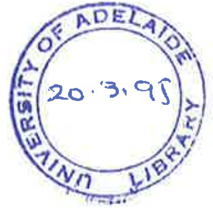

THE PATHOGENESIS OF POST-MENOPAUSAL OSTEOPAENIA USING THE OOPHORECTOMISED RAT MODEL

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Doctor of Philosophy

in

The University of Adelaide
(Faculty of Science)

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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ABSTRACT

After the menopause in the human female, bone turnover is increased and bone density is reduced leading to increased fracture risk. The mechanisms by which oestrogen deficiency causes this bone loss remains unclear.

The mature oophorectomised rat is a well-recognised model of post-menopausal bone loss, and has been used in this thesis to study the effects of oestradiol on bone cells in vivo.

The immediate effects of oophorectomy (oestrogen-deficiency) were determined by the measurement of biochemical and histomorphometric markers of bone formation, resorption and trabecular bone morphometry from time of operation until 21 days post-operation. From this study a model of oestrogen-deficiency bone loss is proposed whereby the immediate increase in bone resorption results in increased activation frequency of bone turnover units, thus increasing the risk of trabecular perforation. Increased bone formation is delayed in oestrogen deficiency, such that the balance of bone turnover is maintained at the cellular level, however due to trabecular perforation, not all resorbed pits continue through to the formation stage, since bone formation requires a surface to build on.

A study of similar design was carried out to determine the effects of oestradiol on bone loss in oophorectomised rats. Oestrogen treatment of oophorectomised rats from the time of operation delayed trabecular bone loss by inhibiting both formation and resorption. Data from this thesis support a model of a direct inhibitory oestrogen action on both osteoclasts and proliferating osteoblasts as reported in vitro. These direct inhibitory effects of oestradiol appear to suppress the direct stimulatory action of oestrogen on mature osteoblast reported in vitro, and immediately following oestradiol treatment in this study.

The effects of salmon calcitonin and PTHrP(107-139) were also assessed in vivo using similar methods. The effects of these hormones on bone cell activity and trabecular bone loss in oophorectomised rats has been compared to the effects of oestradiol.

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ERRATUM

p30	last line "humeral" should read "humoral"
p31	para 2, line 4 reference number 184 should be inserted between the empty brackets.
p43	Table 2.2 first line (GFR values (ml/day)) should read: 3 days: 99 ± 19 6 days: 175 ± 4 9 days: 189 ± 44 12 days: 176 ± 26
p101a	(insert page following)
p101b	(insert page following) Figure 4.7 (legend) line 1 "pevented" should read "prevented"
p119	para 3, line 7 should read: "Studies in vivo demonstrate *decreased* TGFβ production in oophorectomised rats, and the *decrease* may be prevented by oestradiol treatment (46)."
p127	Figure 5.2, p139 Figure 5.9 and p161 Figure 6.1 y-axis label "mmol/L GF" should read "µmol/L GF"
p129	Figure 5.4 y-axis label "U/L" should read "µg/L"
p131	para 2, line 2 should read "The loss of trabecular bone volume in calcitonin treated oophorectomised rats was not associated with decreased trabecular number, *there was however a slight but non-significant increase in this marker.* Trabecular thickness was..."
p133	Table5.1 (legend) add to end of last line: "^{a}, effect of oophorectomy, $p<0.05$ vs sham. ^{b}, effect of calcitonin treatment, $p<0.05$ vs vehicle treated rats in the same operation group."
p136	Figure 5.7 y-axis label "mmol/L" should read "g/L"
p161	Figure 6.1 (legend) line 7 "effect" should read "affect"

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CHAPTER ONE

THE EFFECTS OF OESTRADIOL AND CALCITONIN ON BONE TURNOVER AND CALCIUM HOMEOSTASIS

1.1 The Role of Oestradiol in Bone Maintenance and Calcium Homeostasis

1.1.1 Bone Loss in Oestrogen Deficiency - The Post-Menopausal Woman

It is well established that considerable bone loss occurs in all women after the menopause. Post-menopausal bone loss was originally described by Albright as "too little bone in the bone" since it is characterised by a reduction in the amount of bony tissue in a given volume of anatomical bone (1). The composition of the remaining bone is normal, but there is a reduction in bone density; that is, a reduction in bone mineral content in a given area.

Bone loss after the menopause is rapid. Significant reductions in bone density and bone mass (the total volume of bone) have been described within the first year of the menopause (2,3). A rapid reduction in bone density has also been described following surgical oophorectomy (4). Bone loss in oophorectomised or post-menopausal women appears to be caused by oestrogen deficiency, since it may be prevented by oestrogen treatment (2,5-8). While bone loss associated with oestrogen deficiency is most rapid in the first few years after menopause, it does continue at a slower rate until the end of life. Estimations from forearm bone mineral density of 485 post-menopausal women indicate that approximately 11% of bone is lost in the first 5 years after menopause and a further reduction of 5% over the following 20 years (9). There are two major components to this loss of bone: a linear, age-related bone loss, beginning approximately at age 54 years, and a self-limiting exponential loss of bone beginning at the menopause (9).

The outer surface of skeletal bone is composed of a densely mineralised layer of cortical bone. An internal network of fine bony trabeculae, known as cancellous bone supports the cortical shell. In post-menopausal women, there is a reduction in both cortical thickness and cancellous bone density, however the reduced density of the cancellous bone appears to be more pronounced (2). When bone density was determined by quantitative computer tomography, a method where cancellous bone density and cortical bone density may be determined as separate components, the linear, age-related component of post-menopausal bone loss was no longer significant (10). This result suggests menopausal bone loss occurs primarily within cancellous bone. The linear, age-related loss of bone however, occurs due to a reduction in cortical bone volume (10). Post-menopausal bone loss may therefore be described by an initial rapid loss of cancellous bone due to the menopause, followed by a slow, linear age-related loss of cortical bone (11).

The incidence of wrist and other peripheral (non-hip) fractures increases greatly in women after the menopause; again this appears to follow an exponential function, reaching a plateau at approximately 10 years after the menopause. These fractures occur mainly in cancellous bone, mirroring the cancellous bone loss described above (11). Conversely, hip fracture incidence appears related to the age-related loss of cortical bone (12,13). Many workers have shown that post-menopausal fracture patients have a lower bone density than age-matched non-fracture controls, indicating an inverse relationship between fracture risk and bone density (14-16); that is, low bone density is associated with increased fracture risk. Menopausal bone loss therefore results in increased fracture risk and incidence, resulting in a major cost to the public health system, particularly as the average age of the general population increases.

1.1.2 The Effect of Menopause on Bone Turnover

Adult bone is continually being remodelled. At specific sites on the bone surface, osteoclastic bone resorption and osteoblastic bone formation are co-ordinated within basic multicellular units (BMUs). The coupling of these processes, which results in bone turnover, allows calcium exchange between the bone and the extracellular fluid (Figure 1.1). Bone turnover is elevated soon after the menopause (3,5,6,17-19), after surgical oophorectomy (4), or following treatment with a gonadotrophin releasing hormone agonist (20). Increased bone turnover appears to be the cause of oestrogen-deficiency bone loss. The disturbance in bone turnover responsible for the bone loss must be considered at the level of the BMU.

Bone turnover begins with recruitment and proliferation of haematopoietic mononuclear phagocytes in the bone marrow. These cells attach to specific sites on the bone surface where they fuse, forming large multinucleated osteoclasts. Mature osteoclasts resorb pits of bone, releasing bone mineral and enzymatically degrading the remaining collagen matrix. The amount of bone removed by osteoclasts during this resorption phase of bone turnover is termed the remodelling space (21).

The resorption phase of bone remodelling is followed by a "reversal" phase. During this phase the pit is lined by mononuclear phagocytes, possibly derived from osteoclastic fission. These cells may be involved in scavenging products left behind by osteoclastic bone resorption. Alternatively, mononuclear phagocytes may synthesise some components of the cement line, a mixture of glycosaminoglycans and glycoproteins which coats the surface of the pit before the next phase of bone remodelling (22).

The remodelling space is occupied by osteoblastic bone formation following formation of the cement line. Osteoblasts derived from mesenchymal stromal cells migrate into the pit and synthesise a substance composed of aggregated proteoglycans and collagen fibrils (osteoid). Osteoblasts also appear to be

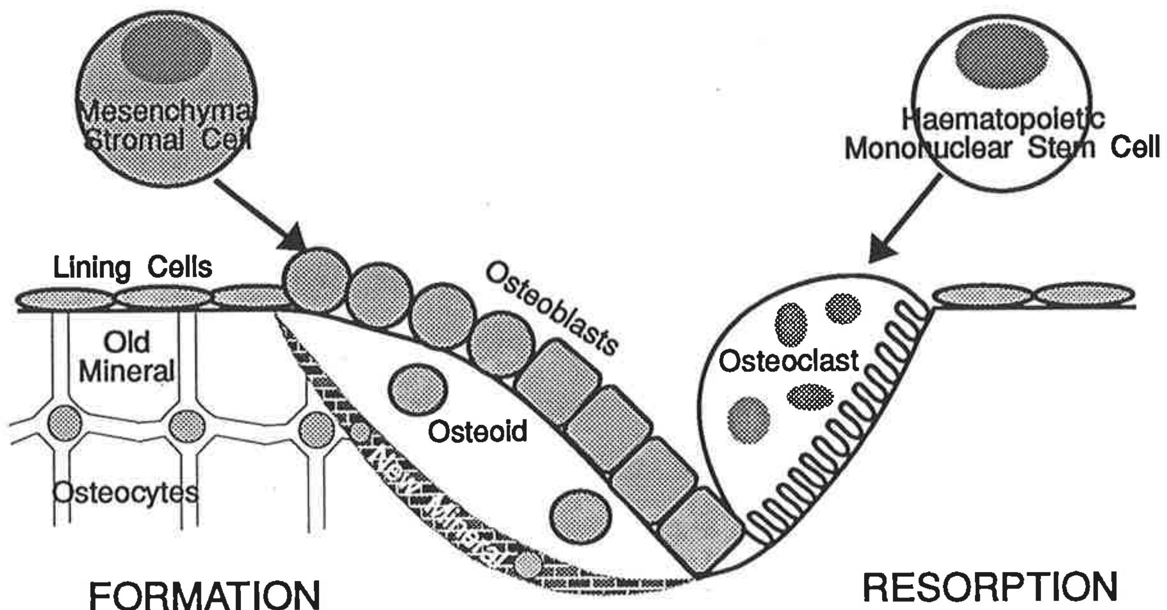


Figure 1.1 The Basic Multicellular Unit of Bone Turnover (BMU). The BMU model describes the normal process of remodelling on the bone surface. When bone remodelling is triggered, mononuclear stem cells of the haematopoietic lineage fuse, forming a large, multinucleated osteoclast. The osteoclast attaches to a specific site on the bone surface and resorbs a pit of bone. After bone resorption, the pit is filled by mononuclear lining cells (not pictured). For brevity, bone formation and resorption have been pictured in the same pit, although they do not necessarily occur at the same time. After the reversal phase, osteoblasts derived from mesenchymal stromal cells migrate into the pit, forming an osteoid layer which eventually becomes mineralised. Some osteoblasts become trapped in the osteoid, eventually forming osteocytes in the mature mineralised bone. Others remain on the surface as flattened lining cells. The cells of the osteoblast lineage are shaded grey.

involved in the mineralisation of osteoid, where calcium and phosphate deposits become complexed to the osteoid layer. During this process, some osteoblasts become embedded in the matrix, and differentiate into osteocytes. Other osteoblasts remain at the bone surface as flat lining cells, leaving a surface indistinguishable from the bone surface prior to remodelling activation. The bone surface remains in this resting phase until turnover is again activated.

In the young adult skeleton, the two processes of bone turnover, resorption and formation are usually coupled temporally such that bone removed during resorption is replaced by an equivalent amount during formation. Increased resorption is usually associated with increased formation, ensuring a constant bone mass. The processes involved in bone growth during childhood are no longer significant in adult bone, such that most of the adult bone mass is controlled by bone remodelling (23).

Calcium kinetic studies in pre- and post-menopausal women have shown that bone turnover appears to be slightly unbalanced at menopausal age, even in pre-menopausal women in favour of bone resorption (5). Some authors have suggested this imbalance is accentuated after the menopause, and is the major cause of the bone loss observed (5,17). Imbalanced bone turnover at the BMU level in the absence of oestrogen has been attributed to an increase in the osteoclast life span (17), resorption of deeper pits by osteoclasts (21), or impaired osteoblast function (17,18,21). These theories would be consistent with a greater level of bone resorption than formation at the BMU level in oestrogen deficiency.

Contrary to popular interpretation however, Heaney's calcium kinetic studies (5) may not indicate that bone turnover is imbalanced at the BMU level such that resorption pits are incompletely filled, as described above. Heaney's study only indicates a predominance of bone resorption compared to the level of

formation occurring throughout the skeleton as a whole. This would include the activities of bone cells on cortical surfaces as well as other areas in which BMU-based remodelling may not occur. No evidence of imbalanced bone turnover at the level of the BMU has been detected in histomorphometric studies of trabecular bone biopsies from post-menopausal women (8). A reduction in wall width at the menopause (23) has been interpreted by many as evidence for remodelling imbalance, however, since resorption depth cannot be reliably measured, these conclusions may be misleading (24).

Post-menopausal bone loss may be caused by an increase in BMU activation frequency (8,17) without alteration in bone remodelling balance. Since the rate of BMU initiation is increased in oestrogen deficiency and contributes to the high level of bone turnover and an increase in bone remodelling space, the likelihood of trabecular perforation by resorption of pits on both sides of one trabecula in the same area would increase. The initial imbalance in this model would normally be transient, however restoration of bone lost due to trabecular perforation may be impaired since there is no longer any cement line coated surface as required for bone formation to proceed. Perforated trabeculae also appear to be preferentially resorbed until they are flattened completely, in both normal and osteoporotic bone (25). Increased resorption of trabecular fragments, and lack of formation due to trabecular perforation as described here would be consistent with the imbalance in whole body bone turnover reported by Heaney (5).

1.1.3 Changes in Calcium Metabolism After the Menopause

There are a number of changes reported in calcium metabolism after the menopause that appear to relate to the increase in bone turnover. Plasma calcium levels are elevated in post-menopausal women probably due to the high level of bone resorption (3,26-28). Calcium exists in the circulation in a number of different states: ionised calcium, complexed calcium (associated

with various cations such as bicarbonate), and protein bound calcium (associated with plasma proteins such as albumin). No change has been detected in the physiologically active ionised calcium after the menopause (26-28), nor in the protein bound fraction (28). Rather, the rise in serum calcium after the menopause occurs mainly in complexed calcium (28). This may follow the elevation in plasma bicarbonate levels (27,28), possibly related to the release of the calcium carbonate fraction from bone during increased bone resorption (11).

The complexed and ionised calcium fractions make up the ultrafiltrable serum calcium fraction. This fraction passes through the renal glomerulus for excretion in the urine. Although the majority of calcium is reabsorbed as it passes through the nephron, it appears the increase in complexed calcium results in an increase in the obligatory urinary calcium loss (3,18,27,29). Increased renal calcium excretion independent of the increased filtered load has also been described in post-menopausal women by comparing the calcium excretion in pre- and post-menopausal women matched for filtered calcium loads (30). It is this increase in urine calcium that has led to the "calcium leak" model of osteoporosis, where it is suggested the bone loss initiated at the menopause is continued due to excessive urinary calcium excretion (3,11,30,31).

Plasma phosphate is also increased after the menopause (27). This may result from increased bone turnover, and may be associated with an increase in renal phosphate reabsorption, since urinary phosphate excretion is not altered at the menopause (32).

Intestinal absorption of calcium from the diet may also be reduced in post-menopausal women (29,229), and this could contribute to the bone loss observed.

The effects of menopause on the kidney and intestine described above have suggested a role for an alteration in production of or sensitivity to systemic calciotropic hormones such as parathyroid hormone (PTH) or calcitonin in the pathogenesis of oestrogen-deficiency bone loss.

The negative calcium balance observed in the first year following the menopause, where urinary calcium loss is increased (3,18,27,29) and absorption of dietary calcium is decreased (29,229) appears to return to the pre-menopausal balance by 20 years after the menopause (11). These transient alterations in calcium metabolism, and the exponential nature of post-menopausal bone loss suggests two phases of post-menopausal bone loss. In the initial phase cancellous bone resorption is increased, leading to raised plasma calcium levels and, by coupling, bone formation is increased. In this phase, cancellous bone is lost in a rapid, exponential manner. As the minimum level of cancellous bone required for structural competence is reached, cancellous bone resorption slows and plasma calcium returns to normal. Age-related cortical bone loss continues in this second phase, and could be mediated by the PTH response to the increased calcium loss in the urine.

1.1.4 Treatment of Post-menopausal Bone Loss with Oestrogen

Post-menopausal bone loss can be prevented by oestradiol treatment either at the menopause or after bone loss has occurred (2,6-8). Oestradiol treatment suppresses the increase in both bone resorption and formation observed in oestrogen-deficiency (5-8). Specifically, oestrogen treatment reduces BMU activation frequency leading to a reduction in the total remodelling space and an increase in the proportion of older, more heavily mineralised bone (8). The decrease in bone remodelling in the presence of oestrogen appears to be associated with a greater relative decrease in bone resorption than formation throughout the skeleton, such that the skeletal remodelling imbalance reported by Heaney is restored to normal balance (5). Bone remodelling balance at the

level of the BMU does not appear to change in response to oestradiol treatment (8).

The correction of bone remodelling activity induced by oestradiol treatment conserves remaining bone. In the remodelling adult skeleton, since bone is not formed without a previous cycle of resorption, oestrogen treatment is not effective in restoring bone density once the bone loss has occurred (5-7).

Oestradiol treatment also prevents the changes in serum calcium reported in oestrogen deficiency. Serum calcium is reduced by oestradiol treatment in post-menopausal women (6,33), although it has not been reported which calcium fraction is affected. Since bicarbonate levels (27) and obligatory calcium loss are reduced by oestradiol treatment (6,27,32,34) without any change in renal calcium reabsorption (27) it seems likely that this reduction may occur in the complexed calcium fraction, however, this change in renal handling of calcium may occur by blockage of the renal calcium leak observed by Nordin et al (30).The post-menopausal reduction in intestinal calcium absorption is also reversed by oestradiol treatment (29,34).

Serum phosphate levels are also returned to pre-menopausal levels by oestradiol treatment (6,32,34,35). Renal tubular phosphate reabsorption is reduced (35) such that urine phosphate remains unchanged (32).

While the changes in calcium and bone metabolism with oestrogen treatment described above are well established, the interactions of oestrogen with bone and other centres of calcium metabolism remain poorly understood. Oestrogen has been proposed to act directly on osteoblasts (36,37), osteoclasts (38), the kidney (39), and the intestine (40). Other models propose indirect effects of oestradiol on a number of calcium homeostatic centres via calcitonin (41,42), parathyroid hormone (PTH) (43,44) or local modulators of bone turnover

(45,46). One of the most versatile models used to investigate these theories has been the oophorectomised rat.

1.1.5 The Oophorectomised Rat - A Model of Post-menopausal Bone Loss

Bone loss in the oophorectomised rat is similar in nature to that described in post-menopausal women (47,48). Oestrogen deficiency bone loss in the rat is associated with increased bone turnover, measured by both biochemical (47,49-51) and histomorphometric markers (52-55). The bone loss follows a self-limited exponential pattern (56,57) and is more pronounced in cancellous compared to cortical bone (47,58-61) as observed in post-menopausal women.

The cancellous osteopaenia observed after oophorectomy appears to be due to conversion of plate to rod trabeculae by trabecular perforation and loss of whole trabeculae, rather than general trabecular thinning (62,63), similar to the changes observed in cancellous bone after the menopause (21). Bone loss occurs in the central area of the long bones, while thick plate trabeculae remain in the periphery, probably maintained in response to mechanical strain (62).

Since the nature of bone remodelling in cortical bone differs from BMU based cancellous bone remodelling, it is not surprising that oophorectomy affects cortical bone in a considerably different manner to cancellous bone. Oophorectomy appears to have little, if any effect on the quantity, quality or composition of cortical bone, even at 18 months after operation (57,60,61). This would explain the stability of bone ash weight after oophorectomy compared to sham operated rats (58,64). There is an increase in endosteal bone resorption (61) and bone formation at both endosteal and periosteal surfaces (59,65). Clearly these effects are balanced, such that cortical bone volume is not influenced by oestrogen status. Any change in cortical volume

may be attributed to ageing (61) as suggested in post-menopausal women (Chapter 1.1.1).

Increased bone turnover and loss of bone in oestrogen deficient rats appear to be self-limiting events (14,52,55,56,63), as observed in oestrogen deficient women (3,66). Bone loss is rapid between the time of operation and 14 days post-operation, but appears to decline thereafter, as the limit of bone loss is reached (52). Dual photon absorptiometric measurements of bone density have shown that the osteopaenia observed in rat femora at 35 days after oophorectomy does not worsen for the next 17 months (57). However, dual photon absorptiometry does not differentiate between cancellous and cortical components of bone, so this stability may relate to the cortical component of these measurements. The increased level of bone turnover is reduced with time (14,52,55,56,63). Bone resorption is higher in the proximal tibial metaphysis of oophorectomised rats only until 100 days after oophorectomy (52). Bone formation also reduces with time, but remains significantly raised at 540 days post-operation, although this may relates to age-related bone loss beginning at 270 days post-operation (56).

The self-limiting nature of bone loss in oestrogen deficiency may relate to an oestrogen-dependent quantum of bone, with its size and rate of loss dictated by menstrual frequency, varying between species such that once this compartment of bone has been lost, the imbalance in bone turnover is restored (57). It seems unlikely however, particularly since only 25% of cancellous bone remains in the proximal tibial metaphysis at 100 days after oophorectomy (56) and oestrogen treatment of post-menopausal women prevents bone loss throughout the skeleton (67). It appears more likely that the limit of bone loss is related to the mechanism of loss, or to a minimal requirement of cancellous bone in areas of greater mechanical strain, whereby further bone loss is prevented by increased bone formation in response to loading (68). Increased

bone formation in response to mechanical strain may also be a partial cause of the high level of bone formation at 540 days after oophorectomy (56).

The increase in bone resorption after oophorectomy is related to an increase in osteoclast progenitor proliferation (69) and osteoclast number (52-54,63).

Increased bone formation after oophorectomy is characterised by increased bone formation rate and forming surface (52-54,63). This apparently results from increased osteoblast precursor proliferation (70) followed by an increase in osteoblast number (52-54). Others have described impaired osteoblast progenitor proliferation within the same time frame (71), but this study was concerned with endosteal osteoblast progenitors which may respond differently to oestrogen deficiency.

Although bone is lost after oophorectomy in both mature and immature rats (72), studies of oestrogen deficiency bone loss may be complicated by effects of age on bone turnover. This is an important consideration since post-menopausal bone loss occurs after skeletal maturity, suggesting models for this loss should also be skeletally mature. Published work include oophorectomy at ages ranging from 3 weeks (73) to 2 years of age (8). Use of 3 week old rats is complicated by the high level of bone turnover and skeletal growth occurring at the time of operation, as well as the rapid decline in bone turnover as animals mature. In two year old rats, although bone growth appears to have ceased, this is very close to the age when ovarian function ceases in the rat (74), and animals of this age are more likely to die due to age related diseases. Bone density and biochemical markers of bone turnover appear to be maintained at a stable low level from 6 months of age onwards (72). This seems an appropriate age for studies of oestrogen deficiency, with bone loss not being complicated by rapid bone growth ending at approximately 3 months (75), or age-related bone loss, beginning at around 9 months (56).

Since the initial rapid bone loss after oophorectomy is coincident with the maximal increase in bone turnover (52), the bone loss is most likely associated with such an increase. It has been generally assumed that osteoblast and osteoclast activities are uncoupled at the BMU level such that the increase in bone resorption in the oophorectomised rat is greater than formation as some have implied from the work of Heaney et al in post-menopausal women (5). Such imbalance has been reported as the cause of the subsequent bone loss (47,56). However, comparisons between markers of bone resorption and formation in the rat tibia indicate the level of formation may actually be greater than resorption in the oophorectomised rat (53,54). The increased level of bone formation was initially explained as an experimental artefact since markers of bone formation used in these studies were static and although osteoblast number was increased, the life span and active resorption of these cells may have been reduced (53). However, since the increase in bone resorption after oophorectomy is transient (14,52,54,55,63) this finding may reflect the longer duration of increased bone formation compared to the duration of increased resorption. It has also been reported that the initial increase in bone resorption precedes the increase in formation by approximately 10 days (63). The time difference between these events may account for the loss of bone after oophorectomy, although the time at which the loss of bone occurs following operation has not been reported.

1.1.6 Changes in Calcium Metabolism in the Oophorectomised Rat

In contrast to the increase in serum calcium consistently reported after the menopause (Chapter 1.1.3), a change in serum calcium has not always been detected in the oophorectomised rat (51,76). Decreased serum calcium has been reported in some studies, however this result was only detected by one group of workers, and not when animals were fed a diet containing 0.1% calcium (47,77). The suggested reduction in serum calcium after oophorectomy is not associated with a change in the ionised calcium fraction

(47,49,51). Levels of complexed calcium have not been reported in the oophorectomised rat to date, however the calculated protein bound fraction of calcium is reduced (51) and this probably relates to a reduction in serum albumin levels (47,51).

Serum phosphate and renal phosphate reabsorption are increased in oophorectomised rats (51,64) and as observed in post-menopausal women, there is no reduction in phosphate excretion. Intestinal calcium absorption is also reduced after oophorectomy (47,78).

The changes in calcium and phosphate metabolism in oophorectomised rats have been cited as evidence for a model of oestrogen deficiency bone loss whereby changes in bone turnover are secondary to changes in general calcium homeostasis. According to this model, bone loss may be caused by altered secretion of or altered sensitivity to systemic calciotropic hormones such as parathyroid hormone (PTH) (43,44), or calcitonin (41,42).

Since circulating PTH levels after oophorectomy are unchanged (44,47), it was suggested that an increase in bone sensitivity to PTH may cause the bone loss observed in oestrogen deficiency. PTH administration in oophorectomised rats resulted in an increase in serum calcium of longer duration (44) and a greater decrease in cortical thickness (43) compared to PTH treated sham operated animals. This finding could be interpreted as a more potent effect of PTH in oestrogen deficiency, however since an untreated oophorectomised group was not included in the latter study, it is unclear whether changes in cortical thickness were due to PTH treatment, or an effect of oophorectomy. In contrast, parathyroidectomised rats exhibit similar changes in calcium metabolism and trabecular bone volume after oophorectomy as non-parathyroidectomised rats (Sims et al, manuscript in preparation), indicating PTH is not necessary for oestrogen deficient cancellous bone loss. Turner et al have suggested PTH or other calcium homeostatic hormones may be involved

in cortical bone loss, since the increased endosteal bone formation after oophorectomy is not suppressed by oestradiol treatment (59).

Decreased serum calcitonin levels, measured by radio immunoassay have been reported after oophorectomy in a number of studies (44,47,79). However, this decrease was not observed when rats were maintained on a low calcium diet and significant bone loss still occurred after oophorectomy (47). Bone loss has also been detected in calcitonin-deficient rats (77), indicating an alteration in sensitivity to or production of this hormone is not necessary for bone loss in oestrogen-deficiency. A change in serum calcitonin levels may be secondary to a direct effect of oophorectomy on bone.

Since the discovery of oestrogen receptors in bone cells (36-38,80), it is accepted that oestrogen may act directly on bone, and oestrogen deficiency is a direct cause of the bone loss observed in the oophorectomised rat. Effects on other calcium homeostatic mechanisms may be either secondary to the changes in bone turnover or due to direct action of oestrogen on the kidney or intestine.

1.1.7 The Effect of Oestrogen Treatment in Oophorectomised Rats

Oestrogen treatment prevents osteopaenia in oophorectomised rats in both short term (50,81-85) and long term studies (50,86) consistent with the effect of oestrogen treatment in post-menopausal women (5-7,34). Bone loss is prevented presumably by suppressing the rise in both bone resorption and formation (50,81,82,84,85,87,88). However, studies of oestradiol treatment in oophorectomised rats have not measured levels of bone resorption and formation over the time period when bone turnover is initially increased or the time when bone loss is first detected after oophorectomy.

The lower level of bone turnover in oophorectomised rats treated with oestradiol is associated with reduced osteoclast and osteoblast numbers on trabecular surfaces (82,84,86,88,89) as well as a reduction in bone cell activity after oestradiol treatment (50,82,84-89).

Withdrawal of oestradiol treatment in oophorectomised rats has much the same effect of oophorectomy, leading to increased bone turnover, and bone loss of a similar magnitude to that observed after oophorectomy (90).

After 40 days of treatment, doses as low as 0.06μg/kg/day 17β-oestradiol reduce serum alkaline and acid phosphatase activities, markers of osteoblast proliferation and osteoclastic resorption respectively in oophorectomised rats compared to untreated oophorectomised controls (85). A higher dose of 2.5μg/kg/day 17β-oestradiol over the same time frame was required to prevent trabecular bone loss in the oophorectomised rat (85,91). Still higher doses, up to 50μg/kg 17β-oestradiol, resulting in circulating oestradiol levels of almost three times the mean level measured in sham operated rats, prevent bone loss, but are not capable of increasing bone mass beyond that of intact rats (82).

Oestrogen treatment in oophorectomised rats 2 months (92) or 3 months (90) after oophorectomy, although possibly preventing further bone loss by reducing bone turnover, could not restore trabecular bone volume to levels observed in intact rats. The continued low level of trabecular bone volume in oophorectomised rats treated with oestradiol after bone loss had occurred suggests oestrogen treatment is not capable of replacing trabeculae lost by trabecular perforation as described in oestrogen deficiency (62,63).

Oestrogen treatment in oophorectomised rats appears to decrease the level of bone turnover as well as eliminating the imbalance in turnover throughout the skeleton. Since bone lost is not restored, it is unlikely that BMU or whole body turnover imbalance is reversed such that bone formation exceeds resorption.

Despite the large volume of work supporting an inhibitory effect of oestradiol on both bone resorption and formation, one group of researchers has consistently reported a stimulatory effect of oestradiol on bone formation (93-95). These findings may relate to the use of intact, rather than oophorectomised rats in these experiments such that circulating oestradiol levels become supra physiological, although circulating oestradiol levels have not been reported in these studies. However, in oophorectomised rats treated with very high doses of oestradiol such that circulating oestradiol levels may exceed those reached in treated intact rats, bone formation is still inhibited. (82). The extent of cancellous bone formation in the studies reporting stimulation of bone formation by oestradiol was determined by the extent of fluorochrome label incorporation into the bone at fronts of bone mineralisation. The extent of fluorochrome label at any time point is dependent on the level of bone resorption, since fluorochrome label is also resorbed along with the bone. It has been suggested that the reduction in bone resorption with oestradiol treatment may result in an overestimate of fluorochrome labelled surface, since less fluorochrome label would be resorbed (88). Since the level of bone resorption was not reported, and only fluorochrome labelled surface, rather than both surface and mineralisation rate (determined by the distance between two fluorochrome labels administered over a known time interval) were measured in these study, the observed increase in bone formation may be an artefact. One study is also complicated by administration of one fluorochrome label before oestrogen was administered (95).

Turner et al have conducted a number of experiments to fully examine the reported anabolic effect of oestradiol on the bone (96). They have shown that the reports of increased bone formation in response to oestradiol are unreliable since non-equivalent sampling sites were used in the treated and untreated groups such that both modelling (growth) and remodelling responses were

measured, and increased bone formation could not be detected in a controlled study of a similar design.

Oophorectomy appears to alter the renal handling of calcium and phosphate as well as reducing intestinal calcium absorption (Chapter 1.1.6). Oestrogen receptors have been localised in both the kidney (39) and intestine (40). There is no published data describing the effects of oestradiol treatment on the changes in calcium and phosphate metabolism in the oophorectomised rat to date.

Systemic administration of 17β-oestradiol to oophorectomised rats suggests a direct inhibitory action of oestradiol on either osteoclasts or osteoblasts, or both, regulating bone cell development and activity such that pre-operative levels of bone turnover are maintained. In contrast, local administration of 17β-oestradiol by infusion into the long bones of oophorectomised rats increases trabecular bone volume by decreasing bone resorption while increasing formation in comparison to non-infused bones (97). The authors suggest the difference in effect of oestradiol may be due to the high dose required for systemic studies. This conclusion suggests a biphasic oestradiol effect as reported for oestradiol stimulation of alkaline phosphatase activity in vitro (98). Alternatively, oestradiol may interact with other autocrine / paracrine factors at the local or systemic level. This possibility will be discussed in more detail in Chapter 1.1.9.

1.1.8 The Effects of Oestrogen in Vitro

Oestrogen receptors have been located in osteoblasts (36,37,99) and osteoclasts (38,100) as well as stromal osteoblast progenitors and unidentified mononuclear haematopoietic cells, which may be osteoclast precursors (80). Recently, non-nuclear oestradiol receptors have also been described in the

osteoblast (101), although their role in oestrogen deficiency bone loss has not yet been investigated.

In the isolated osteoclast, oestradiol treatment directly inhibits bone resorption (38,102) decreasing pit number per osteoclast and lysozyme transcription (103). This is consistent with the effects of oestradiol treatment in vivo in both oophorectomised rats (Chapter 1.1.7) and post-menopausal women (Chapter 1.1.4).

The majority of studies of oestradiol action on cultured osteoblasts and osteoblast-like calvarial cells indicate that oestradiol stimulates osteoblast proliferation (99,104-106). Oestradiol also increases transcription of type I collagen (36,104-106), Transforming growth factor β (TGFβ) (36) insulin-like growth factor-1 mRNA (104), and increases alkaline phosphatase activity (98) in osteoblasts at physiological concentrations. Increased osteoblast activity and proliferation in response to oestradiol treatment in vitro appears to be in direct contrast to effects of systemic oestradiol administration in vivo (50,84).

In the UMR106-06 osteoblastic cell line, oestradiol has been reported to inhibit osteoblast growth during the proliferative phase (98,107). This was observed over the same concentration range as in the calvarial cell studies described above. It is likely the decrease in UMR106-06 proliferation in the presence of oestradiol is due to the high level of proliferation in this cell type. Over a 24 h period, vehicle treated UMR106-06 cells increase in number by approximately 81.4%/day, whereas in calvarial culture, the increase is approximately 42%/day (105). The higher level of osteoblast proliferation in the UMR106-06 cell line may be more indicative of the level of osteoblast proliferation occurring in oestrogen deficiency in vivo, in which case oestrogen inhibition of proliferation is consistent with the effect of oestradiol treatment in oophorectomised rats. The UMR106-06 cell line is a less mature cell line than most other types. This may indicate that oestradiol has different effects at

different stages of bone cell maturation, such that proliferation is inhibited by direct oestradiol action on osteoblasts at an early stage of development.

The possible disparity between the action of oestrogen in vitro and in vivo on osteoblasts may be explained by a number of theories. As mentioned above, oestradiol may have different actions at different stages of osteoblast development (98,228), in different osteoblast sub-populations (104), or in different compartments of bone (47,57,58). Different levels of response may be regulated by varied expression or responsiveness of the osteoblastic oestradiol receptor during changes in cell density, proliferation rate, cell cycle phase or differentiation state. It has been suggested that cells from oophorectomised rats may behave differently to those from intact rats in vitro, however the effects of oestradiol on bone cell cultures prepared from oophorectomised rats do not differ in response to cells from intact rats (104).

The effects of oestradiol in vivo may result from the accumulation of numerous effects, relating also to mediation of oestradiol effects by other locally acting factors. It is relevant to note that in vivo, the effects of oestradiol treatment have not been reported prior to 21 days after commencement of treatment, when transient effects such as those reported in vitro may have already occurred.

While in vivo evidence supports an inhibitory effect of oestradiol on the osteoblast, it is possible that oestradiol may stimulate proliferation of isolated osteoblasts and stimulate bone formation in vitro. In the presence of osteoclasts however, the inhibition of osteoclast function by oestradiol may be dominant, and as a consequence of osteoblast / osteoclast coupling, bone formation would be reduced. Oestradiol in vivo may act directly on osteoclasts to inhibit bone resorption. Oestrogen regulation of the production or secretion of some locally acting factor may result in indirect oestrogen inhibition of osteoblast activity. As well, oestrogen may have an autocrine / paracrine role,

where altered transcription of local factors by bone cells affects bone cell activity.

1.1.9 The Role of Local Factors in Oestrogen Action on Bone

As suggested above, the difference between in vitro studies of oestradiol treatment and the action of systemic oestradiol treatment of oophorectomised may be explained by a role of locally acting factors on bone turnover. The possible contributions of interleukins 1 and 6 and transforming growth factor ß will now be discussed in detail.

1.1.9.1 Interleukin - 1

Interleukin-1 (IL-1) receptors have been located in osteoblasts and osteoblast-like cells (108,109). IL-1 treatment of osteoblasts in vitro results in stimulated proliferation (110-112), as well as increased collagen synthesis (112) and alkaline phosphatase activity (113). However, other studies have demonstrated IL-1 inhibition of osteoblast proliferation (113,114), collagen synthesis (111,112,114) and osteocalcin synthesis (115). This effect may be dose dependent since at higher concentrations of IL-1, bone formation is reduced and the stimulatory effect of IL-1 is only seen at low concentrations (111).

IL-1 also increases bone resorption in vitro (116-120). The effect of IL-1 on osteoclasts appears to be mediated by osteoblasts since disaggregated osteoclasts only respond to IL-1 in the presence of calvarial cells, or UMR106 osteoblast-like cells (120).

The stimulatory effect of IL-1 on bone resorption and formation has also been observed in vivo (121,122). IL-1 also appears to increase production of TGFβ (119) and IL-6 (123) in vitro. These factors also have effects on bone turnover and may be involved in the effect of oestradiol on bone. These effects would

be consistent with a model of IL-1 mediation of increased bone turnover after oophorectomy.

IL-1 is spontaneously released by peripheral mononuclear cells following oophorectomy in humans (124,125). Dynamic markers of bone formation rate at both the tissue and BMU level, and the extent of bone formation surface were significantly greater in subjects with high IL-1 levels, indicating IL-1 may play a role in stimulating bone formation in oestrogen deficiency (125). The increase in circulating IL-1 may also be prevented by ovarian hormone treatment (126).

IL-1 production is also elevated in the oophorectomised rat (127). The rise in IL-1 in oestrogen deficiency may be prevented by oestradiol treatment (127). It is possible therefore that oestradiol modulates bone turnover by inhibiting IL-1 production. Recently it has been demonstrated that an IL-1 receptor antagonist does not inhibit bone loss observed in oophorectomised rats, however the authors suggested the method of IL-1 receptor blockage was not successful, and suggest, IL-1 or some other factor may still be implicated in the pathogenesis of oestrogen deficiency bone loss (128).

1.1.9.2 Interleukin - 6

Interleukin-6 (IL-6) is another locally acting factor produced by haematopoietic cells and osteoblasts (129) and may mediate some effect of oestradiol on bone. Although receptors have not been located yet, IL-6 appears to act directly on osteoclasts, inducing bone resorption on bone slices in vitro (130).

After oophorectomy in the human, IL-6 is spontaneously released into the circulation by peripheral haematopoietic cells (124). There may also be an increase in osteoblastic IL-6 production. A similar occurrence has been suggested in the oophorectomised mouse, where the increased osteoclast

proliferation normally associated with oestrogen deficiency may be prevented by administration of IL-6 antibody (45). In vitro, 17β-oestradiol reversibly inhibits osteoblastic IL-6 production (131,132). These results indicate a possible role of IL-6 in oestradiol modulation of osteoclast development.

1.1.9.3 Transforming Growth Factor-β

Transforming growth factor β (TGFβ) is a growth regulatory peptide abundant in the bone matrix and, like the interleukins, is produced by osteoblasts (230-232). Local TGFβ administration in vivo increases bone formation (134-136). The effects of TGFβ in vitro appear biphasic, depending on cell density in culture (137) and TGFβ concentration. At low concentrations, TGFβ stimulates both osteoblast and osteoclast proliferation (138), as well as increasing transcription of osteopontin, an osteoblast specific protein (139), and the rate of collagen synthesis on vitro (140-142). At very high concentrations TGFβ has been reported to inhibit osteoclast proliferation (143,230) and bone resorption (144).

Oestradiol treatment in vitro stimulates TGFβ production by mature osteoblasts (36,233). This finding is in contrast to increased TGFβ production in oophorectomised rats, and prevention of this increase by oestradiol treatment (46). TGFβ administration to oophorectomised rats also prevents the oophorectomy-induced rise in osteoclast precursor proliferation, although such administration did not prevent bone loss (69), suggesting oestradiol may act on osteoblasts, stimulating TGFβ production, thereby modulating bone cell proliferation.

1.2 The Effects of Calcitonin and PTHrP(107-139) on Bone Metabolism

1.2.1 The Effect of Calcitonin in Vitro

1.2.1.1 The Effect of Calcitonin on the Osteoclast

Time lapse photography has shown that within a few minutes of calcitonin administration to isolated osteoclasts, cell motility is decreased (145). Ruffling of extended processes associated with bone resorption ceases completely and the inactive processes gradually retract (145). The area covered by the osteoclast (cytoplasmic spreading) is also reduced with calcitonin treatment (146).

Calcitonin inhibits osteoclast proliferation in vitro. This appears to occur by slowing fusion of osteoclasts to mononuclear osteoclast precursors (Chapter 1.1.2), such that osteoclast formation is limited to fusion of newly formed mononuclear precursors, a much slower process (147). A decrease in osteoclast nuclei number may also indicate an increase in osteoclastic fission to their mononuclear components (148).

Consistent with decreased osteoclast motility and inhibited osteoclast formation, calcitonin treatment in calvarial culture inhibits induced bone resorption (147,149). Basal osteoclastic resorption on bone slices is also inhibited by calcitonin treatment (150). In the cell suspensions used in these studies osteoclasts are regarded as being apart from the influence of other cell types in bone, further supporting a model of a direct effect of calcitonin on osteoclasts. This finding is consistent with the rapid behavioural change induced by calcitonin (145,146), and localisation of calcitonin receptors in osteoclasts (151).

Addition of calcitonin to pure osteoclast culture results in an initial elevation of adenylate cyclase activity and intracellular cAMP concentration (152). Although the exact mechanism of calcitonin action is not known, this rise in cAMP probably initiates a cascade of biochemical events, starting with protein kinase activation (153). As indicated by behavioural changes, cellular RNA synthesis is decreased (148). In the presence of calcitonin, both synthesis and release of osteoclastic enzymes, such as acid phosphatase, are reduced (154).

Calcitonin inhibition of osteoclastic formation and bone resorption in vitro appears to be transient, even with addition of fresh calcitonin (147,149). This phenomenon, termed "escape", is probably due to down-regulation of osteoclastic calcitonin receptors (155).

1.2.1.2 The Effect of Calcitonin on the Osteoblast

There is evidence that calcitonin also enhances osteoblast development in vitro. Calcitonin receptors are present in UMR106-06 osteoblast-like cells (156), indicating their transient presence early in osteoblast differentiation (156,157). Calcitonin treatment of isolated osteoblast-like UMR106-06 cells increases cAMP production (158,159) and cellular activity of alkaline phosphatase, an osteoblast-specific enzyme involved in bone formation (159). cAMP was also increased in an osteoblastic stem cell line treated with calcitonin (160). Calcitonin treatment also increased ^{3}H-thymidine incorporation into MC-3T3-E1 cells, an osteoblast-like stem cell line (161), providing further evidence for calcitonin regulation of osteoblast proliferation early in development.

1.2.2 Calcitonin Treatment of Post-Menopausal Women

The osteoclast inhibitory properties of calcitonin in vitro has lead to the use of calcitonin in treatment of bone loss following oophorectomy (162) or the

menopause (163-167), particularly in the treatment of post-menopausal osteoporosis (168,169) as an alternative to hormone replacement therapy.

Intranasal salmon calcitonin treatment, at doses between 50 - 100 IU / day has significant effects after 12 months of treatment. Further bone loss is prevented in the lumbar spine of post-menopausal women (164,166,167), although such an effect is not observed at peripheral sites or throughout the skeleton as a whole (166). Calcitonin treatment for 12 months at 50 IU/day appears to prevent bone loss by reducing bone resorption as indicated by lower levels of urinary hydroxyproline excretion, a product of collagen breakdown released during bone resorption (167). At a higher dose of 100 IU/day markers of bone formation are also reduced after 12 months of treatment (164). The reduction in both resorption and formation is not consistent with the stimulatory effect of calcitonin reported in osteoblasts in vitro and may result from inhibition of resorption and coupling between resorption and formation in vivo. A decrease in bone turnover in women treated with calcitonin at 100 IU / day is not consistently reported however (166), suggesting calcitonin treatment may induce transient uncoupling of bone resorption and formation. Alternatively, the decrease in bone resorption may occur some time before the decrease in bone formation, and may explain the transient increase in bone content reported (164,167). There is little information however on the temporal effects of calcitonin on bone turnover in post-menopausal women, and significant alterations in bone turnover have not been reported prior to 12 months after commencement of treatment.

It has been suggested that response to calcitonin treatment is enhanced in oestrogen deficiency since the reduction in serum calcium induced by a single calcitonin injection was amplified after hysterectomy or oophorectomy (42). This may not necessarily indicate a role of calcitonin in the pathogenesis of post-menopausal bone loss (Chapter 1.1.6) but may indicate a variation in the response of bone to elevated levels of calcitonin. This probably relates to the

elevation in bone cell activity in oestrogen deficiency. Alternatively, this may result from inhibition of bone resorption and a greater proportion of serum calcium in oophorectomised women arising from the elevated bone resorption.

