on the mechanical features of the healing fractures was seen at either two or four weeks post fracture in the statin group versus placebo. There was also no apparent effect on the expression of the growth factors investigated. The lack of any effect raised the possibility that the simvastatin was not reaching the bone in an active form but this is contradicted by several factors. Firstly an increase in the proportion of woven bone in the statin group calluses was seen at four weeks suggesting an effect on callus maturity. Secondly, effects of simvastatin administration were seen in the ovariectomised animals. Finally, the technique of drug administration and the rat model were the same as those used by previous workers who have reported drug effects. It is technically possible to assay bone for inhibition of protein prenylation and hence confirm a skeletal effect of simvastatin administration. This was not however, included in the method of this study as, for the above reasons, it was not felt to be necessary.

The results from the present study therefore show that simvastatin at 20mg/kg daily for two weeks post fracture results in an increased level of immature bone in the sham model at four weeks post fracture but that this does not have any significant mechanical consequence. Further, no evidence was found for altered growth factor expression at the times studied.

It is only possible to speculate whether a different dose or timetable of administration would have resulted in different findings. The dose of simvastatin does seem to be critical according to the findings of Maritz *et al.*⁷². Their work showed that doses lower

than 20mg/kg given for six weeks resulted in a reduction in bone density due to an increased resorptive state. 20mg/kg increased both bone production and resorption with no net effect on bone mineral density. The same simvastatin dose (20mg/kg) did result in increased BMP-2 production and bone formation in the rat model of Mundy *et al.*⁶⁵ It is important to note, however, that their model of the ovariectomised rat was in a skeletally resorptive state whereas a fracture in the early stages is anabolic. It is possible that no effect was seen on BMP-2 or other growth factor expression in the fracture callus because it was already maximal. Skoglund *et al.*¹⁸³ did not investigate growth factor expression so it remains unknown if massive doses of simvastatin promote the already high levels of BMP-2 expression in the early stages of fracture healing. They did show, however, that while the callus was larger in the statin group, no histological difference existed compared with controls. If the primary mechanical effect of simvastatin is enlargement of callus then this effect may have been masked by the relatively smaller callus produced in the cancellous fracture model in this study. If the many differences which confound comparison between the study of Skoglund *et al.*¹⁸³ and this study are not considered it is reasonable to conclude that the dose of simvastatin is highly important in fracture healing. A very high but potentially toxic dose leads to a larger and stronger callus in normal bone but a lower, more therapeutically realistic dose, hinders callus maturity. There is an obvious need to investigate the effect of statin doses between 20 and 50mg/kg, above which toxicity has been reported (see Section 2.2.2.2).

Much clearer effects of simvastatin administration were seen in the osteopenic, ovariectomised group. Once again the fracture callus at four weeks had a higher

proportion of immature bone present compared with placebo but this was also related to a significantly smaller callus area. The smaller callus withstood less load than might be expected but the intrinsic properties of the callus in the drug group were also impaired relative to control. The mechanism for this deleterious action of simvastatin did not appear to be due to a pronounced effect on BMP-2 or 6 expression. An increased level of Smad1 expression in the chondrocytes two weeks post fracture in the simvastatin group did suggest a possible mechanism for the drug effect. The increased Smad1 levels may have reflected a promotional effect of simvastatin on chondrocyte activity which would fit with the increased levels of immature bone produced by endochondrial ossification. A prolongation of the endochondral phase would result in a more immature callus but does not account for the reduced size of the callus overall. Interestingly, Fukuyama *et al.*²⁴³ have shown that statins inhibit osteoblast migration *in vitro* which may well result in delayed osteogenesis in a situation such as in the osteopenic model where osteogenesis is already impaired. This effect appeared to be due to statins' inhibitory effect on protein geranylgeranylation, a down stream product of the HMGCoA pathway.