1.2.3 Calcitonin in the Intact Rat

Administration of a single dose of calcitonin to intact rats results in a rapid decrease in serum calcium (170-172). This effect lasts for approximately 5 h (173). At 8 h the change in serum calcium is no longer detectable (174) and levels of serum calcium are increased at 24 h after calcitonin injection (170). This rebound in serum calcium is probably due to an increase in PTH levels detected at 5 h after calcitonin administration (173) due to the hypocalcaemia, suggesting a dominant effect of PTH over calcitonin.

When calcitonin is present continuously, as in an infusion, the reduction in serum calcium is also transient. In one study, osmotic minipumps were used to infuse calcitonin at a rate of approximately 0.72 IU / day for one week (175). After one day of salmon calcitonin infusion, rats were hypocalcaemic. For the remainder of the experiment however, serum calcium had returned to normal levels despite the continued presence of calcitonin such that circulating calcitonin levels were the same as at day 1.

The transient nature of the response of serum calcium to continuous calcitonin treatment is consistent with the "escape" phenomenon described in vitro (155). It would be more likely however in the intact rat for a number of homeostatic mechanisms to be available to prevent a continued hypocalcaemia. Homeostatic control mechanisms are not apparent immediately, such that the reduction in serum calcium induces an elevation in PTH secretion, returning serum calcium to normal levels or greater, as observed after a single bolus injection. The role of other homeostatic mechanisms in the intact rat is supported by recurrence of the hypocalcaemia

observed in calcitonin-infused rats when parathyroid glands were removed (175).

Osteoclast nuclei number are decreased at the same time as the reduction in serum calcium and is significantly lower for the first 2 h after calcitonin injection (170-172). The reduction in osteoclast nuclei number appears to be caused by fission of existing osteoclasts to their mononuclear component cells (172), consistent with inhibition of osteoclast precursor fusion reported in vitro (147). Osteoclast inhibition in the presence of calcitonin also appears to be transient since osteoclast number is increased at 5 h, 8 h and 24 h after calcitonin injection (170,173,174). Again, PTH appears responsible for this rebound effect since parathyroidectomy prevented the secondary increase in osteoclast number (173). It is likely that although bone resorption may initially have decreased after calcitonin treatment in these studies, the hypocalcaemia induced by calcitonin may stimulate resorption overall via increased PTH secretion, such that serum calcium is increased.

Daily calcitonin treatment in vivo causes a reduction in bone formation, indicated by decreased levels of serum osteocalcin, and tetracycline labelled bone surface after 42 days of treatment (173). The lower level of bone formation detected in calcitonin treated rats in the presence of a high level of bone resorption suggests calcitonin treatment uncouples osteoclast and osteoblast activities, possibly by a specific calcitonin effect on osteoclasts alone, or direct and different effects on both osteoclasts and osteoblasts, as suggested by in vitro studies. However, the effect of calcitonin on osteoblasts in vivo is inconsistent with increased alkaline phosphatase production in the presence of calcitonin in vitro (159). The contrast between results obtained in vitro and in vivo may be explained by the presence of systemic calciotropic hormones. The initial hypocalcaemia caused by calcitonin treatment may elicit an increase in PTH secretion in an attempt to restore normocalcaemia. Parathyroidectomy appears to prevent the reduction in bone formation

observed after calcitonin injection in vitro, suggesting the effect of PTH on bone turnover induced by hypocalcaemia dominates the effect of calcitonin on bone turnover in the long term.

Daily calcitonin treatment appears to result in a negative calcium balance. The increase in bone resorption and formation observed in the calcitonin treated rat results in a reduction in trabecular bone volume after 42 days of treatment (173). This result is inconsistent with the effect of calcitonin both in vitro, and in the post-menopausal woman (163,164,166,167). The difference between the in vitro and in vivo results may again be explained by the role of PTH in maintaining serum calcium levels in vivo, since parathyroidectomy prevented the reduction in trabecular bone volume caused by calcitonin administration. It is puzzling then, that PTH does not appear to have the same effect on bone turnover and bone volume changes induced by calcitonin in the post-menopausal woman. The reason for this is unclear, however it may be more useful to examine the effect of calcitonin in the oophorectomised rat compared to sham animals to determine whether bone responds differently to calcitonin / PTH in the absence of oestrogen.

1.2.4 Calcitonin Treatment in the Oophorectomised Rat

In the oophorectomised rat, calcitonin treatment of 16 IU on alternate days from time of operation caused decreases in both bone resorption and formation. Osteoclast, osteoblast and osteoid surface were reduced to levels observed in sham operated rats, and bone loss in the early oestrogen deficient state was prevented (177). While this is consistent with effects on the early post-menopausal bone loss (163,164,166,167), the decrease in bone resorption and maintenance of bone volume is inconsistent with the increased level of resorption and bone loss observed in intact rats (173,174). Wronski et al have suggested calcitonin may suppress bone turnover only when the level of bone turnover is elevated, as in oestrogen deficiency (177). However, in

vitro studies have shown that calcitonin is effective not only in suppressing stimulated levels of bone resorption (147), but also in inhibiting the basal level of bone resorption (150).

The difference between the response of oophorectomised and sham operated animals to calcitonin treatment may be due to the number of active osteoclasts on the bone surface. In oophorectomised rats, active osteoclast surface is increased (Chapter 1.1.2), such that the effect of calcitonin in oophorectomised rats will be more pronounced than in sham operated rats, resulting in enhanced hypercalcaemia following calcitonin injection in oophorectomised rats compared to ovary-intact controls (79,176).

Alterations in response to calcitonin in sham operated and oophorectomised rats may also be explained by changes in the calcitonin receptor number. Osteoblastic production of TGFβ is inhibited in oophorectomised rats, but not in oophorectomised rats treated with oestradiol (46), consistent with effects of oestradiol on TGFβ production in vitro (36). As well as regulating bone cell activity as discussed in Chapter 1.1.9.3, high concentrations of TGFβ have been shown to result in down regulation of osteoblastic calcitonin receptors in vitro (178). This may indicate that in the oophorectomised rat, where TGFβ levels are reduced, the calcitonin receptor may respond differently to the response in sham operated animals where TGFβ levels are high.

This concept has not been properly investigated in vivo however, and no studies have directly compared the effect of daily calcitonin administration on bone metabolism in intact and oestrogen deficient rats.

1.2.5 The Effect of Parathyroid Hormone Related Protein (107-139) on Bone

Parathyroid hormone related peptide (PTHrP) is produced by a variety of human tumours and causes the clinical syndrome of humeral hypercalcaemia

of malignancy (179,180). Homology of the peptide with PTH is restricted to the amino-terminal region, where 8 of the 13 amino acids are identical to the amino-terminal region of PTH. Such homology allows PTHrP to cause hypercalcaemia by interacting with osteoblastic PTH receptors to indirectly stimulate bone resorption, and interact with renal PTH receptors, to restrict calcium excretion as well as promoting phosphate and cAMP excretion (181-183).

The effect of PTHrP on mixed osteoblast / osteoclast culture appears biphasic however. At lower concentrations, PTHrP stimulates bone resorption, whereas at high concentrations bone resorption is inhibited (184). Also, in pure osteoclastic preparations, PTHrP inhibits bone resorption (). These differing activities of PTHrP appear to be located in different regions of the peptide. The amino terminal region (residues 1 - 34) stimulates bone resorption via osteoblastic PTH receptors (185). The carboxy terminal region however (residues 107 - 139) appears to inhibit bone resorption specifically suggesting the presence of osteoclastic receptors (186). PTHrP(107-139) reduces the number of resorption pits per osteoclast as well as decreasing osteoclastic spreading (184), an effect similar to that of calcitonin on osteoclasts (146). In contrast to the actions of calcitonin, PTHrP(107-139) has no effect on osteoblastic cells (187).

To date, the effects of PTHrP(107-139) have not been investigated in vivo. I have used this peptide in an attempt to inhibit the level of bone resorption in the oophorectomised rat.

1.3 Hypotheses and Aims

1.3.1 The Effects of Oestrogen Deficiency and Oestradiol Treatment on Bone Turnover and Calcium Homeostasis

In this thesis, the 6 month old oophorectomised rat has been used as a model to study the initial rapid phase of cancellous bone loss in oestrogen deficiency. It is hypothesised that the increase in bone remodelling previously reported in the oophorectomised rat is the cause of cancellous bone loss, and the rise in bone resorption and formation is anticipated to occur prior to or concurrent with the reduction in cancellous bone volume. It was aimed to characterise the time frame and nature of the disturbance in bone turnover over the course of the initial loss of bone. This was investigated at both the systemic level, by measuring biochemical markers of bone turnover, and by histomorphometric analysis in the femur. Morphometric markers of cancellous bone volume were used to determine the nature of the bone loss in the distal femur in oestrogen deficiency.

Changes of renal calcium and phosphate metabolism have been hypothesised to be involved in oestrogen deficiency bone loss, although such changes may be secondary to changes in bone turnover. Serum and urine calcium and phosphate levels were examined in both the oophorectomised rat, and after oestradiol treatment, to investigate the manner in which changes in renal calcium metabolism relate to the initial phase of cancellous bone loss in oestrogen deficiency. Levels of the various serum calcium fractions were also measured in oophorectomised rats.

Oestrogen deficiency bone loss can be prevented by treatment with oestradiol. While the changes in calcium and bone metabolism with oestrogen treatment described above are well established, the interactions of oestrogen with bone or other centres of calcium metabolism remain poorly understood. It is

hypothesised that oestradiol acts directly to inhibit both osteoblast and osteoclast activity. The effects of oestradiol treatment in oophorectomised rats on levels of bone resorption and formation over the time period when bone turnover is initially increased or during the time when bone loss is first detected after oophorectomy have not been reported and such studies have been performed in this thesis. Biochemical and Histomorphometric variables were measured. In vitro studies have indicated that oestradiol may have various effects at different stages of osteoblast maturation, a number of markers of osteoblast activity were used to investigate this further. The effect of oestradiol treatment on trabecular bone morphometry was also investigated.

Oestradiol treatment also prevents the changes in the calcium economy reported in oestrogen deficiency. There are no published data describing the effects of oestradiol treatment on the changes in calcium and phosphate metabolism in the oophorectomised rat to date. Consequently, such changes were characterised over the time frame of the initial increase in bone turnover and loss of bone.

1.3.2 The Effects of Calcitonin and PTHrP(107-139) on Bone Turnover and Calcium Homeostasis

Calcitonin has been reported to inhibit osteoclastic bone resorption and stimulate osteoblast development in vitro. These properties of calcitonin have lead to its use in treatment of oestrogen deficiency bone loss. However, studies in the rat model have suggested the effects of calcitonin depend on ovarian status. In intact rats, calcitonin has been reported to inhibit bone formation, and stimulate bone resorption, whereas in the oestrogen-deficient rat, both bone resorption and formation are reduced with calcitonin treatment. No studies have directly compared the effect of daily calcitonin administration on bone metabolism in intact and oestrogen deficient rats. This thesis has investigated in a controlled study the nature of the effects of calcitonin in

oophorectomised and ovary-intact rats. The effects of calcitonin have also been compared to the effects of oestradiol, both remodelling inhibitors, on bone turnover, bone loss and calcium metabolism in oestrogen deficiency.

PTHrP(107-139) has been reported to specifically inhibit bone resorption without any effect on osteoblast activity in vitro. To date, the effects of PTHrP(107-139) have not been investigated in vivo. The effects of this peptide on bone turnover, bone loss and calcium homeostasis have also been investigated and compared with the effects of calcitonin and oestradiol in the oophorectomised rat.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Animals

Six month old female Sprague Dawley rats (Gilles Plains Animal Resource Centre, Gilles Plains, South Australia) were used for all experiments. At this age, bone turnover has reached a stable adult level (47,72,75). All animal procedures were approved by the IMVS Animal Ethics Committee.

2.2 Diet

Rats were supplied with tap water ad libitum and 15 g/day commercial rat chow containing 0.76% calcium, 0.46% available phosphorus and 2000 IU/kg vitamin D_3 (Milling Industries, South Australia) between 0900 and 1200 h. The amount of food supplied was slightly below the average daily food consumption of 16.3 g/rat/day determined over a 2 week period. Dietary restriction was particularly important in this study since oophorectomised rats tend to eat more food and gain more weight than sham operated controls (47,53,58). Even pair feeding of oophorectomised rats with sham rats does not prevent the increase in body weight associated with oophorectomy (188). Weight gain does not appear to stimulate bone loss in oophorectomised rats, the increase in weight appears to protect, albeit incompletely against bone loss, probably by increasing mechanical strain and stimulating bone formation in some areas of the skeleton (188).

2.3 Interpretation of Biochemical Markers of Bone Turnover

Levels of a number of parameters measured in the serum and urine may be used as biochemical markers of bone turnover and calcium homeostasis

throughout the body. These markers indicate total levels of bone turnover throughout the body, in contrast to histomorphometric markers that are measured in specific sites of the skeleton.

Biochemical markers are used extensively in the diagnosis and management of metabolic bone disease in humans. All markers used in this thesis are well established in routine use, and have been shown to be sufficiently reproducible in post-menopausal women to be clinically and physiologically meaningful (189). In animal-based experiments, the use of biochemical markers enables examination of bone turnover at a number of time points without requiring the killing of a large number of animals.

The total serum calcium is made up of a protein bound fraction and an ultrafiltrable fraction comprising complexed and ionised components. The ionised calcium is under homeostatic control by the parathyroid hormone / vitamin D system, and possibly calcitonin. Complexed calcium is bound to citrate, phosphate, lactate, sulphate, bicarbonate and other anions. Protein bound calcium is bound to albumin and globulin.

Urinary calcium excretion is dependent on dietary calcium absorption and renal reabsorption. To avoid the dietary influence on urine calcium, levels were measured in specimens collected after food had been withdrawn for 18 h.

Serum phosphate concentration is a function of phosphate input and renal tubular reabsorption of phosphate and a negative function of glomerular filtration rate. Measurement of the urine and serum phosphate simultaneously allows calculation of the renal tubular reabsorption of phosphate (TmP).

The most commonly used marker of bone resorption is urinary hydroxyproline excretion. Hydroxyproline is a major constituent of bone, making up approximately 50% of the content of bone. Hydroxyproline is released into the

circulation from collagen breakdown during osteoclastic bone resorption. Although approximately 80% of hydroxyproline is metabolised by the liver, the excretion of the residual amount reflects the level of bone resorption. A proportion of hydroxyproline is derived from other sources such as the skin or the liver, but again, production from these sources does not appear as variable as that derived from bone.

Expression of urine excretion of hydroxyproline, phosphate and calcium as the ratio to urine creatinine corrects for dilutional errors. However, where either muscle mass or glomerular filtration rate varies the urine excretion has been calculated by multiplying the ratio to urine creatinine by the plasma creatinine level. Such a variable expresses the urine excretion per litre of glomerular filtrate (GF) and allows assessment of excretion independently of the glomerular filtration rate.

Alkaline phosphatase is produced by osteoblasts during the early differentiation stage of osteoblast development (190). Serum alkaline phosphatase arises from both bone and liver, particularly in disease states. When liver disease is not present, any rise in alkaline phosphatase activity indicates a rise in bone formation.

Osteocalcin is found in considerable quantities in mature bone and is produced by osteoblasts during bone mineralisation (190). Serum osteocalcin represents the fraction of newly synthesised osteocalcin that fails to bind to hydroxyapatite in the extracellular matrix before diffusing away from the mineralising site into the extracellular fluid. Serum osteocalcin does not arise from resorption of the extracellular matrix of bone (191). Osteocalcin is cleared rapidly by the kidney, with a half-life in the circulation of about 5 minutes, and therefore may detect transient fluctuations in bone metabolism (192).

2.4 Serum and Urine Analyses

Serum levels of total sodium and potassium were determined by ion selective electrodes. Serum chloride was determined in samples mixed with an equilibrium solution of ferric, mercuric, and thiocyanate ions, which undergoes a double displacement reaction, forming the red-brown complex, ferric thiocyanate. The end-point measurement was read at 476 nm. Bicarbonate levels were measured in acidified samples, such that gaseous carbon dioxide was liberated and passed through an alkaline buffer where bicarbonate ions were formed and detected by phenolphthalein indicator. Serum phosphate was measured by the formation of a phospho-molybdate complex, measured photometrically at 340 nm. Serum calcium was measured by colourimetry with cresolphthalein complexone, a metal complexing dye, in the presence of 5-hydroxy-quinoline, used to prevent interference by binding magnesium. Bromocresol green was used to preferentially bind serum albumin at pH 4.2, causing a shift in the absorption spectrum, measured after 30 seconds at 604 nm. Total serum protein was determined in an alkaline copper sulphate solution by the level of copper ion complex formed. The shift of spectral absorption was measured photometrically. Creatinine was detected by reaction with picric acid in an alkaline medium to produce a red coloured complex, and the rate of formation of this complex was measured at 500 nm on a first-order reaction curve. Alkaline phosphatase activity (ALP) was detected by hydrolysis of p-nitrophenyl phosphate to form p-nitrophenol. The rate of p-nitrophenol formation was followed by absorption at 404 nm. The above analyses were performed on Technicon SMAC II (Tarrytown, New York, USA).

Serum osteocalcin was measured by radioimmunoassay, based on the method of Price and Nishimoto (193), as described by Morris et al (51). Bovine osteocalcin antisera were raised in rabbits by multiple injections of osteocalcin / bovine serum albumin conjugate. Standard concentrations were established by quantitative amino acid analysis. Parallel displacement of bovine osteocalcin standards by rat osteocalcin confirmed cross reactivity. The lower limit of

detection of the assay was 1.1μg/L and the interassay coefficient of variation at 14.4μg/L was 15%.

Serum 17β-oestradiol was measured in samples extracted with 60% cyclohexane / 40% ethyl acetate and reconstituted in human charcoal stripped serum using a commercial radioimmunoassay kit (Spectria, Orion Diagnostica, Espoo, Finland). N-terminal parathyroid hormone levels (INS-PTH) were measured in serum using a commercial radioimmunoassay kit (Nichols Institute Diagnostics, San Juan Capistrano, California, USA).

Urine creatinine was measured in fresh specimens on Olympus Reply (Integrated Sciences, Sydney, Australia) by the picric acid reaction. The remaining urine specimen was acidified to pH < 1 to dissociate complexed calcium and phosphate. Urine hydroxyproline was determined following acid hydrolysis by the colourimetric method of Bergman and Loxley (194). Urine phosphate was determined in acidified samples by colourimetry with molybdate (195) on Roche Cobas Bio (Roche, Switzerland) centrifugal analyser. Urine calcium was determined in acidified samples by atomic absorption spectroscopy.

2.5 Biochemical Calculations

Anion gap was calculated as the difference between the sum of serum sodium and potassium and the sum of serum chloride and bicarbonate. Globulin was calculated as the difference between serum total protein and albumin. Serum calcium fractions were calculated from serum levels of calcium, albumin, globulin, bicarbonate, and anion gap using an iterative computer program, and the following formulae derived from linear regression analysis of data from post-menopausal women (28).

Ionised calcium = total serum calcium - protein bound calcium - complexed calcium

Where:

$$\text{Protein bound calcium} = \frac{0.01257 \times (\text{ionised calcium}) \times \text{albumin}}{1 + (0.01257 \times (\text{ionised calcium}))} + \frac{0.0049 \times (\text{ionised calcium}) \times \text{globulin}}{1 + (0.0049 \times (\text{ionised calcium}))}$$

and:

$$\text{Complexed calcium} = \frac{0.00835 \times (\text{ionised calcium}) \times (\text{anion gap})}{1 + (0.00835 \times (\text{ionised calcium}))} + \frac{0.00759 \times (\text{ionised calcium}) \times \text{bicarbonate}}{1 + (0.00759 \times (\text{ionised calcium}))}$$

and ultrafiltrable calcium = complexed calcium + ionised calcium.

(Serum albumin and globulin are in mg/L, all other units are mmol/L)

Urinary excretion of hydroxyproline (OHPrE) and excretion of phosphate (PE) were calculated by multiplying the ratio to urine creatinine by serum creatinine levels. Glomerular filtration rate (GFR) was calculated by the ratio of the 24 h urine creatinine to serum creatinine expressed in ml/min. Maximal renal tubular phosphate reabsorption (TmP) was calculated by the method of Marshall (196) as follows:

$$\text{TmP (mmol/L)} = \frac{(\text{serum phosphate - phosphate excretion})}{1 - (0.01 \times \log_e \left(\frac{\text{serum phosphate}}{\text{phosphate excretion}}\right)}$$

2.6 Blood and Urine Sampling Protocol

The effect of collecting blood and 24 h urine specimens at 3 day intervals, on the reproducibility of biochemical variables was determined by collecting specimens from 10 rats at 3 day intervals for a total of 12 days.

Food was withheld from animals for 44 h before blood collection and 20 h before a 24 h urine collection was commenced. Urine samples were collected from rats housed in individual metabolic cages. Unlimited water was supplied to all animals. Animals were weighed after the 24 h urine specimen was collected. Blood was collected from the tail vein under halothane anaesthesia. When animals were killed, blood was collected by cardiac puncture under halothane anaesthesia. Whole blood was centrifuged to obtain serum and serum and urine samples were stored at -70°C until analysis. Blood and urine specimens were analysed as described above.

Rats lost approximately 20 g body weight over the 12 day collection period, with a significant decrease in serum albumin and alkaline phosphatase levels (Table 2.1). The changes were exacerbated as blood sampling was continued. Serum levels of calcium and phosphate were significantly increased but osteocalcin remained unchanged (Table 2.1). Calcium excretion was

Table 2.1 Change in weight and serum biochemistry at 3 day intervals over 12 days.

	3 days	6 days	9 days	12 days
Weight (g)	300 ± 8	291 ± 8[a]	285 ± 8[a]	278 ± 8[a]
Anion Gap (mmol/L)	16.6 ± 0.4	15.4 ± 0.5[a]	18.1 ± 0.3[a]	15.7 ± 0.3[a]
Albumin (g/L)	39.3 ± 0.7	37.9 ± 0.6[a]	38.0 ± 0.6[a]	36.4 ± 0.4[a]
Calcium (mmol/L)	2.45 ± 0.02	2.57 ± 0.03[a]	2.56 ± 0.02[a]	2.54 ± 0.02[a]
Phosphate (mmol/L)	1.41 ± 0.06	1.62 ± 0.05[a]	1.75 ± 0.05[a]	1.64 ± 0.08[a]
ALP (U/L)	81.0 ± 5.4	64.7 ± 6.4[a]	71.0 ± 6.0[a]	65.2 ± 4.1[a]
Osteocalcin (μg/L)	18.27 ± 0.95	17.43 ± 1.02	16.91 ± 1.12	19.03 ± 0.75

Values are mean ± SEM weight and serum biochemical markers measured in specimens collected 36 h after food was withdrawn at three day intervals. ALP is serum alkaline phosphatase activity. a, $p<0.05$ vs. day 3 value.

Table 2.2 Change in urinary biochemistry at 3 day intervals over 12 days

	3 days	6 days	9 days	12 days
GFR (ml/day)	70 ± 13	30 ± 6	63 ± 20	61 ± 15
OHPrE (mmol/L GF)	0.50 ± 0.05	0.61 ± 0.11	0.49 ± 0.05	0.47 ± 0.04
PE (mmol/L GF)	0.14 ± 0.02	0.14 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
CaE (μmol/L GF)	18.0 ± 4.6	25.7 ± 6.0	43.6 ± 9.8[a]	41.5 ± 6.8[a]

Values are mean ± SEM urinary biochemical variables measured in 24 h urine samples collected 12 h after food was withdrawn at three day intervals. GFR is glomerular filtration rate; OHPrE, hydroxyproline excretion. PE, phosphate excretion; CaE, calcium excretion. a, $p<0.05$ vs. day 3 values.

increased on the last day of sampling (Table 2.2), but kidney function did not appear to be affected, with glomerular filtration rate as well as excretion of hydroxyproline and phosphate remaining unchanged for the duration of the experiment (Table 2.2).

These data indicate poor reproducibility of a number of serum and urine analyte levels when sampling at 3 day intervals. The changes observed include decreased serum albumin levels and reduced alkaline phosphatase activity and may relate to stress experienced by the animals due to this rigorous sampling protocol. The length of time when food was withheld from the animals required in this protocol was probably excessive, particularly since animals did not appear to drink as much water when housed in metabolic cages for urine collection. It was therefore necessary to alter the blood and urine sampling protocol.

To minimise stress in the experiments to follow, food was withdrawn 36 h before blood collection, and 12 h before 24 h urine collection was commenced. Animals were also injected intra-peritoneally with 2 ml physiological saline when blood samples were collected to replace lost fluid and prevent dehydration.

2.7 Oophorectomy and Sham Operations

Oophorectomy was performed under halothane anaesthesia. When fully anaesthetised, animals were placed in a supine position with a face mask to maintain anaesthesia and the abdominal region was shaved. A small incision (5 - 10mm) was made centrally in the abdominal wall and peritoneum. Both ovaries were exposed, clamped, occluded with surgical silk and removed with a scalpel. For sham operations, ovaries were exposed but not removed. The peritoneum was secured with Vicryl 5-0, and the skin with 3-0 surgical silk (Johnson and Johnson, Australia). The incision area was bathed in iodine, and

animals recovered in a separate cage from non-operated animals. The incision area was checked daily for the first 3 days after operation.

2.8 24 h Urinary Calcium Excretion as a Measurement of Obligatory Calcium Excretion

In humans, the minimum obligatory calcium excretion may be determined as the level of urinary calcium excretion following a 12 h fast. The level of calcium excreted in urine collected under these conditions is not affected by diet, and is determined by the glomerular filtration rate, the tubular reabsorption of calcium, and the filtered load of calcium; that is, the ultrafiltrable fraction of serum calcium, made up of the complexed and ionised calcium fractions.

One effect of the menopause observed in humans is an increase in the obligatory calcium loss (3). This increase is not simply a result of an increase in the filtered load of calcium, such as an increase in the ultrafiltrable calcium fraction (28), since it has been shown that calcium excretion is increased independent of the level of filtered calcium (30).

A significant change in renal calcium handling has not been detected in the oophorectomised rat (51,58). It has been questioned whether calcium levels in urine specimens collected from the rat reflect the obligatory calcium loss. Two factors may influence calcium excretion levels: the excessive level of calcium in the rat diet resulting in a further more labile compartment of calcium stored in bone, and the time taken for clearance of dietary calcium from the circulation.

2.8.1 The Effect of a Low Calcium Diet on 24 h Urinary Calcium Excretion

A low calcium, semi-synthetic rat diet was prepared according to the American Institute of Nutrition (AIN76) recommendations (197) modified to a calcium level of 0.15%. 10 rats were fed standard Milling Industries chow diet (0.76% Ca), and 10 rats fed the modified AIN76 diet (0.15% Ca) for 3 weeks. Food was withdrawn for 12 h before 24 h urine specimens were collected. This was followed by collection of a blood specimen.

Neither serum calcium nor urinary calcium excretion were reduced on the AIN76 semi-synthetic diet (Figure 2.1). However, levels of serum albumin and alkaline phosphatase were significantly increased in animals fed the AIN76 diet. Serum anion gap and creatinine were also significantly increased. These data may be early indications of renal failure or liver dysfunction, suggesting AIN76 diet is unsuitable for Sprague Dawley rats. The AIN76 diet has been associated with hepatic lesions and steatosis in male Fischer 344 rats (198,199), and renal calcification in young female Sprague Dawley rats (200). This is probably due to the high sucrose level in the AIN76 diet (50%) since replacement with starch prevents the changes in liver and kidney observed when rats are fed the standard AIN76 diet (201). The use of the AIN76 diet was not continued in this study.

2.8.2 Duration of Food With-holding Before Dietary Influences are Removed from Urine Specimens

The level of calcium in Milling Industries rat chow is 0.76%. At 15 g/day for each 250 g rat, the daily dietary calcium intake is 460 mg calcium per kilogram body weight, a value equivalent to approximately 40 times the recommended daily allowance for humans (202). Withdrawal of food 12 h before commencing urine collection may not provide sufficient time for removal of all dietary calcium from the circulation.

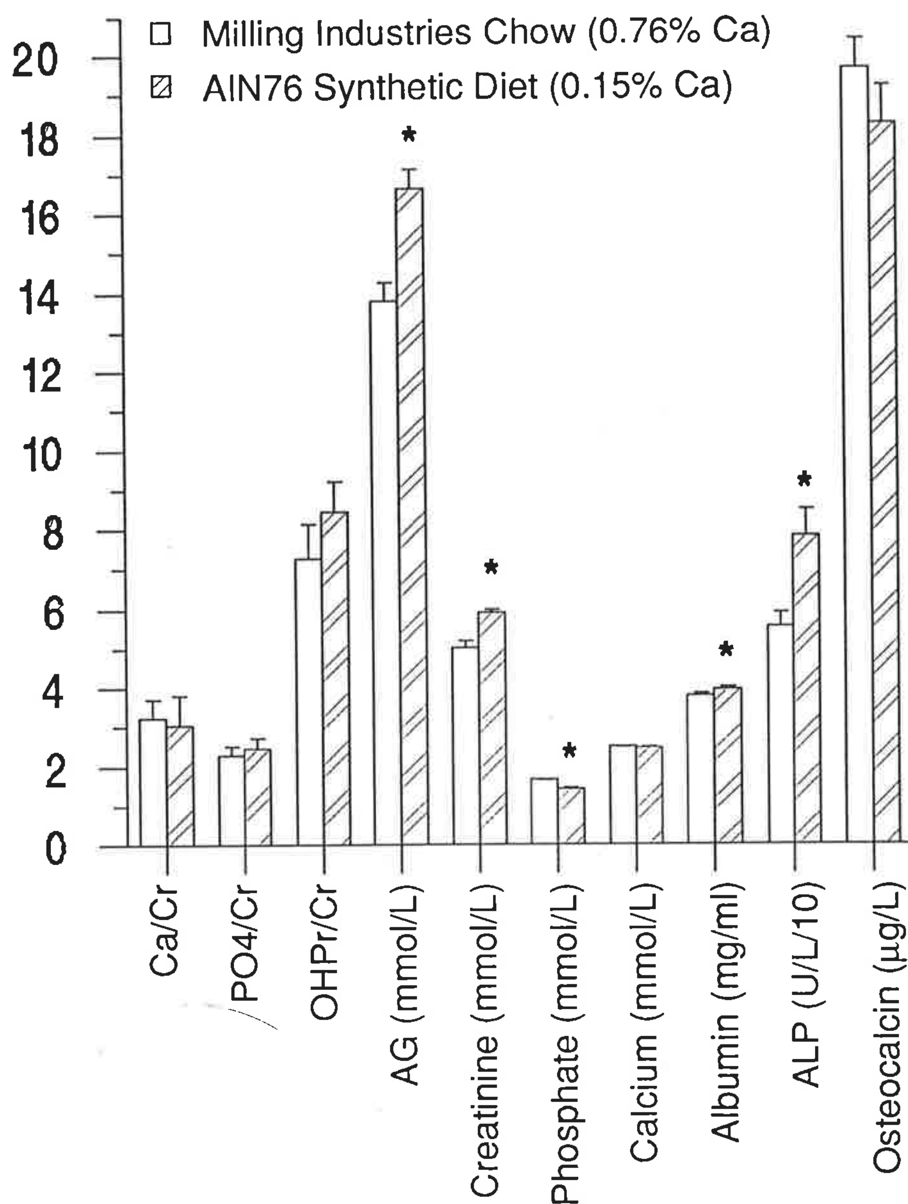


Figure 2.1. Effect of AIN76 Synthetic low calcium diet on rat serum and urine biochemistry. Urinary calcium / creatinine ratio (Ca/Cr), phosphate / creatinine ratio (PO4/Cr), and hydroxyproline / creatinine ratio (OHPr/Cr) were not affected by the change in diet. Serum anion gap (AG), creatinine, phosphate (PO4), albumin and alkaline phosphatase activity (ALP) were all altered by the change in diet. Serum calcium (Ca) and osteocalcin were not affected. Values are mean + SEM. *, $p<0.05$ vs Milling Industries Chow fed rats.

The duration of food with-holding required to remove the effect of dietary calcium from urinary calcium excretion was determined in six oophorectomised and six sham operated rats. One month after operation, food was withdrawn from rats for 12 h prior to urine collection. 24 h urine specimens were collected for 4 days in succession. Rats were supplied with unlimited water during the urine collection period, but were not supplied with food. Urinary excretions of calcium, phosphate and creatinine are expressed as ratios to urine creatinine for this experiment since blood specimens were not collected.

There was a significant reduction in urine calcium creatinine ratio (Ca/Cr) from 60 to 84 h without food in both sham operated and oophorectomised rats (Figure 2.2). Ca/Cr was lower in oophorectomised rats compared to sham at 60 and 84 h without food. Although there was some variation, there was no significant alteration in urine hydroxyproline or phosphate with time (Figure 2.3). Urine hydroxyproline/creatinine ratio was significantly higher in oophorectomised rats compared to sham at 60 and 118 h without food. Urine phosphate/creatinine was significantly greater in oophorectomised rats compared to sham at 118 h.

The late reduction in urine Ca/Cr implies the high level of dietary calcium was not fully cleared from the extracellular fluid until after 84 h, indicating that 24 h urine calcium measurements after only a 12 h period without food (a total of 36 h) do not provide accurate determinations of obligatory urinary calcium excretion. However, it is not reasonable to deprive animals of food for 84 h within the design of the following experiments, so measurements of urine calcium were omitted from the protocol.

In contrast to the urine calcium levels, the 24 h urine hydroxyproline and phosphate levels determined on specimens collected after a 12 h period

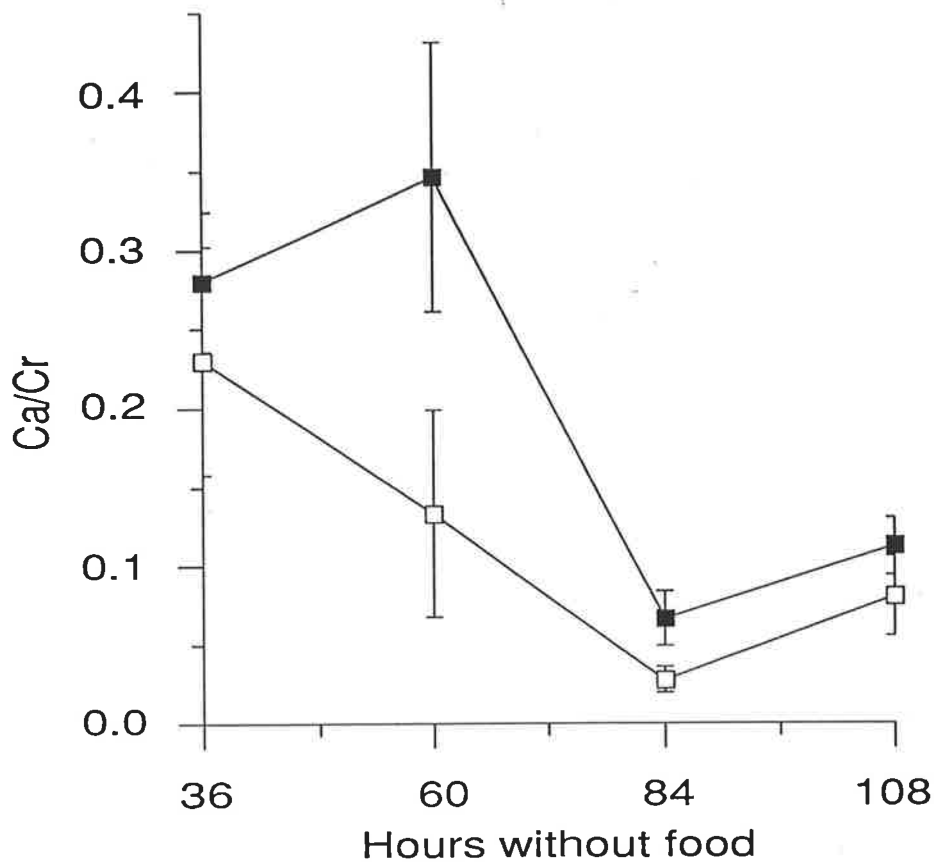


Figure 2.2 Effect of starvation on urinary calcium excretion in sham operated (■) and oophorectomised (□) rats. Urinary calcium / creatinine ratio (Ca/Cr) is shown after 36 - 108 hours of continuous starvation. There was a significant reduction in Ca/Cr between 60 and 84 h without food in both sham operated and oophorectomised rats. Ca/Cr was lower in oophorectomised rats compared to sham at 60 and 84 h. Values are mean ± SEM. *, $p<0.05$ vs 36 h, +, $p<0.05$ vs sham.

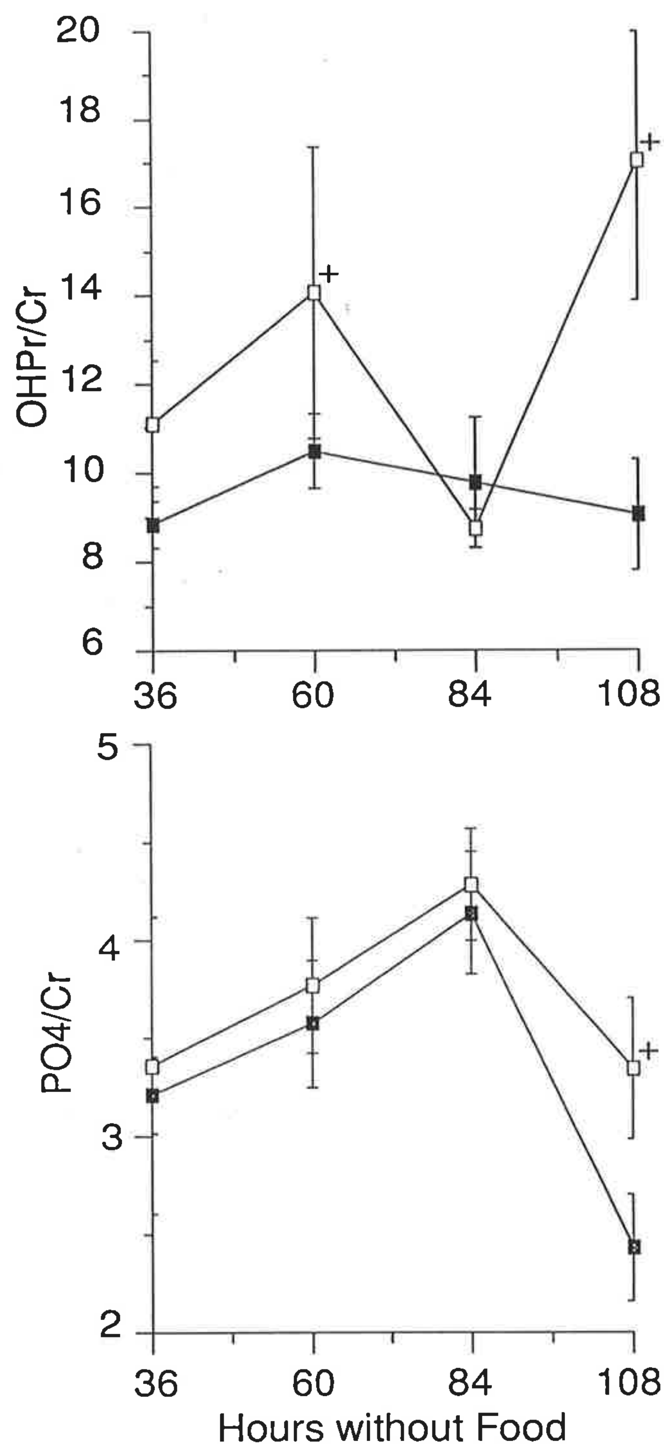


Figure 2.3 Effect of starvation on urinary hydroxyproline and phosphate excretion in sham operated (■) and oophorectomised (□) rats. Urinary hydroxyproline / creatinine ratio (OHPr/Cr) and urinary phosphate / creatinine ratio (PO4/Cr) are shown after 36 - 108 hours of continuous starvation. There were no significant changes in either variable with time in either operation group. OHPr/Cr was significantly greater in oophorectomised rats compared to sham at 60 and 108 h. PO4/Cr was significantly greater in oophorectomised rats compared to sham at 108 h. Values are mean ± SEM. +, $p<0.05$ vs sham.

without food appear to be valid since neither hydroxyproline nor phosphate excretion altered significantly with respect to time. A difference between sham operated and oophorectomised rats was not detected in these parameters after the initial 12 h fast, although in the experiments described in Chapters 4 through 7 significant differences were detected. Samples were collected one month after operation, whereas in the later experiments the time frame was shorter. Since the increase in bone resorption may be a limited event (Chapter 1.1.2), the increase in hydroxyproline excretion may no longer be significant at this time point.

Even when the effect of dietary calcium was removed, following 84 h without food oophorectomised rats still did not have a higher level of urine calcium compared with sham operated rats, in fact urine calcium appeared to be reduced in oophorectomised rats compared to sham. It has been suggested that bone loss after the menopause is caused, at least in part, by an increase in the obligatory calcium loss (31). Since urine calcium is not increased in oophorectomised rats, even 108 h after food was withdrawn, an increase in urine calcium does not appear to be a requirement for bone loss in oestrogen deficiency in this species.

2.9 Histomorphometric Techniques

2.9.1 Bone Processing - Glycol Methyl Methacrylate and K Plast Resin

For glycol methyl methacrylate embedding of bone specimens, femora and lumbar vertebrae fixed in 10% buffered formol saline overnight. Each vertebral sample and the distal third of each femur were bisected in the sagittal plane using a low speed saw (Buehler, USA), with a diamond tipped cutting blade. One half was processed as follows: 70% ethanol for 24 h, 95% ethanol for 24 h, 3 changes of 100% ethanol for 24 h each, chloroform for 24 h, 100% ethanol for 4 h, 50:25:25 mixture of 100% ethanol:glycol methacrylate:methyl

methacrylate for 4 h, followed by 2 changes of 50:50 glycol methacrylate:methyl methacrylate (GMMA) for 24 h each. Specimens were embedded overnight in GMMA resin with 0.01% initiator (benzoyl peroxide) at 37°C in plastic moulds. 5μm thick longitudinal sections were cut using a Jüng K motorised microtome (Reichert, Germany). Sections were cut in tap water to soften the resin and prevent breaks in the sections.

GMMA resin embedding caused some problems as a result of its hydrophobicity and swelling occurred while sections were cut. To overcome this difficulty, a modification to this procedure was made in experiments described in Chapter 5 when bones were embedded in K Plast (Medim-Medizinische Diagnostik, Germany), a commercial methacrylate resin (203). This resin not only eliminated the problem of swelling while sections were cut, but produced flatter sections, and allowed staining by the von Kossa method on the slide, possibly preventing trabecular breakage that may have occurred when stained as free-floating GMMA sections. The soluble property of K Plast also allowed re-embedding of sections if problems had occurred when processing.

For K Plast resin embedding, bones were fixed in cold 10% formol buffered saline for 4 hours at 4°C, then bisected as above. Halves were dehydrated in 70% acetone for 1 h, 95% acetone for 1 h, 2 changes of 100% acetone for 1 h each. Dehydrated bone specimens were infiltrated with 2 changes of K Plast solution A mixed with 8% volume solution B. Specimens were embedded in the infiltration solution containing 1.0% initiator. Polymerisation occurred in sealed polypropylene tubes overnight in a 37°C water bath. 5μm sections were cut in 50% alcohol, and stored in tap water.

K Plast sections were lifted from tap water onto gelatin coated slides, dipped in 50% ethanol, followed immediately by dipping into spreading solution (30:70 ethylene glycol mono-ethyl ether : 70% ethanol) heated to 70-75°C.

Sections were carefully teased flat and covered with a square of bagging plastic dipped in the spreading solution. Slides were separated by a square of filter paper, clamped together and dried overnight in a 37°C oven to adhere sections to slides. Acid phosphatase sections were adhered to slides after staining.

2.9.2 Use of the Quantimet 520 Image Analysis System to Determine Trabecular Bone Morphology

Photographic staining of bone sections by the von Kossa method (204), results in a high contrast section, where trabecular bone is stained dark brown, distinct from other components of the section (Figure 2.4).

Sections were placed in a 2% silver nitrate solution (1% silver nitrate for K Plast sections), and exposed to UV light for approximately 30 minutes, or until bone was dark brown in colour. The stain was fixed with 1% hypo-sodium thiosulphate for one minute. The high contrast sections produced by this method were examined using a grey level Quantimet 520 Image Analysis System (Cambridge Instruments, Germany) interfaced with an Olympus BH-2 microscope (Tokyo, Japan).

The Quantimet 520 system measures features of the bone section by creating a digital representation of the original image on an X by Y pixel array. This is done by setting the binary threshold to a grey level capable of detecting all the bone matrix without detecting any background staining. This level is selected by the operator. The Quantimet 520 calculates the total pixel count of the digitised image, as well as the perimeter pixel count and the total measuring frame size. In this way the total bone area and perimeter of the

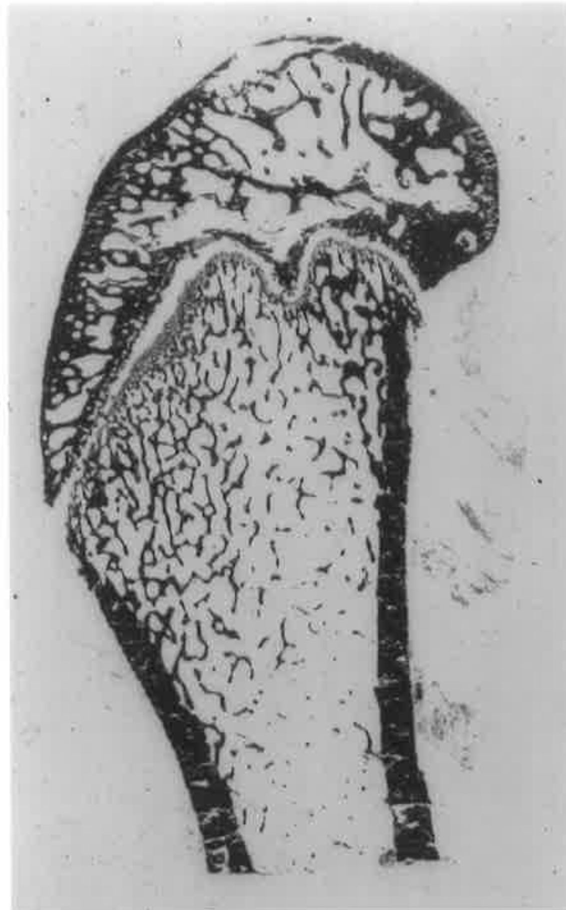


Figure 2.4 The distal femoral third of a rat femur stained by the Von Kossa silver method. Bone tissue is stained dark brown, while other tissue remains unstained. Sections such as this were used with the Quantimet 520 Image Analysis System to determine trabecular bone volume, thickness and number.

total bone area were derived. From these data trabecular bone volume (BV/TV) trabecular number (Tb.N) and trabecular thickness (Tb.Th) were calculated as follows (205):

BV/TV = Bone Area / Frame Area x 100

Tb.Th = (BV/TV / 100) / 0.5 x (Perimeter / Frame area x 100)

Tb.N = (BV/TV / 100) / Tb.Th

Care was taken when preparing sections for image analysis to ensure that background staining was minimal. Generation of bone dust during gross sectioning before bones are embedded, fracturing of trabeculae during microtome sectioning or handling during the staining process, and ineffective washing of staining solution from the section, unclean slides and air bubbles may leave small, optically dense particles within the sections. The detection level of the system may be altered such that some of these particles are below the detection threshold, but this should be avoided, particularly in low density specimens, where alterations in the detection level may significantly alter results obtained. It is more appropriate to set the pixel sizing to 2 pixel to ensure only relevant bone structure is measured (Parkinson IH, Fazzalari NL, manuscript in preparation).

The area in which trabecular morphometry is measured is also subject to operator variation, and for this reason, I carried out all measurements myself. Bone loss due to oestrogen deficiency appears to occur throughout the distal femur, although at variable rates in different areas (206). Trabecular bone loss in areas such as the epiphysis, and the distal metaphysis however (Figure 2.5) appears to be limited by stress induced bone formation due to increased mechanical forces on remaining trabeculae with decreasing

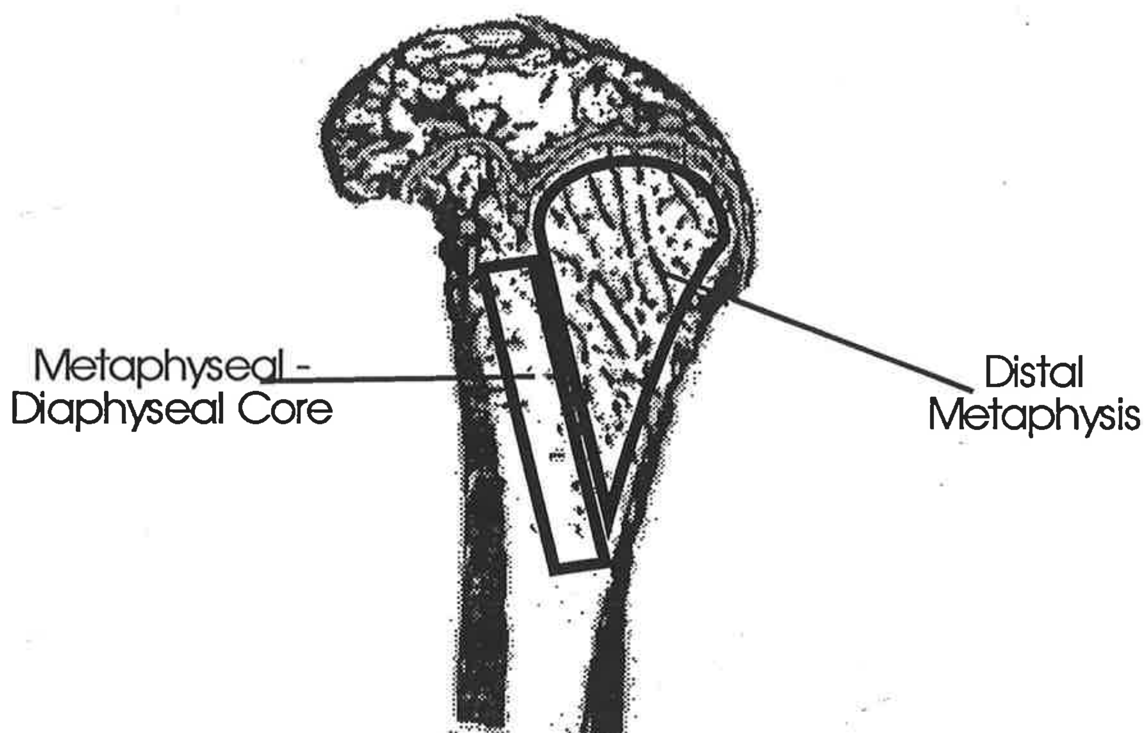


Figure 2.5. The distal third of a rat femur from an oophorectomised rat stained by a modified Von Kossa method. Morphometric parameters were measured in the metaphyseal diaphyseal core. Osteoclast surface, mineral appositional rate, extent of osteoid surface and double fluorochrome labelled surface were measured in the distal metaphysis.

trabecular number (62). For this reason, trabecular morphometry was measured in the anterior diaphyseal-metaphyseal core area, 1 mm from the growth plate as described in Figure 2.5. The mean frame area ± SEM measured was 8.54 ± 1.90 mm, and ranged from 5.612 -12.540 mm. It was important to allow variation in frame area, such that a representative area could be measured in each section regardless of the size of the anatomical bone.

Measurements were performed using a 2 x objective on the BH-2 microscope. This low level of magnification allowed detection of trabecular bone throughout the diaphyseal-metaphyseal core area without need for moving the specimen, and possible overlap of some areas. All measurements were carried out at the same magnification since increased magnification causes a rise in detected perimeter of the stained area, while stained volume remains constant (207). A rise in the detected perimeter may result in a falsely elevated trabecular number value as well as a low trabecular thickness value.

2.9.3 Detection of Bone Mineralisation Using Fluorochrome Labels

Systemically administered fluorochrome labels bind to mineralisation fronts on the bone surface by chelating calcium deposited while the fluorochromes are in the circulation. These labels, once incorporated, may be viewed under UV light (Figure 2.6), and quantified as a measure of bone mineralising surface. Administration of two different labels over a known time period allows determination of mineralisation rate.

Calcein (Sigma Chemical Co, USA) was dissolved 10 mg/ml in 0.9% (physiological) saline containing 20 mg/ml sodium bicarbonate. Demeclocycline (Lederle Laboratories, Australia) was dissolved 10 mg/ml in 0.9% saline. Animals were injected intraperitoneally with fresh calcein and

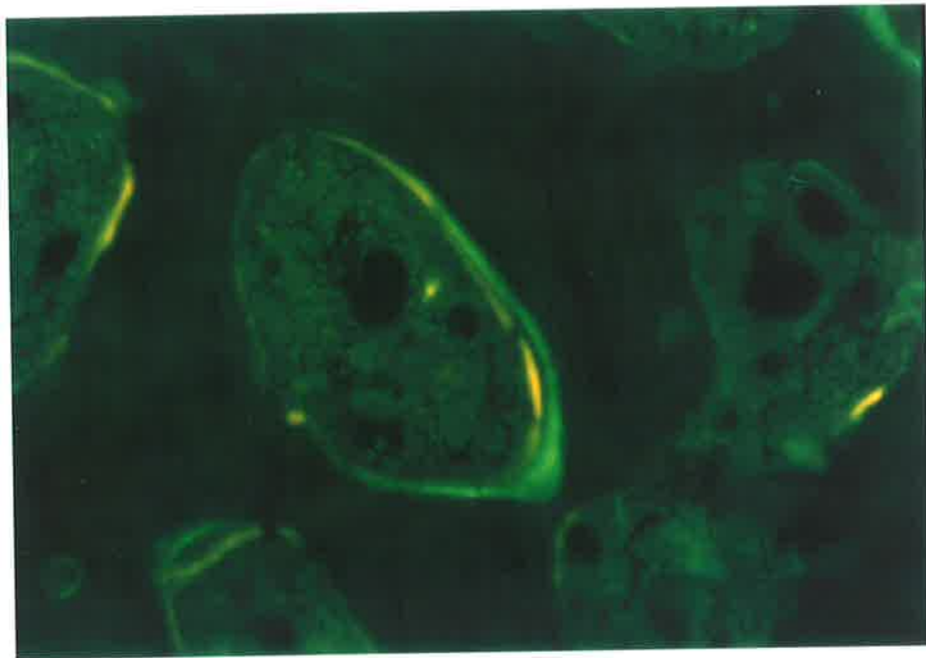


Figure 2.6 Incorporation of fluorochrome labels viewed in unstained sections under a fluorescence microscope. Calcein (green) and demeclocycline (orange) labels have become incorporated at fronts of bone mineralisation. Extent of labelled surface was determined as a percentage of total bone surface. Mineral appositional rate was determined from the distance between the two labels divided by the time between label administration.

demeclocycline at 10 mg/kg body weight at 3 and 9 days respectively before killing.

Inter label distance was measured at 2 - 6 evenly spaced sites along all double labelled trabecular surfaces detected in the distal metaphysis (≥8 surfaces in each section) using a Zeiss fluorescence microscope with an ocular-mounted micrometer at 400 x magnification. Mineral appositional rate (MAR) was calculated by dividing the mean inter label distance of the measured area by the time lapsed between label administration (6 days).

Extent of double labelled, single labelled, and unlabelled trabecular surfaces were determined using an ocular mounted graticule (Weibel II) superimposed on the sections. A magnification of 100 x was used, and 7-12 fields were analysed for each section. The number of intercepts at double, single and unlabelled trabecular surfaces were counted in the anterior metaphyseal surface. These values were expressed as a percentage of the total number of trabecular intercepts.

To determine whether single or double labelled surface was the more appropriate measure of bone forming surface, a group of 6 oophorectomised rats and 6 sham operated rats were killed 21 days post-operation, a time at which bone forming surface is increased in oophorectomised rats (52). Femora were processed as described above, and extent of labelled surface was determined on unstained sections. Double (dLS) and single (sLS) labelled surfaces were determined as a percentage of total bone surface. Total labelled surface (tLS) was calculated as the sum of double labelled surface and half single labelled surface as a percentage of total bone surface. These variables were measured in both the distal metaphysis and the anterior metaphyseal-diaphyseal core (Figure 2.5).

In the distal metaphysis, the increase in labelled surface in oophorectomised rats was only significant when tLS and dLS were measured, but not with sLS (Figure 2.7). Single labelled surface would include trabecular surfaces at a number of different stages, including a stage when bone formation was nearing completion at 9 days before killing, or when bone formation was commencing at 3 days. Thus, the high level of single labelled surface might be predicted and would contribute to an elevation of total labelled surface measurements also.

Labelled surface was increased in both anatomical areas where measurements were made, indicating a systemic increase in bone formation in oestrogen deficiency. It was considered unlikely therefore that the changes in labelled surface after oophorectomy were site specific. However measurements made in the metaphyseal-diaphyseal core were more varied than measurements made in the distal metaphysis, particularly in oophorectomised rats, probably as a result of the bone loss and limited bone remaining in the core area. Since double labelled surface in the distal metaphysis displayed the least variation, this marker was used throughout this thesis as a marker of the extent of trabecular bone forming surface.

2.9.4 Measurement of Bone Surface Osteoid and Osteoclast Surface

Sections were stained for osteoid with buffered aqueous eosin in von Kossa stained sections followed by a Harris haematoxylin counter stain (Figure 2.8). Extent of bone surface osteoid was determined in the distal metaphysis as a percentage of total bone with an ocular mounted graticule (Weibel II) superimposed on the sections. A magnification of 200 x was used, and 15 - 25 fields were analysed for each section. The number of intercepts at sites of osteoid surface was counted and expressed as a percentage of the total number of trabecular intercepts.

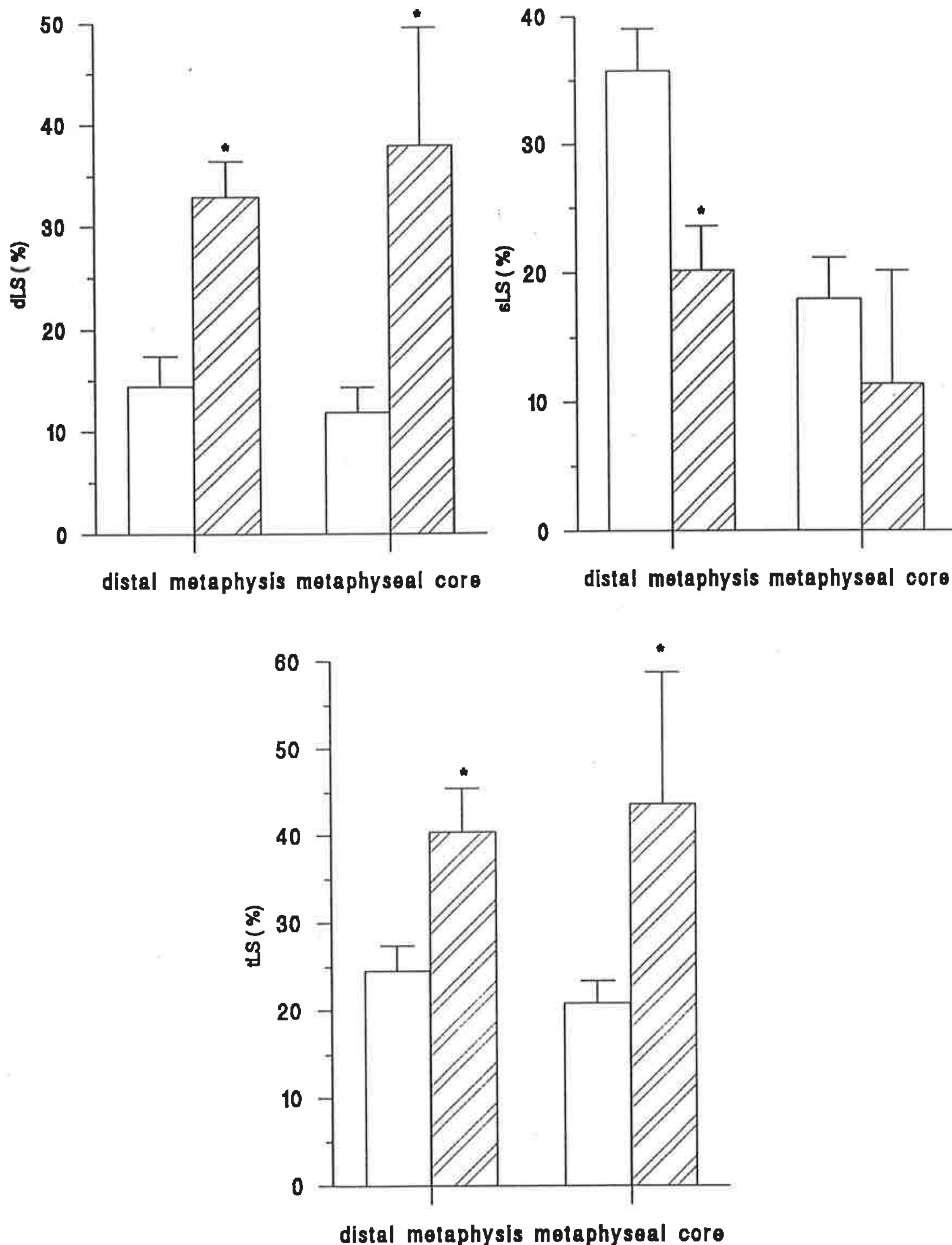


Figure 2.7 Measurement of fluorochrome labelled surface as a marker of bone formation in sham operated and oophorectomised rats. Double (dLS), single (sLS) and total (tLS) labelled surface were determined in the distal metaphysis and metaphyseal-diaphyseal core in distal femoral specimens at 21 days post-operation. dLS and tLS were increased in oophorectomised rats in both regions, consistent with other markers of bone formation. Variation was least in the distal metaphysis. Values are mean±SEM. *, $p<0.05$ vs sham.

Acid phosphatase is an enzyme specific for osteoclasts. The enzyme was reactivated in a pH 5 acetate buffer for 1 h, then stained with an azo dye coupling method. Naphthol AS-BI phosphate was used as the substrate, with 5% basic Fuchsin in 10% hydrochloric acid (hexasotised pararosanilin) as the dye, and incubated for 1 h at 37°C (208). Sections were counterstained with Harris haematoxylin, and blued with lithium carbonate (Figure 2.8). Osteoclast surface (Oc.S) was determined in the same area and by a similar method to osteoid surface with the number of intercepts at sites of acid phosphatase staining being counted.

2.10 Statistical Methods

Levels of each variable measured in each experiment were tested for normal distribution by the signed rank sum test. The only variable that was not normally distributed was serum 17β-oestradiol. For this variable, the median value and range of values are presented. To determine the statistical significance of differences in serum oestradiol existed between operation groups, the non-parametric Kruskal Wallis test was used. A $P<0.05$ was considered significant.

Normally distributed data are presented in graphs and tables as the mean value and the standard error of the mean. Analyses of variance were performed on these data to determine the statistical significance of differences in levels between operation groups. In experiments where only one time point was sampled, such as the histological components of Chapters 6 and 7, a one-way analysis of variance was used. In experiments where data were collected at a number of time points, a two-way analysis of variance was used to determine significant changes associated with both operation and time. When analyses of variance revealed a significant difference between operation or time groups, Tukey's post hoc test was used to identify mean values which were significantly different from each other.

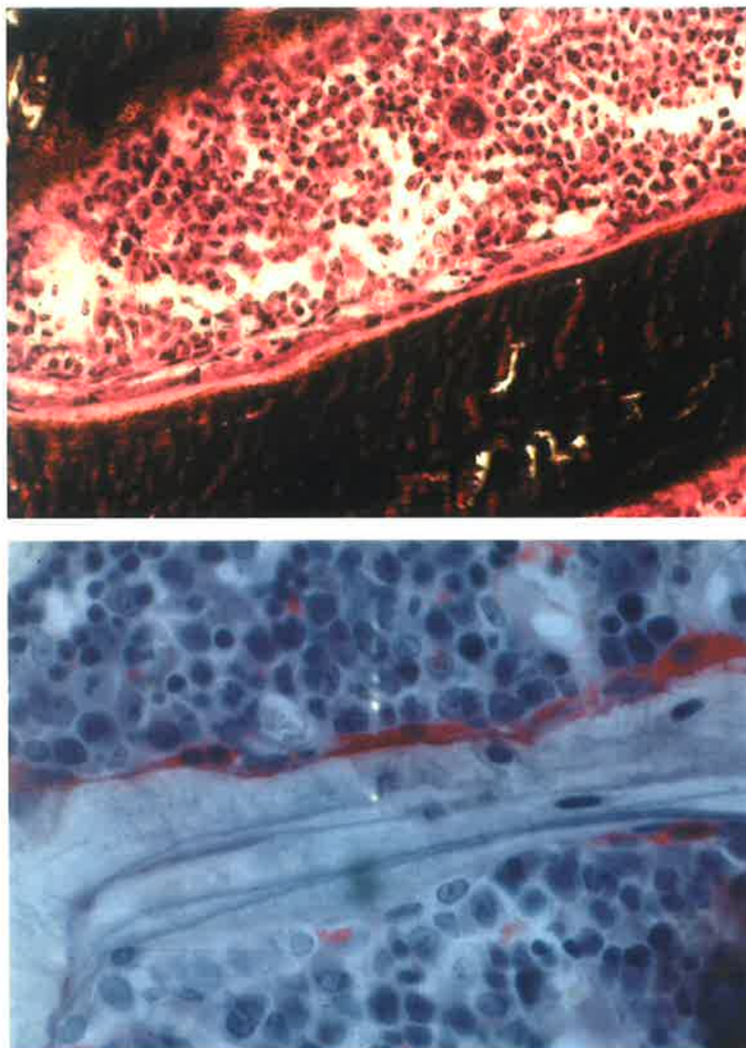


Figure 2.8 Bone stained for osteoid surface and acid phosphatase activity. a. A section of bone stained by the von Kossa silver method, followed by haemotoxylin and eosin. Osteoid appears as a pink band on the surface of the dark brown mineralised bone. Extent of osteoid surface was determined as a percentage of total trabecular surface. b. A section of bone stained by an enzyme histochemical method for acid phosphatase activity, counterstained with haematoxylin. Along the upper surface of the trabecula is a number of osteoclasts stained bright red, indicating positive acid phosphatase activity. Extent of osteoclast surface was determined as the extent of acid phosphatase positive surface as a percentage of total trabecular surface.

Where a significant difference was found between operation groups at the time of operation, the change from time of operation was calculated for each rat at each time point. These values were analysed by two-way analysis of variance and Tukey's post hoc test as described above.

To determine the relationship between biochemical and histomorphometric markers of bone turnover, data were paired randomly at each time point within each operation group since at some time points, different animals were used for histomorphometric and biochemical analyses. These data were graphed and analysed by a simple linear regression and analysis of variance. To determine relationships between two biochemical or two histomorphometric variables, the complete data set was used.

All analyses were performed using the Minitab 8.0 statistics package on an IBM compatible personal computer.

CHAPTER THREE

TEMPORAL RELATIONSHIP BETWEEN BONE RESORPTION AND FORMATION FOLLOWING OOPHORECTOMY

Several studies report increased bone turnover (47,49-55) and bone loss (47,52,53,56-58) in the oophorectomised rat. The time course of the changes in bone turnover immediately following operation has not been well characterised. In this chapter the time frame of increased bone turnover and trabecular bone loss in the distal femur of the mature rat is described over the first 42 days following oophorectomy. An increase in bone resorption was detected prior to increased formation and the reduction in trabecular bone volume suggesting the transient imbalance between resorption and formation is the primary cause of bone loss in the oophorectomised rat.

It has been proposed that bone loss in the post-menopausal woman may relate to an increase in renal calcium excretion (3,30,31). Similarly, it has been suggested that bone sensitivity to calcium regulating hormones is altered in the oophorectomised rat and is a major cause of oestrogen-deficiency bone loss (41,42,44). Short term alterations in serum and urinary parameters of calcium and phosphate metabolism and their relationship to the bone loss after oophorectomy are also described.

3.1 Protocol

126 Female Sprague Dawley rats at 6 months of age were used. The experimental protocol is presented in Figure 3.1. Six non-operated animals were killed as a baseline group. The remaining animals were randomly selected for sham operation (Sham) or oophorectomy (Oophx), both performed under halothane anaesthesia. At 6, 9, 15, 18, 21 and 42 days post-operation, 10 sham operated and 10 oophorectomised rats were killed by cervical dislocation

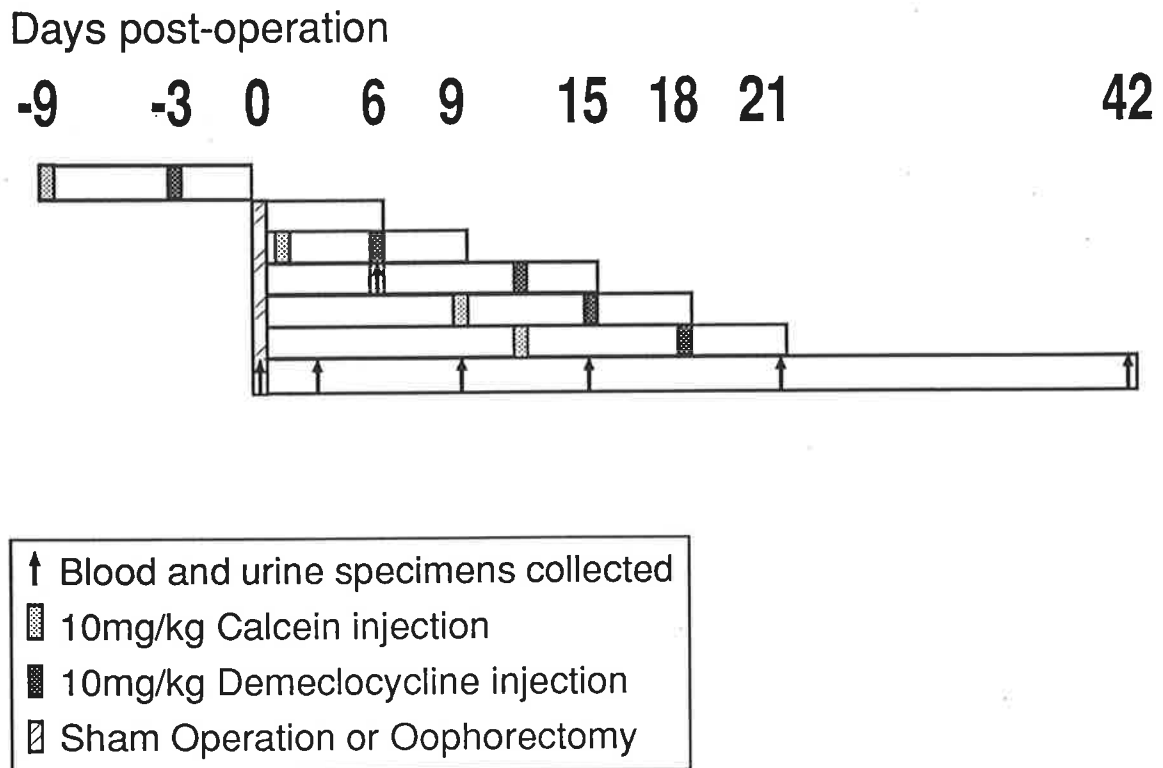


Figure 3.1 Protocol to determine short term effects of oophorectomy. Six animals were killed as a baseline group. The remaining 120 were sham operated (60) or oophorectomised (60) at day 0, shown by the striped bar. Calcein and demeclocycline labels were administered at days indicated by the pale and dark stripes respectively. Blood samples were collected at the arrowed time points. Animals were killed in groups of 10 from each operation group at 6, 9, 15, 18 and 21 days post-operation, and femora removed for analysis.

under halothane anaesthesia, and femora were removed. Lumbar vertebrae were removed from animals killed at day 18. Calcein and demeclocycline (10 mg/kg body weight) were administered intraperitoneally at 9 and 3 days respectively before killing to unoperated rats, and those killed at 9, 15 and 18 days post-operation.

The inclusion of an histological component in this study precluded a longitudinal design. Therefore animals were grouped such that sham operated and oophorectomised rats were paired at each time point. Rats killed at each time point were from the same breeding group, and were treated identically in each section of the experiment, allowing a cross-sectional analysis while accounting for slight variations in age or handling between operation groups.

Blood and 24 h urine specimens were collected at 3, 9, 15, 21 and 42 days post-operation from animals killed at 42 days, and at 6 days post-operation from rats killed at day 15. Blood was collected from the tail vein under halothane anaesthesia, followed by a 2 ml intra-peritoneal saline injection to replace fluid removed. At day 42, when animals were killed, blood was collected by cardiac puncture under halothane anaesthesia.

Serum was analysed for sodium, potassium, chloride, bicarbonate, creatinine, albumin, total protein, alkaline phosphatase activity, osteocalcin and 17β-oestradiol (Chapter 2.3). Serum anion gap, globulin and calcium fractions were calculated (Chapter 2.3). Urinary hydroxyproline and phosphate excretions and TmP were determined in 24 h urine specimens (Chapter 2.3).

Femora and vertebral bodies were embedded in GMMA resin (Chapter 2.6.1). Trabecular bone volume, number and thickness were determined in the diaphyseal-metaphyseal core of the femur, and the lumbar vertebrae using the Quantimet 520 Image Analysis System on von Kossa stained sections (Chapter 2.6.2). Extent of double fluorochrome labelled surface and mineral appositional

rate were determined in unstained sections (Chapter 2.6.3). Extent of bone surface osteoid was determined on von Kossa stained sections counterstained with haematoxylin and eosin (Chapter 2.6.4), and osteoclast surface was determined on sections stained for acid phosphatase (Chapter 2.6.4).

3.2 Results

Serum 17β-oestradiol levels were unevenly distributed in sham operated rats. Most levels were low, but some very high levels were obtained. In oophorectomised rats, 17β-oestradiol levels were significantly lower than levels in sham operated rats at 32 days post-operation. Median values and ranges were: Sham, 27.5, (8 - 230) pmol/L; Oophx, 6.5, (1 - 9) pmol/L.

Hydroxyproline excretion was significantly lower in oophorectomised rats compared to sham operated rats before operation. Mean values (mmol/L GF) ± SEM were: Sham, 0.36 ± .04; Oophx, 0.26 ± 0.03. To eliminate this factor from analyses, the change in hydroxyproline from pre-operative levels was calculated for each rat, and used for analysis (Chapter 2.6.5). Hydroxyproline excretion increased significantly in oophorectomised rats, and was significantly greater compared to sham operated rats from 6 days post-operation onwards (Figure 3.2).

Extent of osteoclast surface in the distal metaphysis of the femur was reduced significantly with time in sham operated rats, but no reduction was seen in oophorectomised rats (Figure 3.2). Osteoclast surface increased significantly following oophorectomy and was significantly greater in oophorectomised rats compared to sham operated controls from 6 days post-operation onwards; values were significantly greater than baseline at days 15 and 18 post-operation (Figure 3.2). At 42 days post-operation, no difference was detected between sham operated and oophorectomised rats in osteoclast surface in the femoral distal metaphysis.

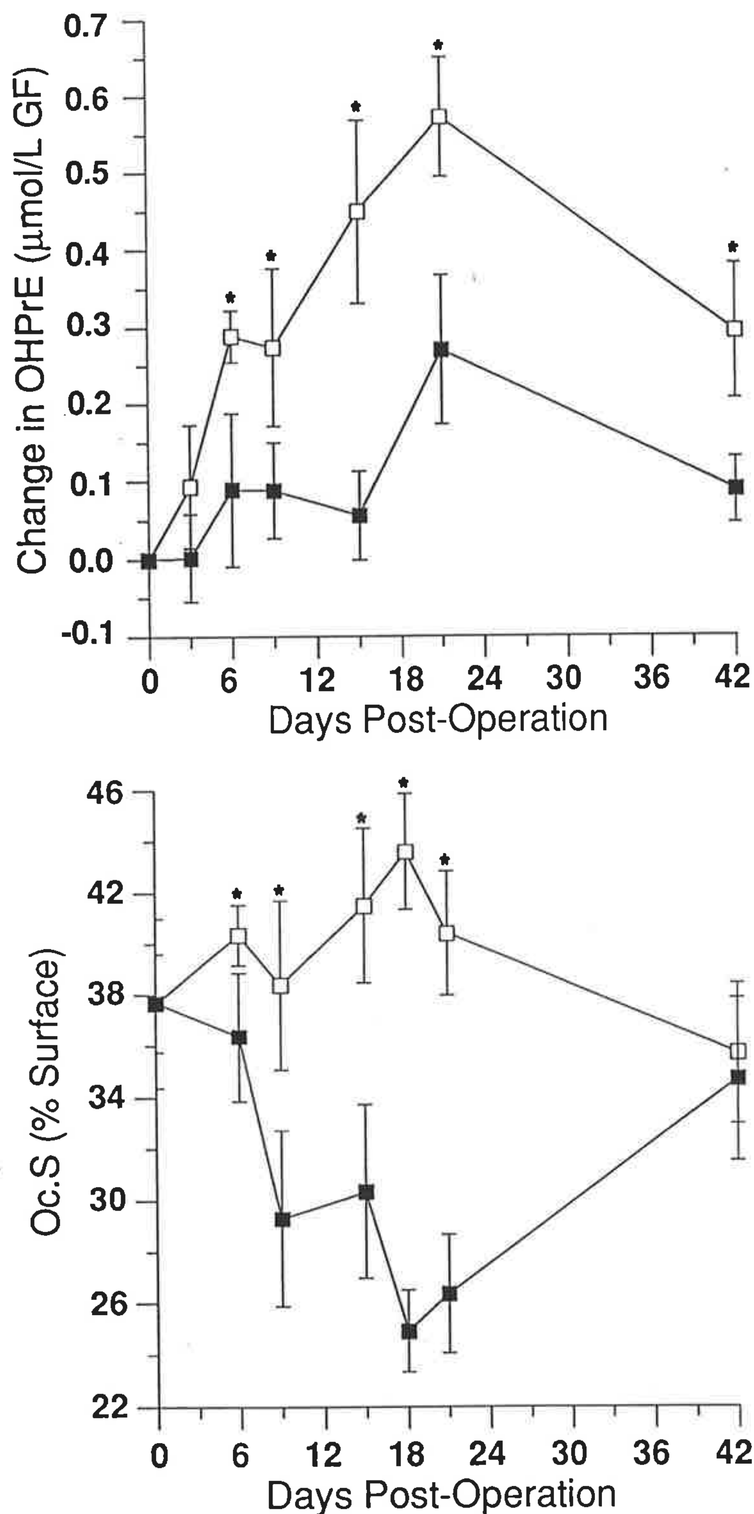


Figure 3.2 Biochemical and histomorphometric markers of bone resorption were increased by 6 days after oophorectomy. Urinary hydroxyproline excretion (OHPrE - µmol/L GF) and femoral osteoclast surface (Oc.S - %) were significantly greater in oophorectomised (□) compared to sham operated rats (■) from 6 days post-operation. OHPrE values are mean change calculated from day 0 ± SEM. Oc.S values are mean ± SEM. *, $p<0.05$ vs sham by two-way analysis of variance and Tukey post-hoc test.

There was a significant positive correlation between extent of femoral osteoclast surface and urinary hydroxyproline excretion in oophorectomised rats. The relationship was not significant in sham operated rats (Figure 3.3).

Serum osteocalcin was significantly increased in both sham operated and oophorectomised rats at 3 and 6 days post-operation. At 9 days post-operation, serum osteocalcin levels in sham operated rats returned to pre-operative levels, remaining stable for the duration of the experiment. In oophorectomised rats, serum osteocalcin continued to rise, reaching a plateau at 15 days post-operation. This elevated level of osteocalcin was maintained for the duration of the experiment.

Serum alkaline phosphatase activity was significantly reduced compared to pre-operative levels in oophorectomised rats at 6 days post-operation and was slightly (though not significantly) depressed in sham operated rats at this time point (Figure 3.4). At 9 days post-operation alkaline phosphatase activity was significantly greater in oophorectomised rats compared to sham operated controls, and remained so for the duration of the experiment.

Mineral appositional rate in the femoral distal metaphysis was significantly increased in both sham operated and oophorectomised rats at 6 days post-operation (Figure 3.5). In sham operated rats, mineral appositional rate was returned to pre-operative levels at 12 days post-operation, whereas in oophorectomised rats, this marker continued to rise remaining greater than levels in sham operated rats for the duration of the experiment. Double fluorochrome labelled surface in the femoral distal metaphysis was increased in oophorectomised rats compared to stable sham operated levels from day 12 post-operation (Figure 3.5).

Alkaline phosphatase activity and double fluorochrome labelled surface were significantly correlated in oophorectomised rats, but not in sham operated rats

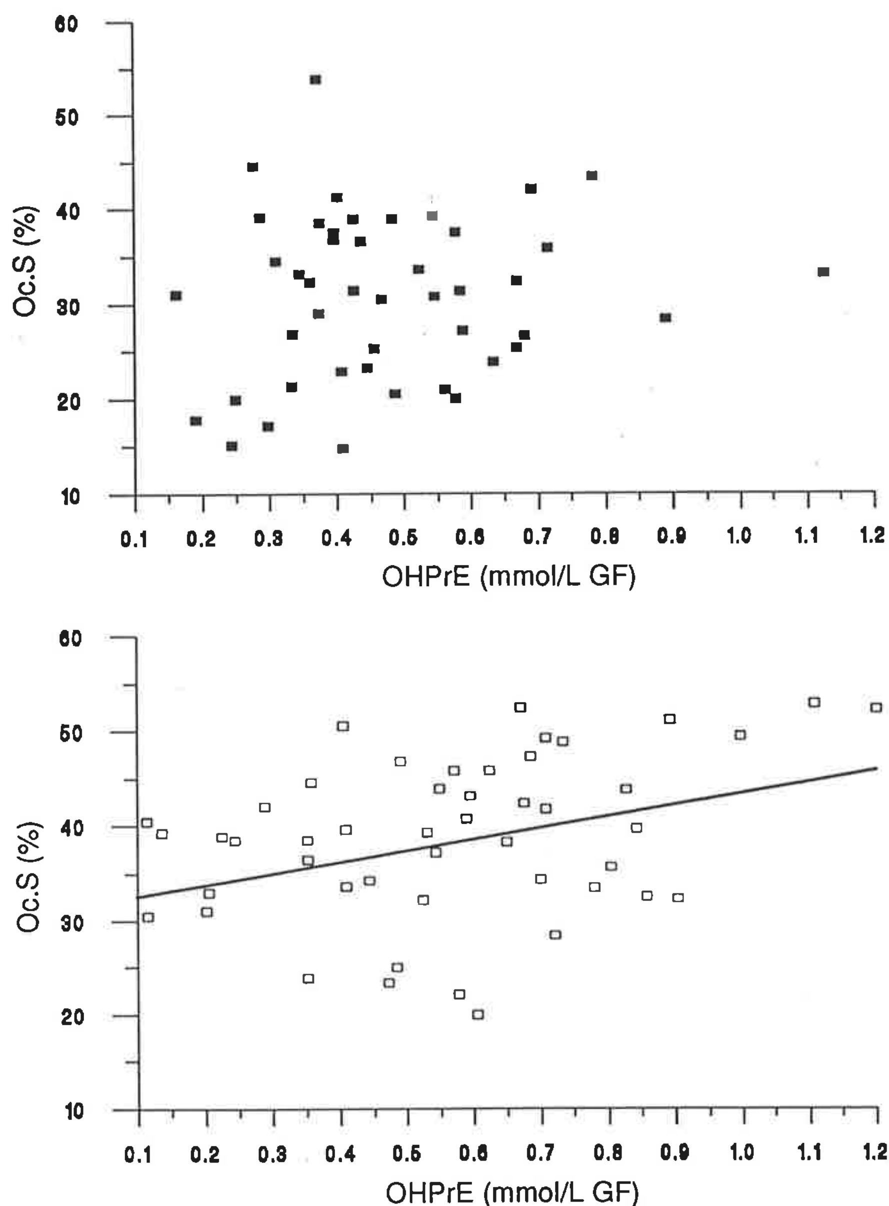


Figure 3.3 Relationship between histomorphometric and biochemical markers of bone resorption. Levels of femoral osteoclast surface in the distal metaphysis (Oc.S) and urinary hydroxyproline excretion (OHPrE) are plotted in sham operated (■) and oophorectomised (□) rats. There was no significant relationship between Oc.S and OHPrE in sham operated rats. In oophorectomised rats, Oc.S and OHPrE are positively related. The straight line represents a simple linear regression equation derived from the data presented:

Oc.S = 32.6 + 10.8 x OHPrE; R = 0.115; p = 0.025.

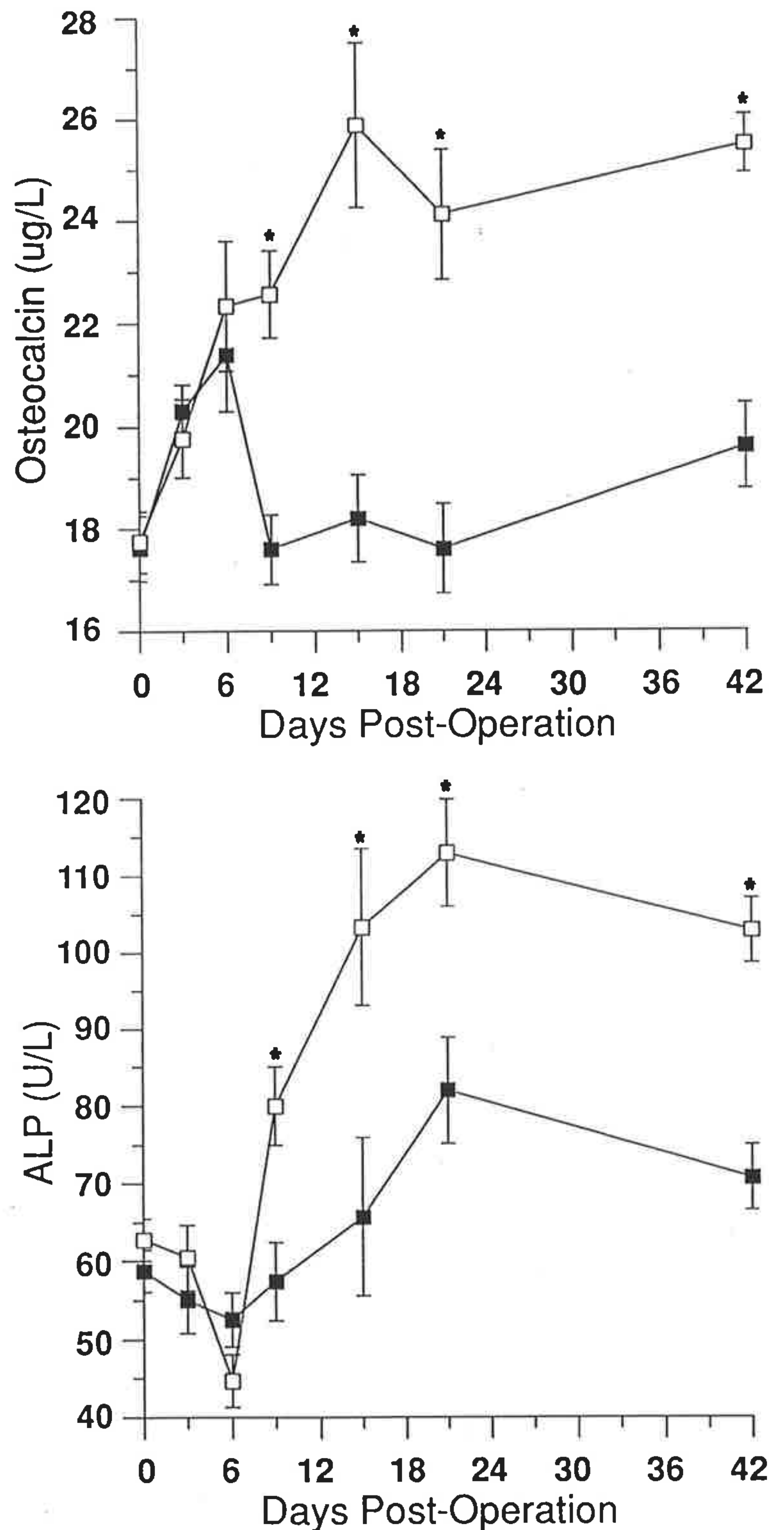


Figure 3.4 Biochemical markers of bone formation were increased by 9 days after oophorectomy. Serum osteocalcin (µg/L) and serum alkaline phosphatase activity (ALP - U/L) increased in oophorectomised rats (□) compared to sham operated rats (■), significant from 9 days post-operation until the end of the experiment. Values are mean±SEM. *, p<0.05 vs sham by two-way analysis of variance and Tukey post-hoc test.

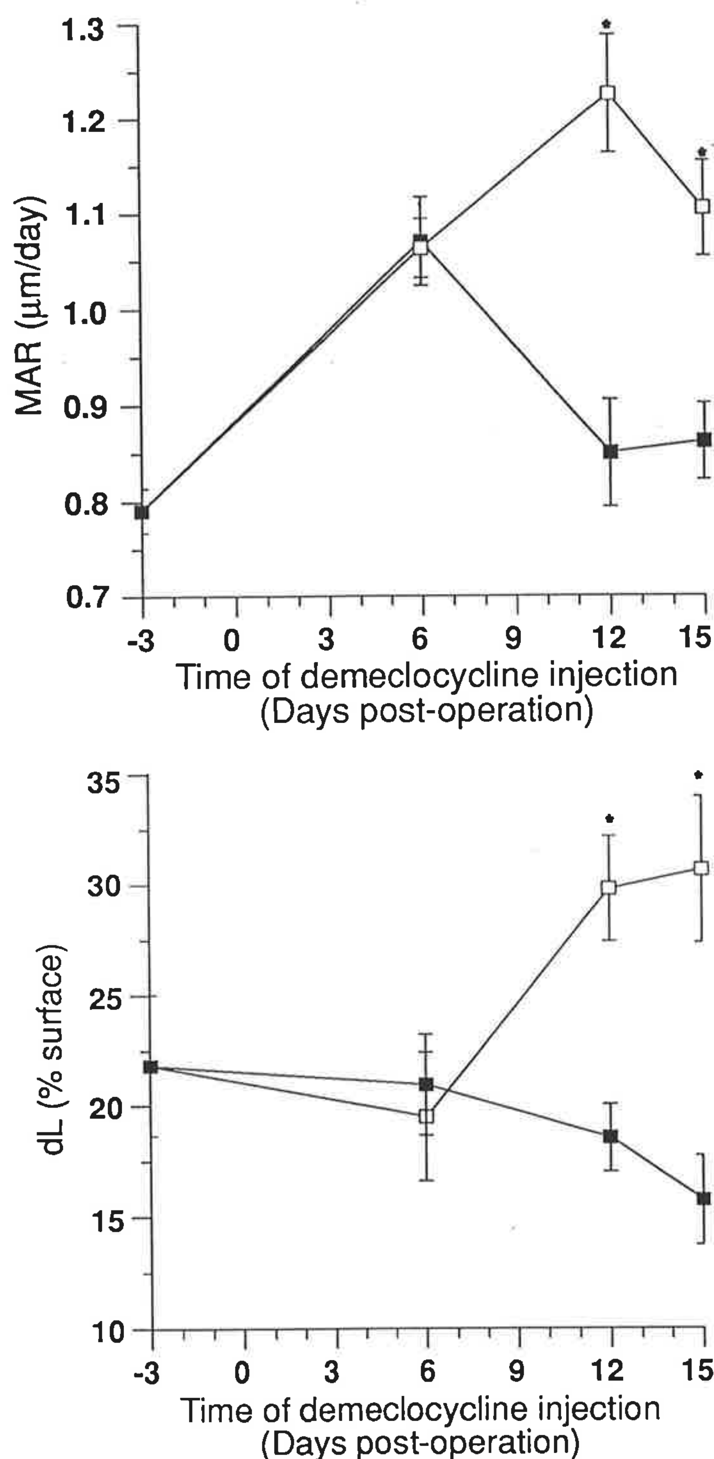


Figure 3.5 Histomorphometric markers of bone formation increased at 12 days after oophorectomy. Mineral Appositional Rate (MAR - μm/day) and double labelled trabecular surface (dLs - %) in the distal metaphysis were increased significantly in oophorectomised rats (□) compared to sham operated (■) at 12 days post-operation. Values are mean±SEM. *, $p<0.05$ vs sham by two-way analysis of variance and Tukey post-hoc test.