As has been noted already, callus in the osteopenic animals appeared to be more immature than in animals with normal bone density but simvastatin at the dose given appears to make the situation worse. If this was due to a purely anti-resorptive effect then immature bone would remain present but a larger callus would be expected. This was the effect shown by Cao *et al.*²¹³ on callus formation following administration of the anti-resorptive bisphosphonate alendronate in an osteopenic rat model. The anabolic simvastatin effect proposed by Mundy *et al.*⁷¹ would also be expected to result in a

larger callus albeit possibly of lesser maturity. It may be that while some promotion of endochondrial ossification did occur due to simvastatin in this study, the pro-resorptive anoestrous environment prevented formation of a larger callus. This would result in a callus with poorer mechanical properties as seen in this situation.

Another potentially confounding factor when using the ovx model for the study of statins is the link between the effect of statins on adipocytes and bone. It is well known, and shown in this study, that ovariectomy results in weight gain by production of adipose tissue. It is thought that adipose tissue and bone tissue production have an inverse relationship with increased adiposity of bone marrow being associated with reduced bone density²⁴⁴. The link is perhaps not surprising due to the common mesenchymal stem for both types of connective tissue. Li *et al.*²⁴⁵ reported that lovastatin enhanced osteoblast differentiation at the cost of suppressing fat cell promoter levels and hypothesised that the statin shunted uncommitted osteoprogenitor cells from the adipocytic to the osteoblastic pathways. In the post ovariectomy model it is possible that the pro-adipocytic drive is too influential to allow the dose of statin used to promote osteogenesis.

The peptide hormone leptin appears to be integrally involved with the regulation of adipose tissue and its link with bone density²⁴⁶. Leptin treatment has been shown to increase bone formation in leptin deficient mice²⁴⁴. It is thought though that leptin levels fall in the oestrogen deficient state encountered post ovariectomy or physiological menopause²⁴⁷. Although one study actually reported an increase in serum leptin levels

at day 20 post ovariectomy in the rat²⁴⁸. The role of leptin in the regulation of BMD is further confused by some workers reporting a positive association of leptin with BMD²⁴⁹ and others finding no correlation²⁵⁰. Leptin has also been shown to cause an increase in gene expression for vegf in other tissues²⁵¹ suggesting a pro-angiogenic role. What is clear is that leptin and, by implication adipose tissue, have an influence over bone production and regulation which is very likely to be affected by statin treatment. From the currently available evidence it is not possible to predict how statin therapy in the anoestrous environment would influence serum leptin levels. This may however, provide another explanation for the unexpected effects of statin supplementation in the ovx model in this study and would be an exciting area for further work.

The reason for the poorer mechanical properties of the osteopenic callus compared with control and the apparent worsening of the situation by administration of simvastatin at the dose used is not explained by the findings of this study. This reflects the complexity of the processes involved in fracture healing and the current lack of understanding of the process in relation to osteopenia. The view that simvastatin promoted BMP-2 expression and would therefore prove beneficial in fracture healing has not been supported by this work. Fracture healing is an intricate balance of formation and resorption with the timing of these processes critical to the integrity of the healing fracture. Attempts to manipulate fracture healing pharmacologically or genetically must take this into account.

4.5 Future work

Further attempts to elicit the cause for a difference in the mechanical properties of fracture healing in osteopenia and control are warranted. Establishing the patterns of expression and functional role for the host of growth factors not investigated in this study might lead to the discovery of an exploitable difference between these groups. Of particular interest are the pro-osteoclastic cytokines interleukin 1 and 6 and tumour necrosis factor and the angiogenic VEGF. Investigation of the initial stages of fracture repair may reveal important differences in the way that osteopenic bone responds to injury and initiates healing. Such information would be beneficial in making the use of evolving techniques such as gene therapy to enhance fracture healing.

Parathyroid hormone is emerging as a promising agent for the treatment of osteoporosis and enhancement of fracture healing. Investigation of its role in osteoporotic fracture healing is certainly merited and has already been undertaken by some investigators. PTH has the potential to fulfil the much needed role of a truly anabolic agent in fracture healing and osteoporosis. However, concerns about the oncogenic potential of PTH must be conclusively addressed for it to have a major future role in this area.