(Figure 3.6). Serum osteocalcin and mineral appositional rate were also significantly correlated in oophorectomised rats, but not sham operated rats (Figure 3.7). There were no other significant relationships between the markers of bone formation.

Trabecular osteoid surface in the femoral distal metaphysis increased significantly with time for the duration of the experiment, but there was no significant difference in levels of osteoid surface between sham operated and oophorectomised rats (Figure 3.8).

At 15 days post-operation, significant bone loss had occurred in the oophorectomised rat femur. This appeared to occur mainly in the diaphyseal - metaphyseal core area (Figure 3.9). Bone loss in the distal metaphysis or epiphysis did not appear as pronounced. Trabecular bone volume in the diaphyseal - metaphyseal core was significantly lower in oophorectomised rats compared to sham operated controls (Figure 3.10). No further trabecular loss occurred after day 15 in this area. The loss of bone volume was accompanied by a concomitant decrease in trabecular number (Figure 3.11). Trabecular thickness did not differ significantly between sham operated and oophorectomised rats, although there was a significant increase with time in both operation groups (Figure 3.11). Trabecular bone volume and trabecular number were non-significantly increased at 6 days post-operation in both sham operated and oophorectomised rats after operation, consistent with the rise in mineral appositional rate and osteocalcin and the decrease in osteoclast surface observed.

At 18 days post-operation, vertebral trabecular bone volume, thickness and number were not significantly altered by oophorectomy. Mean trabecular bone volume (% $\pm$ SEM): Sham 12.1$\pm$1.1, Oophx 12.0$\pm$1.2. Mean trabecular thickness (μm $\pm$ SEM): Sham, 45.9 $\pm$ 3.6; Oophx, 47.3 $\pm$ 2.2. Mean trabecular number (/mm $\pm$SEM): Sham, 2.66 $\pm$ 0.13; Oophx, 2.52 $\pm$ 0.18.

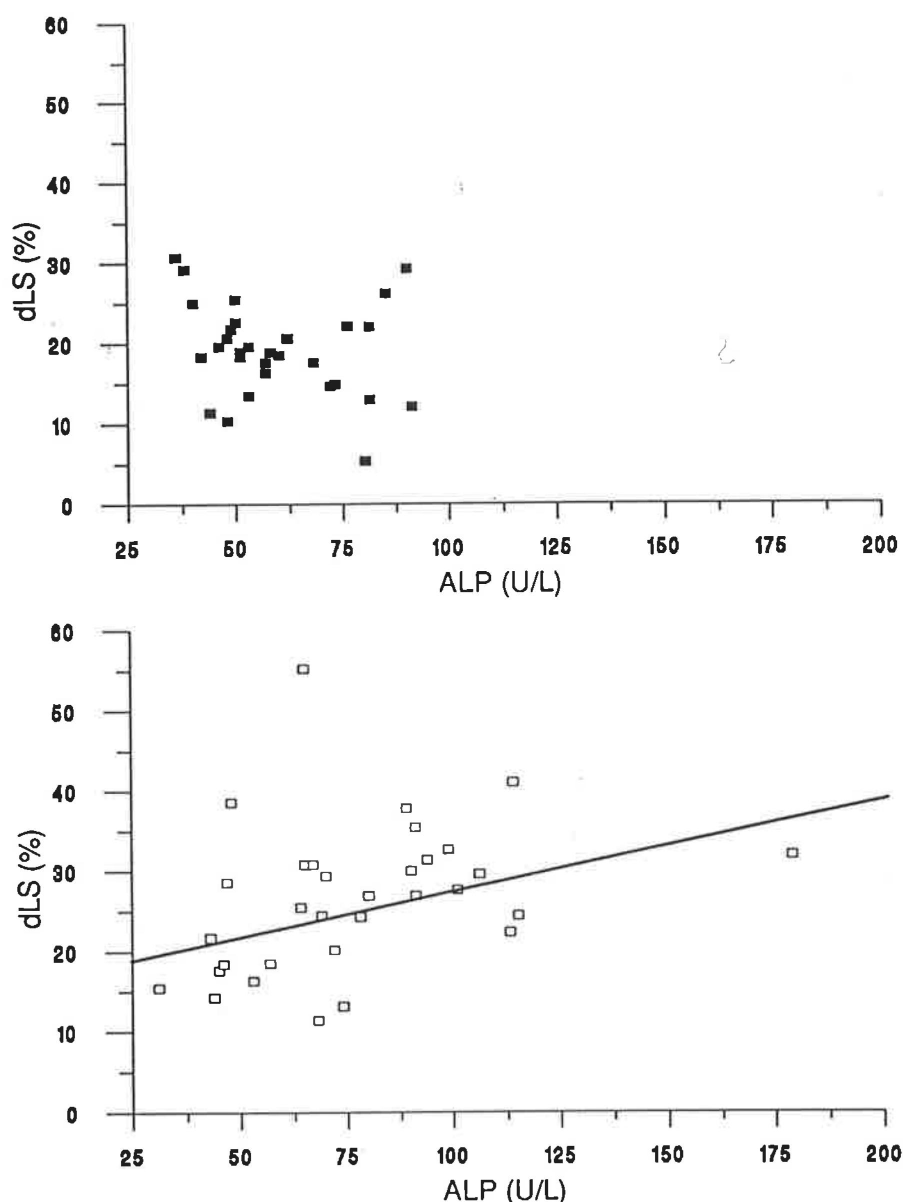


Figure 3.6 Relationship between femoral double fluorochrome labelled surface and serum alkaline phosphatase activity. Values are double fluorochrome labelled surface (dLS) in the femoral distal metaphysis and serum alkaline phosphatase activity (ALP) in sham operated controls (■) and oophorectomised (□) rats. There was no significant relationship between dLS and ALP in sham operated rats. In oophorectomised rats, there was a positive relationship between dLS and ALP, represented by the straight line, a simple linear regression equation derived from the data presented:

dLS = 19.3 + 0.098 x ALP; R = 0.125; p=0.047.

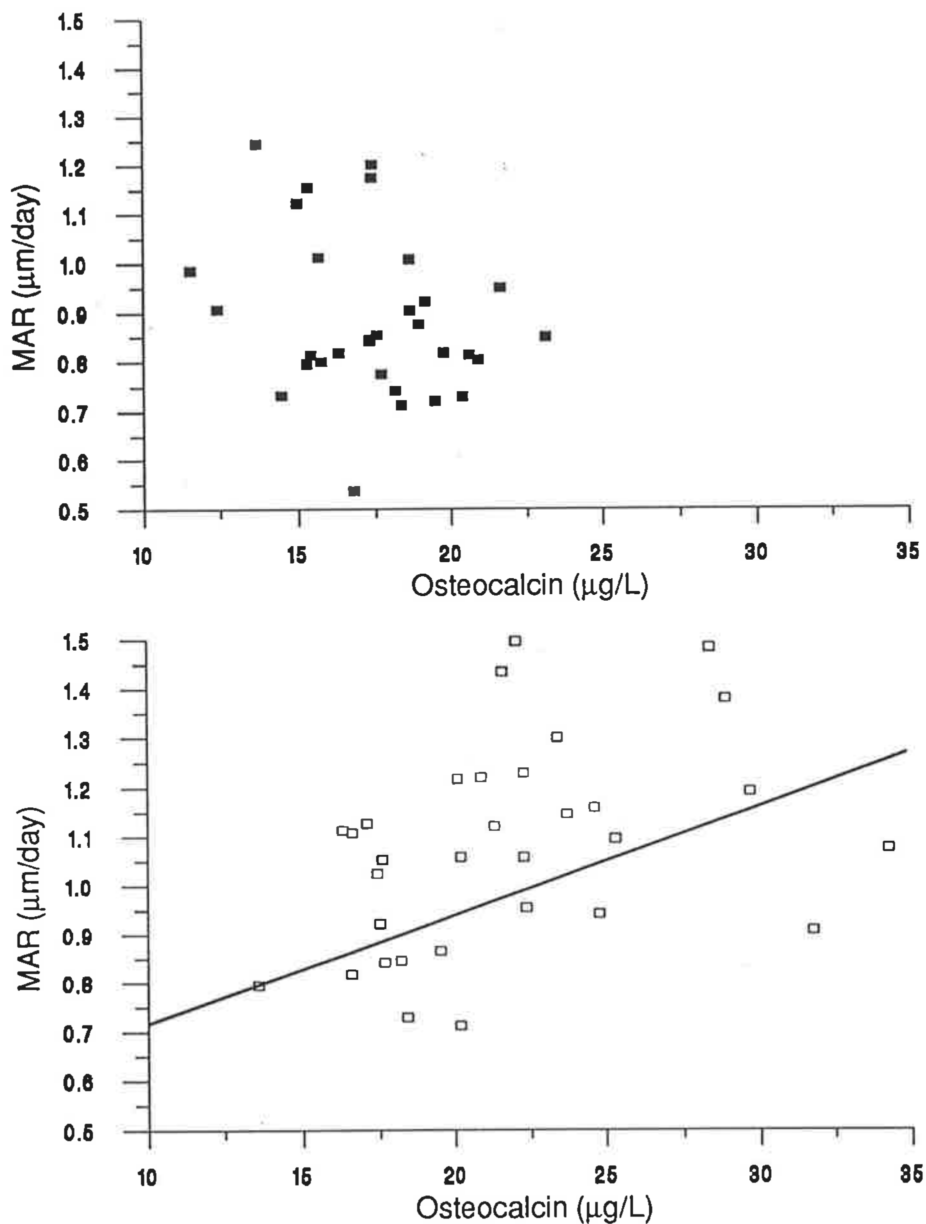


Figure 3.7 Relationship between femoral mineral appositional rate and serum osteocalcin. Values are femoral mineral appositional rate measured in the distal metaphysis (MAR) and serum osteocalcin (µg/L) in sham operated controls (■) and oophorectomised (□) rats. MAR and osteocalcin were not significantly related in shamoperated rats. MAR and serum osteocalcin were positively related in oophorectomised rats. The line represents a simple linear regression equation derived from the data presented:

MAR = 0.72 + 0.015 x Osteocalcin; R = 0.125; p = 0.047.

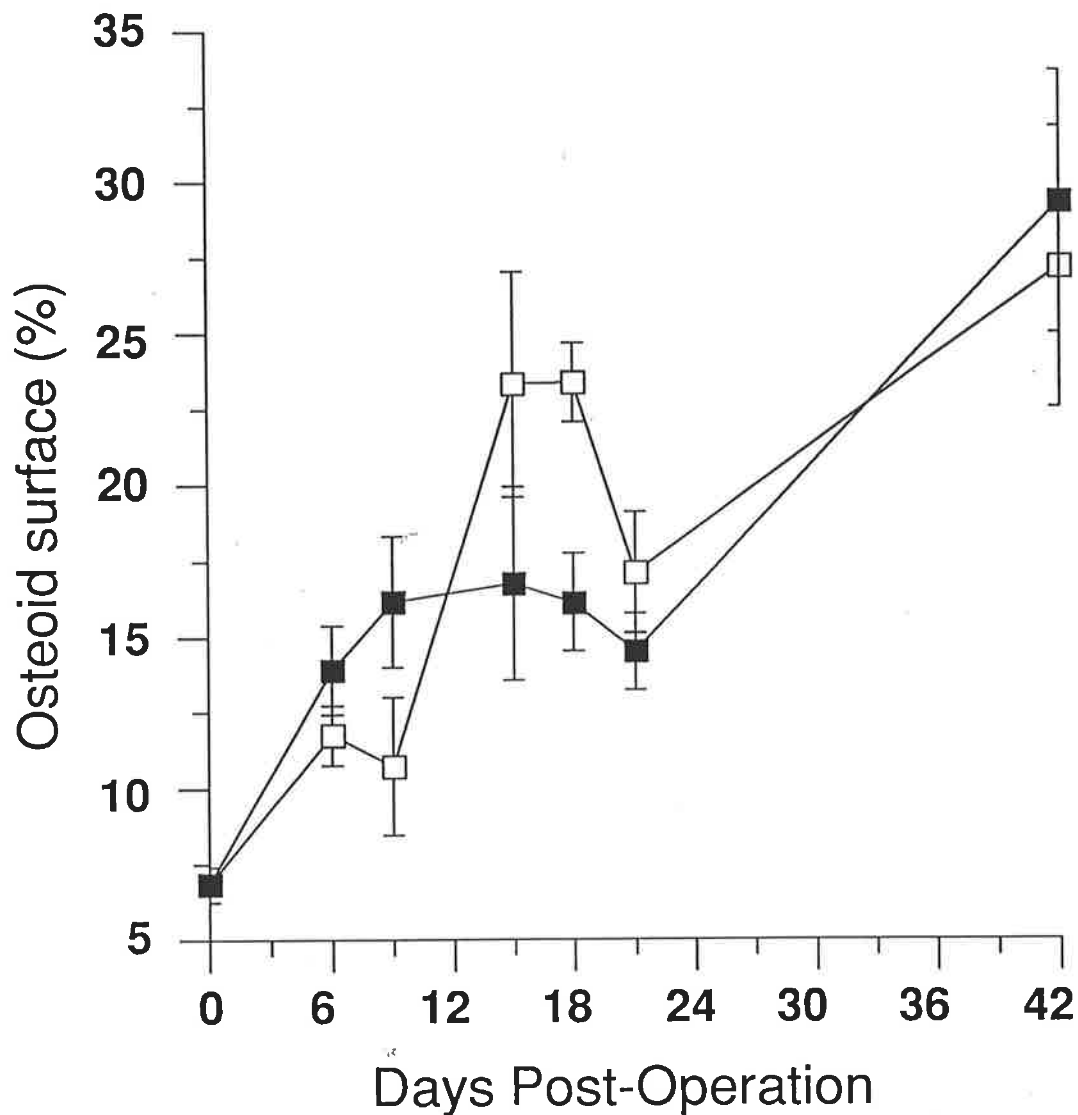


Figure 3.8 Oophorectomy did not significantly affect surface osteoid. Extent of Osteoid Surface (%) was not significantly altered in oophorectomised rats (□) compared to sham operated controls (■) for the duration of the experiment. There was a significant increase in osteoid surface over the course of the experiment. Values are mean± SEM.

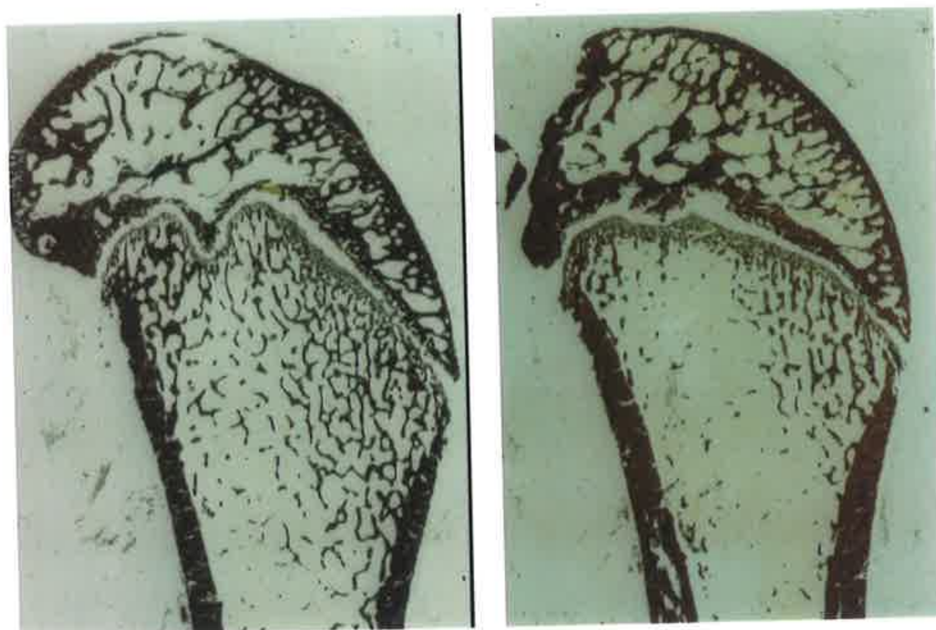


Figure 3.9 The distal third of femora from sham operated and oophorectomised rats. Von Kossa silver sections from sham operated (left) and oophorectomised (right) rats at 15 days post-operation. Note the loss of bone in metaphyseal-diaphyseal core area, but not in the distal metaphysis. Bone loss appears to be due to loss of whole trabecular surfaces rather than general thinning of existing trabeculae. Trabecular bone volume for these femora were approximately the mean value for that operation group.

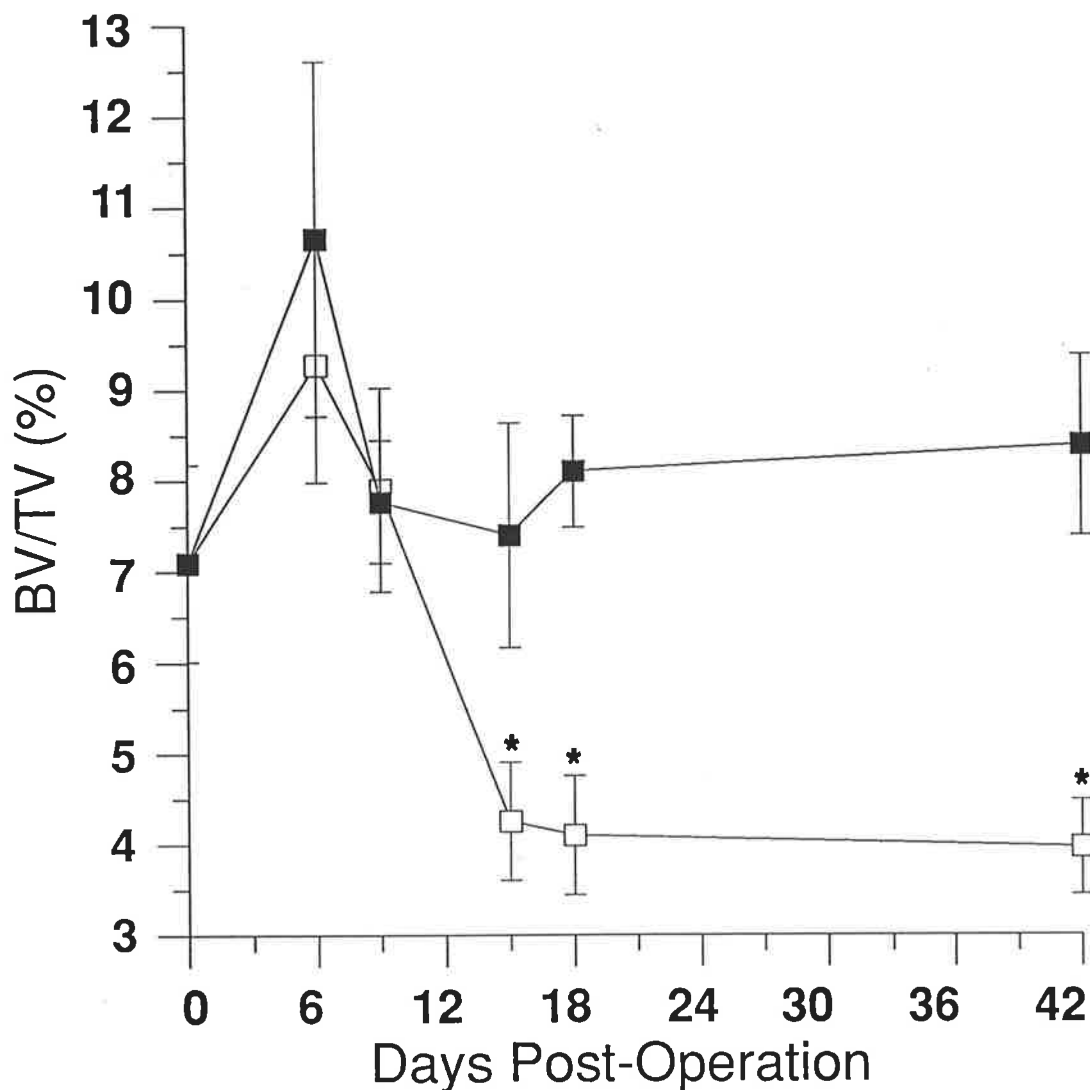


Figure 3.10 Oophorectomy caused a rapid reduction in trabecular bone volume. Trabecular Bone Volume (BV/TV) decreased between 9 and 15 days post opertion in oophorectomised (□) rats compared to sham operated controls (■). BV/TV did not decrease further for the duration of the experiment. Values are mean ± SEM. *, $p<0.05$ vs sham by two-way analysis of variance and Tukey post-hoc test.

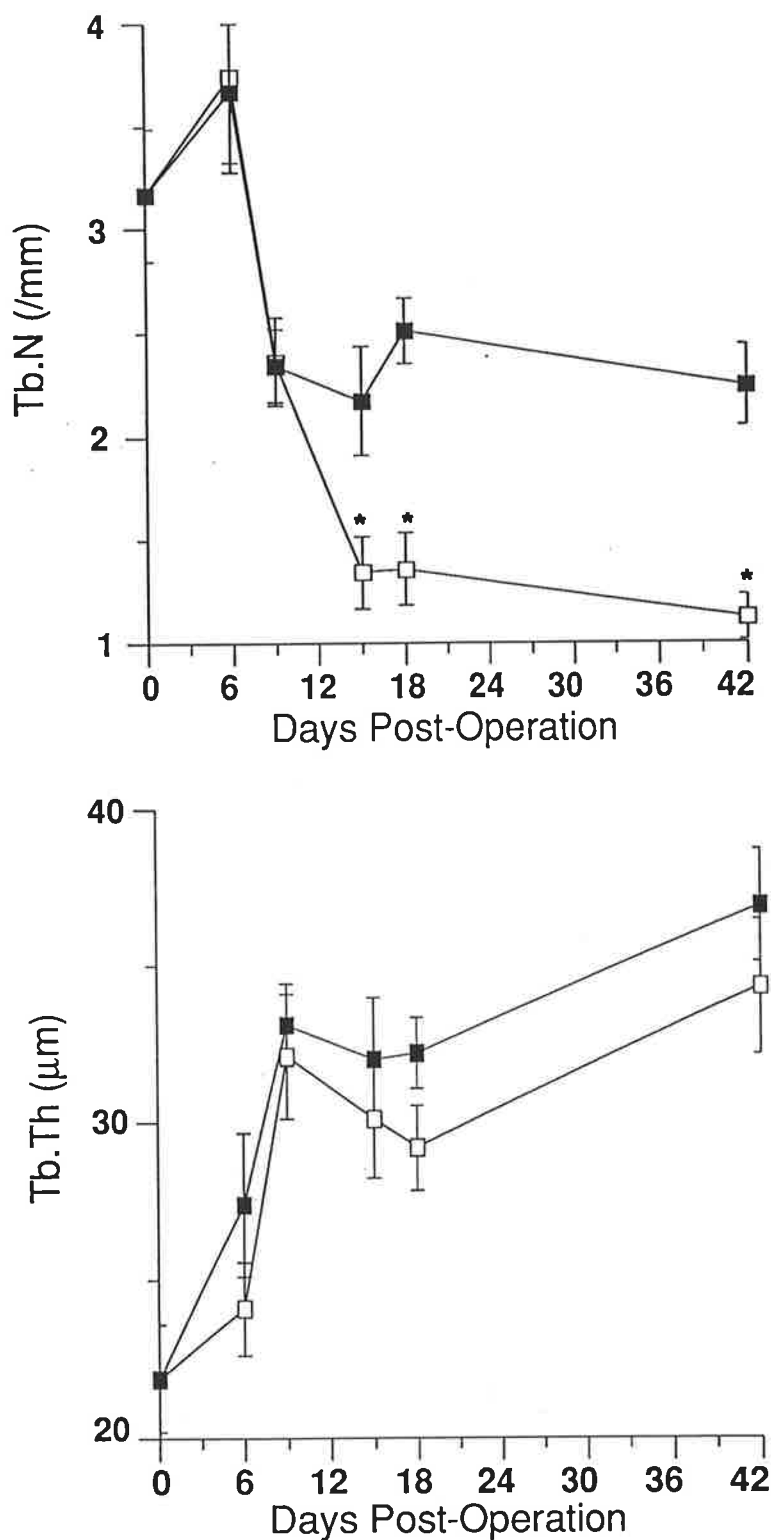


Figure 3.11 Change in trabecular number and thickness in oophorectomised rats. Trabecular Number (Tb.N) was reduced at 15 days post operation in oophorectomised (□) rats compared to sham operated controls (■). Trabecular thickness (Tb.Th) was not significantly altered by oophorectomy. Values are mean ± SEM. *, $p<0.05$ vs sham by two-way analysis of variance and Tukey post-hoc test.

Vertebral osteoclast surface values were significantly lower than levels detected in the femur at day 18. Extent of osteoclast surface in this area was significantly greater in oophorectomised animals compared to sham operated controls. Mean Oc.S (% ± SEM): Sham, 6.3±0.6, Oophx, 10.6±1.1, $p<0.05$.

There was no significant change in either total or ionised serum calcium related to oophorectomy (Table 3.1). The protein bound fraction of serum calcium was significantly greater in oophorectomised rats compared to sham operated controls before operation (mean values (mmol/L) ± SEM: Sham, 0.74 ± 0.01; Oophx, 0.78 ± 0.01), therefore results have been expressed as the change in protein bound calcium. Protein bound calcium was significantly reduced in both sham operated and oophorectomised rats at 3 days post-operation. In sham operated rats, this change was no longer significant from day 6 onwards, whereas in oophorectomised rats, the protein bound fraction was low from day 9 until the end of the experiment. Serum albumin was reduced in both sham operated and oophorectomised rats at 3 days post-operation. However, serum albumin in sham operated rats returned to pre-operative levels by day 21, while levels in oophorectomised rats remained significantly reduced at all times post-operation (Table 3.1).

Serum phosphate and TmP differed significantly between sham operated and oophorectomised rats at time of operation (mean values ± SEM; Phosphate (mmol/L): Sham, 1.50 ± 0.04; Oophx, 1.44 ± 0.02; TmP (mmol/L GF): Sham, 1.78 ± 0.06; Oophx, 1.68 ± 0.03). For this reason, the change in both variables from the time of operation was used for all analyses. Serum phosphate was increased in oophorectomised rats compared to sham operated controls from day 3 post-operation for the duration of the experiment (Table 3.2). There was no significant change in phosphate excretion with time or operation. The increase in renal phosphate reabsorption, indicated by increased TmP from 3 days post-operation onwards in oophorectomised rats was

Table 3.1 Serum calcium, albumin and calculated serum calcium fractions in sham operated and oophorectomised rats

		0 days	3 days	6 days	9 days
Calcium	Sham	2.38±0.02	2.28±0.02	2.55±0.03	2.31±0.03
(mmol/L)	Oophx	2.43±0.03	2.34±0.02	2.48±0.03	2.35±0.03
Ca^{2+}	Sham	1.26±0.01	1.27±0.01	1.35±0.01	1.25±0.01
(mmol/L)	Oophx	1.28±0.02	1.31±0.01	1.36±0.01	1.28±0.02
ΔPBCa	Sham	-	-0.1±.02	-0.03±.04	-0.05±.04
(mmol/L)	Oophx	-	-0.1±.01	0.001±.02	-0.08±.01[a]
Albumin	Sham	35.8±0.6	31.6±0.5	34.6±0.4	33.8±0.6
(g/L)	Oophx	36.4±0.6	30.7±0.4[a]	33.2±0.4[a]	31.9±0.4[a]

		15 days	21 days	42 days
Calcium	Sham	2.37±0.02	2.44±0.02	2.33±0.01
(mmol/L)	Oophx	2.34±0.03	2.45±0.02	2.37±0.01
Ca^{2+}	Sham	1.26±0.02	1.28±0.01	1.28±0.01
(mmol/L)	Oophx	1.27±0.01	1.30±0.01	1.32±0.03
ΔPBCa	Sham	-0.02±.02	0.01±0.01	-0.06±.01
(mmol/L)	Oophx	-0.09±.02[a]	-0.04±.02[a]	-0.10±.01[a]
Albumin	Sham	34.4±0.6	35.5±0.6	32.6±0.4
(g/L)	Oophx	31.9±0.6[a]	33.6±0.5[a]	30.4±0.4[a]

Values are mean ± SEM total serum calcium, calculated serum ionised calcium (Ca^{2+}), calculated serum protein bound calcium, and serum albumin in sham operated (Sham) and oophorectomised (Oophx) rats. *, $p<0.05$ vs sham by two way analysis of variance and Tukey's post hoc test.

Table 3.2 Serum phosphate, urinary phosphate excretion and maximal tubular phosphate reabsorption in sham operated and oophorectomised rats.

		0 days	3 days	6 days	9 days
ΔPO_4	Sham	-	0.12±0.10	0.09±0.09	0.11±0.09
(mmol/L)	Oophx	-	0.05±0.07[a]	0.20±0.09[a]	0.46±0.07[a]
PE	Sham	0.16±0.02	0.12±0.01	0.15±0.02	0.18±0.03
(mmol/L GF)	Oophx	0.14±0.01	0.13±0.04	0.13±0.01	0.14±0.02
ΔTmP	Sham	-	-0.08±0.16	0.16±0.10	0.07±0.13
(mmol/L GF)	Oophx	-	0.21±0.16[a]	0.42±0.11[a]	0.73±0.14[a]

		15 days	21 days	42 days
ΔPO_4	Sham	0.12±0.11	0.24±0.08	0.06±0.07
(mmol/L)	Oophx	0.49±0.10[a]	0.68±0.07[a]	0.54±0.06[a]
PE	Sham	0.17±0.01	0.25±0.03	0.13±0.01
(mmol/L GF)	Oophx	0.17±0.02	0.20±0.03	0.13±0.01
ΔTmP	Sham	0.03±0.13	0.11±0.15	0.18±0.16
(mmol/L GF)	Oophx	0.66±0.20[a]	0.83±0.14[a]	0.87±0.12[a]

Values are mean ± SEM change in serum phosphate (ΔPO_4), mean phosphate excretion (PE) and mean change in maximal tubular phosphate reabsorption (Δ TmP) in Sham operated (Sham) and oophorectomised (Oophx) rats. [a], $p<0.05$ vs baseline in the same operation group by two-way analysis of variance and Tukey's Post Hoc test.

probably responsible for maintenance of the level of phosphate excretion.

Serum phosphate and urinary hydroxyproline excretion were significantly related in oophorectomised rats, but not in sham operated rats. The linear regression equation in oophorectomised rats was:
$PO_4 = 1.61 + 0.435 \times OHPrE$, $R^2 = 0.180$, $p<0.005$.

3.3 Discussion

3.3.1 Serum Oestradiol Levels in Sham Operated and Oophorectomised Rats

17β-oestradiol levels in sham operated rats varied considerably. Most values were low, but in a number of animals, values were high, consistent with the sharp peak of 17β-oestradiol production observed during the 4-5 days oestrous cycle of the rat (209). Levels were significantly lower in oophorectomised rats. Values in oophorectomised rats were actually below the reported detection range for the immunoassay used. It is important to note that the low levels of serum oestradiol observed after oophorectomy were the same as levels reached at some stages of the oestrous cycle.

3.3.2 Effect of Operative Stress on Bone Turnover

Sham operation caused an increase in mineral appositional rate and osteocalcin, as well as a reduction in osteoclast surface, but not hydroxyproline excretion. These changes are consistent with the non-significant increase in trabecular bone volume and trabecular number observed at day 6 post-operation, and probably result from post-operative stress. Local production of growth factors may have been increased such that bone formation was stimulated relative to bone resorption.

The effect of sham operation on osteoclast surface lasted for 21 days, and it is possible that the factor inducing osteoclast surface reduction is oestrogen-dependent since osteoclast surface was not significantly reduced in oophorectomised rats with time. Since histomorphometric variables require different animals for each time point, these changes in osteoclast surface may also reflect slight differences in age or handling of animals at different time points. The experimental protocol was designed to allow for such differences (Chapter 2.1).

3.3.3 The Time Course of Increased Bone Turnover and Bone Loss after Oophorectomy

An increase in osteoclast surface has previously been reported at 15 days post-oophorectomy (52), and raised urinary hydroxyproline excretion reported at 21 days (51). We now describe a high level of osteoclast surface and hydroxyproline excretion in oophorectomised rats compared to sham operated rats by 6 days post-operation. Even at 3 days post-operation, hydroxyproline excretion was already increased, although the increase did not reach statistical significance until later, indicating the onset of increased bone resorption in oestrogen deficiency is very rapid. Markers of osteoclast function increased further until 21 days post-oophorectomy before beginning to decline between 21 and 42 days post-operation towards levels detected in sham operated rats. The apparent reduction in bone resorption at this stage is consistent with previous data where hydroxyproline excretion declined between 3 and 9 weeks post-operation (51).

Increased osteoclast surface and hydroxyproline excretion may indicate stimulated osteoclastic recruitment resulting in increased bone resorption after oophorectomy. Increased osteoclast recruitment in oophorectomised rats would be associated with increased BMU activation frequency and a greater whole body remodelling space, as previously suggested by studies in post-

menopausal women (8,17). As activation frequency is increased, the risk of trabecular perforation would also increase since the likelihood of resorption occurring on both sides of a single trabecula would rise (62,63).

At 6 days post-oophorectomy, when hydroxyproline excretion and osteoclast surface were elevated, there were no changes detected in either histomorphometric or biochemical markers of bone formation. In contrast, alkaline phosphatase activity and osteocalcin were not increased until 9 days after oophorectomy, continuing to rise until 21 days when levels reached a plateau. Mineral appositional rate and double fluorochrome labelled surface were significantly increased at 12 days post-oophorectomy, consistent with the rise in alkaline phosphatase activity and osteocalcin. Previous studies have reported an increase in serum osteocalcin by 14 days post-oophorectomy (49), and increased osteoblast surface and active formation surface at 15 days post-oophorectomy (52). It appears therefore that bone resorption increases prior to formation in the oophorectomised rat.

No significant change in osteoid surface was detected in oophorectomised rats although an increase in osteoid surface has been reported at 10 months after oophorectomy (64). Since a cycle of bone formation takes approximately 33 days in the rat (210), it may not be surprising that a change in osteoid surface was not detected in the present short term study.

The increase in bone resorption prior to formation supports the model of a direct effect of oestradiol on osteoclasts or osteoclast precursors via specific receptors (38,103). However, other direct effects of oestradiol may not be excluded, such as stimulation of osteoblast precursor proliferation however, or an effect on osteoclasts via production of a locally acting factor by osteoblasts.

The temporary imbalance between bone resorption and formation appears to be the primary cause of the dramatic reduction in trabecular bone volume in the metaphyseal core region. Loss of bone after oophorectomy was rapid, occurring soon after the increase in bone turnover, and in this area the bone loss was limited, with no further reduction in bone volume detected between 15 and 42 days post-operation. The delayed increase in bone formation following increased resorption, and the limited nature of the subsequent bone loss suggest a model of an initial disturbance of bone turnover after oophorectomy, rather than a continued negative balance of bone resorption and formation at the BMU level (17,23). This is particularly evident since bone turnover remained high for the duration of this experiment without further bone loss.

Our observation of the time course of increased histomorphometric markers of increased bone turnover and bone loss after oophorectomy is limited to only one area, that is cancellous bone morphometry in the femur. The increase in urinary hydroxyproline excretion prior to the increases in serum osteocalcin and alkaline phosphatase activity indicate that throughout the skeleton bone resorption is increased prior to bone formation. In the present study however, histomorphometric variables were measured in the anterior metaphyseal core of the femur. Loss of bone in this area occurs over a very short time period, probably because of the architecture in this area. In other areas of the skeleton, for example in the vertebrae, bone cell activity was increased, but loss of bone had not occurred in the time frame of this experiment. It is likely that the rate of bone loss in different areas of the skeleton varies with the architecture of the bone, relative activity of bone cells in the area, and the mechanical forces acting on the bones.

The proposed model of a temporary imbalance in bone turnover in favour of bone resorption is supported by the exponential character of menopausal bone loss (9,10,66) and other reports of rapid and limited bone loss in the

oophorectomised rat (14,52,55,56,63). Wronski et al have reported rapid bone loss between the time of operation and 14 days post-operation, which declines, remaining at a plateau from 100 days post-oophorectomy onwards as the limit of trabecular bone loss is reached with continuation of the study (52). Another report by that group has shown femoral bone loss at 35 days after oophorectomy does not worsen for the next 17 months, although this study was based on dual photon absorptiometry measurements of bone density which include a cortical component, possibly masking any further trabecular bone loss (57).

The increase in bone formation appeared to last for longer than the increase in resorption, consistent with the greater level of formation than resorption reported at 35 days after oophorectomy by Wronski et al. (53,54). The longer duration of the high level of bone formation may prevent any further loss of bone in response to greater mechanical strain as trabecular bone volume is reduced, as described in the metaphyseal core (62). The duration of increased bone resorption and formation we have detected in response to oophorectomy may be characteristic of the femoral distal metaphysis and metaphyseal - diaphyseal core regions only. The level of bone resorption may remain elevated for longer in other parts of the skeleton since elevated bone resorption has been reported 100 days after oophorectomy in the proximal tibial metaphysis (52). Bone formation in the same area however, is still raised at 540 days post-operation, although this result is confounded by the age-related bone loss beginning at 270 days post-operation (56).

Trabecular number reduced concomitantly with decreased trabecular bone volume between 9 and 15 days post-oophorectomy, without a detectable change in trabecular thickness. Data presented here are consistent with a model of bone loss after oophorectomy due primarily to perforation and loss of whole trabeculae (62,63) in the metaphyseal core. The early increase in bone resorption, and increased activation frequency is likely to result in rapid

trabecular perforation, followed by continued resorption of remaining termini and complete removal of trabecular fragments which are not weight bearing. Gradual trabecular thinning may be involved in later bone loss or age-related loss after oophorectomy. Thinning has been reported at other femoral sites after oophorectomy (211), but was not noted here, possibly being obscured by preferential resorption of thinner trabecular plates.

Vertebral trabecular bone volume, trabecular number and thickness were not altered at 18 days post-operation, a time when femoral bone loss had already occurred. A reduction in vertebral trabecular bone volume in the oophorectomised rat has been reported by others at 6 months post-operation (56). Vertebral osteoclast surface, although significantly greater in oophorectomised rats indicating a systemic effect of oestrogen deficiency on bone metabolism, was considerably lower than levels of femoral osteoclast surface. The lower level of osteoclast activity in vertebral bodies and the greater trabecular bone volume in this region indicates a much lower proportion of bone turnover to bone volume in the vertebral body compared to the femur and may explain why no bone loss was detected at this stage.

While these data fit with a model of increased BMU initiation, and maintenance of the normal temporal relationship of bone resorption and formation in bone remodelling, a direct effect of oestrogen deficiency on osteoblast recruitment compared to the rapidly increased osteoclast recruitment cannot be ruled out as contributing to the bone loss following oophorectomy. Tabuchi et al have described impaired proliferative potential of marrow cells in oophorectomised rats, without any alteration in their osteogenic potential (71). Alternatively, osteoblasts and osteoclasts may be recruited simultaneously but osteoblast function may be impaired and bone formation delayed (212). Since both bone formation rate and surface were increased, an impairment in osteoblast function in oestrogen deficiency appears the most unlikely model to fit our observations. Osteoblast impairment has been suggested by others to relate

more to the age related loss of bone also occurring in post-menopausal women (18).

Hydroxyproline excretion and osteoclast surface were closely related in this experiment as previously described in post-menopausal women (208). We have now also demonstrated a significant relationship between mineral appositional rate and serum osteocalcin and between double fluorochrome labelled surface and alkaline phosphatase activity. Since alkaline phosphatase activity and osteocalcin are expressed at different stages of osteoblast development and function (190) it is likely that mineral appositional rate and double fluorochrome labelled surface also indicate different stages of osteoblast activity in the process of bone formation.

Alkaline phosphatase is produced during the early differentiation stage of osteoblast development (190). It follows that in oestrogen deficiency increased serum alkaline phosphatase activity levels indicate increased osteoblast number, consistent with bone formation at a greater number of BMUs and confirmed by increased double fluorochrome labelled surface. Increased double fluorochrome labelled surface was not significantly related to mineral appositional rate, implying the elevation in mineral appositional rate after oophorectomy may not relate directly to an increase in osteoblast number, but to increased mineralising activity per osteoblast. Osteocalcin is produced by osteoblasts during bone mineralisation (190) and increased serum osteocalcin in oestrogen deficiency was related to increased mineralising rate, but not to the increased bone mineralising surface. These data suggest two simultaneous events may be involved in increased bone formation in oestrogen deficiency; increased osteoblast proliferation, indicated by rises in double fluorochrome labelled surface and alkaline phosphatase activity, and increased activity per osteoblast, indicated by rises in osteocalcin and mineral appositional rate. These events follow the increase in osteoclast proliferation in an attempt to refill BMU pits resorbed during the rise in bone resorption.

3.5 Effect of Oophorectomy on Calcium and Phosphate Metabolism

No detectable change in serum calcium, or the ionised fraction of serum calcium was detected following oophorectomy in this experiment despite the increase in bone resorption, consistent with a number of other studies (47,51,76). Kalu et al have reported a decrease in serum calcium after oophorectomy, however this was only a minor fall and was only detected at a higher level of dietary calcium than used in this study (47). There does not appear to be any evidence for a reduction in serum calcium prior to bone loss in the oophorectomised rat. The increase in bone resorption does not therefore appear to occur in response to a change in serum calcium. It is possible that residual calcium from the high level of dietary calcium may conceal changes in serum calcium levels, however, a reduction in the level of serum calcium by use of a synthetic diet did not significantly alter serum calcium levels (Chapter 2.5.2).

Serum albumin levels are decreased rapidly after oophorectomy. This decrease was associated with a reduction in the protein bound fraction of serum calcium, and may indicate a direct effect of oestradiol on hepatic albumin production. This change appears to be due to decreased albumin synthesis rather than albumin leakage from dysfunctional liver cells (213), and may be related to the role of plasma proteins in binding blood oestradiol indicating some feedback mechanism whereby oestradiol regulates albumin production.

The oophorectomy induced increase in serum phosphate was related to increased renal phosphate reabsorption and may reflect a direct oestrogen action on specific renal receptors (39). Changes in serum phosphate may also relate to an increase in renal PTH sensitivity, as suggested in bone (43) or slight hyperparathyroidism in the oophorectomised rat (44). This model seems unlikely however, since parathyroidectomy of oophorectomised rats does not

prevent the oophorectomy induced changes in renal phosphate handling (Sims et al, in press). Serum phosphate was significantly related to bone resorption, consequently release of phosphate from the extracellular matrix during increased resorption may contribute to the increased level in serum.

CHAPTER FOUR

IMMEDIATE EFFECTS OF OESTRADIOL TREATMENT ON BONE TURNOVER IN THE OOPHORECTOMISED RAT

4.1 Clearance of Injected 17β-Oestradiol From the Circulation

4.1.1 Introduction

Treatment of oophorectomised rats with 5μg/kg/day or 10μg/kg/day 17β-oestradiol produces supraphysiological circulating 17β-oestradiol levels (82,85). Oestradiol treatment at these doses was effective in preventing bone loss after oophorectomy however, since oestradiol levels are not normally distributed, the actual circulating level required for this action is not clear. Over the 4-5 day oestrous cycle of the rat serum oestradiol levels fluctuate from approximately 17 to 88 pg/ml (209), a range that includes the mean oestradiol levels reported for the oestradiol treated rats above. With a half life of 8 h in the circulation, daily injections of oestradiol may be cleared some time before the next injection. It is not apparent therefore whether reported levels of oestradiol relate to the peak level attained after a bolus injection, or circulating oestradiol levels at another time. Published studies have not reported the time after injection at which circulating oestradiol levels were measured.

Circulating 17β-oestradiol levels have been reported over 168 h after a single intravenous injection in the mouse, and levels of serum oestradiol resulting from doses of 1.3 mg/kg - 16.7 mg/kg had all dropped to below the range of values obtained in sham operated mice at 168 h after injection (214). At 33 mg/kg oestradiol, circulating oestradiol levels did not drop below the sham operated range, because oestradiol accumulated in the circulation after repeated doses. This treatment level, which also has significant side effects is considerably higher than the level required to prevent bone loss in

oophorectomised mice, determined to be 7.5μg/kg (215). Direct comparisons of clearance time in this study on mice cannot be applied to work done in the rat.

A short-term study was performed to determine changes in circulating 17β-oestradiol levels in oophorectomised rats over the 24 hours following single bolus injections of 8μg/kg and 20μg/kg 17β-oestradiol compared with levels in rats receiving corn oil vehicle.

4.1.2 Protocol

This experiment was carried out within the protocol of the long-term experiment described in Chapter 4.2. Oophorectomised rats were treated with oil vehicle, 8μg/kg/day or 20μg/kg/day 17β-oestradiol by daily injections. 17β-Oestradiol (Sigma, St Louis, MO, USA) was dissolved 1 mg/ml in 100% ethanol, and diluted in corn oil for 0.1 ml injections. Injections were administered subcutaneously at 0900 h from the day of operation. At 2, 4 or 24 h after injection blood specimens were obtained from the tail vein from 6-8 animals in each treatment group. Serum samples were analysed for 17β-oestradiol (Chapter 2.3).

4.1.3 Results

Figure 4.1 shows the levels of oestradiol in treated rats compared to the range of oestradiol levels measured in untreated sham operated and oophorectomised rats in Chapter 2. A single bolus injection of oestradiol at 8μg/kg or 20μg/kg significantly increased serum oestradiol to levels well in excess of the normal range of intact rats (Figure 4.1). Oestradiol was cleared such that levels were within the oophorectomised range by 24 h after injection. At 2 h after injection, serum oestradiol levels in vehicle treated oophorectomised rats were slightly raised compared to levels in untreated oophorectomised rats. The

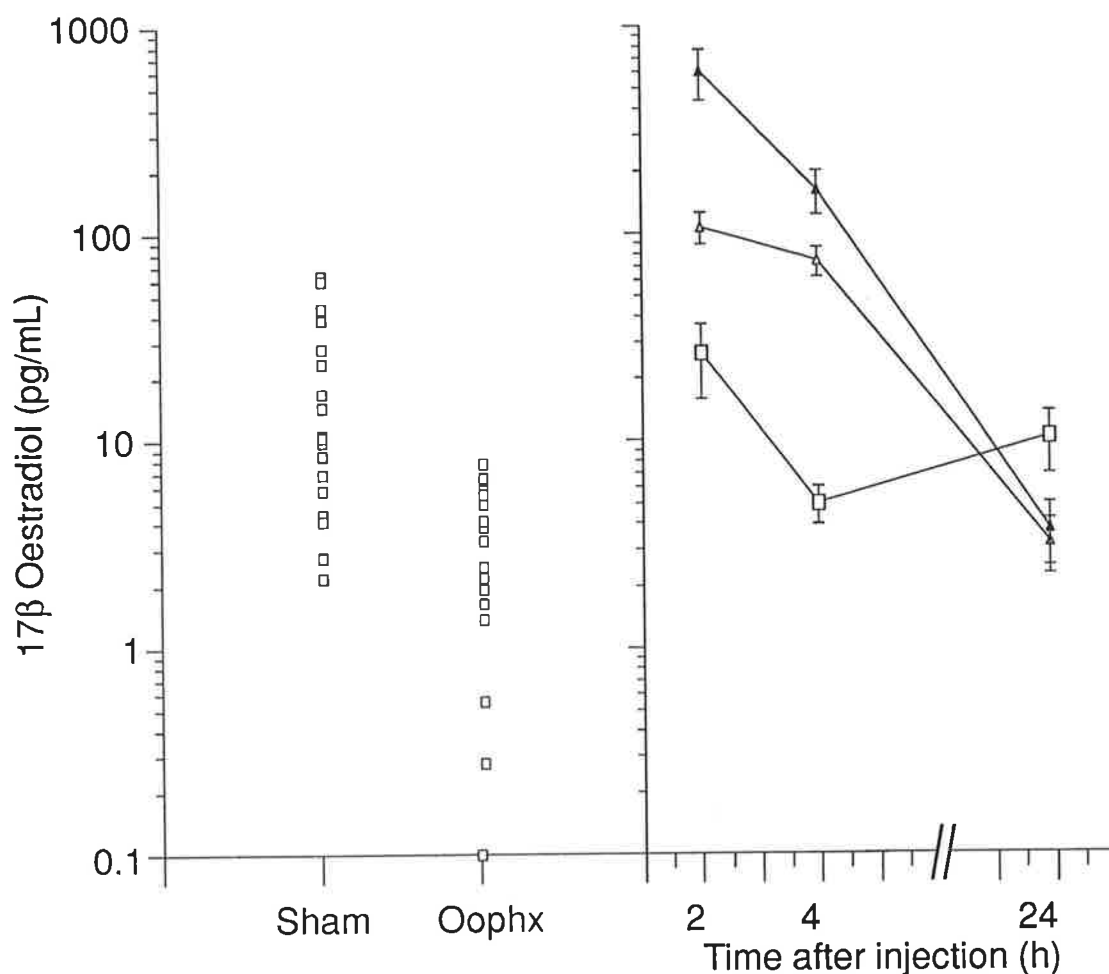


Figure 4.1 Clearance of injected 17β-oestradiol from the circulation. Serum 17β-oestradiol levels (mean pg/ml ± SEM) in oophorectomised rats treated with oil vehicle (□), 8μg/kg/day (△) or 20μg/kg/day (▲) 17β-oestradiol at 2, 4 and 24 h after a single bolus injection (right) in comparison to 17β-oestradiol levels in untreated sham operated and oophorectomised rats (left). In oestradiol treated rats, circulating oestradiol exceeded the range of levels observed in sham operated rats for the first 4 h after injection. Levels returned to those observed in untreated sham operated and oophorectomised rats at 24 h.

reason for this is unclear, but did not appear to alter the effect of oophorectomy on bone turnover (Chapter 4.4 and 4.5).

4.2 Immediate Effects of Oestradiol Treatment on Bone Turnover in the Oophorectomised Rat

It is well established that systemic oestradiol treatment prevents increased bone turnover (50,81,82,84,85,87,88) and bone loss in oophorectomised rats (50,81-86) however the mechanism of this effect is unclear. This section describes oestradiol suppression of increased bone turnover and bone loss in oophorectomised rats during the first 21 days following oophorectomy, at the time of the initial increase in bone turnover, and trabecular bone loss described in Chapter 3.

4.2.1 Protocol

102 female Sprague Dawley rats at 6 months of age were used. 6 non-operated animals were killed under halothane anaesthesia as a baseline group. The remaining animals were oophorectomised and separated into three groups: oil vehicle treatment (n=30), 8μg/kg oestradiol (low dose, n=34), 20 μg/kg oestradiol (high dose, n=32). 17β-Oestradiol (Sigma, St Louis, MO, USA) was dissolved 1 mg/ml in 100% ethanol, and diluted in corn oil for 0.1 ml injections. Subcutaneous injections were administered at 0900 h from the day of operation until animals were killed.

6 - 8 animals from each treatment group were killed by cervical dislocation under halothane anaesthesia and femora were removed at 6, 9, 15 and 21 days after operation. Blood and urine specimens were collected at the time of operation and at 3 or 6, 9, 15 and 21 days post-operation from animals killed at 15 and 21 days. At the end of the experiment, prior to killing, blood was collected by cardiac puncture under halothane anaesthesia. Calcein and

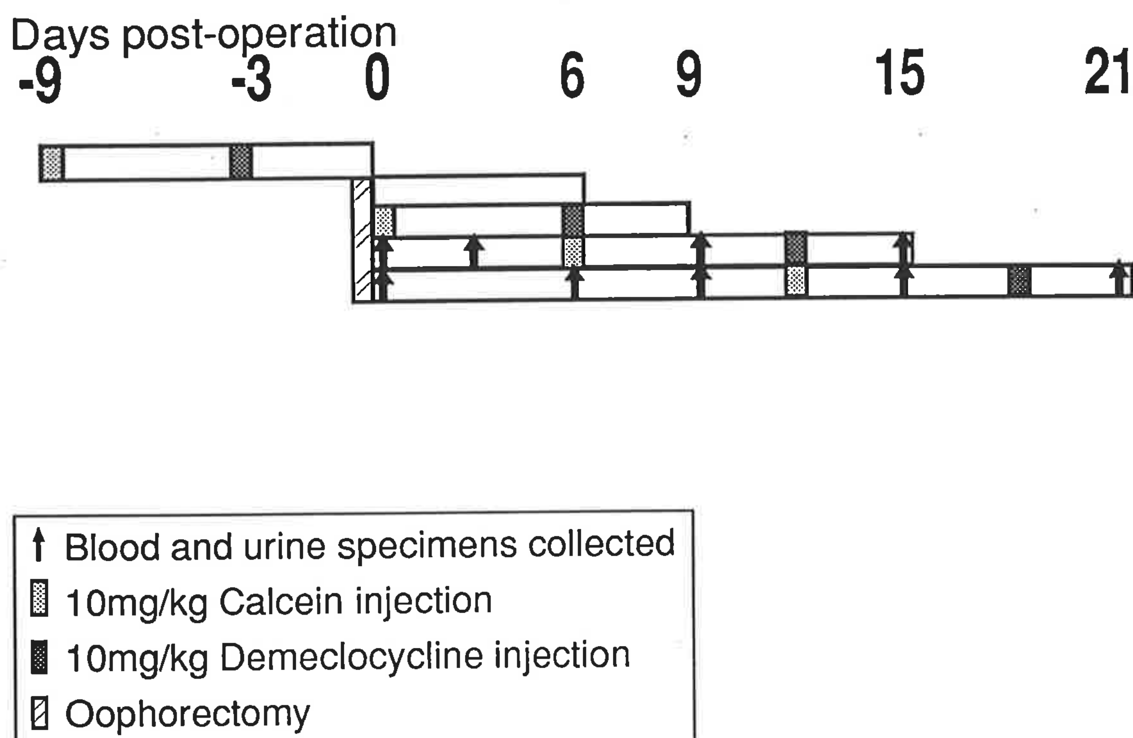


Figure 4.2 Protocol to determine short term effects of oestradiol treatment in oophorectomised rats. Six animals were killed as a baseline group. The remaining 96 were oophorectomised at day 0, shown by the striped bar. Injections of oil vehicle (n=30), 8μg/kg (n=34) or 20μg/kg 17β-oestradiol (n=32) were administered daily from day 0. Calcein and demeclocycline labels were administered at days indicated by the pale and dark stripes respectively. Blood samples were collected at the arrowed time points. Animals were killed at 6, 9, 15, 18 or 21 days post-operation, and femora removed for analysis.

demeclocycline (10 mg/kg body weight) were administered intraperitoneally at 9 and 3 days respectively before killing to all rats, except those killed at day 6 post-operation. This protocol is represented in Figure 4.2.

Serum was analysed for sodium, potassium, chloride, bicarbonate, creatinine, albumin, alkaline phosphatase activity and osteocalcin (Chapter 2.3). Serum anion gap, globulin and calcium fractions were calculated (Chapter 2.3). Urinary hydroxyproline and phosphate excretion and TmP were determined in 24 h fasting urine specimens (Chapter 2.3).

Femora were embedded in K Plast resin (Chapter 2.6.1). trabecular bone volume, trabecular number and trabecular thickness were determined in the diaphyseal-metaphyseal core using the Quantimet 520 Image Analysis System on von Kossa stained sections (Chapter 2.6.2). Extent of double fluorochrome labelled surface and mineral appositional rate were determined in unstained sections (Chapter 2.6.3). osteoclast surface was determined on sections stained for acid phosphatase (Chapter 2.6.4).

4.2.2 Results

The initial effect of oestradiol treatment in oophorectomised rats was a transient rise in trabecular bone volume and trabecular number observed at either dose at 6 days post operation (Figure 4.3). In the oophorectomised rats receiving the oil vehicle only, this experiment confirmed the reduction in trabecular bone volume and trabecular number between 9 and 15 days post-operation described in Chapter 3. Oestradiol treatment delayed bone loss at either dose only until 21 days post-operation. Oestradiol treatment also significantly increased trabecular thickness at 15 and 21 days post-operation (Figure 4.4).

The relationship between trabecular bone volume and trabecular number was

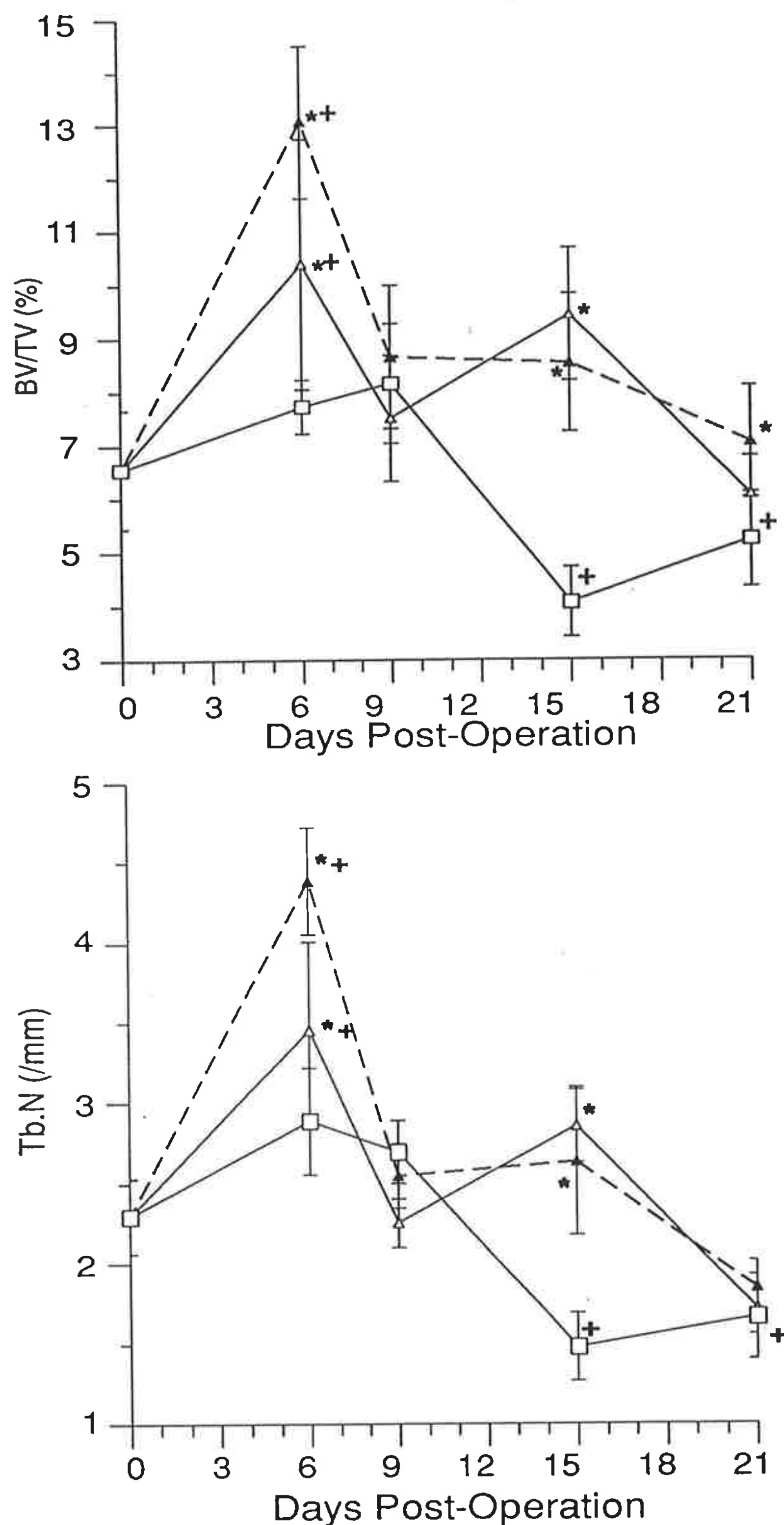


Figure 4.3 Loss of trabecular bone is delayed by oestradiol treatment. The reduction in trabecular bone volume (BV/TV) and trabecular number (Tb.N) observed in oophorectomised rats (□) was delayed by both 8 µg/kg/day (△) and 20µg/kg/day (▲) oestradiol. There was also a transient increase in BV/TV and Tb.N at 6 days post-operation, before bone loss had occured. Values are mean ± SEM. *, $p<0.05$ vs oophorectomised rats; +, $p<0.05$ vs baseline rats (day 0) by two-way analysis of variance and Tukey post-hoc test.

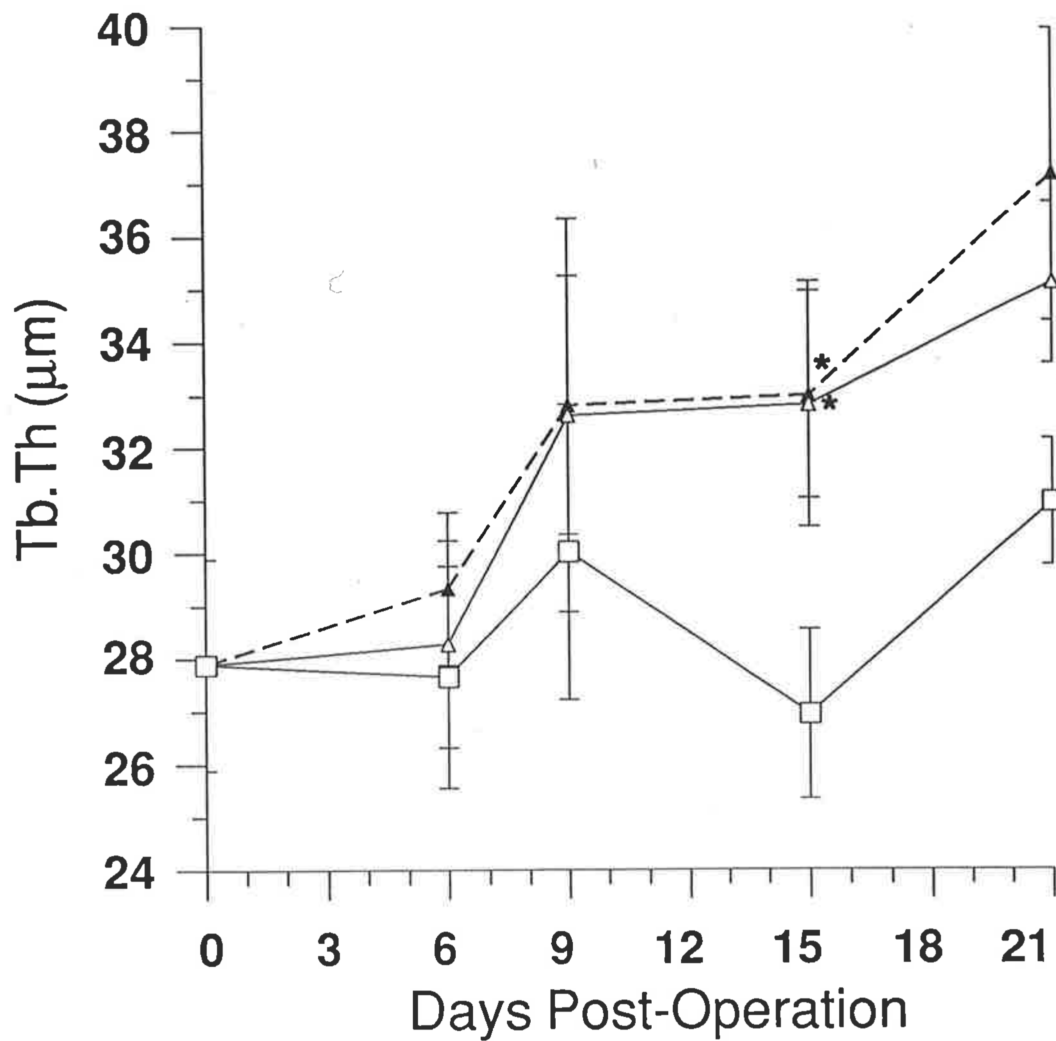


Figure 4.4 Effect of oestradiol treatment on trabecular thickness. Trabecular thickness (Tb.Th) was not affected by oophorectomy (□). Oestradiol treatment at both 8 μg/kg/day (△) and 20μg/kg/day (▲) significantly increased Tb.Th from 15 days post-operation. Values are mean ± SEM. *, $p<0.05$ vs oophorectomised rats by two-way analysis of variance and Tukey post-hoc test.

stronger than that between trabecular bone volume and trabecular thickness in both oestradiol and vehicle treated rats (Figure 4.5). At the high dose of oestradiol, the relationship between trabecular bone volume and trabecular thickness was not significant (Table 4.1)

High dose oestradiol treatment completely prevented the rise in urinary hydroxyproline excretion observed in oophorectomised rats. Hydroxyproline excretion in oestradiol treated rats was decreased to below pre-operative levels at 21 days post-operation (Figure 4.6). Low dose oestradiol treatment was partially effective, with a rise in hydroxyproline excretion at 6 days and 9 days being suppressed at 15 days post-operation. Hydroxyproline excretion returned to baseline levels at 21 days post-operation.

High dose oestradiol treatment only partially prevented the rise in osteoclast surface (Figure 4.6), such that at 9 days post-operation the level of osteoclast surface was equivalent to the level observed in untreated oophorectomised rats. Low dose oestradiol treatment significantly reduced osteoclast surface only at 15 days post-operation. It is difficult to determine whether the effects of oestradiol on osteoclast surface were significant at 21 days post-operation however due to the unexpected fall in osteoclast surface in both vehicle and oestradiol treated rats observed at this time.

Serum alkaline phosphatase activity was suppressed by both low and high dose oestradiol treatment at 6 days post-operation to levels lower than baseline (Figure 4.7). This suppression occurred prior to the time when elevated alkaline phosphatase activity was observed in vehicle treated oophorectomised rats at 9 days post-operation. The suppression of alkaline phosphatase activity was maintained for the duration of the experiment. High dose oestradiol also suppressed double fluorochrome labelled surface to levels below baseline until 12 days post-operation (Figure 4.7). Low dose oestradiol treated rats had a significantly lower level of double fluorochrome labelled

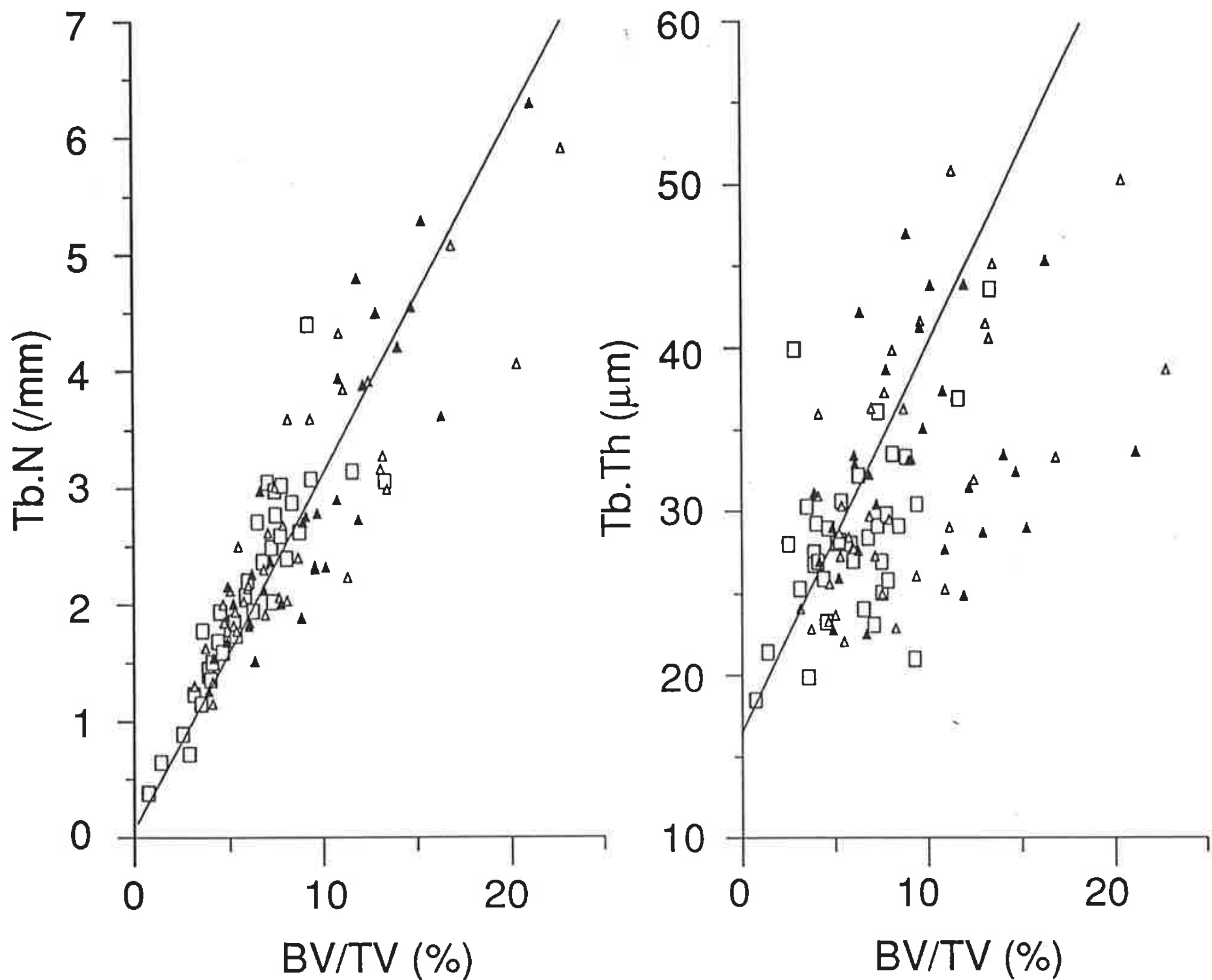


Figure 4.5 Linear relationships between femoral morphometric variables. Values are trabecular bone volume (BV/TV), trabecular number (Tb.N) and trabecular thickness (Tb.Th) from oophorectomised rats treated with oil vehicle (□), 8ug/kg/day 17β-oestradiol (△), or 20ug/kg/day 17β-oestradiol (▲). The straight lines represent simple linear regression equations derived from the data presented:

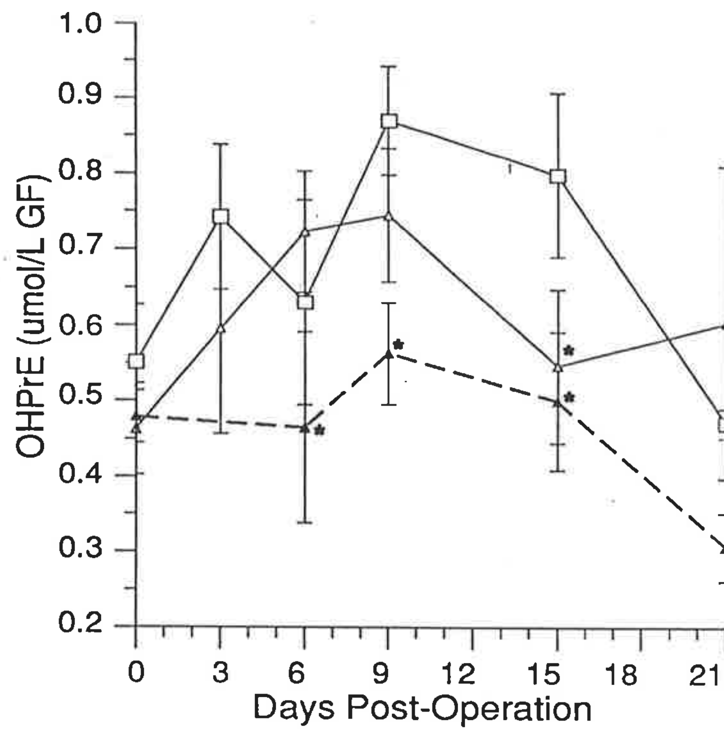
BV/TV = -0.407 + 3.3 x Tb.N; R = 0.789; $p<0.001$.

BV/TV = - 2.20 + 326 x Tb.Th; R = 0.288; $p<0.001$.

Table 4.1 The relationship between trabecular bone volume and trabecular number or trabecular thickness in vehicle treated or oestradiol treated rats

Treatment Group	Regression Equation	R^2	p value
Combined	BV/TV = - 0.407 + 3.3 x Tb.N	0.789	$p<0.001$
	BV/TV = - 2.20 + 326 x Tb.Th	0.288	$p<0.001$
Vehicle Treated	BV/TV = 0.152 + 2.8 x Tb.N	0.773	$p<0.001$
	BV/TV = - 1.48 + 262 x Tb.Th	0.236	$p=0.002$
8μg/kg/day	BV/TV = - 1.27 + 3.7 x Tb.N	0.763	$p<0.001$
17β-oestradiol	BV/TV = - 3.16 + 370 x Tb.Th	0.375	$p<0.001$
20μg/kg/day	BV/TV = 0.87 + 3.0 x Tb.N	0.815	$p<0.001$
17β-oestradiol	BV/TV = 3.55 + 175 x Tb.Th	0.046	$p=0.128$

Linear regression equations were derived from trabecular bone volume (BV/TV), thickness (Tb.Th) and number (Tb.N) in oophorectomised rats treated with oil vehicle, 8μg/kg/day or 20μg/kg/day 17β-oestradiol at all times post-operation.



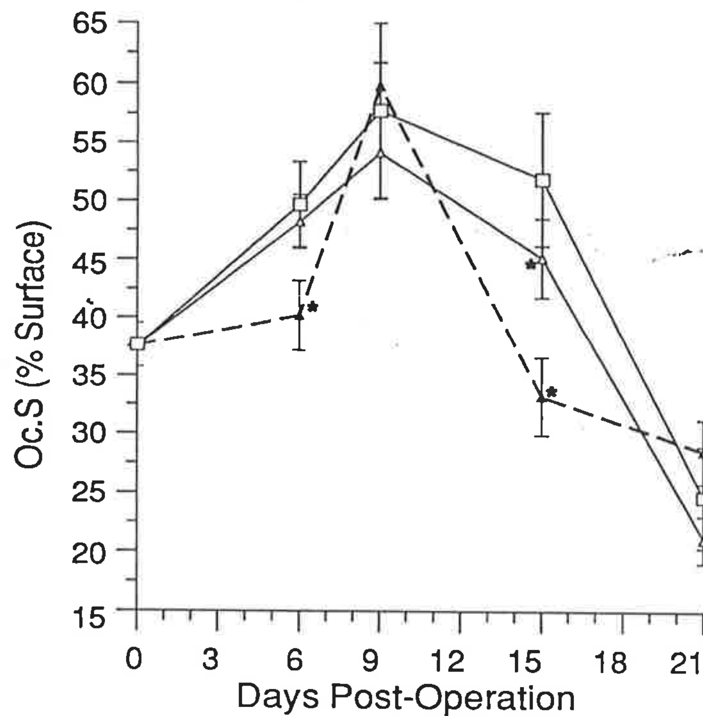
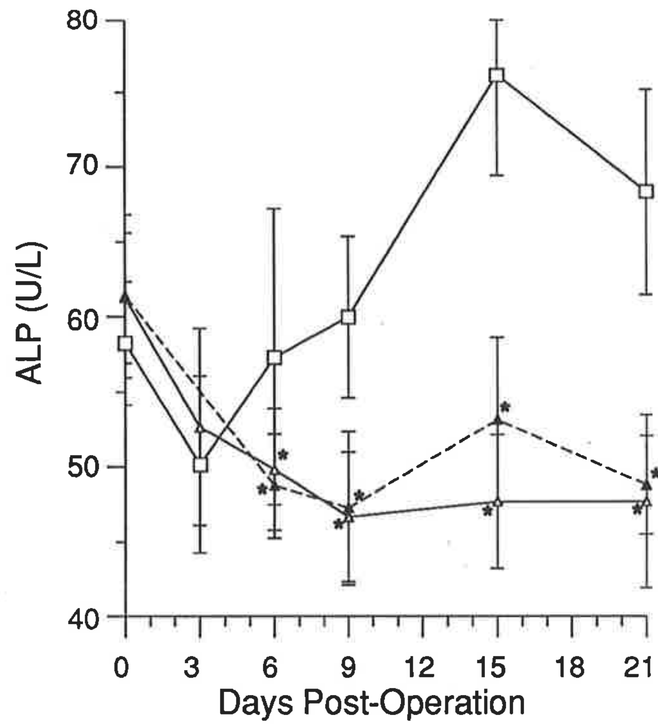


Figure 4.6 Oestradiol treatment prevented the increase in bone resorption observed in oophorectomised rats. The oophorectomy-induced rise (□) in urinary hydroxyproline excretion (OHPrE) was completely blocked by 20µg/kg/day oestradiol (▲). The increase in osteoclast surface (Oc.S) was only significantly lower in oestradiol treated rats at 6 and 15 days post-operation. Oestradiol treatment at 8µg/kg/day (△) inhibited markers of resorption at 15 days post-operation only. Values are mean ± SEM. *, $p<0.05$ vs vehicle treated oophorectomised rats by two-way analysis of variance and Tukey post-hoc test.



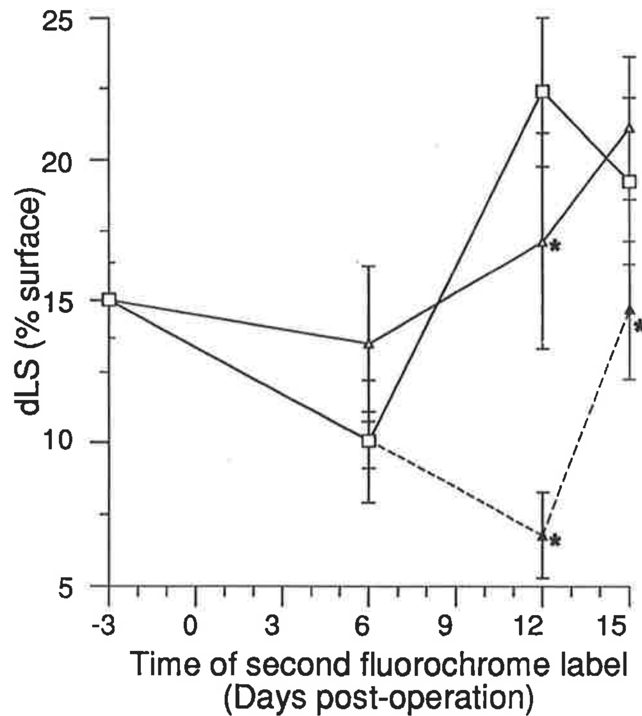


Figure 4.7 Oestradiol treatment pevented the rise in markers of osteoblast maturation observed in oophorectomised rats (□). Alkaline phosphatase activity (ALP) was suppressed to a minimum from 6 days post-operation at both 8µg/kg/day (△) and 20µg/kg/day oestradiol (▲) for the duration of the experiment. Extent of double fluorochrome labelled surface (dLS) was reduced from day 12 by 20µg/kg/day. Treatment with 8µg/kg/day reduced dLS at day 12 only. Values are mean ±SEM. *, p<0.05 vs vehicle treated oophorectomised rats by two-way analysis of variance and Tukey post-hoc test.

surface compared to vehicle treated rats at 12 days post-operation only.

Oestradiol treatment did not prevent the rise in serum osteocalcin detected at 6 days post-oophorectomy (Figure 4.8). This increase in bone formation is possibly related to the stress of operation, since it was also observed in sham operated rats in Chapter 3.2. A dose related suppression of serum osteocalcin by oestradiol treatment was observed from 15 days post-operation. Osteocalcin levels returned to baseline in oestradiol treated rats at 21 days post-operation (Figure 4.8). Mineral appositional rate was suppressed by oestradiol treatment in a dose dependent manner at 12 days post-operation (Figure 4.8).

There was no significant change in serum total calcium or ionised calcium levels detected in this experiment with either oophorectomy or oestradiol treatment (Table 4.2). Oestradiol treatment prevented the oophorectomy-induced reduction in serum albumin, and consequently, the reduction in the protein bound calcium fraction associated with oophorectomy (Table 4.2).

Oestradiol treatment prevented the increase in serum phosphate observed after oophorectomy (Table 4.3). Renal tubular phosphate reabsorption however, was only partially reduced by oestradiol treatment, remaining at the high levels induced by oophorectomy (Table 4.3). The level of urinary phosphate excretion was not significantly altered by oestradiol treatment.

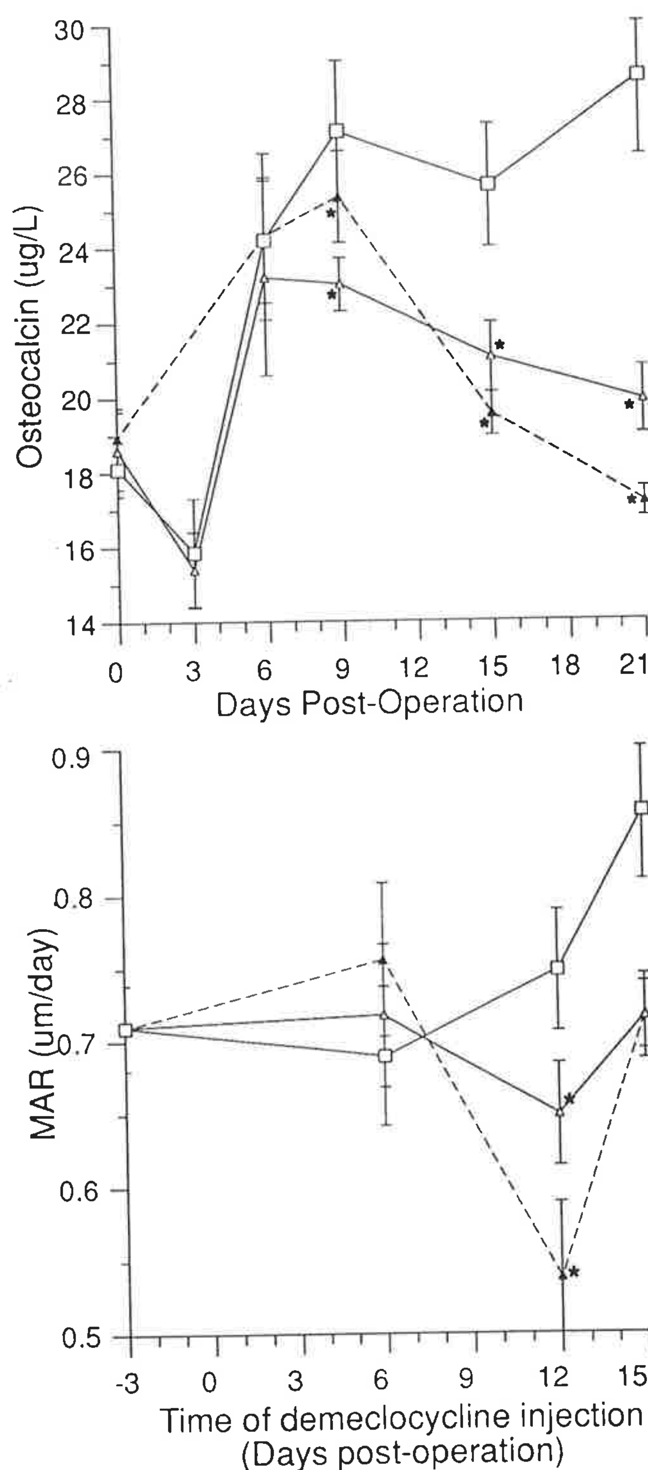


Figure 4.8 Oestradiol treatment at both low and high doses prevented the oophorectomy-induced rise (□) in markers of bone mineralisation. There was an initial rise in serum osteocalcin, but this was lowered from 9 days post-operation by oestradiol treatment at both 8μg/kg/day (△) and 20μg/kg/day oestradiol (▲). Mineral appositional rate (MAR) was suppressed in a dose-dependent manner from day 12. Values are mean ± SEM. *, $p<0.05$ vs vehicle treated controls by two-way analysis of variance and Tukey post-hoc test.

Table 4.2 Serum calcium, albumin and calculated serum calcium fractions in oophorectomised rats treated with vehicle or 20μg/kg/day oestradiol

		0 days	6 days	9 days	15 days	21 days
Calcium	Control	2.41±0.04	2.37±0.04	2.35±0.04	2.37±0.03	2.35±0.01
(mmol/L)	Treated	2.41±0.03	2.38±0.03	2.49±0.01	2.39±0.03	2.34±0.02
Ca^{2+}	Control	1.27±0.01	1.28±0.02	1.27±0.02	1.26±0.01	1.29±0.01
(mmol/L)	Treated	1.27±0.02	1.28±0.01	1.32±0.01	1.25±0.01	1.25±0.01
ΔPBCa	Control	-	-0.11±.04	-0.09±.02	-0.11±.02	-0.11±0.02
(mmol/L)	Treated	-	-0.04±.02[a]	-0.02±.02[a]	-0.04±.02[a]	-0.06±.02[a]
Albumin	Control	38.5±1.1	31.7±0.2[a]	32.9±0.4[a]	32.2±0.5[a]	30.8±0.4[a]
(g/L)	Treated	38.5±1.0	33.1±0.6[a,b]	35.1±0.6[a,b]	36.0±0.8[a,b]	33.3±0.4[a,b]

Values are mean ± SEM serum total calcium, ionised calcium (Ca^{2+}), protein bound calcium, and albumin in oophorectomised rats treated with oil vehicle (Control) or 20μg/kg/day 17β-oestradiol (Control). Data from 8μg/kg/day oestradiol treated rats are not presented since values were not significantly different from 20μg/kg/day. [a], $p<0.05$ vs. day 0 of same operation group; [b], $p<0.05$ vs. vehicle treated group at same time, by two-way analysis of variance and Tukey's post-hoc test.

Table 4.3 Serum phosphate, urinary phosphate excretion and maximal tubular phosphate reabsorption in sham operated and oophorectomised rats.

		0 days	6 days	9 days	15 days	21 days
Phosphate	Control	1.34±0.11	1.63±0.11[a]	1.65±0.06[a]	1.75±0.07[a]	1.81±0.08[a]
(mmol/L)	Treated	1.33±0.07	1.58±0.04[a]	1.56±0.07[a]	1.55±0.03[a,b]	1.51±0.03[a,b]
PE	Control	0.12±0.01	0.19±0.03	0.17±0.02	0.18±0.03	0.10±0.01
(mmol/L GF)	Treated	0.14±0.02	0.12±0.03	0.21±0.06	0.12±0.02	0.12±0.02
TmP	Control	1.62±0.12	1.89±0.21	1.89±0.07	2.03±0.12[a]	2.42±0.12[a]
(mmol/L GF)	Treated	1.54±0.09	2.10±0.15[a]	1.78±0.22	1.95±0.09[a]	1.85±0.10[a]

Values are mean ± SEM serum phosphate, mean phosphate excretion (PE) and mean maximal tubular phosphate reabsorption (TmP) in oophorectomised rats treated with oil vehicle (Control) or 20μg/kg/day 17β-oestradiol (Control). Values from oophorectomised rats treated with 8μg/kg/day 17β-oestradiol are not presented since values were not significantly different to 20μg/kg/day 17β-oestradiol at any time point. [a], $p<0.05$ vs. day 0 of same operation group; [b], $p<0.05$ vs. vehicle treated group at same time, by two-way analysis of variance and Tukey's post-hoc test.

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4.2.3 Discussion

4.2.3.1 Oestradiol Treatment Prevents Bone Loss by Preventing Loss of Whole Trabeculae

Oestradiol treatment provided dosages resulting in serum oestradiol levels significantly greater than those observed in ovary-intact rats immediately following administration returning to basal levels before the next injection. The initial effect of these levels of oestradiol was to stimulate bone formation such that trabecular bone volume was elevated above pre-operative levels at 6 days post-operation, which is prior to the loss of bone induced by oophorectomy (Chapter 3). The increase in trabecular bone volume was associated with increased trabecular number, suggesting synthesis of new trabeculae. This is consistent with the non-significant rise in trabecular bone volume and trabecular number observed at 6 days post-operation in sham operated and oophorectomised rats in Chapter 3, and could in part be related to post-operative stress, although the increase with oestradiol treatment was greater.

It is unlikely that the increased trabecular number with oestradiol treatment arises from a rapid effect of oestradiol on chondrocytes and formation of new trabeculae. The growth plate has been reported to form bone in 3 month old rats at a rate equivalent to 0.1 mm over 6 days (210). In 6 month old rats bone turnover is less rapid (72) and therefore this rate may be slower. Bone morphometry in this experiment was measured at distances greater than 1 mm from the growth plate, therefore it is unlikely that new trabeculae arising from the growth plate are detectable at this site at 6 days post-operation. Rather, the increase in trabecular number may be due to extension of trabecular surfaces at the plane above or below the section we have taken, or preservation of trabeculae normally lost over this time period in ovary-intact rats due to ageing.

At 6 days post-operation, oestradiol treatment resulted in suppression of alkaline phosphatase activity to levels below baseline. Hydroxyproline excretion and osteoclast surface were also reduced at 6 days post-operation in oestradiol treated rats compared to oophorectomised control rats. These effects suggest reduced osteoblast proliferation and osteoclastic resorption with oestradiol treatment. Serum osteocalcin was increased compared to baseline levels at 6 days post-operation, possibly resulting from post-operative stress since this effect was also observed in sham operated rats (Chapter 3). Mineral appositional rate was also slightly increased above baseline at 6 days post-operation in high dose oestradiol treated rats, but this increase did not reach statistical significance. The increase in these markers of bone formation must have been greater than that observed due to the stress of operation, since an increase in trabecular bone volume was detected in oestradiol treated rats, suggesting a rapid, and possibly a direct stimulatory effect of oestradiol on mature osteoblasts. Direct stimulation of mature osteoblast activity is consistent with the reported effects of oestradiol on mature osteoblast culture (99,104-106). Less mature osteoblasts are inhibited by oestradiol (98,107), and the overall effect of oestradiol in this experiment at later time points was an inhibition of bone turnover, suggesting opposing direct effects of oestradiol at different stages of osteoblast proliferation or maturation.

Between 9 and 15 days post-oophorectomy, trabecular bone volume was reduced in vehicle treated rats by a loss of whole trabeculae consistent with the findings described in Chapter 3. This loss of bone was delayed in rats treated with either dose of oestradiol. This effect is consistent with longer term studies of oestradiol treatment in the oophorectomised rat (81,82,84-86,89,91).

The reduction in trabecular bone volume and trabecular number reported in oophorectomised rats was delayed until 21 days post-operation with low dose oestradiol treatment, suggesting oestradiol levels achieved with the low dose

regimen were not sufficient to prevent the loss of trabeculae in the long term. This result was unexpected, since peak circulating oestradiol levels achieved by the low dose of oestradiol treatment were similar if not greater than levels observed in the ovary-intact animals (Chapter 4.1) and markers of bone turnover were still depressed in these rats until 21 days post-operation. Wronski et al have shown prevention of bone loss for up to 360 days post-operation with 10μg/kg/day oestradiol in the proximal tibial metaphysis and in lumbar vertebrae (86). The apparent bone loss observed at 21 days post-operation in the femoral metaphyseal-diaphyseal core reported here may have been a result of the short term nature of the experiment, where continual handling of the animals resulted in a change in bone turnover. However, no alterations in biochemical markers of bone turnover were detected that would be consistent with this hypothesis. A reduction in bone volume due to handling at 21 days is also at variance with the increased trabecular bone volume and trabecular number observed at 6 days post-operation in all treatment groups contributed to by the stress of operation.