The future for statins in the field of fracture healing is not clear. Very little work has been done on their role in fracture healing with the only other study reporting contradictory results. As discussed above there are many potential reasons why

contradictory results are being reported in this field. This simply reflects the lack of understanding of the fracture healing process in osteopenic bone and the complexities of the links between different systems and metabolic pathways such as those between adipose tissue and bone. More work is required on the role of leptin in osteoporosis and its link with statins. This study has provided enough evidence to show that statins do influence fracture repair and there is therefore more to be gained by persevering with the study of statins in this field. In particular the timing, delivery and perhaps most importantly, the biologically available dose, seem likely to be critical factors. The introduction of newer more lipophilic statins should not be ignored as these are likely to have more of an effect on bone than earlier generation statins.

4.6 Conclusions

This study has shown that ovariectomy resulted in poorer mechanical properties of the healing tibial fracture at four weeks post fracture compared with control. This provides supportive evidence for the hypothesis that the anoestrous environment is deleterious to the early stages of fracture healing. The callus in the osteopenic model appeared to have a lower density of bone as early as four weeks post fracture representing either an impairment of bone formation or inappropriately accelerated bone resorption. Additionally, the presence of higher levels of undifferentiated tissue in these fractures suggested a general impairment of callus maturation. These differences were not associated with any changes in the expression of the growth factors studied or in the replication of key cell populations.

Contrary to previous work the dose and method of delivery of simvastatin in this study had no apparent effect on the mechanical properties of the healing tibial fractures in normal bone. This dose of simvastatin had no effect on the expression of BMP-2 or related factors in the fracture calluses from normal bone at the time points studied.

Simvastatin did have a deleterious effect on the properties of healing fractures in the osteopenic model with a reduction in callus size and maturity and the ability of the healing fractures to withstand load. The reduced levels of smad-1 in the chondrocytes and the retardation of bone maturation in this group suggests simvastatin has an undesirable effect on both chondrogenesis and bone remodelling at this dose

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Appendices

6 Appendices

Appendix 1

A1.1 Specimen Automated Processing Protocol

Specimens processed in Vacuum Infiltrated Processor (VIP, Tissue Tech VIP, E300 series, Miles Inc)

Alcohol 50%, 2 hours at 35°C

Alcohol 80%, 1 hour at 35°C

Alcohol 95%, 1 hour at 35°C

Alcohol 100%, 1 hour at 35°C

Alcohol 100%, 1 hour at 35°C

Alcohol 100%, 2 hours at 35°C

Xylene 100%, 3 changes of 1 hour each

Paraffin, 4 changes of 1 hour each at 60°C

Specimens moulded in paraffin blocks

A1.2 Haematoxylin and Eosin Staining

Materials

Harris Haematoxylin (6765004)
Eosin (CI 453380)
Pertex mounting resin

SHANDON
SHANDON
CELL PATH

Method

- 1) Dewax and rehydrate sections through graded alcohols to water
- 2) Immerse in haematoxylin for 3 minutes
- 3) Wash sections through 3 changes of tap water 1 minute each
- 4) Immerse in Scott's tap water for 30 seconds to blue
- 5) Immerse in eosin for 30 seconds
- 6) Wash sections in tap water for 1 minute
- 7) Dehydrate sections through graded alcohols to absolute alcohol (2 changes)
- 8) Sections through 3 changes of xylene and mount

Nuclear staining appears blue/purple. Cytoplasm, collagen, keratin and erythrocytes stain pink.