Although other workers have demonstrated that oestradiol treatment consistently prevents the oophorectomy-induced reduction in trabecular bone volume (50,81-86), the question of whether this effect occurs by preventing the loss of whole trabeculae that occurs following oophorectomy has not previously been examined. A decrease in trabecular number following oophorectomy was delayed by both high and low dose oestradiol treatment, until 21 days post-operation as discussed above, suggesting bone loss is delayed in oestradiol treated rats by preventing loss of trabeculae, consistent with the reduction in trabecular number associated with bone loss after oophorectomy. In another study, trabecular number in the proximal tibial metaphysis of rats treated with 50μg/kg/day oestradiol were reduced to that of oophorectomised rats although trabecular bone volume was not reduced (89). However, the omission of a baseline group in this study leaves open the

conclusion as to whether trabeculae were lost, or trabecular number remained unchanged over the course of the experiment.

Oestradiol treatment at either dose significantly increased trabecular thickness at 15 and 21 days post-operation. This may indicate a greater level of formation than resorption with oestradiol treatment, as suggested by some studies in post-menopausal women (5), although this may not occur at the BMU level (8).

Bone loss after oophorectomy did not result in any change in trabecular thickness in the metaphyseal - diaphyseal core (Chapter 3) although some change in thickness may occur in other areas (211). The strong relationship between trabecular bone volume and trabecular number observed in the present study in all treatment groups indicates trabecular bone volume may be determined by trabecular number rather than trabecular thickness in the metaphyseal-diaphyseal core. Even in oestradiol treated rats when trabecular thickness was significantly increased, the relationship between trabecular bone volume and trabecular number was stronger than that between trabecular bone volume and trabecular thickness. These data suggest therefore, that oestradiol inhibits bone loss by maintaining trabeculae, perhaps by preventing the trabecular perforation reported after oophorectomy in the rat (62,63). Perforation appears to be a direct result of increased bone resorption and increased BMU activation frequency after oophorectomy (Chapter 3). Oestradiol has been shown in the present study to prevent the increase in bone resorption, and may prevent perforation. Oestradiol has also been shown to prevent bone loss in this manner in post-menopausal women, by preventing the increase in activation frequency at the BMU level, thereby reducing the risk of trabecular perforation (8).

While the peak level of oestradiol used in this experiment was in excess of normal peak oestradiol levels detected by others in the rat (209), it did not

appear to inhibit the increase in bone formation completely. A surge of oestradiol of a longer duration may be required to completely prevent any increase in bone turnover. In the long term however, this appears to be of little consequence since lower doses have been shown to inhibit bone loss after oophorectomy (50,81,82,85,87).

It cannot be ascertained from these data whether bone responds to the peak level of oestradiol reached after a single bolus injection, or a lower average over the time between injections. Sustained release pellet or minipump administration of oestradiol at the systemic level, or measurement of bone turnover at various times after oestradiol injection may provide answers to this question.

4.2.3.2 Inhibition of Increased Bone Turnover and Bone Loss in Oophorectomised Rats by Oestradiol Treatment

The first event reported after oophorectomy in the rat is a transient rise in bone resorption at 6 days post-operation, achieving a maximum at 18 - 21 days followed by a gradual decrease from the maximal level (Chapter 3). High dose oestradiol treatment from the time of operation completely inhibited the rise in markers of bone resorption until both hydroxyproline excretion and osteoclast surface were reduced to below pre-operative levels at day 21. It is not clear why oestradiol treatment was not effective in inhibiting osteoclast surface at 9 days post-operation.

Inhibition of increased bone resorption associated with oestrogen deficiency is consistent with long term studies where osteoclast surface (82,84) and urine hydroxyproline levels (85) were reduced with oestradiol treatment. The speed of this effect of oestradiol on bone resorption is consistent with direct inhibition of bone resorption via specific osteoclastic receptors (38,103) or receptors in osteoclast precursors (80).

Oestradiol treatment at either dose completely blocked the oophorectomy-induced rise in alkaline phosphatase activity, reducing levels to below the baseline level even at the low dose, indicating a high sensitivity of alkaline phosphatase-producing osteoblasts to oestradiol. The rise in double fluorochrome labelled surface induced by oophorectomy, and significantly related to the rise in alkaline phosphatase activity observed in oophorectomised rats (Chapter 3), was also inhibited by oestradiol treatment at 12 days post-operation, although this was not maintained in low dose oestradiol treated rats.

The effects of oestradiol on serum osteocalcin and mineral appositional rate however, were slightly different. The increase in osteocalcin in oophorectomised rats was not immediately suppressed by oestradiol treatment; there was still an initial rise in this marker of bone formation, and levels did not return to pre-operative values until day 15. Oestradiol treatment inhibited the rise in mineral appositional rate completely. Mineral appositional rate and osteocalcin both displayed a dose-dependent response to oestradiol. These effects, coupled with the later effect of oestradiol on osteocalcin, appear to indicate that osteoblasts at the stage of alkaline phosphatase production are more sensitive to oestradiol than osteocalcin-producing osteoblasts associated with mineralisation.

Isolation of mRNA from calvarial culture has shown alkaline phosphatase is expressed immediately following osteoblast proliferation, whereas osteocalcin expression increases at the onset of bone mineralisation (190). This suggests alkaline phosphatase is involved in preparation of the extracellular matrix for mineral deposition, whereas osteocalcin may be involved in the onset and progression of bone mineralisation. In situ hybridisation studies support such a model, showing alkaline phosphatase expression in osteoblasts and pre-

osteoblasts, and osteocalcin expression exclusively in osteoblasts in contact with the bone surface or osteocytes embedded in the bone matrix (216).

Results presented here suggest that at an early stage of osteoblast maturation, oestradiol has an inhibitory effect. This occurs in addition to stimulation of the mature osteoblast resulting in increased trabecular number at 6 days post-operation. Inhibition of osteoblast maturation would result in a reduced number of mature osteoblasts involved in bone mineralisation. Such a model is consistent with the reduction of osteocalcin levels some time after serum alkaline phosphatase activity is reduced by oestradiol treatment. While less mature (alkaline phosphatase-producing) osteoblasts are inhibited by oestradiol, the more mature cells at the bone surface are stimulated, and are producing osteocalcin at a high level. Osteocalcin levels reduce as proliferation and maturation of new osteoblasts is inhibited, and the number of cells reaching the osteocalcin producing stage of bone cell maturation is reduced.

The inhibitory effect of oestradiol on immature osteoblasts is consistent with in vitro studies. The UMR106-06 osteoblastic cell line has been used in studies of oestradiol action on bone cells. This cell line produces alkaline phosphatase, but not osteocalcin. Oestradiol treatment of this cell line inhibits bone cell proliferation (98,107). The stimulatory effect of oestradiol on osteocalcin producing osteoblasts observed at day 6 is also supported by the finding that oestradiol stimulates bone formation in more mature cell lines in vitro (36,99,104,105). It is likely that oestradiol inhibits osteoblast proliferation at the stage of alkaline phosphatase production or earlier, such that alkaline phosphatase activity levels and the extent of bone formation surface are reduced. However, at a mature stage of differentiation the effect of oestradiol may be stimulatory, as observed when oestradiol was administered by infusion in the femur of oophorectomised rats (97). At 6 days post-operation a relative rise in bone formation was observed in the present study in oophorectomised rats treated with systemic oestradiol compared to vehicle treated rats,

indicated by a higher level of trabecular bone volume. At later time points, such an effect may be difficult to detect because of the inhibition of osteoblast development by oestradiol and the lower number of mature osteoblasts remaining after oestradiol treatment (82) or may be confounded by coupling of osteoblast activity to osteoclasts inhibited by a direct action of oestradiol.

Oestrogen receptors have been detected in both osteoblasts (36,37,99) and osteoclasts (38,100) as well as their precursors (80,217). The effect of oestradiol on the osteoclast was detected earlier than the effect on the osteoblast. This may relate to the higher concentration of oestradiol receptors in osteoclasts and indicate a greater sensitivity to oestradiol (38) but since a higher dose of oestradiol was required to inhibit bone resorption compared to that required to inhibit formation this seems unlikely. It is possible that this early effect of oestradiol on bone resorption simply relates to the fact that bone resorption is a more rapid process, and osteoclast function is increased before bone formation in response to oophorectomy (Chapter 3). The early effect of oestradiol on the osteoclast does not necessarily point to a more direct effect of oestradiol in this cell type compared to osteoblasts however. Since responses in both osteoclasts and osteoblasts are very rapid, these data suggest oestradiol may act directly on both cell types, with the osteoclast being less sensitive since higher doses were required. The inhibitory effect of oestradiol on the osteoclast would also inhibit BMU-based bone formation via coupling of osteoblast activity to that of the osteoclast. Results from the present study therefore support a model of a stimulatory effect of oestradiol on mature osteoblasts, but a dominant inhibitory effect of oestradiol on osteoblast and osteoclast proliferation. These data do not rule out a role for other systemic and local factors in the response of bone to oestradiol, however.

Interleukin-1 (IL-1) is one locally acting factor which has been implicated in the effect of oestradiol on bone. IL-1 has been shown to stimulate osteoblast proliferation and activity in vitro at low concentrations (110-113). At higher

concentrations IL-1 inhibits osteoblast proliferation and activity (111-115). IL-1 also increases bone resorption in vitro (116-120) via its effect on osteoblasts (120). In vivo, IL-1 has been shown to stimulate both bone resorption and formation (121,122). Serum levels of IL-1 are raised after oophorectomy in both the human female (124,125) and the oophorectomised rat (127). Such a rise appears to be associated with the increased level of bone formation observed in oestrogen deficiency (125) and may be prevented by oestradiol treatment in both the human (126) and the rat (127). Oestradiol may then inhibit bone turnover by inhibiting IL-1 production.

Interleukin-6 (IL-6) is another locally acting factor produced by haematopoietic cells and osteoblasts (129). IL-6 stimulates bone resorption in vitro (130). After oophorectomy in the human, circulating IL-6 levels are increased (124). In the oophorectomised mouse, the increased osteoclast proliferation normally associated with oestrogen deficiency may be prevented by administration of IL-6 antibody (45). In vitro, 17β-oestradiol reversibly inhibits osteoblastic IL-6 production (131,132). These results indicate a possible role of IL-6 in oestradiol modulation of osteoclast development.

TGFβ is produced by osteoclasts (133), immune cells and platelets. Local TGFβ administration in vivo increases bone formation (134-136). At low concentrations in vitro, TGFβ stimulates both osteoblast and osteoclast proliferation and activity (138-142). At very high concentrations TGFβ inhibits osteoclast proliferation (143) and bone resorption (144). Oestradiol treatment in vitro stimulates TGFβ production in both proliferating (107) and mature osteoblasts (36). Studies in vivo however, demonstrate increased TGFβ production in oophorectomised rats, and the increase may be prevented by oestradiol treatment (46). TGFβ administration to oophorectomised rats also prevents the oophorectomy-induced rise in osteoclast precursor proliferation, although this did not prevent bone loss (69), suggesting oestradiol may

stimulate osteoblastic TGFβ production and thereby reduce osteoclast proliferation.

4.2.3.3 Effect of Oestradiol Treatment on Calcium and Phosphate Homeostasis

The increase in serum phosphate observed after oophorectomy was completely suppressed by oestradiol, but the increase in TmP was only partially suppressed. This suggests that the oophorectomy induced increase in serum phosphate was only partially due to increased renal phosphate reabsorption. Since increased hydroxyproline excretion was significantly related to increased serum phosphate levels in the oophorectomised rat, it is likely that the reduction of serum phosphate in oestradiol treated oophorectomised rats is partially due to suppression of the high level of bone resorption associated with oophorectomy. It is also likely that besides an indirect effect of oestradiol on serum phosphate by inhibiting bone resorption, oestradiol may act directly on the kidney via specific receptors (39).

The effects of oestradiol on renal phosphate handling have not previously been reported in the rat, but in post-menopausal women treated with oestrogen both serum phosphate (34) and TmP are reduced (7). It had been suggested these changes in renal phosphate handling were due to a primary increase in PTH activity (7). This concept has largely been disregarded, however, since oestradiol receptors have been localised in bone cells (36-38,80). Altered renal PTH sensitivity in oestradiol treated rats is also unlikely since parathyroidectomy does not alter renal phosphate handling in oestradiol treated (218) or oophorectomised rats (Sims et al, manuscript in preparation).

Oestradiol also blocked the decrease in serum albumin observed in oophorectomised rats. This change is probably caused by increased albumin synthesis (213). Whether oestradiol acts directly on the liver via specific

oestradiol receptors (219) or indirectly by increasing expression of the vitamin D receptor (220) is not known, but oestradiol treatment of a human liver cell line does not affect albumin secretion (88). It has been reported that IL-1, IL-6 and TGFβ all inhibit albumin mRNA expression in hepatic cells in vitro (221-224). Since production of these cytokines is altered in the presence of oestradiol (36,45,107,124,126,127,131,132) it is likely that this effect of oestradiol treatment may also be mediated at least in part by locally acting cytokines.

CHAPTER FIVE

THE EFFECT OF SALMON CALCITONIN ON BONE TURNOVER IN SHAM-OPERATED AND OOPHORECTOMISED RATS

5.1 Introduction to Salmon Calcitonin Treatment

Calcitonin treatment of bone cells in vitro elicits a direct inhibitory effect on osteoclast development and activity (145-152,154), and directly stimulates osteoblast development (156-161). Salmon calcitonin (sCT) has been used, with some success as treatment for post-menopausal bone loss (163-169) as an alternative to hormone replacement therapy.

The effects of salmon calcitonin treatment in the rat however, appear to depend on oestrogen status. In the intact rat, daily calcitonin treatment increases bone resorption and decreases formation (173), whereas in the oophorectomised rat, both resorption and formation are inhibited (177). These studies however may not be directly comparable since different rat strains, time frames and levels of calcitonin dose were used.

To investigate the relationship between the effect of calcitonin treatment on bone turnover and oestrogen status, I have compared the effects of calcitonin on bone turnover and calcium metabolism in sham operated and oophorectomised rats and examined the dose response of biochemical and histomorphometric markers of bone turnover to calcitonin treatment in the oophorectomised rat.

5.2 Protocol

The first part of this experiment was performed to determine whether the response of bone to calcitonin treatment is influenced by ovarian status. This

was done by comparing changes in markers of bone turnover and calcium metabolism in a group of sham operated and oophorectomised rats treated with the same dose of calcitonin.

In the second part of this experiment, the effect of different doses was examined in oophorectomised rats to determine whether the influence of calcitonin in oestrogen-deficient rats was dose dependent. Calcitonin prevents vertebral bone loss in post-menopausal women at a dose of 50 or 100 IU/day (164,166,167), which is equivalent to approximately 0.7 - 1.4 IU/kg/day. The lower doses we have used may more accurately reflect the effect of salmon calcitonin on bone turnover than previous studies using high doses. We have also included 24 IU/kg/day to compare with previous studies carried out in vivo.

5.2.1 Effect of Salmon Calcitonin in Sham Operated and Oophorectomised Rats

Female Sprague Dawley rats were sham operated or oophorectomised at 6 months of age. Rats were injected daily at 0900 h with salmon calcitonin (Auspep, Parkville, Australia) by subcutaneous injection at 1.2 IU/kg/day (0.3μg/kg/day) or normal saline vehicle from the day of operation.

Fasting serum and urine specimens were collected at time of operation, and at 6, 15 and 21 days after operation, 24 h after the last calcitonin injection. Rats were injected with calcein and demeclocycline at 12 and 6 days before killing respectively (Figure 5.1). Femora were removed at time of killing, fixed in 10% formol buffered saline and stored in 70% ethanol.

5.2.2 Dose Response to Salmon Calcitonin in Oophorectomised Rats

Female Sprague Dawley rats were sham operated or oophorectomised at 6

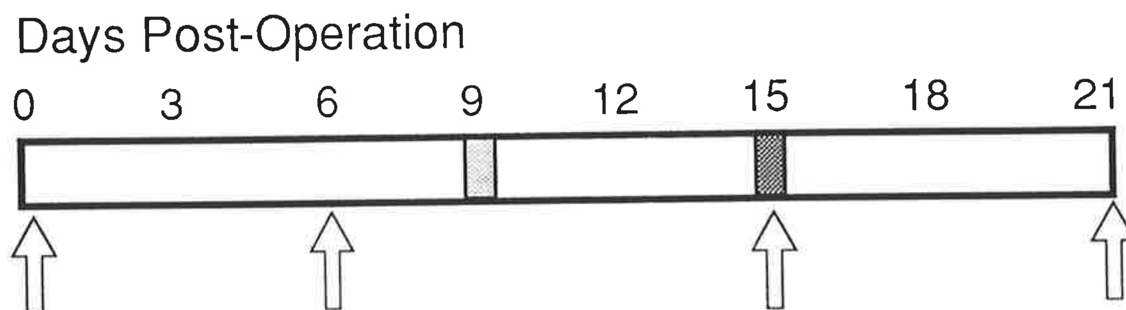

Figure 5.1 Short-term effects of calcitonin treatment in the oophorectomised rat - experimental protocol. Rats were injected with salmon calcitonin daily from time of operation at doses described in the text. Blood and urine samples were collected at the arrowed points. Calcein and demeclocycline were administered at times indicated by the pale and dark stripes respectively. Animals were killed at day 21, and femora were collected for analysis.

months of age. Rats were injected daily at 0900 h with salmon calcitonin (Auspep, Parkville, Australia) at doses of 0.4 IU/kg (0.1μg/kg), 0.8 IU/kg (0.2μg/kg), 1.2 IU/kg (0.3μg/kg), 2 IU/kg (0.5μg/kg), and 24 IU/kg (6μg/kg), or normal saline vehicle from the day of operation.

Fasting serum and urine specimens were collected at time of operation, and at 6, 15 and 21 days after operation, 24 h after the last calcitonin injection (Figure 5.1). Rats were injected with calcein and demeclocycline at 12 and 6 days before killing respectively. Femora were removed at time of killing, fixed in 10% formol buffered saline and stored in 70% ethanol.

5.2.3 Analyses Performed

Serum was analysed for levels of sodium, potassium, chloride, bicarbonate, creatinine, albumin, alkaline phosphatase activity and osteocalcin (Chapter 2.3). Serum anion gap, globulin and calcium fractions were calculated (Chapter 2.3). Urinary hydroxyproline and phosphate excretion and TmP were determined in 24 h fasting urine specimens (Chapter 2.3).

Femora were embedded in GMMA resin (Chapter 2.6.1). trabecular bone volume, trabecular number and trabecular thickness were determined in the diaphyseal-metaphyseal core using the Quantimet 520 Image Analysis System on Von Kossa stained sections (Chapter 2.6.2). Extent of double fluorochrome labelled surface and mineral appositional rate were determined in unstained sections (Chapter 2.6.3). osteoclast surface was determined on sections stained for acid phosphatase (Chapter 2.6.4).

5.3 Results

5.3.1 Effect of Salmon Calcitonin on Bone Turnover in Sham Operated Rats

Treatment of sham operated rats with salmon calcitonin significantly increased hydroxyproline excretion for the duration of the experiment (Figure 5.2). Osteoclast surface was also increased in sham operated rats treated with calcitonin (Figure 5.2).

Salmon calcitonin treatment increased serum alkaline phosphatase activity in sham operated rats (Figure 5.3). This effect was only significant at 21 days post-operation. At 21 days post-operation, double fluorochrome labelled surface was also significantly increased in response to calcitonin treatment (Figure 5.3). Serum osteocalcin was increased significantly at days 6 and 21 post-operation in sham operated rats treated with calcitonin (Figure 5.4). There was no significant change in mineral appositional rate, although levels in calcitonin treated rats appeared slightly lower than in non-treated controls.

Trabecular bone volume was unchanged in sham operated rats treated with calcitonin (Figure 5.5). Although neither trabecular bone volume nor trabecular thickness were significantly altered by calcitonin treatment, there was a significant reduction in trabecular number.

5.3.2 Effect of Salmon Calcitonin on Bone Turnover in Oophorectomised Rats

In oophorectomised rats treated with calcitonin, hydroxyproline excretion was significantly greater than in vehicle treated oophorectomised rats at 6 days post-operation, but by 15 days post-operation when maximal levels of hydroxyproline excretion are achieved there was no longer any difference observed between the two groups (Figure 5.2); osteoclast surface however, was not significantly altered by calcitonin treatment in oophorectomised rats (Figure 5.2).

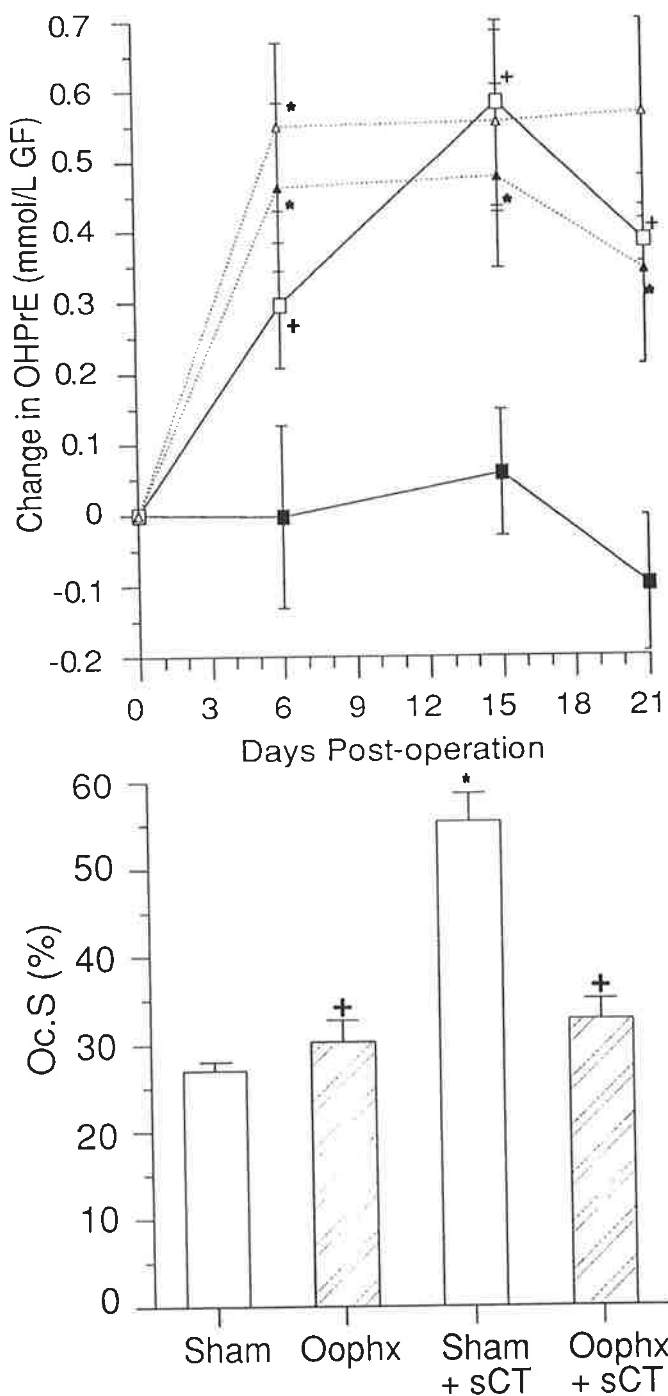

Figure 5.2 Effect of salmon calcitonin treatment on bone resorption in sham operated and oophorectomised rats. Salmon calcitonin (sCT) treatment of oophorectomised rats (△) at 1.2 IU/kg/day did not alter the rise in urinary hydroxyproline excretion (OHPrE) observed in oophorectomised rats (□). In sham operated rats sCT treatment (▲) significantly increased OHPrE compared to levels in vehicle treated sham rats (■). Osteoclast surface (Oc.S) at 21 days post-operation was significantly increased by sCT treatment in sham operated rats, but not in oophorectomised rats.Values are mean ± SEM *, significant effect of sCT, $p<0.05$ vs vehicle treated rats in the same operation group. +, significant effect of oophorectomy, $p<0.05$ vs vehicle treated sham.

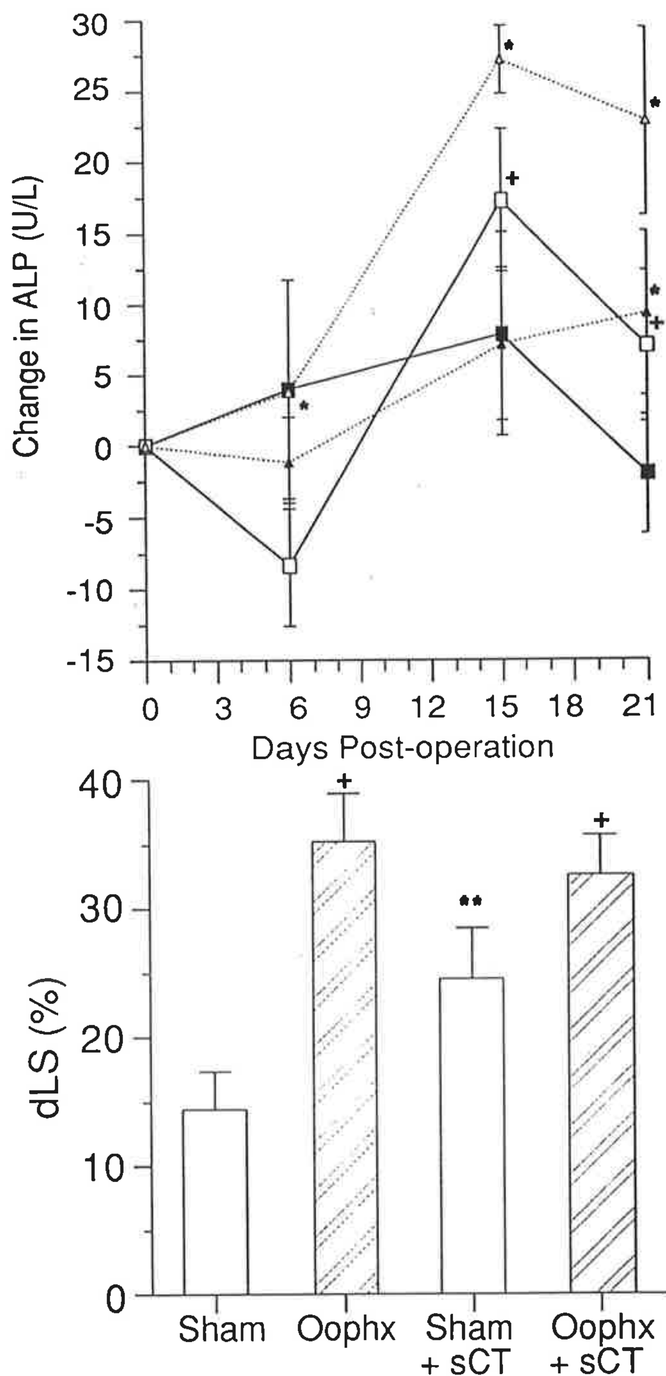

Figure 5.3 Effect of salmon calcitonin on alkaline phosphatase activity and double labelled surface in sham operated and oophorectomised rats. Salmon calcitonin (sCT) treatment at 1.2IU/kg/day increased serum alkaline phosphatase activity (ALP) in oophorectomised (△) rats compared to vehicle treated oophorectomised (□) controls. The same dose in sham operated rats (▲) increased ALP at only 21 days post operation compared to vehicle treated sham rats (■). Values are mean ± SEM *, significant effect of sCT, $p<0.05$ vs vehicle treated rats with the same operation. +, significant effect of oophorectomy, $p<0.05$ vs vehicle treated sham.

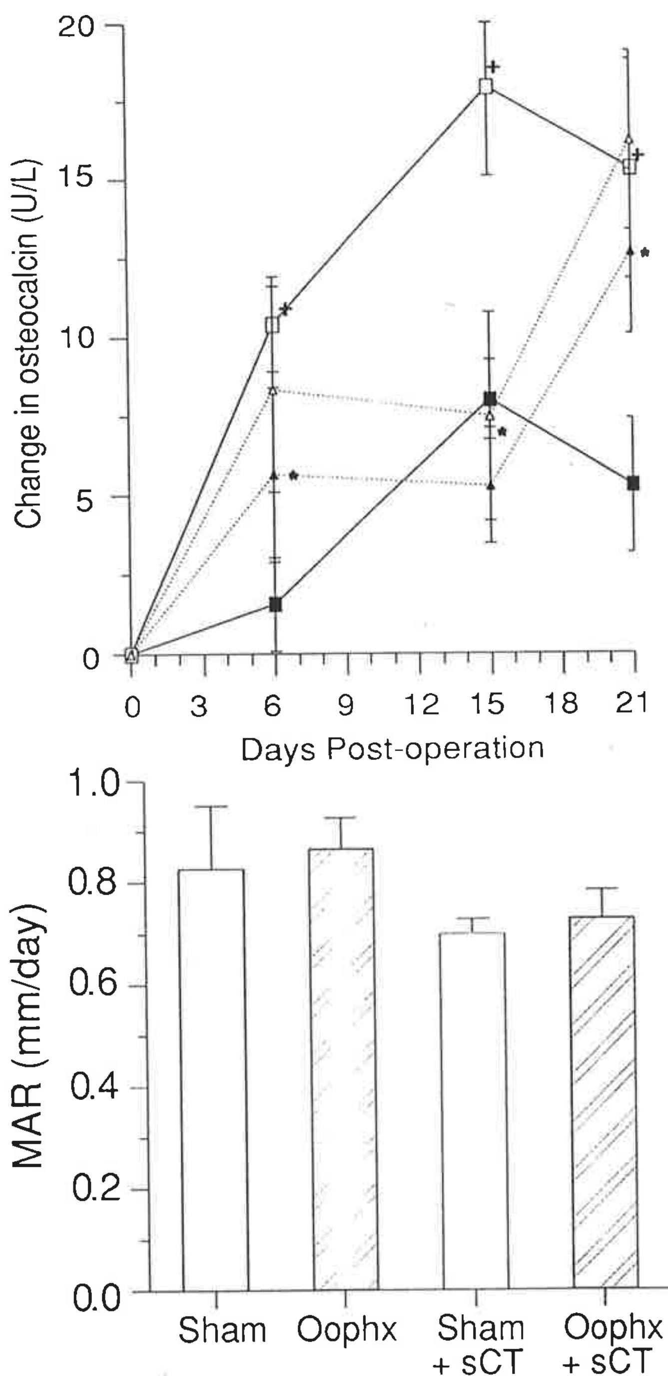

Figure 5.4 Effect of salmon calcitonin treatment on osteocalcin adn mineral appositional rate in sham operated and oophorectomised rats. Salmon calcitonin (sCT) treatment of oophorectomised rats (△) at 1.2 IU/kg/day delayed the oophorectomy-associated rise in serum osteocalcin (□) until 21 days post-operation. In sham operated rats sCT treatment (▲) significantly increased serum osteocalcin at 6 and 21 days compared to vehicle treated sham rats (■). Mineral appostional rate (MAR) was not significantly altered by oophorectomy or sCT treatment in this experiment. Values are mean ± SEM *, significant effect of sCT, $p<0.05$ vs vehicle treated rats in the same operation group. +, significant effect of oophorectomy, $p<0.05$ vs vehicle treated sham.

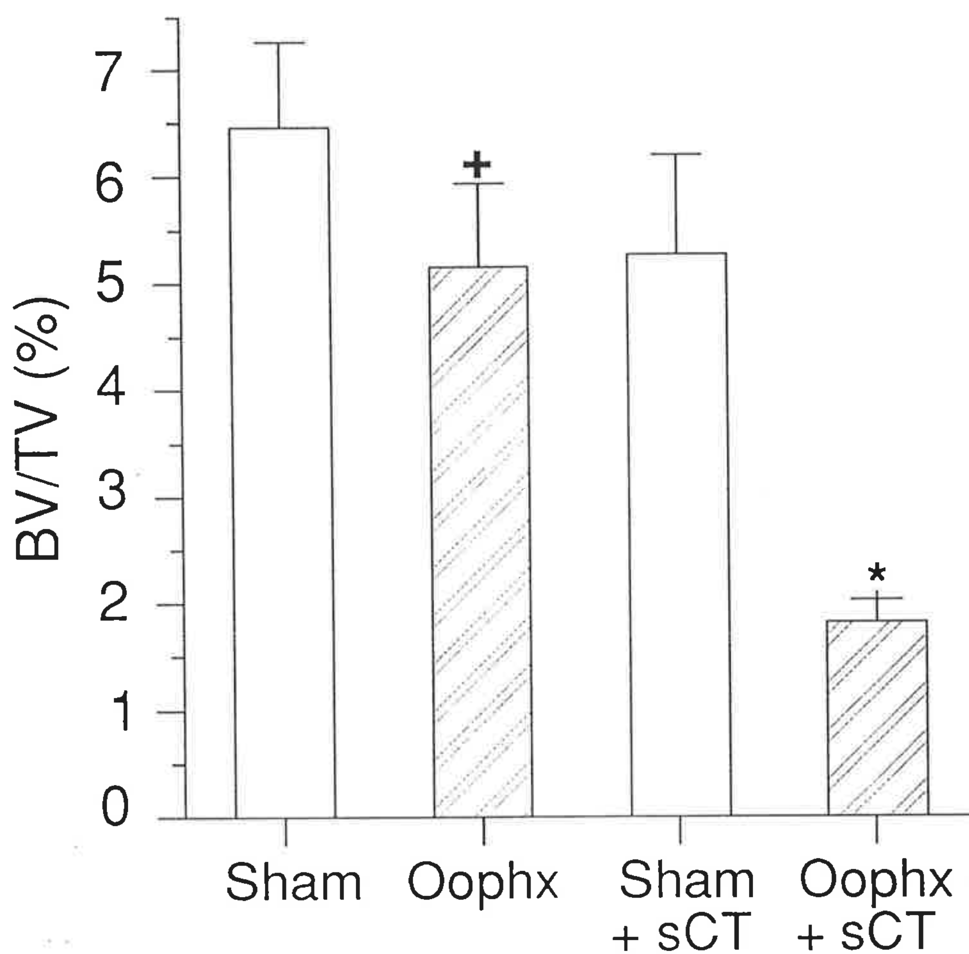

Figure 5.5 Effect of Salmon Calcitonin treatment on trabecular bone volume in sham operated and oophorectomised rats. Salmon calcitonin (sCT) treatment at 1.2 IU/kg/day exaggerated the reduction in trabecular bone volume (BV/TV) normally observed in oophorectomised rats. There was no effect of sCT detected on BV/TV in sham operated rats. Values are mean ± SEM. *, significant effect of sCT, $p<0.05$ vs vehicle treated rats with the same operation. +, significant effect of oophorectomy, $p<0.05$ vs vehicle treated sham.

Salmon calcitonin treatment increased serum alkaline phosphatase activity in oophorectomised rats in addition to the increase induced by oophorectomy and this effect was significant for the duration of the experiment (Figure 5.3). Double fluorochrome labelled surface was not significantly altered in calcitonin treated oophorectomised rats compared to oophorectomised controls at 15 days post-operation (Figure 5.3). Serum osteocalcin in oophorectomised rats was significantly decreased with calcitonin treatment at day 15 post-operation only (Figure 5.4). There was no significant change in mineral appositional rate with calcitonin treatment, although levels in calcitonin treated rats appeared slightly lower than in non-treated controls.

In oophorectomised rats calcitonin treatment caused a dramatic reduction in trabecular bone volume at 21 days post-operation (Figure 5.5). The loss of trabecular bone volume in calcitonin treated oophorectomised rats was not associated with decreased trabecular number. Trabecular thickness was slightly lower in oophorectomised rats treated with calcitonin compared to vehicle treated controls, but this was not significant (Table 5.1).

5.3.3 The Effect of Salmon Calcitonin on Serum Calcium Phosphate and Albumin in Sham Operated and Oophorectomised Rats

Serum calcium was raised in both oophorectomised and sham operated rats treated with 1.2 IU/kg/day calcitonin compared to vehicle treated controls. In oophorectomised rats treated with calcitonin, the increase in serum calcium was significantly lower than the rise observed in sham operated rats treated with calcitonin (Figure 5.6).

Serum phosphate was raised by salmon calcitonin treatment in sham operated and oophorectomised rats (Table 5.2). This increase in serum phosphate due to calcitonin treatment occurred more rapidly than the increase in serum phosphate induced by oophorectomy alone. The increase in serum phosphate

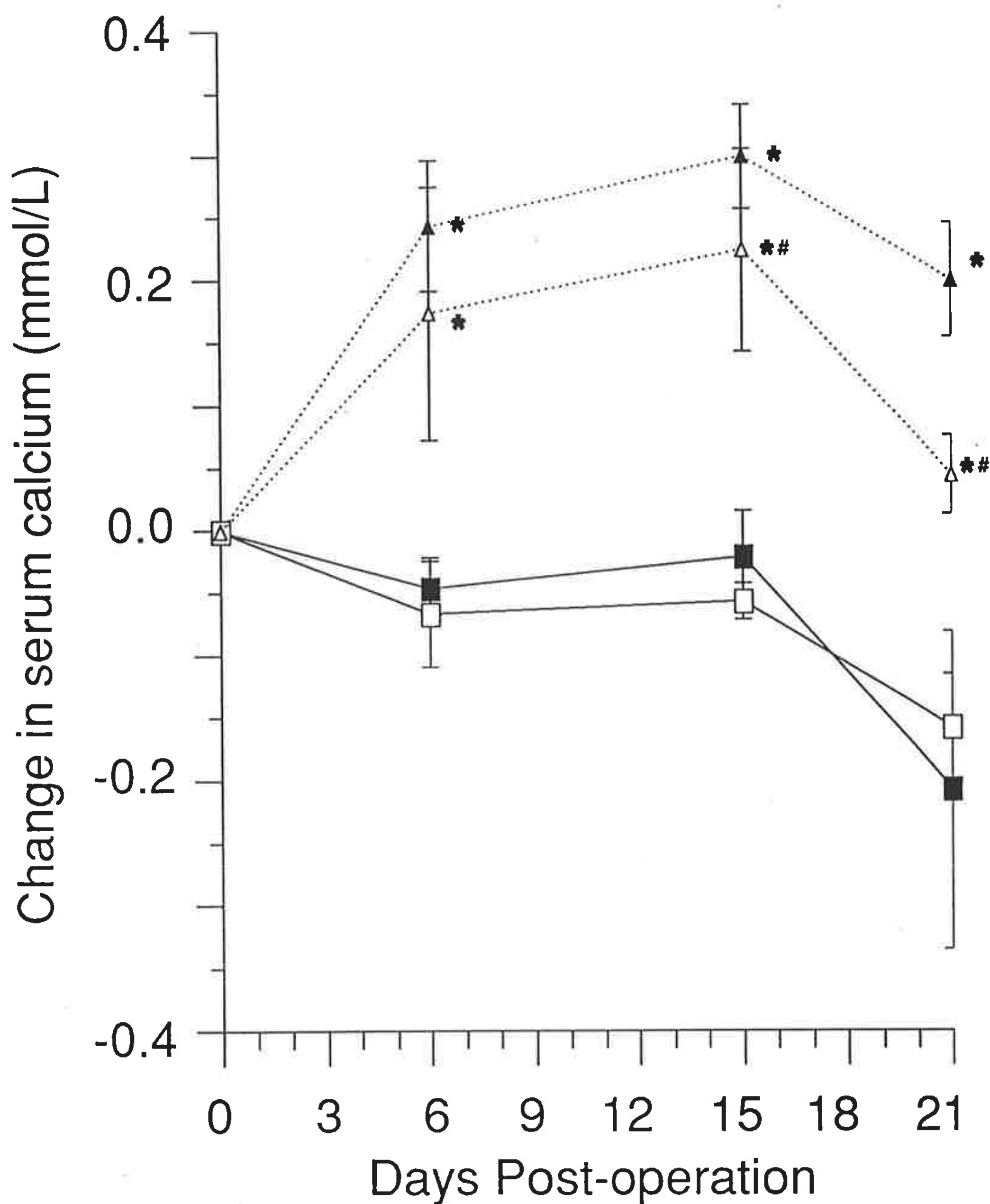

Figure 5.6 Serum calcium is increased in sham operated and oophorectomised rats treated with salmon calcitonin. Salmon calcitonin (sCT) treatment at 1.2IU/kg/day increased serum calcium in sham operated (▲) and oophorectomised (△) rats compared with vehicle treated sham operated (■) and oophorectomised (□) rats. The rise in serum calcium was significantly lower in oophorectomised rats compared to that observed in sham operated rats treated with sCT. Values are mean ± SEM *, $p<0.05$ vs vehicle treated rats with the same operation; #, $p<0.05$ vs sham operated rats treated with sCT.

Table 5.1 Effect of salmon calcitonin treatment on trabecular number and thickness in sham operated and oophorectomised rats at 21 days post-operation.

	Sham	Oophx	Sham + sCT	Oophx + sCT
Tb.N (/mm)	1.97 ± 0.14	1.50 ± 0.22[a]	0.64 ± 0.10[b]	1.81 ± 0.22
Tb.Th (μm)	32.4 ± 2.8	34.2 ± 2.4	28.4 ± 1.6	28.0 ± 2.2

Values are mean ± SEM trabecular number (Tb.N) and thickness (Tb.Th) in the femoral metaphyseal - diaphyseal core. Rats were treated with 1.2 IU/kg/day salmon calcitonin (sCT) from day of sham operation or oophorectomy (Oophx).

Table 5.2 Change in serum phosphate, and renal phosphate handling in sham operated and oophorectomised rats treated with 1.2 IU/kg/day salmon calcitonin (sCT)

	days	Sham	Oophx	Sham+sCT	Oophx+sCT
ΔPhosphate	6	-0.18 ± 0.11	0.05 ± 0.07[a]	0.21 ± 0.08[b]	0.45 ± 0.14[b]
(mmol/L)	15	0.10 ± 0.09	0.19 ± 0.12	0.36 ± 0.07[b]	0.48 ± 0.12[b]
	21	0.11 ± 0.13	0.40 ± 0.12[a]	0.35 ± 0.07[b]	0.51 ± 0.06[b]
ΔPE	6	-0.04 ± 0.03	0.05 ± 0.05	0.05 ± 0.07	0.09 ± 0.02
(mmol/L GF)	15	0.05 ± 0.03	0.08 ± 0.02	0.08 ± 0.03	0.07 ± 0.03
	21	0.17 ± 0.06	0.14 ± 0.03	0.10 ± 0.08	0.07 ± 0.02
ΔTmP	6	-0.15 ± 0.13	-0.06 ± 0.17	-0.04 ± 0.08	0.37 ± 0.17[b]
(mmol/L GF)	15	0.00 ± 0.12	0.03 ± 0.13	0.11 ± 0.31	0.45 ± 0.23[b]
	21	-0.21 ± 0.17	0.13 ± 0.16[a]	0.21 ± 0.25[b]	0.58 ± 0.07[b]

Values are mean ± SEM change in serum phosphate, urinary phosphate excretion (PE) and renal maximal phosphate reabsorption (TmP) from pre-operative levels in sham operated (Sham) and oophorectomised (Oophx) rats treated with vehicle or 1.2 IU/kg/day salmon calcitonin (sCT). Specimens were collected 24 h after last calcitonin injection. [a], effect of oophorectomy, $p<0.05$ vs. sham. [b], effect of calcitonin treatment, $p<0.05$ vs. vehicle treated rats in the same operation group.

was related to an increase in TmP in both sham operated and oophorectomised rats treated with calcitonin.

Serum albumin was significantly reduced in oophorectomised rats, but not in sham operated controls as previously observed (Chapter 3.2). Calcitonin treatment increased serum albumin levels to a similar extent in both sham operated and oophorectomised rats (Figure 5.7).

5.3.4 Dose Related Effects of Salmon Calcitonin in Oophorectomised Rats

Serum calcium was increased in rats treated with all doses of calcitonin (Figure 5.8). With the high dose calcitonin treatment (24 IU/kg/day) the increase in serum calcium was not as great as that achieved with the lower doses at 15 days post-operation, and was not detectable at 21 days post-operation. Serum intact PTH at 21 days post-operation, and 24 h after the previous calcitonin injection, was not significantly altered by calcitonin treatment at any dose (Table 5.3).

At 15 days post-operation, there was also a rise in serum phosphate associated with calcitonin treatment. Again, the effect of 24 IU/kg/day calcitonin was not as strong as that of lower doses (Table 5.3). The increase in serum phosphate was associated with increased phosphate excretion with renal phosphate reabsorption (TmP) maintained at a normal level (Table 5.3). Calcitonin treatment at 0.8 IU/kg/day did cause an alteration in TmP, and phosphate excretion was significantly increased. Renal handling of phosphate was not altered by 24 IU/kg/day calcitonin (Table 5.3).