A1.3 Immunohistochemistry with the Avidin-Biotin-Complex (ABC) method

Materials

Hydrogen peroxide H_2O_2 (H1009)	SIGMA
Avidin / Biotin Block (Sp2001)	VECTOR
Dako Protein Block (X0909)	DAKOCYTOMATION
Desired primary antibody	
Biotinylated rabbit-anti-goat secondary a/b(E0466)	DAKOCYTOMATION
RTU ABC Vecastain Kit (Pk-7100)	VECTOR
DAB & Substrate (K3468)	DAKOCYTOMATION

Method

- 1) Dewax and rehydrate sections through graded alcohols to distilled water
- 2) Block endogenous peroxidase by immersion of slides in 3% H_2O_2 for 10mins
- 3) Wash in PBS
- 4) Perform desired antigen retrieval
- 5) Wash in PBS and mount slides in sequenza
- 6) Add 100ul avidin block for 10 minutes
- 7) Wash PBS
- 8) Add 100ul biotin block for 10 minutes
- 9) Wash PBS
- 10) Add 100ul Dako protein block for 10 minutes
- 11) Add 125ul of primary antibody at desired concentration and incubate for desired period
- 12) Wash PBS
- 13) Add 125ul of biotinylated secondary antibody at concentration of 1:400 and incubate for 30 minutes at room temperature
- 14) Wash PBS
- 15) Add 100ul RTU ABC Vecastain and incubate for 30 minutes at room temperature.
- 16) Wash PBS
- 17) Add 125ul of DAB + substrate chromogen and incubate for 5 minutes at room temperature
- 18) Wash in PBS
- 19) Transfer to staining rack and counterstain in haematoxylin
- 20) Dehydrate slides through graded alcohols to absolute alcohol and mount slides from xylene

Positively stained structures appear brown when viewed under direct light microscopy

A1.4 Trypsin Antigen Retrieval**Materials**

Trypsin (porcine type 2, pancreas) T 7409	SIGMA
Calcium Chloride (CaCl ₂) C3881	SIGMA

Preparation

Dissolve 0.5g of trypsin and 0.5g CaCl₂ in 500 ml of distilled water
Correct pH to 7.7 with TRIS at room temperature

Method

Preheat solution to 37⁰C in water bath
Immerse dewaxed, hydrated slides in solution for desired length of time. Remove slides and rinse on PBS. Proceed with assay.

A1.5 EDTA Antigen Retrieval**Materials**

Ethylene Diamide Tetra Acetic Acid EDTA(C ₁₀ H ₁₆ N ₂ O ₈)	SIGMA
Sodium Hydroxide (NaOH)	SIGMA
Tween -20 (C ₅₈ H ₁₁₄ ,O ₂₆) 437082Q	BDH

Preparation

Prepare 1mM solution of EDTA in 1l of distilled water. Add 0.1% by volume Tween-20 and correct pH to 8 with NaOH.

Method

Preheat solution to 65⁰C on a stirrer. Immerse dewaxed and hydrated slides in solution and leave overnight. Place lid on beaker to prevent evaporation and drying out of slides. Next morning remove from hotplate and cool to room temp. Rinse slides in tap water and proceed with assay.

A1.6 Immunohistochemistry with ARK – (Animal Research Kit) DAKO

The following must be calculated prior to beginning the assay:

- a) Working volume = no. of slides x 100ul
- b) Volume of concentrated primary antibody = (a) x final dilution
- c) Volume of biotinylated reagent = primary stock conc. / 100 x (b)
- d) Volume of blocking agent = (a) / 25
- e) Volume of diluent = (a)- (b + c + d)

Preparation of biotinylated primary antibody

Add primary antibody (b) to biotinylated reagent (c) and diluent (e). Incubate for 15 minutes at room temperature. Add blocking solution (d) and incubate for at least 5 minutes.

Biotinylated reagent - biotinylated anti-mouse IgG containing TRIS-HCL, protein and sodium azide

Blocking agent - normal mouse serum in TRIS-HCL buffer, protein and sodium azide

Diluent – ChemMate Antibody Diluent, (Dako Cytomation) containing Tris buffer, 15mmol sodium azide and protein at pH 7.2

Method

- 1) Sections prepared and antigen retrieval performed as described in relevant method section
- 2) Sections washed in PBS and load into sequenza (Shandon) ensuring absence of air bubbles and good seal with cover slip
- 3) 100ul of peroxidase block (0.03% hydrogen peroxide containing sodium azide) added to each slide for 5 minutes at room temperature.
- 4) Slides washed with PBS
- 5) 100ul of biotinylated primary antibody added for desired time
- 6) Slides washed with PBS
- 7) 100ul of streptavidin-peroxidase added for 15 minutes at room temperature
- 8) Slides washed in PBS
- 9) 100ul of 3,3-diaminobenzidine (DAB) + Substrate chromogen added for 5 minutes at room temp.
- 10) Slides washed with distilled water
- 11) Slides transferred to staining rack and immersed in haematoxylin for 30 seconds
- 12) Wash in tap water
- 13) Immerse in Scott's Tap Water for 30 seconds
- 14) Wash in tap water
- 15) Dehydrate through alcohol 64 OP, 74 OP, absolute, 1 minute each
- 16) Mount from xylene

Positive staining is represented by brown coloured precipitate at the antigen site.

A1.7 ZOCOR (Simvastatin, Merck USA)

Active ingredient:

Simvastatin $C_{25}H_{38}O_5$

Inactive ingredients:

Butylated hydroxyanisole

Ascorbic acid

Citric acid monohydrate

Cellulose

Starch

Magnesium stearate

Lactose

Hypromellose

Hydroxypropylcellulose

Titanium dioxide

Talc

Iron oxide red

Zocor 40mg (weight 400mg) = 40mg simvastatin

Appendix 2

Intra / interobserver error for immunohistochemical analysis

As the data were ordinal, a linear weighted Kappa was calculated using Vasser Stats software (Richard Lowry 2001, Poughkeepsie, USA).

Intra-observer Error:

Kappa = 0.796

SE = 0.089

95% CI = 0.621, 0.970

Inter-observer Error:

Kappa = 0.752

SE = 0.099

95% CI = 0.560, 0.946

Appendix 3.1 Results of tibial ash weight, volume and bone mineral density calculation for all specimens

Sham				OVX			
Specimen	Ash weight(g)	Volume (cm ³)	BMD (g/cm ³)	Specimen	Ash weight(g)	Volume (cm ³)	BMD (g/cm ³)
A11	0.314	0.44	0.714	A21	0.31	0.43	0.721
A12	0.332	0.48	0.692	A22	0.323	0.55	0.587
A13	X	X	X	A23	0.314	0.52	0.604
A14	0.348	0.50	0.696	A24	0.319	0.45	0.709
A15	0.313	0.42	0.745	A25	0.335	0.46	0.728
A31	0.33	0.47	0.702	A41	0.325	0.49	0.663
A32	0.347	0.49	0.708	A42	0.301	0.44	0.684
A33	0.3	0.41	0.732	A43	0.279	0.40	0.698
A34	0.315	0.42	0.750	A44	0.300	0.52	0.577
A35	0.312	0.45	0.693	A45	0.298	0.44	0.677
B11	0.315	0.44	0.715	B21	0.360	0.49	0.735
B12	0.336	0.45	0.747	B22	0.297	0.50	0.594
B13	0.346	0.49	0.706	B23	0.328	0.48	0.683
B14	0.313	0.40	0.783	B24	0.300	0.44	0.682
B15	0.322	0.41	0.785	B25	0.266	0.39	0.682
B31	0.328	0.42	0.781	B41	0.301	0.38	0.792
B32	0.298	0.39	0.764	B42	0.337	0.55	0.613
B33	0.312	0.42	0.743	B43	0.332	0.49	0.678
B34	0.290	0.40	0.726	B44	0.305	0.48	0.635
B35	0.274	0.38	0.721	B45	0.306	0.62	0.494
Mean	0.318	0.436	0.731	Mean	0.312	0.476	0.662
SD	0.018	0.034	0.029	SD	0.020	0.054	0.062
SEM	0.004	0.015	0.013	SEM	0.004	0.011	0.013
Upper 95% CI	0.310	0.421	0.719	Upper 95% CI	0.303	0.452	0.635
Lower 95% CI	0.326	0.451	0.744	Lower 95% CI	0.320	0.500	0.689