Urinary hydroxyproline excretion was not significantly altered in oophorectomised rats treated with calcitonin at any but the high dose, where hydroxyproline excretion was increased in excess of the normally elevated levels observed in oophorectomised rats (Figure 5.9). The effect of calcitonin

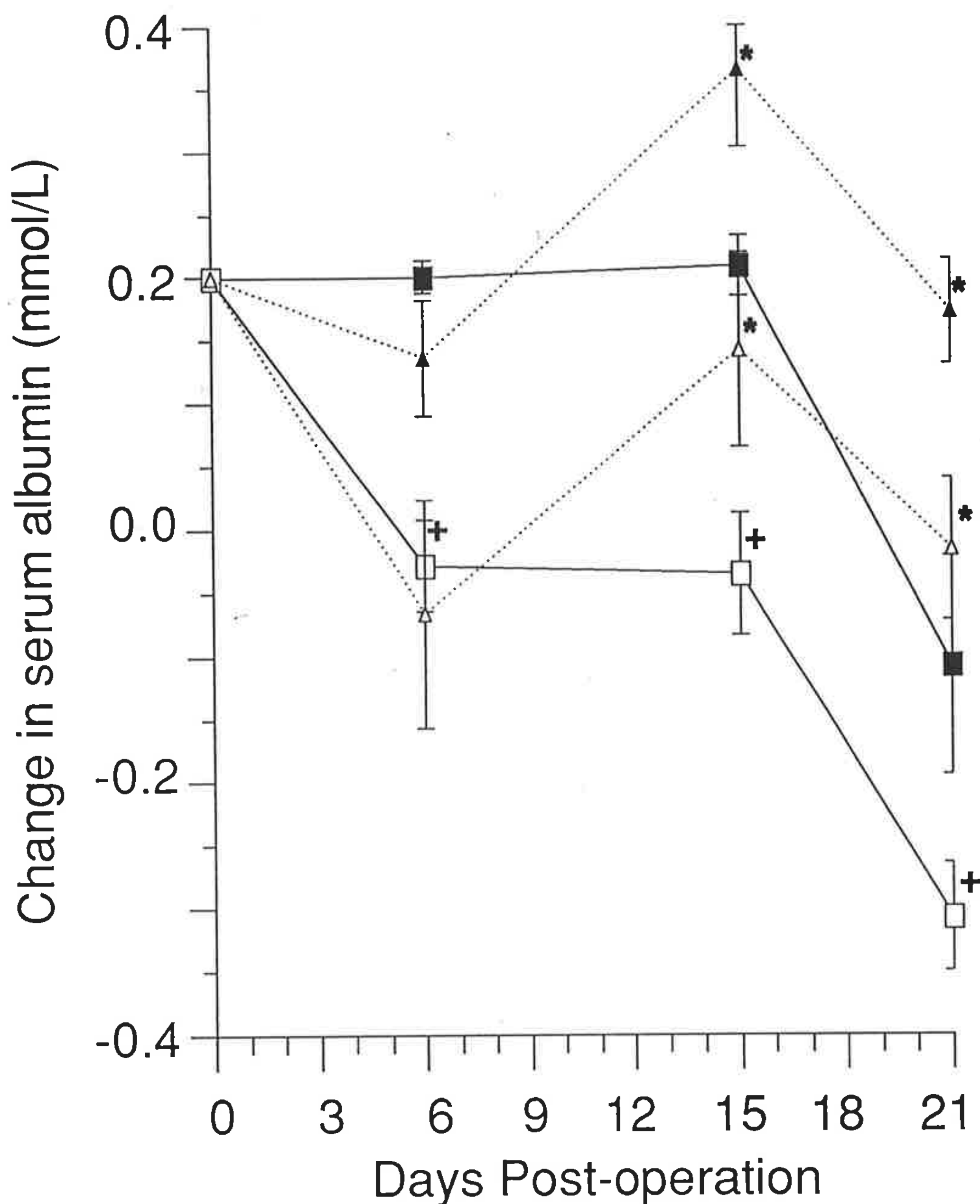

Figure 5.7 Salmon calcitonin treatment prevented the decrease in serum albumin in sham operated and oophorectomised rats. Salmon calcitonin (sCT) treatment at 1.2 IU/kg/day in sham operated (▲) and oophorectomised (△) rats prevented the reduction in serum albumin observed in vehicle treated sham operated (■) and oophorectomised (□) rats. Values are mean ± SEM *, $p<0.05$ vs vehicle treated rats in the same operation group.

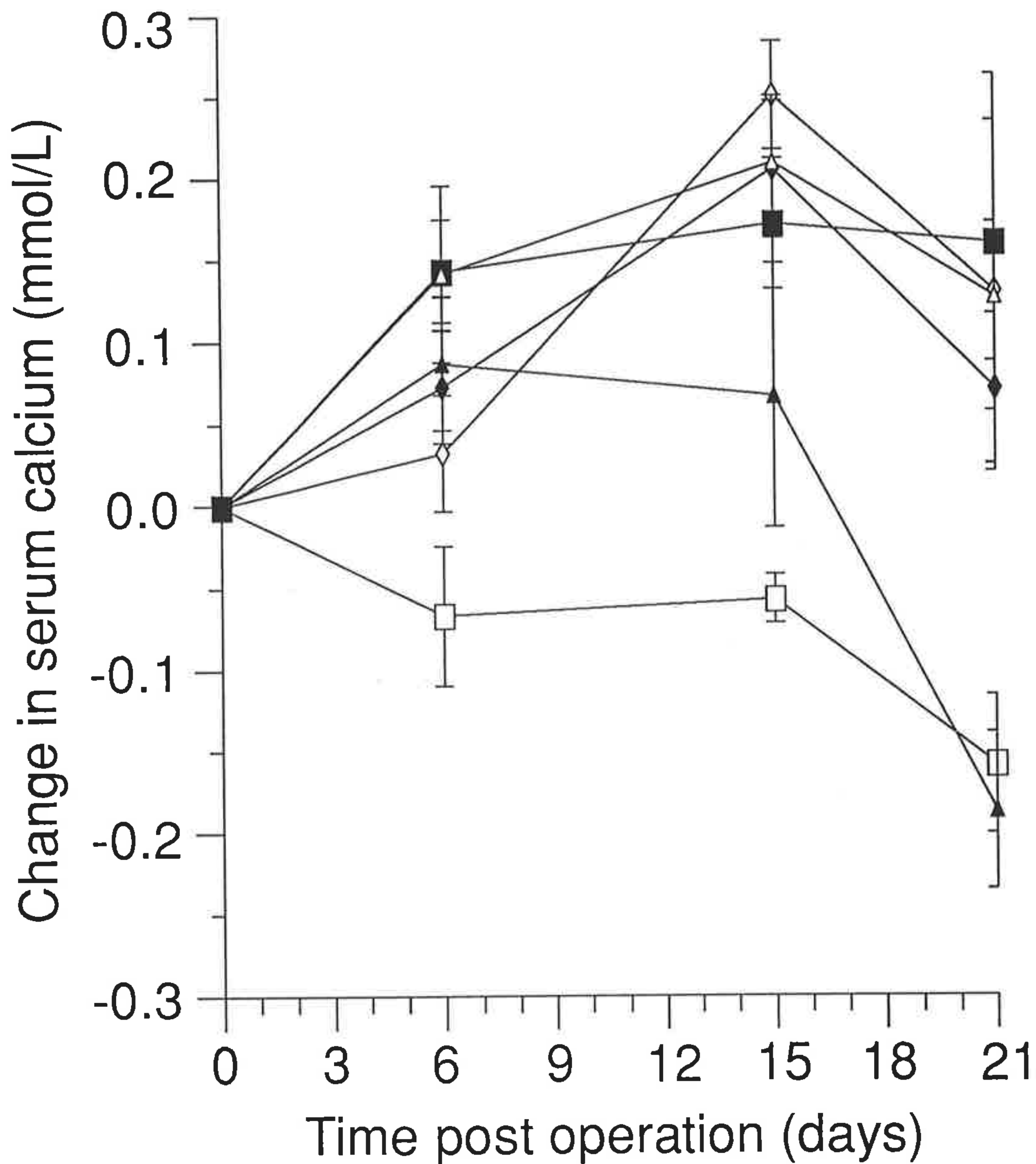

Figure 5.8 The effect of salmon calcitonin treatment on serum calcium in oophorectomised rats. Measurements were taken 24 h after previous sCT injection. □ , control vehicle; ■ , 0.4 IU/kg/day; ◇ , 0.8 IU/kg/day; ◆ , 1.2 IU/kg/day; △ , 2.0 IU/kg/day; ▲ , 24 IU/kg/day. Serum calcium was significantly elevated at all time points and for all doses except 24IU/kg/day at day 21. Values are mean ± SEM.

Table 5.3 Change in serum albumin, phosphate and renal phosphate handling in oophorectomised rats treated with salmon calcitonin, 24 h after last injection at 15 days post-operation.

	control	0.4 IU	0.8 IU	1.2 IU	2.0 IU	24 IU
PTH (ng/L)	35.3 ±3.5	34.3 ±2.2	27.0 ±2.3	32.5 ±2.5	33.3 ±3.4	24.8 ±2.5
ΔAlbumin	-4.71 ±0.66	-5.83 ±1.13	-1.14 ±1.05[a]	-4.08 ±1.22	-3.57 ±1.25	-2.71 ±0.78[a]
ΔPO_4	0.19 ±0.12	0.48 ±0.13[a]	0.47 ±0.09[a]	0.49 ±0.07[a]	0.50 ±0.08[a]	0.26 ±0.09
ΔTmP	0.03 ±0.13	0.19 ±0.17	0.51 ±0.14[a]	0.25 ±0.11	0.17 ±0.14	0.01 ±0.38
ΔPE	0.07 ±0.02	0.17 ±0.03[a]	0.07 ±0.05	0.20 ±0.06[a]	0.23 ±0.06[a]	0.07 ±0.02

Values are mean change from pre-operative levels ± SEM at 15 days post-operation, 24 h after the last salmon calcitonin injection in oophorectomised rats; ΔAlbumin, change from day 0, (g/L); ΔPO_4, change in serum phosphate (mmol/L); ΔTmP, change in renal maximal phosphate reabsorption (mmol/L GF); ΔPE, change in phosphate excretion (μmol/L GF). [a], $p<0.05$ vs. control treated rats at 15 days post-operation.

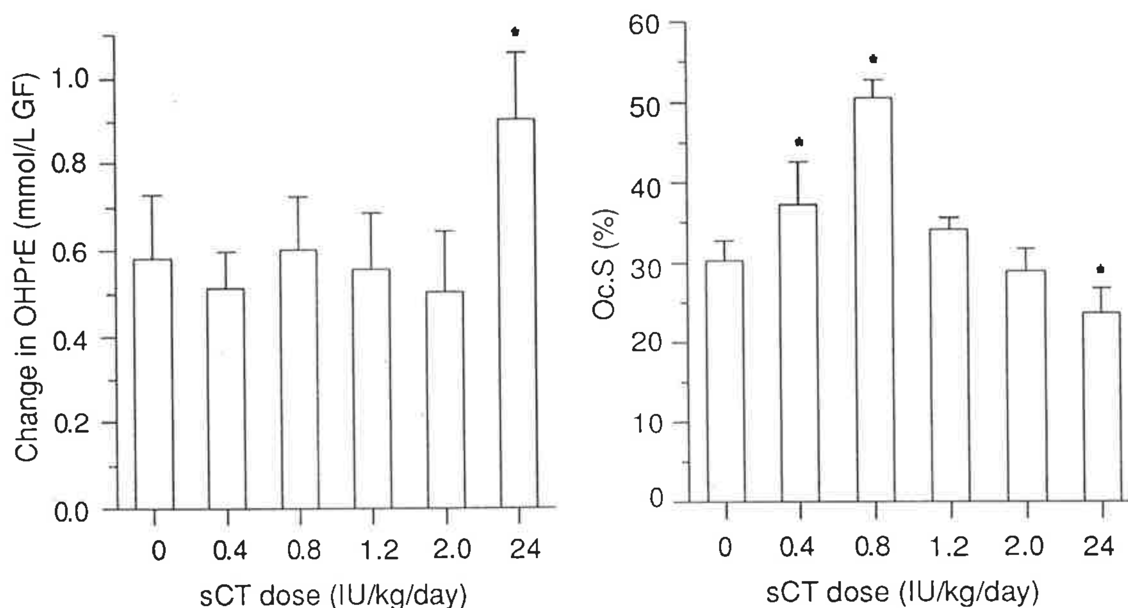

Figure 5.9 The effect of salmon calcitonin treatment on markers of bone resorption in oophorectomised rats. Urinary hydroxyproline excretion (OHPrE) at 15 days post-operation was not affected by sCT except at 24 IU/kg/day, when OHPrE was significantly reduced. Osteoclast surface (Oc.S) at 21days post-operation was significantly elevated by 0.4 and 0.8 IU/kg/day sCT, and inhibited by 24 IU/kg/day sCT. Change in OHPrE was calculated for each rat as the change from day 0. Values are mean + SEM *, $p<0.05$ vs control treated rats.

on osteoclast surface appeared biphasic. Levels of osteoclast surface were greater than levels observed in oophorectomised controls when oophorectomised rats were treated with calcitonin at doses of 0.4 and 0.8 IU/kg/day. Osteoclast surface was unchanged by calcitonin treatment at 1.2 and 2.0 IU/kg/day. At the high dose of 24 IU/kg/day, calcitonin prevented the rise in osteoclast surface associated with oophorectomy.

There was no significant change in serum alkaline phosphatase activity at any dose of calcitonin treatment (Figure 5.10). Double labelled surface (Figure 5.10), serum osteocalcin and mineral appositional rate (Figure 5.11) were all lower in calcitonin treated oophorectomised rats at low doses (0.4 -2.0 IU/kg/day) compared to vehicle treated controls. High dose calcitonin did not suppress the increase in osteocalcin or mineral appositional rate. The rise in double fluorochrome labelled surface associated with oophorectomy was the only change in a marker of bone formation prevented by the high dose of calcitonin (24 IU/kg/day).

Low dose calcitonin treatment in oophorectomised rats caused a further reduction in trabecular bone volume below that observed in vehicle-treated oophorectomised rats (Figure 5.12) reflecting the reduction in bone formation, as well as increased bone resorption at 0.4 and 0.8 IU/kg/day. This was accompanied by a decrease in trabecular number, but no change in trabecular thickness (Table 5.4)

High dose calcitonin treatment (24 IU/kg/day) prevented the oophorectomy-induced reduction in trabecular bone volume (Figure 5.12). The high trabecular bone volume in rats treated with 24 IU/kg/day was associated with a greater trabecular number than in oophorectomised rats. Trabecular thickness was unchanged (Table 5.4).

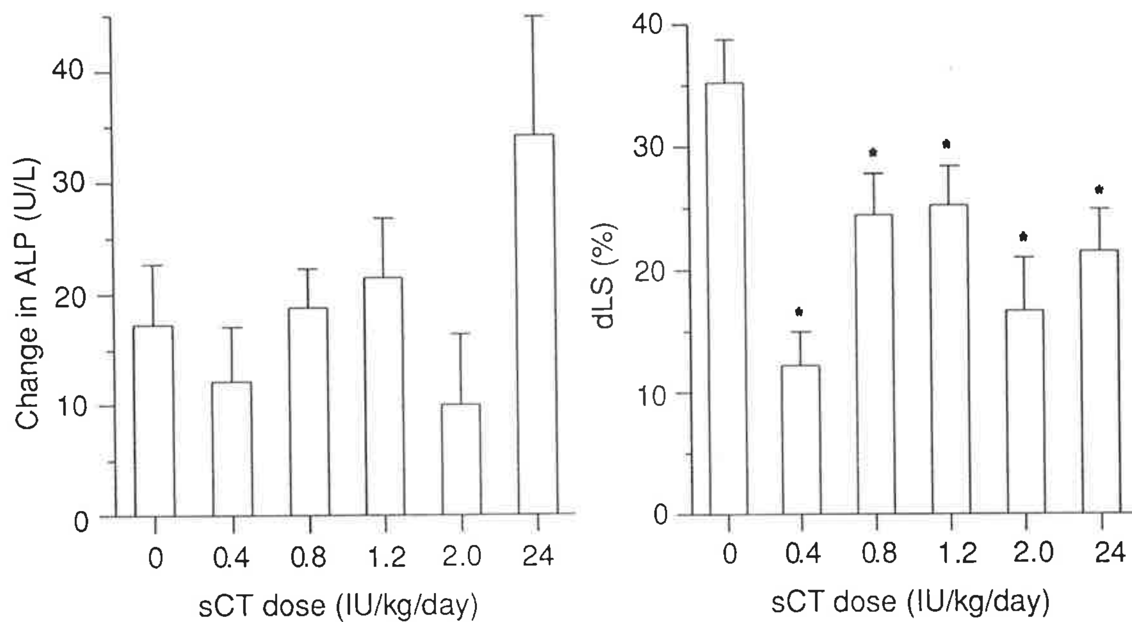

Figure 5.10 The effect of salmon calcitonin treatment on alkaline phosphatase activity and double labelled surface in oophorectomised rats. Alkaline phosphatase activity (ALP) at 15 days post-operation was not significantly affected by sCT administration. Extent of double labelled surface (dLS) at 15 days post-operation was significantly reduced by all doses of sCT. Change in ALP was calculated for each rat from day 0. Values are mean + SEM. *, $p<0.05$ vs control treated rats.

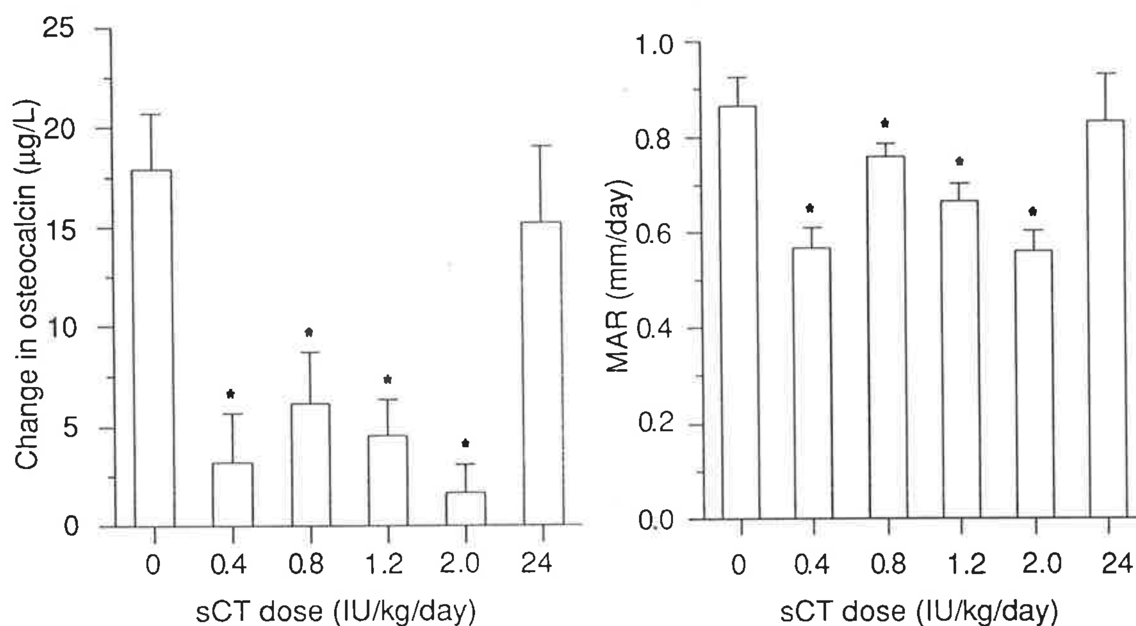

Figure 5.11 The effect of salmon calcitonin treatment on osteocalcin and mineral appositional rate in oophorectomised rats. Serum osteocalcin at 15 days post-operation was significantly reduced by all but 24 IU/kg/day sCT. Mineral appositional rate (MAR) at 15 days post-operation was significantly reduced by all doses but 24 IU sCT. Change in osteocalcin was calculated for each rat from day 0. Values are mean + SEM. *, $p<0.05$ vs vehicle treated rats.

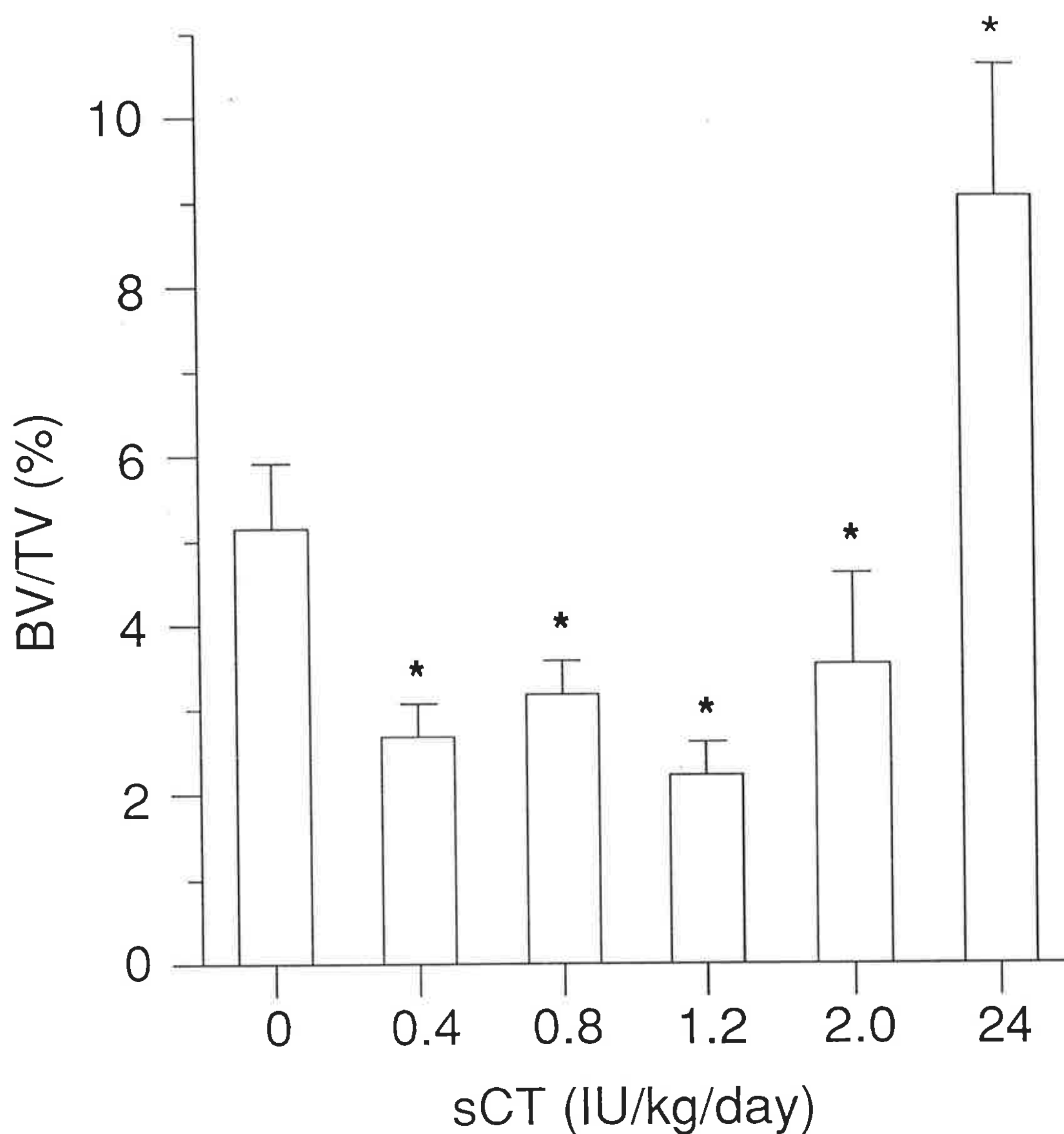

Figure 5.12 The effect of salmon calcitonin treatment on trabecular bone volume (BV/TV) at 21 days post-operation in oophorectomised rats. sCT treatment at the low doses caused a decrease in BV/TV. At the high dose of 24IU/kg/day sCT, BV/TV was increased significantly. Values are mean + SEM. *,p<0.05 vs vehicle treated rats.

Table 5.4 Effect of salmon calcitonin (sCT) on trabecular number and thickness in oophorectomised rats at 21 days post-operation, 24 h after the last calcitonin injection.

	control	0.4 IU	0.8 IU	1.2 IU	2.0 IU	24 IU
Tb.N (/mm)	1.50±0.21	1.04±0.19[a]	1.06±0.14[a]	0.79±0.13[a]	0.84±0.18[a]	2.3±0.2[a]
Tb.Th (μm)	34.2±2.8	29.0±4.3	28.6±1.9	33.1±7.8	40.3±6.8	38.2±3.8

Values are mean ± SEM at 21 days post-operation, 24 h after the last salmon calcitonin injection in oophorectomised rats; Tb.N, trabecular number; Tb.Th, trabecular thickness, measured on the Quantimet 520 image analysis system on von Kossa silver stained sections of the distal femur. [a], $p<0.05$ vs. control treated oophorectomised rats.

5.4 Discussion - Salmon Calcitonin in Sham Operated and Oophorectomised Rats

Calcitonin treatment directly inhibits osteoclast activity (145-150,154) and proliferation in vitro (147) via specific osteoclastic receptors (151). Calcitonin receptors are also present early in osteoblast differentiation (156,157). Calcitonin treatment of osteoblast-like cells and osteoblast stem cells in vitro increases cellular activity (158-160).

The primary inhibitory effect of calcitonin on osteoclasts in vitro has also been observed in short term studies in the intact rat and results in pronounced hypocalcaemia (170-172). The hypocalcaemia induced by calcitonin treatment stimulates PTH secretion in vivo such that a series of secondary alterations in calcium metabolism are induced (173). The increased level of circulating PTH induces a secondary increase in bone resorption, stimulating calcium release from the bone as well as an increase in bone formation (173). For this reason, it has been important to consider which effects of calcitonin treatment detected in vivo arise from the primary action of calcitonin, or the secondary effects of PTH.

5.4.1 Effect of Calcitonin Treatment on Bone Turnover in Sham Operated and Oophorectomised Rats

Daily treatment of intact rats with 20 IU/kg salmon calcitonin has been reported to increase bone resorption and reduce bone formation (173). In contrast, Wronski et al. have shown that 16 IU/kg calcitonin administered on alternate days reduces both bone resorption and formation in the oophorectomised rat (177). In the present study treatment of intact rats with 1.2 IU/kg/day salmon calcitonin increases both bone resorption and formation, whereas in oophorectomised rats, the same dosage increased bone resorption and reduced bone formation, resulting in a loss of trabecular bone.

The increased bone resorption observed in both sham operated and oophorectomised rats treated with calcitonin is in direct contrast to effects observed in vitro (145-152,154), and short term in vivo studies (170-172). The difference between the primary inhibitory effect of calcitonin observed in vitro, and the stimulatory effect on bone resorption seen here is probably due to calcium homeostatic mechanisms stimulated by the hypocalcaemia immediately following calcitonin treatment (170-172). The transient hypocalcaemia after calcitonin treatment is observed soon after calcitonin treatment in vivo and causes an increase in PTH secretion (173). Increased PTH secretion is likely to induce bone resorption, and in the present study, this secondary stimulation appears to dominate the initial reduction in bone resorption detected 4 h after calcitonin administration by others (170-172). A rebound increase in bone resorption is consistent with increased levels of serum calcium detected 24 h after calcitonin injection in the present study.

Although a rise in bone resorption following calcitonin treatment has been reported in one other in vivo study (173), such an effect was not observed by Wronski in the oophorectomised rat (177), or in studies of post-menopausal women (163-169). The different effect of calcitonin treatment on bone resorption in these studies does not appear to relate to the difference in ovarian status since in the present study, a secondary increase in bone resorption was detected in both sham operated and oophorectomised rats treated with calcitonin. It is likely that the difference in response to calcitonin may be dose-related.

In Wronski's study of oophorectomised rats, 16 IU/kg salmon calcitonin was administered on alternate days (177), considerably higher than the daily dose of 1.2 IU/kg used in the present study. The low dose of calcitonin used in the present study was selected in an attempt to administer calcitonin at an equivalent dose to that used in post-menopausal women, however since a

different effect of calcitonin was observed, it appears a simple weight / volume ratio is not appropriate for cross-species comparisons. This may relate to the different routes of administration; bioavailability is greater via a subcutaneous route, than by intranasal spray. Alternatively, the higher level of bone turnover in the rat compared to the human may necessitate use of a different dose of calcitonin. The effect of different calcitonin doses in the oophorectomised rat will be discussed in more detail in Chapter 5.5.

The different effect of calcitonin on bone resorption observed by Wronski in the oophorectomised rat may also relate to the time at which bone specimens were collected. Since the time of calcitonin injection and bone specimen collection were not reported by Wronski, it is possible that Wronski has detected the primary decrease in osteoclast number, whereas the increased osteoclast surface detected in the present study, and detected by Glajchen in the intact rat relates to the secondary effect of PTH.

The effect of calcitonin on bone resorption in both sham operated and oophorectomised rats was very rapid. At 6 days post-operation, when markers of bone resorption are increased in oophorectomised rats, the level of hydroxyproline excretion was even greater in oophorectomised and sham operated rats treated with calcitonin. Bone resorption was maintained at the high level for the duration of the experiment. The effects of calcitonin treatment and oophorectomy on bone resorption did not appear additive, suggesting a maximal level of osteoclast activity was reached.

The level of osteoclast surface was considerably greater in sham operated rats treated with calcitonin than oophorectomised rats (both treated and untreated groups). Although the effects of calcitonin treatment and oophorectomy on osteoclast activity, indicated by hydroxyproline excretion appeared equal, the difference in osteoclast surface may indicate a more pronounced effect of calcitonin on osteoclast proliferation. Osteoclast surface was measured at 21

days post-operation, a time when the elevated osteoclast proliferation induced by oophorectomy may be falling, since this change is transient (Chapter 3). The secondary increase in bone resorption induced by calcitonin treatment may occur daily as PTH levels increase in response to the primary hypocalcaemia induced by calcitonin. In this way, the increased bone resorption could continue indefinitely, as long as calcitonin treatment was continued.

Salmon calcitonin treatment appeared to increase serum alkaline phosphatase activity in oophorectomised rats. However, serum osteocalcin was reduced in the oophorectomised rat treated with calcitonin at 15 days post-operation. This apparent discrepancy may indicate that in the oophorectomised rat, calcitonin stimulates osteoblast proliferation, rather than stimulating the activity of mature osteoblasts. In oophorectomised rats, it is possible that osteoblast activity is already at a maximal level, due to the coupling of osteoblast and osteoclast activities, such that calcitonin treatment cannot increase this further. This was not the case in sham operated rats, where both alkaline phosphatase activity and serum osteocalcin were increased by calcitonin treatment.

Histomorphometric markers of bone formation in the distal metaphysis were not significantly affected by calcitonin treatment in the oophorectomised rat at 21 days post-operation. This may relate to the very low volume of trabecular bone remaining at this time point, particularly as double fluorochrome labelled surface was elevated in sham operated rats, where calcitonin treatment did not result in trabecular bone loss.

The changes in bone turnover induced by calcitonin treatment caused a decrease in trabecular bone volume in addition to that associated with oestrogen deficiency. The bone loss due to calcitonin treatment in oophorectomised rats may occur by a different mechanism to oestrogen

deficiency bone loss, since bone resorption and formation were not increased beyond levels observed in untreated oophorectomised rats. Alkaline phosphatase activity was increased and serum osteocalcin was reduced significantly at 15 days, and this may indicate bone loss in the calcitonin treated rat is due to inhibition of mature osteoblastic activity by calcitonin.

Calcitonin treatment did not alter trabecular bone volume in sham operated rats. The increased bone resorption in sham operated rats appears to be compensated by increased osteoblast proliferation and activity in intact rats. The nature of the increased bone turnover in response to calcitonin treatment must differ from the increased bone turnover associated with oestrogen deficiency however, since calcitonin treatment did not result in trabecular bone loss in the metaphyseal diaphyseal core.

5.4.2 Effect of Calcitonin Treatment on Serum Calcium and Phosphate in Sham-Operated and Oophorectomised Rats

Since blood specimens were collected 24 h after calcitonin injection, the hypercalcaemia detected in calcitonin treated rats was probably due to the secondary effect associated with raised serum PTH (170). The low serum calcium level observed soon after calcitonin administration (170-172) results in hyperparathyroidism, such that in the intact animal, serum calcium is increased via an increase in bone resorption. This is supported by evidence that parathyroidectomy results in reappearance of the calcitonin-induced hypocalcaemia (175).

The effect of calcitonin on serum calcium differed in sham operated rats compared to oophorectomised rats treated with the same dose. The hypercalcaemia detected after calcitonin treatment was less pronounced in oophorectomised rats compared to ovary-intact controls. The lower level of serum calcium in oophorectomised rats treated with calcitonin compared to

calcitonin treated intact rats may occur in response to the greater level of bone turnover in oestrogen-deficiency, and increased flow of calcium from the skeletal reserves. However, it cannot be ruled out that altered PTH sensitivity in oestrogen deficient rats may also contribute to this difference (41-44).

Hypocalcaemia immediately following a single calcitonin injection is more pronounced in oophorectomised rats compared to ovary-intact controls (79), suggesting a more dramatic primary effect of calcitonin in oestrogen deficiency. In post-menopausal women, levels of cAMP and serum calcium following a calcitonin stimulation test were lower after oophorectomy, and sensitivity to calcitonin was returned to normal when patients were treated with hormone replacement therapy (42), further supporting the concept that the effect of calcitonin on the osteoclast is enhanced in oestrogen deficiency. However, it cannot be ruled out that the more pronounced hypocalcaemia in oophorectomised rats treated with calcitonin is due to the greater proportion of serum ionised calcium arising from bone as a result of the high level of bone turnover. Although an increased level of serum ionised calcium has not been detected in oophorectomised rats (Chapter 3), serum calcium may be described as being more mobile in oestrogen deficiency, since there is a greater level of bone turnover, and this may be the cause of the greater response of serum calcium to calcitonin injection.

Calcitonin treatment appeared to prevent the reduction in serum albumin associated with operation in both sham operated and oophorectomised rats. This effect has not been reported by others, and may relate to a direct effect of calcitonin on the liver.

It is not clear whether the increase in serum phosphate in response to calcitonin treatment is affected by ovarian status, since serum phosphate is also increased by oestrogen-deficiency. The increase in serum phosphate may relate to the increased TmP observed in calcitonin treated animals from both

operation groups at 21 days post-operation, or this may compensate for the rise in serum phosphate, and maintain serum phosphate excretion levels. The increased renal reabsorption of phosphate contrasts with that reported by Heath in his review of the pharmacological effects of calcitonin, however as with the changes in serum calcium, the effects of calcitonin on serum phosphate reported here may be secondary, and the effects reported by Heath may be primary (234). The increase in serum phosphate observed in calcitonin treated animals may relate to an increase in bone resorption, or may occur due to a direct effect of calcitonin on the kidney to increase renal phosphate reabsorption .

5.5 Discussion - Dose Response Curve of Calcitonin in Oophorectomised Rats

5.5.1 Dose Response Effect of Calcitonin on Serum Calcium, Parathyroid Hormone, Phosphate and Albumin

As discussed in Chapter 5.4.2, serum calcium 24 h after calcitonin injection was elevated in oophorectomised rats treated with calcitonin. This was observed at all doses, and is probably due to a secondary rebound in serum PTH levels induced by the primary hypocalcaemia caused by calcitonin. Hypercalcaemia was less pronounced in oophorectomised rats treated with 24 IU/kg/day at 15 days post-operation, and was not detected at this dose at 21 days.

The gradual lowering of serum calcium in rats treated with 24 IU/kg/day may relate to the phenomenon of escape. Other workers have shown that infusion of salmon calcitonin to intact rats resulted in hypocalcaemia for the first 20 minutes of treatment only, and was then no longer detectable (175). Similarly, after 36 to 48 hours of continual calcitonin treatment of bone cell culture, the level of osteoclast function increases, even when fresh calcitonin is added (147,149,155). The phenomenon of escape is not caused by a change in

osteoclastic receptor affinity for calcitonin, rather it appears to be mediated by a decreased number of free osteoclastic calcitonin binding sites, largely due to poor dissociation of tightly bound receptor / hormone complexes (155). The high dose of salmon calcitonin used in the present study may have been incompletely cleared from the circulation before administration of the next injection, such that calcitonin was continually present in the circulation, resulting in down-regulation of calcitonin receptors (155). This is less likely to have occurred in the study of Wronski et al (177), since the dose of 16 IU/kg/day was administered on alternate days. Changes in other biochemical markers of calcium metabolism and bone turnover were also diminished with time in rats treated with 24 IU/kg/day, and were undetectable at 21 days post-operation. Since histomorphometric variables were only measured at 21 days post-operation, it is not clear whether effects of calcitonin on these markers diminished with time. Double fluorochrome labelled surface and mineral appositional rate reflect the bone forming activity at the time fluorochrome labels were administered, not the time of killing, so the reduction in double labelled surface reflects a reduction at 15 days post-operation. There was still a significant effect of high dose calcitonin on osteoclast surface at 21 days post-operation, but since this marker was not measured at other time points, it is not clear whether this effect depleted with time.

Since a change in serum calcium in response to 24 IU/kg/day calcitonin was not detected at 21 days post-operation, and the effects of oophorectomy on bone turnover appear to have reached a plateau at 15 days post-operation (Chapter 3), biochemical markers of bone turnover are presented as the change from pre-operative levels at 15 days post-operation only. Double fluorochrome labelled surface and mineral appositional rate are presented at 15 days post-operation also. Osteoclast surface and morphometric data are presented at 21 days post-operation, the only time point at which these data were collected.

The secondary hypercalcaemia observed in calcitonin treated rats occurs in response to increased PTH secretion following the primary decrease in serum calcium. A significant alteration in circulating PTH was not detected in calcitonin treated rats in this experiment, however. Measurements were made 24 h after the previous calcitonin injection, and it is possible the rise in serum PTH associated with serum calcium rebound was transient and levels had returned to normal at 24 h after calcitonin administration.

Serum phosphate was elevated in oophorectomised rats treated with calcitonin. This may result from increased bone resorption following the stimulation of PTH secretion. As well, increased serum phosphate was associated with an increase in TmP, but phosphate excretion was not altered by calcitonin treatment. As observed with serum calcium, the effect of 24 IU/kg/day calcitonin on serum phosphate was not significant, further supporting the possibility of calcitonin receptor down-regulation at this high dosage. Renal handling of phosphate was not altered by 24 IU/kg/day calcitonin. The effect of 0.8 IU/kg/day calcitonin appeared to differ from all other doses such that TmP was not altered by calcitonin treatment, and phosphate excretion was significantly increased. The reason for this is unclear.

5.5.2 Dose Response Effect of Salmon Calcitonin on Bone Turnover in Oophorectomised Rats

In this study the high level of bone resorption observed in oophorectomised rats was not affected by calcitonin treatment at 15 days post-operation. This is consistent with the first part of the experiment, where hydroxyproline excretion in oophorectomised rats was only significantly greater in calcitonin treated rats prior to the maximal elevation of bone resorption due to oophorectomy. It is important to note that hydroxyproline excretion was measured over a 24 h period. Since the primary hypocalcaemia induced by calcitonin causes a rise in PTH (170), it is likely that the primary decrease in

bone resorption was followed by a secondary increase of a longer duration such that, over the 24 h period the total effect detected was an elevation in bone resorption. The increased in bone resorption induced by calcitonin observed in this study is consistent with the effect of calcitonin on bone resorption reported in the intact rat by Glajchen et al (173), but not that of Wronski in the oophorectomised rat (177). However, Wronski has not described the time at which bone specimens were collected after calcitonin was administered, and it is possible that these workers have detected the primary decrease in osteoclast number, whereas the increased osteoclast surface detected in the present study relates to the secondary effect of PTH.

Studies performed in vitro have shown that calcitonin treatment inhibits osteoclast formation in vitro by slowing osteoclastic fusion (147), and inhibits bone resorption (147,149). It may be expected then that calcitonin treatment in vivo would be effective in inhibiting the increased bone resorption associated with oophorectomy. However, due to the secondary effect of PTH, this did not appear to be the case with doses administered in this study.

Calcitonin treatment appeared to have a biphasic effect on osteoclast surface in oophorectomised rats. At the lowest doses tested, osteoclast surface was increased, whereas the dose of 24 IU/kg/day reduced osteoclast surface. In contrast, hydroxyproline excretion was elevated in oophorectomised rats treated with 24 IU/kg/day, but not at the lower doses. The reduction in osteoclast surface observed in oophorectomised rats treated with 24 IU/kg/day salmon calcitonin is consistent with Wronski's oophorectomised rat study using 16 IU/kg sCT on alternate days (177). The result of the present study is also consistent with the reduced level of bone resorption in post-menopausal women treated with calcitonin (164,167).

The apparent discrepancy between the response of osteoclast surface and hydroxyproline excretion to calcitonin treatment may relate to the nature of

these two markers of bone resorbing activity. As discussed above, hydroxyproline excretion indicates changes in bone resorption over a 24 h period throughout the body, whereas osteoclast surface reflects bone resorption in a specific area, the femoral distal metaphysis, at the time of killing. If it were not for the increase in hydroxyproline excretion, it may be suggested that at the high dose of calcitonin treatment, the primary effect of calcitonin has been detected without interference of the secondary rebound. However, this is not the case since at some stage in the 24 h following calcitonin treatment, bone resorption was increased, resulting in increased hydroxyproline excretion. It is possible that the timing of changes in bone turnover is altered in rats treated with the high dose of salmon calcitonin. Hypocalcaemia may occur more rapidly at a high dose, and the secondary rise in PTH may last for less than 24 h, such that bone resorption is increased within the 24 h following calcitonin injection, but at 24 h after injection, osteoclast surface is increased. The effect may also relate, in part, to the possible down regulation of calcitonin receptors as discussed above, although it appears that since some response was detected that this process is not complete at 21 days post-operation.

The oophorectomy induced rise in bone formation was significantly inhibited in calcitonin treated animals other than the high dose treated group. The lower doses of calcitonin treatment prevented the rise in all markers of bone formation due to oestrogen-deficiency other than alkaline phosphatase activity. Serum osteocalcin is cleared rapidly by the kidney from the circulation. The half-life of osteocalcin in serum is only 4-5 minutes (191). It can be stated therefore that the low level of osteocalcin is detected 24 h after the previous calcitonin injection (the time of sampling), although this may not be the primary effect of calcitonin treatment. In contrast, mineral appositional rate reflects the level of bone formation over a 6 day period. Double fluorochrome labelled surface would reflect the total surface on which bone was being formed at the time of each injection. Both these histomorphometric markers of

bone formation indicate a reduction of bone formation over 6 days of calcitonin treatment in oophorectomised rats. Alkaline phosphatase activity was unchanged over the course of the experiment. It is likely that the effects of calcitonin treatment would vary over the 24 h period from time of injection, as discussed above. Although calcitonin treatment inhibits bone formation overall, there may be increased levels of bone formation at some stage after calcitonin injection. Although the half-life of alkaline phosphatase has not been determined in the rat, one study in Pagetic patients subjected to plasmaphoresis has indicated a half-life of between 1.12 and 2.15 days in the human (225). If the clearance time in the rat is similar, and bone formation had been raised at some stage over the 24 h period, alkaline phosphatase may not have cleared from this transient rise in formation when samples were collected (24 h after injection). Bone formation may therefore rise transiently after calcitonin administration, due to the direct effect of calcitonin on osteoblasts (156-161). In contrast, a low level of serum osteocalcin has been detected in the present study, indicating a secondary decrease in bone formation lasting at least until 24 h after injection of calcitonin. The decreased levels of mineral appositional rate and double fluorochrome labelled surface indicate this secondary decrease in bone formation is of longer duration or greater magnitude relative to the primary increase in bone formation induced by calcitonin.

Calcitonin inhibition of bone formation in oophorectomised rats is consistent with Wronski's study, demonstrating decreased markers of bone formation which included osteoblast surface, osteoid surface, mineral appositional rate and mineralising surface (equivalent to double fluorochrome labelled surface) in response to 16 IU/kg calcitonin on alternate days. (177).

The high dose of calcitonin used in this study (24 IU/kg/day) resulted in a decrease in double fluorochrome labelled surface only. This effect was observed at 15 days post-operation, and the reduction in double fluorochrome

label at the high dose was not as great as that observed with the lower doses of calcitonin. This may reflect incomplete down-regulation of calcitonin receptors in the continued presence of calcitonin, or changes in the timing of osteoclast response to calcitonin as described above.

In post-menopausal women calcitonin treatment has not consistently been reported to inhibit bone formation. In one study where calcitonin was administered in three weekly doses of 200 IU, alkaline phosphatase activity was decreased significantly after two years of treatment (165). Intranasal administration of 200 IU on alternate days resulted in decreased serum osteocalcin after one year of treatment (162). However, in two other studies, where calcitonin dosage was lower (50 IU or 100 IU / day intranasal) neither serum alkaline phosphatase activity nor osteocalcin were altered (166,167). The difference in these studies may reflect the different doses of calcitonin used, different times of specimen collection (no study in post-menopausal women has reported the time of collection), or administration of supplementary calcium in the latter studies.

In oophorectomised women, calcitonin has been administered at 100 IU by intramuscular injection on alternate days from 7 days after surgery (162). In this study, serum osteocalcin was initially decreased, then gradually increased. However, since data was not compared to untreated oophorectomised controls, in which serum osteocalcin levels also increase, it is difficult to determine whether the increase in osteocalcin with calcitonin treatment was physiologically significant.

The decrease in bone formation observed when oophorectomised rats were treated with calcitonin contrasts with the primary effect of calcitonin observed in vitro. In the osteoblast-like cell line, UMR-106-06, calcitonin treatment increases intracellular adenylate cyclase activity and intracellular free calcium concentration leading to stimulated alkaline phosphatase production (159).

Similarly, alkaline phosphatase production and ^{3}H-thymidine incorporation and proliferation are increased in the mature osteoblastic cell line MC-3T3-E1 (161,226). It must be remembered at this point, that bone formation is a considerably slower process than bone resorption, and while changes in bone resorption may occur rapidly, changes in bone formation are likely to occur over a considerably longer time period. It is possible that, in vivo, the direct stimulatory effect of calcitonin on the osteoblast is obscured by a decrease in bone formation associated with the initial and direct effect of calcitonin on osteoclastic resorption.

Calcitonin treatment at low doses did not prevent the oophorectomy-induced decrease in bone volume. In fact, calcitonin treatment resulted in a further reduction in trabecular bone volume and trabecular number compared to levels in vehicle treated controls. This may be due to the secondary increase in bone resorption coupled with reduced bone formation at the higher doses, and at the lowest doses, increased bone resorption without an associated increase in bone formation.