X – denotes excluded specimen

	1	2	3	4	5
Osteoblasts					
A1	0	3	0	x	x
A2	3	2	3	0	0
A3	3	5	2	9	x
A4	5	6	3	8	2
B1	0	1	3	x	x
B2	3	2	6	3	x
B3	3	6	8	6	3
B4	9	6	8	8	5
Chondrocytes					
B1	3	5	3	x	x
B2	3	3	6	6	x
B3	6	3	2	3	3
B4	3	3	3	6	3

Appendix 3.2 BMP-2 immunohistochemistry scores for all specimens

x- denotes exclusions

Osteoblasts	1	2	3	4	5
A1	3	3	1	x	x
A2	2	6	3	3	2
A3	3	2	6	6	x
A4	5	6	3	8	2
B1	3	0	3	x	x
B2	3	2	5	6	x
B3	6	6	2	7	6
B4	6	5	2	6	9
Chondrocytes					
B1	3	3	2	x	x
B2	2	2	1	3	x
B3	3	6	2	3	0
B4	3	3	5	3	3

Appendix 3.3 BMP-6 immunohistochemistry scores for all specimens

x- denotes exclusions

	1	2	3	4	5
Osteoblasts					
A1	3	3	1	x	x
A2	0	3	3	0	0
A3	0	0	2	2	x
A4	2	0	0	2	0
B1	4	3	5	x	x
B2	3	5	6	3	x
B3	3	3	3	2	5
B4	3	4	2	2	3
Chondrocytes					
B1	3	2	3	x	x
B2	2	3	3	2	x
B3	3	3	3	3	3
B4	3	3	3	3	3

Appendix 3.4 BRDU immunohistochemistry scores for all specimens

x- denotes exclusions

	1	2	3	4	5
Osteoblasts					
A1	3	3	0	x	x
A2	0	3	3	0	3
A3	2	3	0	3	x
A4	3	2	3	0	3
B1	3	3	0	x	x
B2	3	0	3	2	x
B3	6	3	2	3	5
B4	3	5	6	6	3
Chondrocytes					
B1	0	3	0	x	x
B2	3	2	2	0	x
B3	5	2	3	5	2
B4	6	9	5	6	6

Appendix 3.4 Smad-1 immunohistochemistry scores for all specimens

x- denotes exclusions

Group	Description	Post #	Score for presence of fracture line on radiographs										Median
			3	3	3	2	2	2	2	1	1	x	
A1	Sham – placebo	4 weeks	3	3	3	2	2	2	1	1	x	x	2.5
A2	Ovx – placebo	4 weeks	3	3	2	3	4	3	4	4	4	2	3.0
A3	Sham – statin	4 weeks	3	4	4	3	3	2	3	4	3	x	3.0
A4	Ovx – statin	4 weeks	3	4	4	3	2	4	4	3	3	3	3.0
B1	Sham – placebo	2 weeks	1	1	1	1	1	1	1	1	1	1	1
B2	Ovx – placebo	2 weeks	1	1	1	1	1	1	1	1	1	1	1
B3	Sham – statin	2 weeks	1	1	1	1	1	1	1	1	1	1	1
B4	Ovx – statin	2 weeks	1	1	1	1	1	1	1	1	1	1	1