At the high dose of 24 IU/kg/day, the reduction in trabecular bone volume was prevented, apparently by the reduction in osteoclast surface. This probably results from the rapid fission of osteoclasts described in short term studies in vivo (172), and inhibition of osteoclast fusion described in vitro (147). These data are consistent with the work of Wronski et al (177), who reported inhibition of bone loss in oophorectomised rats treated with 16 IU/kg/day calcitonin, via a reduction in osteoclast surface, and compensatory reduction in bone formation, although there was no reduction in bone formation observed in this study. Treatment of intact rats with 4 IU/kg/day or 20 IU/kg/day, however results in decreased bone serum osteocalcin, and double fluorochrome labelled surface, and an increase in trabecular osteoclast number (173) as observed in this study in oophorectomised rats treated with the lower doses of calcitonin.

CHAPTER SIX

PRELIMINARY STUDY OF PTHrP (107-139) TREATMENT OF OOPHORECTOMISED RATS

6.1 Introduction

Parathyroid hormone related peptide (PTHrP) has been purified from a number of different human and animal tumours and, as such, is thought to be responsible for the clinical syndrome of humoral hypercalcaemia of malignancy (179,180). The carboxy terminal region of PTHrP (residues 107 - 139) specifically inhibits bone resorption via a direct action on osteoclasts (184,186), with no effect on osteoblastic cells in vitro (187). In this study the biological activity of PTHrP(107-139) in vivo has been evaluated in the oophorectomised rat.

6.2 Protocol

28 rats were oophorectomised at 6 months of age. From the day of operation until killing, these rats were injected daily subcutaneously with normal saline vehicle (n=10), 5μg/kg PTHrP(107-139) (n=6) or 50μg/kg PTHrP(107-139) (n=12). Blood and urine specimens were collected at time of operation, and at 3, 9 and 15 days post-operation. At days 6 and 12 post-operation, animals were injected intraperitoneally with calcein. At 15 days, animals were killed by cervical dislocation under halothane anaesthesia, and femora were collected.

Serum was analysed for levels of sodium, potassium, chloride, bicarbonate, creatinine, albumin, alkaline phosphatase activity and osteocalcin (Chapter 2.3). Serum anion gap, globulin and calcium fractions were calculated (Chapter 2.3). Urinary hydroxyproline and phosphate excretion and TmP were determined in 24 h fasting urine specimens (Chapter 2.3).

Femora were embedded in GMMA resin (Chapter 2.6.1). trabecular bone volume, trabecular number and trabecular thickness were determined in the diaphyseal-metaphyseal core using the Quantimet 520 Image Analysis System on Von Kossa stained sections (Chapter 2.6.2). Extent of double fluorochrome labelled surface was determined in unstained sections (Chapter 2.6.3). osteoclast surface was determined on sections stained for acid phosphatase (Chapter 2.6.4).

6.3 Results

There was considerable variation in all biochemical variables at time of operation, and consequently, data is expressed as change from day 0 (Chapter 2.6.5).

Oophorectomised rats treated with 5μg/kg/day PTHrP(107-139) tended to have lower levels of urinary hydroxyproline excretion compared to levels in oophorectomised rats (Figure 6.1). This effect was significant at 3 and 15 days post-operation. At the higher dose of PTHrP(107-139) hydroxyproline excretion levels were not different to levels reached in control oophorectomised rats. Levels of osteoclast surface did not differ significantly between operation groups (Figure 6.1).

Oophorectomised rats treated with 5μg/kg PTHrP(107-139) had slightly lower levels of alkaline phosphatase activity and double fluorochrome labelled surface than untreated controls (Figure 6.2). This effect did not reach significance, however. Serum osteocalcin was significantly affected by 5μg/kg/day PTHrP(107-139). At this low dose, the increase in osteocalcin normally observed after oophorectomy was inhibited by PTHrP(107-139) treatment (Figure 6.3). At the 50μg/kg/day dose, osteocalcin levels were also lower than in vehicle treated controls, but this did not reach significance in the time frame of this experiment.

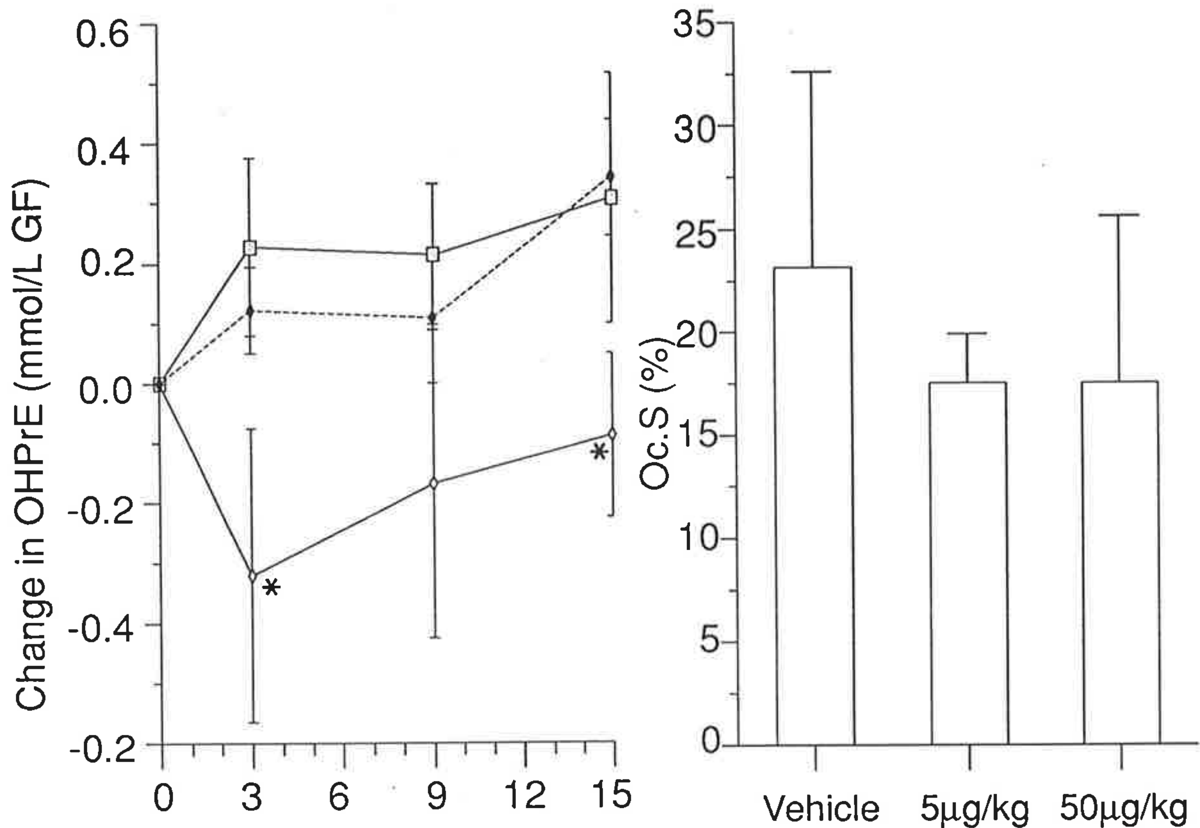

Figure 6.1 PTHrP(107-139) treatment signficiantly inhibits bone resorption in the oophorectomised rat (□). PTHrP(107-139) at 5µg/kg/day (◇) inhibited the rise in urinary hydroxyproline excretion (OHPrE) associated with oophorectomy. OHPrE is expressed here as the mean change from day 0. Osteoclast surface (Oc.S) was not significantly altered by PTHrP(107-139). 50µg/kg/day PTHrP (◆), did not significantly effect either marker. Values are mean ± SEM. *,$p<0.05$ vs oophorectomised control

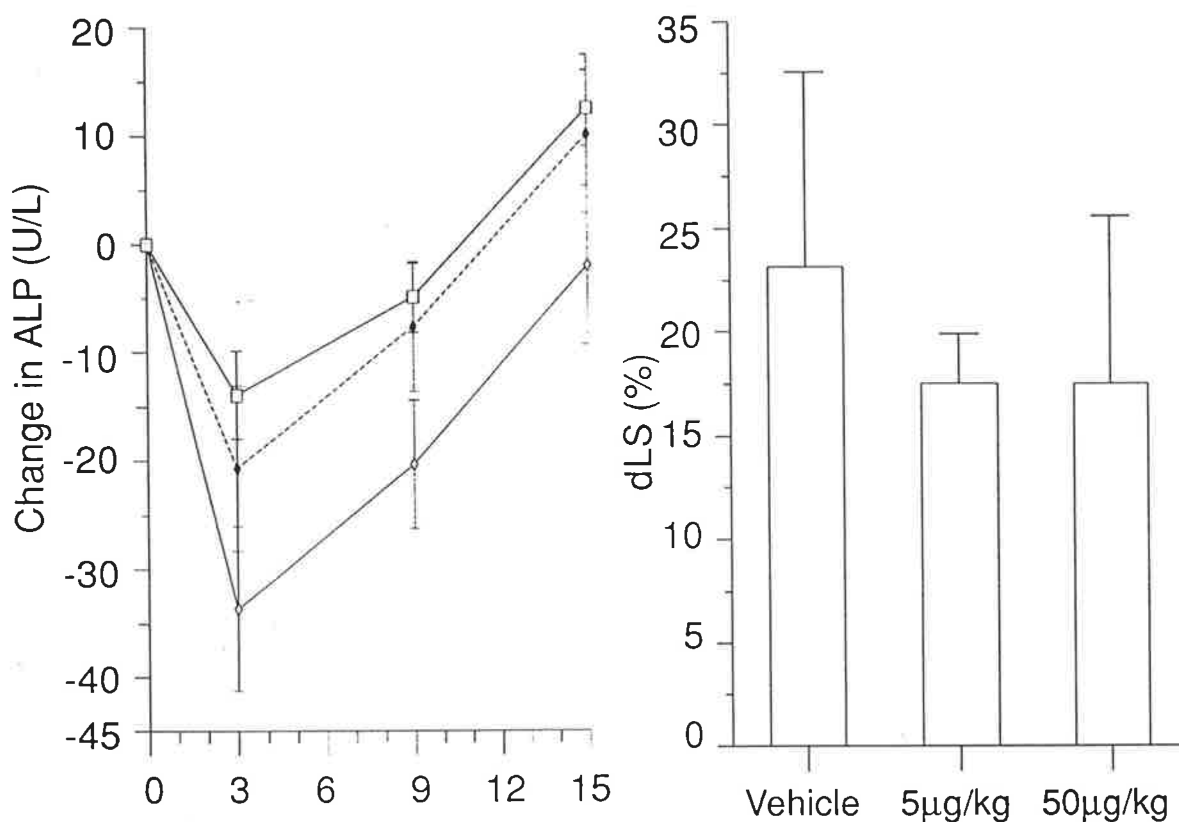

Figure 6.2 PTHrP(107-139) inhibited the increase in bone formation associated with oophorectomy. The rises in serum alkaline phosphatase activity (ALP) and double fluorochrome labelled trabecular surface (dLS) normally observed after oophorectomy (□) were slightly, but non-significantly inhibited by PTHrP(107-139) treatment at 5µg/kg/day (◇). 50mg/kg/day PTHrP(107-139) administered to oophorectomised rats (◆) did not appear to inhibit either marker of bone formation. Values are mean ± SEM.

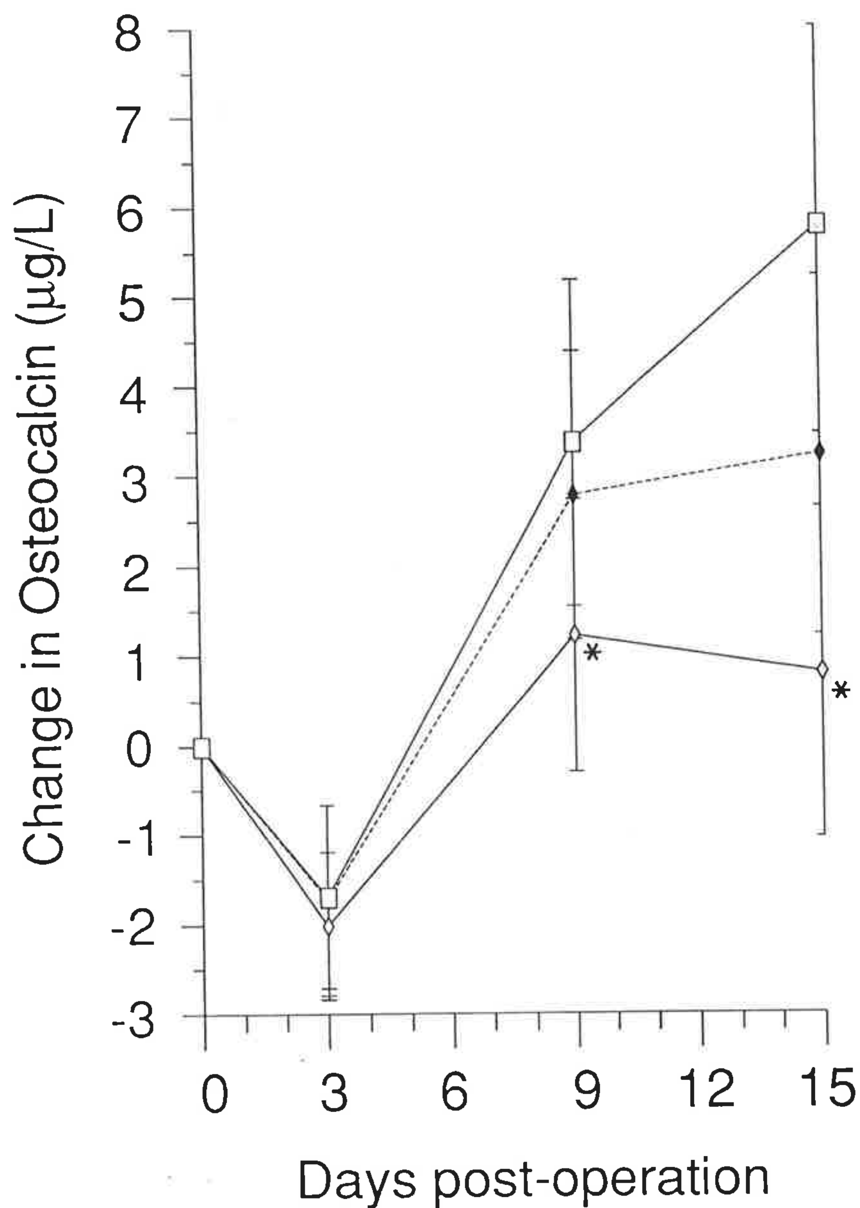

Figure 6.3. PTHrP(107-139) treatment inhibited the rise in serum osteocalcin observed after oophorectomy. Levels of serum osteocalcin were significantly lower in oophorectomised rats treated with PTHrP(107-139) at 5µg/kg/day (◇) compared to vehicle-treated oophorectomised rats (□). This effect did not reach significance at the dose of 50µg/kg/day PTHrP(107-139) (◆). *,$p<0.05$ vs vehicle treated oophorectomised rats. Values are mean change from day 0 ± SEM.

PTHrP(107-139) at either dose did not prevent the reduction in trabecular bone volume and trabecular number associated with increased bone remodelling after oophorectomy (Figure 6.4). There was also no significant effect of PTHrP(107-139) on trabecular thickness (Figure 6.5).

No other biochemical analytes measured were altered by PTHrP(107-139). There was a tendency towards an increase in serum calcium in oophorectomised rats treated with PTHrP(107-139) at 21 days post-operation (Figure 6.6).

6.4 Discussion

In this study, PTHrP(107-139) at the low dose of 5μg/kg/day prevented the rise in serum osteocalcin and urinary hydroxyproline excretion observed in oophorectomised rats.

The reduction in bone resorption is consistent with effects of PTHrP(107-139) observed in vitro, where this fragment of PTHrP reduces osteoclast spreading and nuclei number (184) as well as inhibiting osteoclast proliferation and bone resorption via specific osteoclastic receptors (186). This effect of PTHrP(107-139) on osteoclasts probably inhibits the activity of the mature osteoblast via coupling, as indicated by PTHrP(107-139) inhibition of serum osteocalcin. This does not appear to occur with osteoblasts during the proliferative phase, since no significant alteration in alkaline phosphatase activity was detected.

The data presented in the present study cannot preclude a direct effect of PTHrP(107-139) on the osteoblast. This seems unlikely however, since in vitro studies indicate no significant effect of PTHrP(107-139) on the isolated osteoblast (187).

The effect of PTHrP(107-139) observed in vivo is in contrast to the effects of

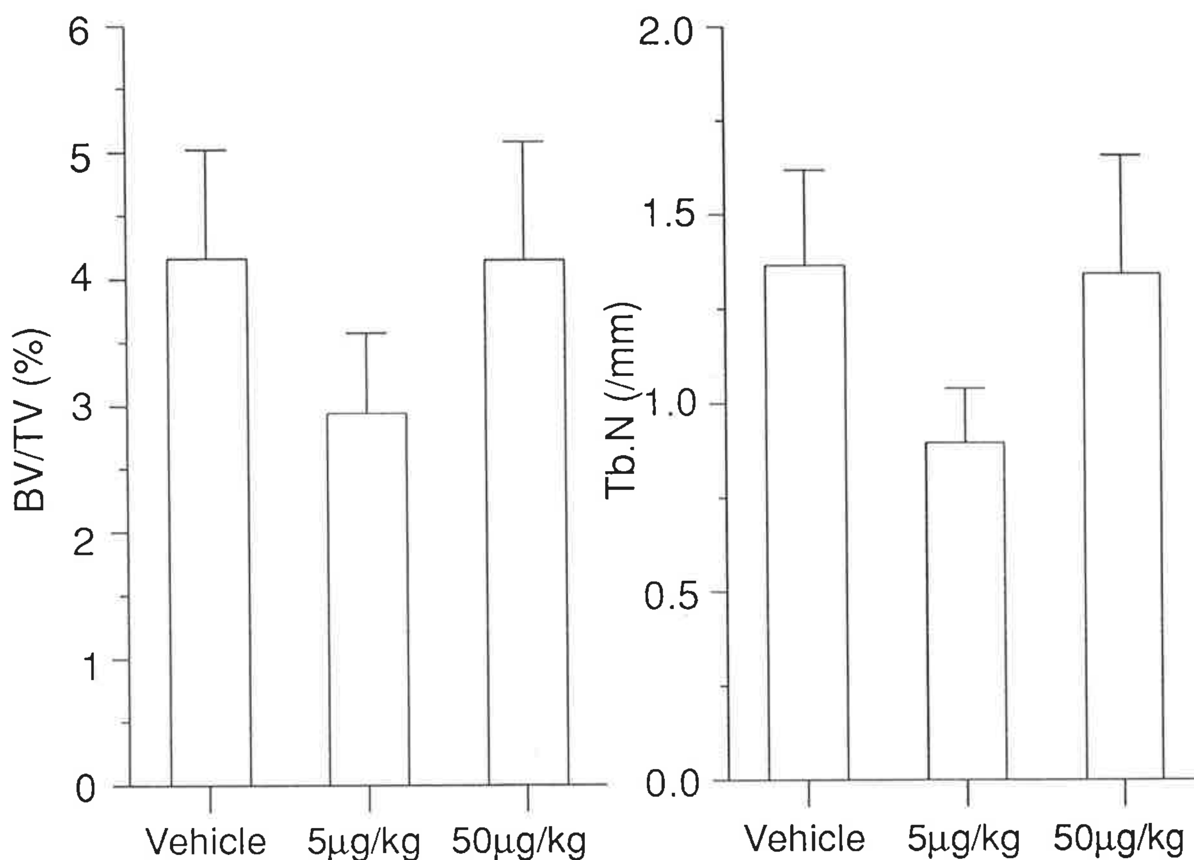

Figure 6.4. PTHrP(107-139) treatment did not prevent trabecular bone loss in the oophorectomised rat. Neither trabecular bone volume (BV/TV) nor trabecular number (Tb.N) were significantly altered by PTHrP(107-139) treatment at either 5µg/kg/day or 50µg/kg/day. There was a slight decrease in both parameters with the lower dose used, but this did not reach significane. Values are mean ± SEM.

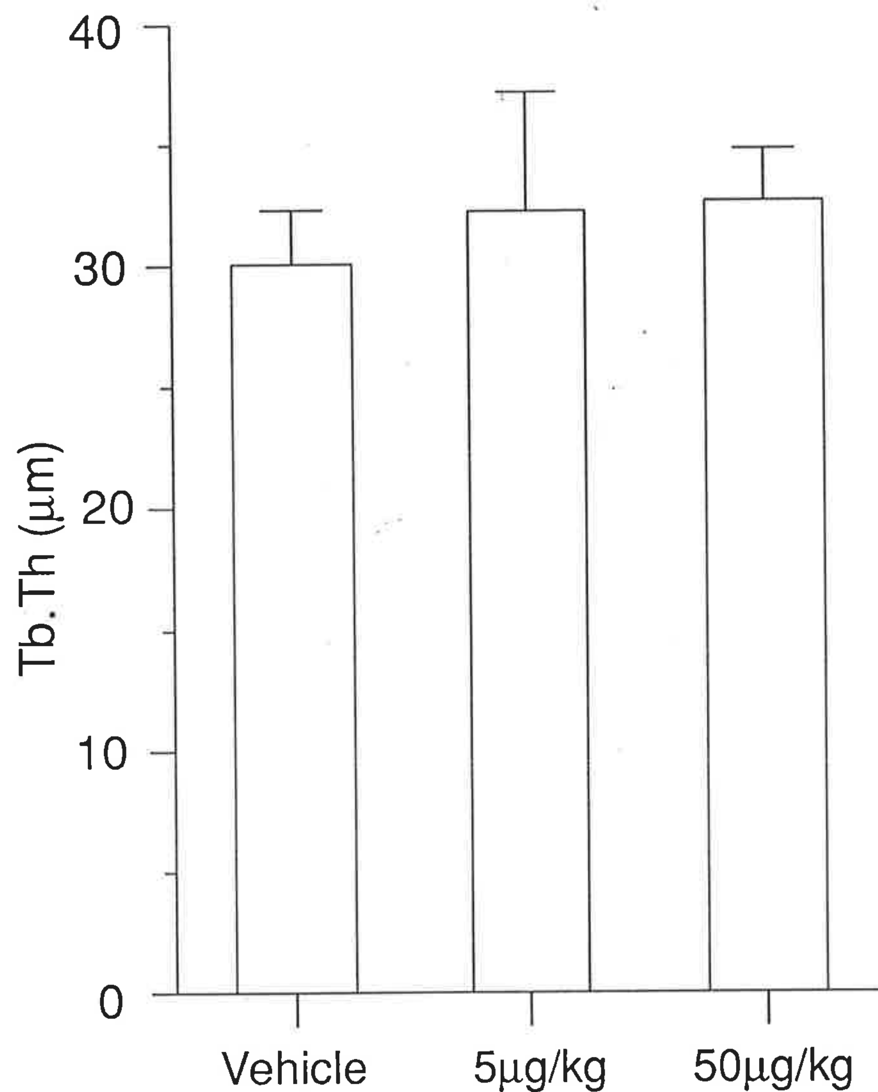

Figure 6.5. Trabecular thickness in the oophorectomised rat was not significantly altered by PTHrP(107-139) treatment. PTHrP(107-139) treatment of oophorectomised rats at 5μg/kg/day or 50μg/kg/day did not significantly alter trabecular thickness (Tb.Th) in the femoral metaphyseal - diaphyseal core. Values are mean ± SEM.

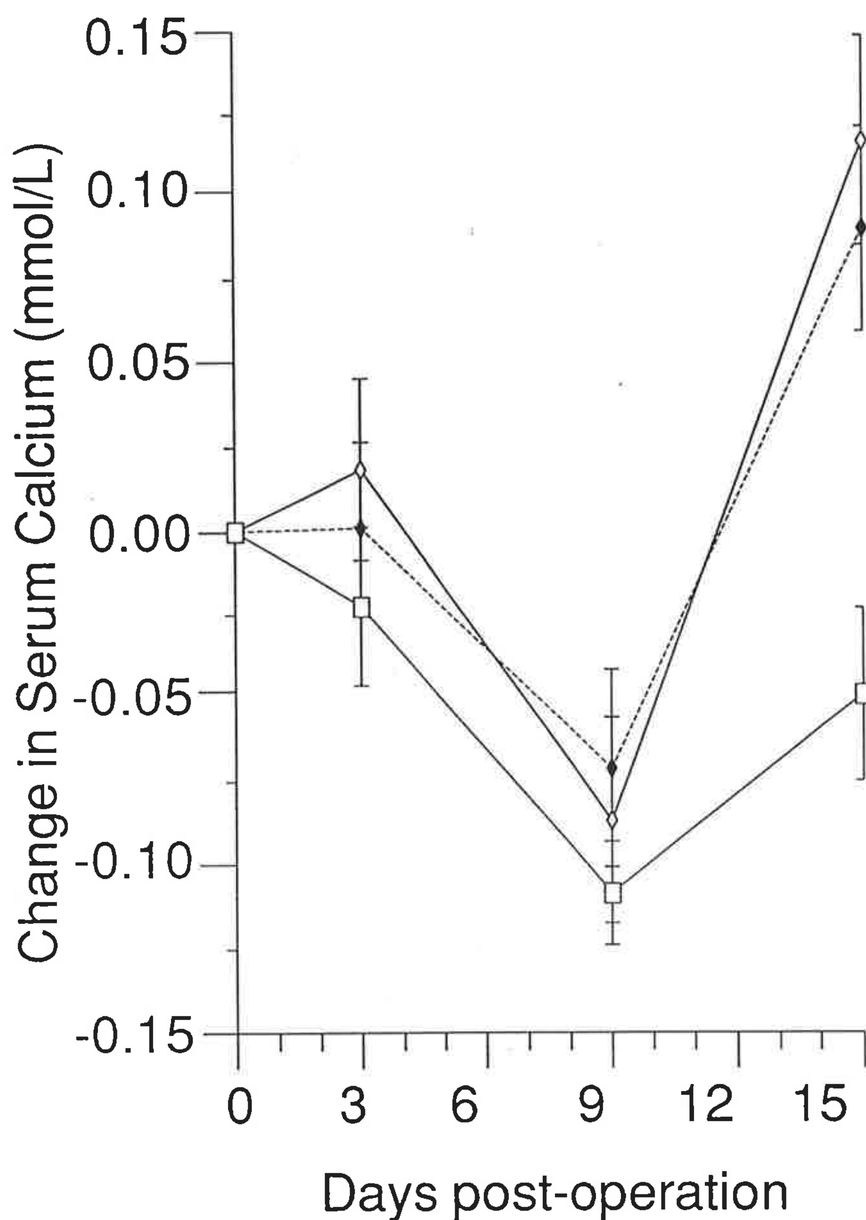

Figure 6.6. Serum calcium was slightly increased by PTHrP(107-139) treatment. At 21 days post-operation, serum calcium was increased in oophorectomised rats treated with 5μg/kg/day (◇) or 50μg/kg/day (◆) PTHrP(107-139), however this effect did not reach significance. Values are mean ± SEM.

the amino terminal PTHrP region (1-34) which stimulates bone resorption both in vivo (181,227) and in vitro (185) via osteoblastic PTH receptors (185).

Inhibition of bone turnover in oophorectomised rats by PTHrP(107-139) was not sufficient to prevent the loss of cancellous bone observed after oophorectomy. In fact, administration of 5μg/kg/day PTHrP(107-139) appeared to result in a further reduction of trabecular bone volume and trabecular number, but this was not significant. This may indicate that although bone turnover is reduced by PTHrP(107-139), the initial temporal imbalance between the increase in osteoclast and osteoblast activity which causes oestrogen-deficiency bone loss has not been prevented by these doses.

PTHrP(107-139) may then be a useful agent to prevent the increased bone resorption observed in oestrogen deficiency. However, these data are only preliminary. A larger study with a wider range of doses and more animals would be required to determine if PTHrP(107-139) will act in vivo inhibit the effects of oophorectomy on bone turnover completely.

CHAPTER SEVEN

SUMMARY AND CONCLUSIONS

7.1 The Mechanism of Trabecular Bone Loss in Oestrogen-Deficiency

Oophorectomy in the rat, a well recognised model for post-menopausal bone loss, results in a rapid increase in osteoclast proliferation in the femoral distal metaphysis at 6 days post-operation. The rise in markers of bone resorption after oophorectomy appeared transient with levels declining towards those of sham operated rats by 42 days post-operation. Increased osteoclast recruitment would be associated with increased BMU activation frequency.

Increased bone resorption after oophorectomy is followed by an increase in osteoblast proliferation and mineralising activity at 9 to 12 days post-operation. The elevated level of bone formation appears to be of longer than duration than the elevation in bone resorption, consistent with longer term studies (53,54). The longer duration of the high level of bone formation may have prevented any further loss of bone, possibly occurring in response to greater mechanical strain as trabecular bone volume is reduced, although this may be a site-specific observation.

The rapid increase in osteoclast proliferation and activity suggest a direct stimulation of the osteoclast in oestrogen deficiency, followed by an increase in osteoblast activity. The delayed increase in bone formation compared to the rapid increase in bone resorption may relate to the normal temporal relationship between bone resorption and formation in the remodelling cycle. Sequential bone remodelling activity in the 3 month old rat has a resorption cycle of 1.4 days followed by a reversal phase of 4.2 days and a formation cycle of 33 days in the vertebral secondary spongiosa (210). The delayed increase in bone formation in oophorectomised rats compared to the rapid increase in bone

resorption is consistent with such a lag time, suggesting a model where initiation of basic multicellular units and consequent bone resorption are increased prior to an increase in bone formation, thus following the normal cycle of bone remodelling. However, these data do not rule out a model of broad cellular activation in oestrogen deficiency, where proliferation and activity of both osteoclasts and osteoblasts are stimulated, but detection of the rise in osteoblast activity is delayed.

The temporary imbalance between bone resorption and formation appears to be the primary cause of trabecular bone loss in the femoral metaphyseal core region. Loss of bone after oophorectomy was rapid, occurring soon after the increase in bone turnover, and in this area the bone loss was limited, with no further reduction in bone volume detected between 15 and 42 days post-operation. The limited loss of bone suggests oestrogen-deficiency bone loss is a finite, once-only event due to a single disturbance in bone turnover, that is, increased resorption prior to formation rather than a continued negative balance of bone resorption and formation at the BMU level.

The data presented in this thesis support a model whereby the increased level of bone resorption after oophorectomy results in increased BMU activation frequency. The risk of trabecular perforation would increase with activation frequency since the likelihood of resorption occurring on both sides of a single trabecula would rise (63). The increase in bone formation following bone resorption, by nature of BMU-based trabecular bone remodelling, is not capable of repairing trabecular perforations since the trabecular surface on which formation would normally occur has been lost. It is also likely that non-weight bearing trabecular fragments which remain are preferentially resorbed, as suggested by Dempster et al (63). In this model the balance of bone turnover at the level of the BMU is maintained, however due to trabecular perforation, not all resorbed pits continue through to the formation stage, since bone formation requires a surface to build on. This model of bone loss in

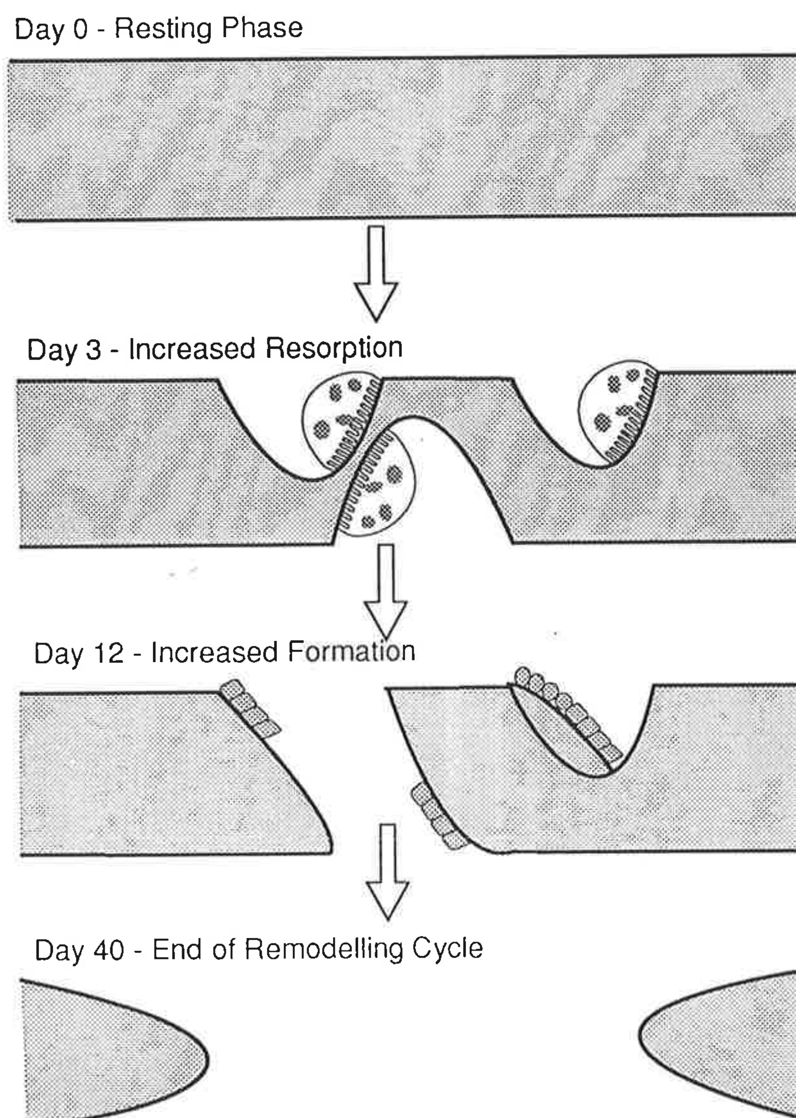

Figure 7.1 A Model for Bone Loss in the Oophorectomised Rat. At 3 days after oophorectomy, increased osteoclast recruitment leads to an increase in activation frequency of new BMUs. The increased number of active BMUs results in increased risk of trabecular perforation. At 12 days post-operation, following the reversal phase of the bone remodelling cycle, bone fomation is increased. Bone formation is only effective where trabecular surface remains, therefore trabecular perforations cannot be refilled. In this way, bone resorption and formation throughout the bone are unbalanced. The overall imbalance of bone turnover, coupled with resorption of remaining spicules results in a loss of trabecular bone by the end of the cycle. For clarity, lining cells and BMUs initiated after 3 days post-operation have not been pictured.

oophorectomised rats is summarised in Figure 7.1.

In the present study, changes in histomorphometric markers of bone turnover in oestrogen deficiency have been described in a specific region of bone. It is likely that the timing of the increased bone resorption and subsequent formation varies at different bone sites, such that, throughout the skeleton, total bone mineral content is reduced more gradually.

The mechanism of bone loss at the menopause in the human female may follow a similar pattern to that described in the present study. A similar model of bone loss has been proposed by Parfitt, whereby increased activation frequency of multicellular units is a major cause of post-menopausal bone loss (17). This has been supported by measurements of increased activation frequency in post-menopausal biopsy specimens compared to pre-menopausal and oestrogen treated controls by Steiniche et al. (8). In post-menopausal women, however, bone loss is likely to be more gradual in any anatomical area since the reduction in circulating levels of oestradiol at the menopause is a slow process. Parfitt has suggested an impairment of osteoblast activity also contributes to oestrogen-deficiency bone loss (17,21). In the oophorectomised rat, however, bone formation is maintained at a high level, suggesting proliferation or activity of osteoblasts is not impaired per se, but the surfaces on which bone may be formed are reduced as bone is lost.

This study is unique not only in the time frame over which changes in bone metabolism have been observed after oophorectomy, but also in the relationships derived between histomorphometric and biochemical markers of bone formation and resorption. Hydroxyproline excretion and osteoclast surface have now been shown to be closely related in oophorectomised rats as previously described in post-menopausal women (208). A significant relationship was also demonstrated between mineral appositional rate and serum osteocalcin and between double fluorochrome labelled surface and

alkaline phosphatase activity, suggesting that the histomorphometric markers, like their biochemical counterparts also indicate different stages of osteoblast activity in the process of bone formation.

7.2 A Model of Oestrogen Action on Bone

Loss of trabecular bone and reduced trabecular number due to oophorectomy was delayed in oestradiol treated rats, although the bone loss was not prevented completely. Oestradiol treatment also increased trabecular thickness suggesting a greater level of bone formation than resorption with oestradiol treatment. The strong relationship between trabecular bone volume and trabecular number observed in the present study in all treatment groups suggests oestradiol inhibits preventing trabecular perforation due to oophorectomy (62,63).

The initial effect of oestradiol treatment was stimulation of bone formation at 6 days post-operation, prior to the loss of bone induced by oophorectomy. This increase in bone volume, associated with an increase in markers of mature osteoblast activity, suggested a direct stimulatory effect of oestradiol on bone mineralisation, consistent with the reported effects of oestradiol on mature osteoblast culture (99,104-106).

Proliferating osteoblasts at the stage of alkaline phosphatase production appeared to be more sensitive to oestradiol than osteoblasts associated with mineralisation. It appears that at an early stage of osteoblast maturation, oestradiol has an inhibitory effect on osteoblast proliferation in addition to stimulating mature osteoblast activity. While less mature osteoblasts are inhibited by oestradiol, the more mature cells at the bone surface are stimulated, and are producing osteocalcin at a high level. Osteocalcin levels reduce as proliferation and maturation of new osteoblasts is inhibited, and the number of cells reaching the osteocalcin producing stage of bone cell

maturation is reduced. The inhibitory effect of oestradiol on the activity of immature osteoblasts is consistent with oestradiol inhibition of the proliferating osteoblast cell line UMR106-06 (98,107).

The increase in markers of bone resorption normally associated with oophorectomy was inhibited by oestradiol treatment, consistent with other in vivo studies (82,84,85), as well as direct inhibition of bone resorption reported in isolated osteoclasts (38,103). The inhibitory effect of oestradiol on the osteoclast would also inhibit bone formation via coupling of osteoblast activity to that of the osteoclast. Results from the present study therefore support a model of a stimulatory effect of oestradiol on mature osteoblasts, but a dominant inhibitory effect of oestradiol on osteoblast and osteoclast proliferation.

A model is proposed, summarised in Figure 7.2 whereby, in oophorectomised rats, the direct inhibitory effect of oestradiol on osteoblast proliferation and oestradiol-induced reduction in osteoclastic bone resorption obscures the direct stimulation of mature osteoblasts such that both bone resorption and formation are reduced to levels observed in intact rats, and bone loss is prevented for the duration of oestradiol treatment. Bone turnover may be reduced further with oestradiol treatment by modulation of local factors involved in the regulation of bone turnover or bone cell production, such as TGFβ, IL-1 or IL-6.

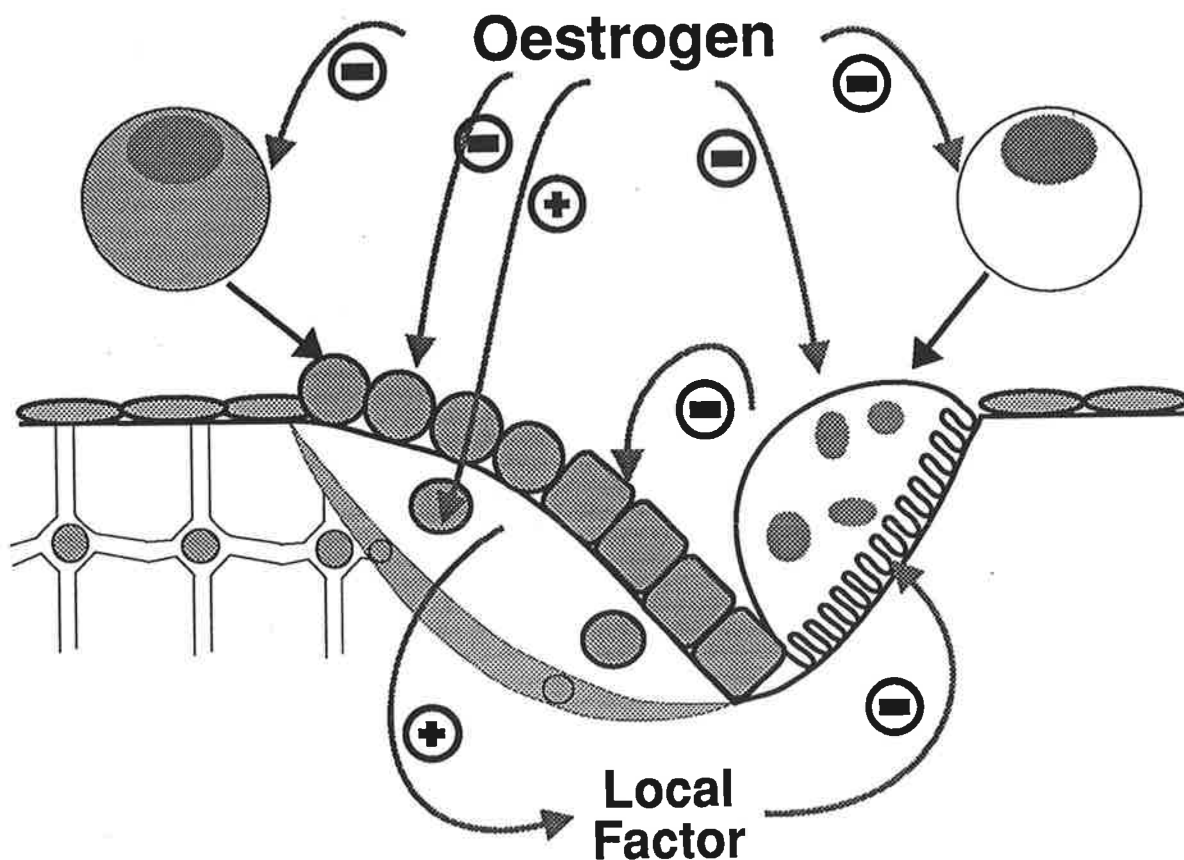

Figure 7.2 A Model of Direct Oestrogen Action on Bone. Oestrogen appears to act directly on osteoclasts and osteoblasts via specific receptors. Osteoclast activity and osteoblast proliferation are both directly inhibited. The activity of the mature osteoblast however, is directly stimulated by oestrogen. The stimulatory effect of oestrogen on mature osteoblasts is obscured by its inhibitory actions on other cell types, such that only inhibiton of bone turnover is observed in the long term. Osteoblast production of local factors such as TGF-β, IL-1 or IL-6 may also be modulated, further inhibiting bone turnover in an autocrine / paracrine manner.

7.3 A Model for Calcitonin Effect on Bone - Comparison with Oestradiol

The primary inhibitory effect of calcitonin on osteoclasts in vitro has also been observed in short term studies in the intact rat and results in pronounced hypocalcaemia (170-172). This hypocalcaemia stimulates PTH secretion and a secondary increase in bone resorption is induced, stimulating calcium release from the bone as well as an increase in bone formation (173). For this reason, the effects of calcitonin in vivo are quite complex, with some primary and secondary responses being detected in this study.

Treatment of intact rats with 1.2 IU/kg/day salmon calcitonin resulted in a secondary increase in both bone resorption and formation. In oophorectomised rats treated with the same dose, bone resorption was increased however, bone formation was reduced, resulting in an exacerbated loss of trabecular bone.

The increase in bone resorption observed in both sham operated and oophorectomised rats treated with calcitonin is in direct contrast to effects observed in vitro (145-152,154). The primary hypocalcaemia induced by calcitonin treatment causes a secondary increase in PTH secretion (173), which in turn, induces bone resorption. In the present study, the secondary PTH-related increase in bone resorption appears to dominate the primary reduction in bone resorption detected 4 h after calcitonin administration (170-172).

The oophorectomy induced rise in bone formation was significantly inhibited in calcitonin treated animals. The decrease in bone formation observed in oophorectomised rats treated with calcitonin contrasts with the direct effect of calcitonin observed in vitro (159,161,226). It appears that in vivo, the direct stimulatory effect of calcitonin on the osteoblast is obscured by decreased bone formation associated with the primary effect of calcitonin on osteoclastic resorption.

Since both calcitonin and oestradiol are osteoclast inhibitors in vitro (38,102,103,145-152,154), it is interesting that the secondary effect of PTH is not observed in oophorectomised rats treated with oestradiol. The reason for this appears to be that oestradiol treatment does not reduce serum calcium levels (Chapter 5), such that PTH secretion is not stimulated. In vitro studies have shown that oestradiol treatment inhibits osteoblastic activity at an early stage of development (98,107) whereas mature osteoblasts are stimulated by oestradiol treatment. calcitonin treatment however directly stimulates the proliferation of mature osteoblastic cell lines (158-161) as well as UMR106-06 osteoblastic cells (156) the same immature cell line in which oestradiol inhibits proliferation. The increased bone formation in the presence of decreased resorption, immediate effects of calcitonin treatment, are the causes of hypocalcaemia. In oestradiol-treated rats however, both resorption and formation are reduced, such that serum calcium is unaltered. These data then, also support the model proposed whereby direct oestradiol inhibition of both bone resorption and osteoblast proliferation eliminates the stimulatory effect of oestradiol on mature osteoblasts reported in vitro.

7.4 A Model of PTHrP(107-139) Effect on Bone - Comparison with Oestradiol

PTHrP(107-139) at the low dose of 5μg/kg/day prevented the rise in serum osteocalcin and urinary hydroxyproline excretion observed in oophorectomised rats. The reduction in bone resorption is consistent with effects of PTHrP(107-139) observed in vitro (184,186). This effect of PTHrP(107-139) on osteoclasts may inhibit mature osteoblastic activity via coupling. This does not appear to occur during the proliferative phase. PTHrP(107-139) treatment did not suppress the loss of cancellous bone associated with oophorectomy, suggesting a direct inhibition of osteoblast activity is required. This further supports to a model of a direct inhibitory effect of oestradiol on proliferating osteoblasts.

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