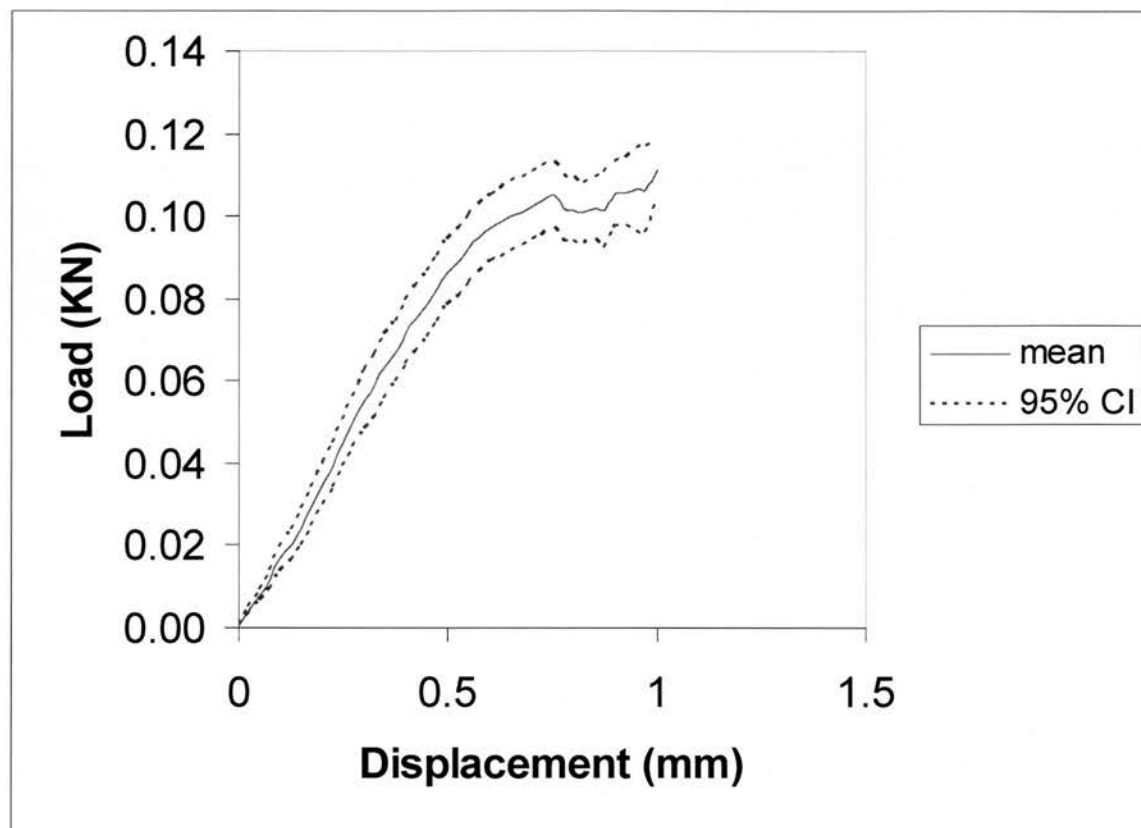
Appendix 3.5 Results of scoring for presence of fracture line seen on radiographs post euthanasia in all groups
x - denotes excluded specimen

	Sham groups				OVX groups			
	A1	A3	B1	B3	A2	A4	B2	B4
1	1.00	1.00	0.86	0.95	0.47	0.52	0.80	0.67
2	1.00	0.78	1.06	0.84	0.48	0.46	0.60	0.74
3	x	0.90	0.94	1.14	0.49	0.55	0.60	0.50
4	0.80	0.80	1.20	0.65	0.42	0.41	0.80	0.47
5	0.76	0.70	0.97	0.85	0.55	0.57	0.64	0.40
6	0.89	0.88	1.20	0.70	0.48	0.42	0.65	0.57
7	0.81	0.89	1.03	0.97	0.52	0.46	0.60	0.40
8	0.76	0.89	1.10	0.84	0.57	0.51	0.53	0.41
9	0.99	1.00	1.05	0.82	0.52	0.47	0.45	0.52
10	0.99	1.10	1.05	1.10	0.42	0.51	0.70	0.39
Mean	0.89	0.89	1.05	0.89	0.49	0.49	0.64	0.51
SD	0.11	0.12	0.11	0.16	0.05	0.05	0.11	0.12
Mean of all groups	0.93				0.53			
SD of all groups	0.14				0.11			

Appendix 3.6 Uterine weights (g) for all specimens

X – denotes excluded specimen

Appendix 3.7 Standard curve for load to failure of normal rat tibiae under four-point bending (n=30)



Mean load at yield	112 N (95% CI 111.4, 112.6)
Mean Load at failure	108N (95% CI 101.5, 114.5)
Linear regression analysis of data to yield point`	
Correlation coefficient (r)	0.9241
ANOVA	P<0.001
Slope	0.1219 (95% CI 0.1526, 0.1653)