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Dose-Dependent Effects of Salmon Calcitonin on Bone Turnover in Ovariectomized Rats.

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Dose-dependent Effects of Salmon Calcitonin on Bone Turnover in Ovariectomized Rats

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by

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In the United States, osteoporosis results in about 1.5 million annual fractures, costing approximately $15 billion. Calcitonin is safe and effective in slowing osteoporotic bone loss, but its effect is transient. The current studies were designed to explore the dose-dependent effects of salmon calcitonin on bone turnover in ovariectomized rats and to determine if the decrease in therapeutic effectiveness of calcitonin demonstrated over time with higher doses is due to oversuppression of bone turnover. Doses of 5, 15, & 50 IU/kg BW/day of calcitonin were compared to placebo in 12-week-old ovariectomized and sham-ovariectomized Sprague-Dawley rats for 24 weeks. The spinal bone mineral content (BMC) as measured by DXA in ovariectomized subjects receiving 5 & 15 IU/kg of calcitonin was not significantly different from sham-ovariectomized subjects, while spinal BMC of subjects receiving 50 IU/kg was significantly lower than sham-ovariectomized subjects ($p<0.05$). Femoral BMC of ovariectomized subjects was significantly lower than sham-ovariectomized subjects ($p<0.05$), but no significant differences were noted between treatment groups. Scanning electron microscopy (SEM) demonstrated a decrease in number and density of trabeculae and in cortical thickness when comparing femurs from ovariectomized with sham-ovariectomized subjects. SEM of subjects receiving 50 IU/kg displayed greater bone loss than other groups. No significant differences were noted between groups for levels of urinary helical peptides or serum pyridinoline [ELISA], indicators of bone resorption. Urinary calcium excretion [capillary ion electrophoresis] was significantly higher in subjects receiving 50 IU/kg of

calcitonin than other ovariectomized subjects ($p<0.05$). Serum levels of osteocalcin [RIA], an indicator of bone formation, were significantly higher in subjects receiving 5 IU/kg of calcitonin than control subjects and those receiving 50 IU/kg ($p<0.05$). Production of antibodies to calcitonin [ELISA] by subjects in this study did not correlate with changes in bone turnover or bone density. The results of this study do not provide evidence higher doses of calcitonin result in oversuppression of bone turnover. However, urinary calcium excretion affected bone resorption in a reverse dose-dependent manner, suggesting the calciuric effect may be responsible for less effective outcomes seen with higher doses of calcitonin.

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INTRODUCTION

Bone is a complex, dynamic tissue of the human body with both structural and metabolic functions. Structurally the bony skeleton provides a protective framework for the vital internal organs as well as allowing for sophisticated bodily motions and facilitation of locomotion. The metabolic function of bone involves its provision of a mineral reservoir for many of the body's vital functions such as muscle contraction, transmitting nerve impulses, blood clotting, and cell adhesion (Paulsen 1993; Rubin and Rubin 1996). Bone contains 99% of the body's total calcium, 85% of its phosphorous, and 66% of its magnesium. Another vital physiological function of bone is seen in the role of bone marrow as host to precursors of blood cells (Glimcher 1990). Skeletal tissue is constantly adapting to balance these functions.

Balance of these functions occurs through the processes of bone resorption and bone formation. In adults these processes are linked together to provide for continuous bone homeostasis. Bone is constantly being resorbed by osteoclasts in order to release minerals essential for the metabolic functions of life. As bone is resorbed osteotropic factors stored in the bone matrix are released resulting in activation of osteoblasts. These osteoblasts proceed with formation of new bone to restore the bone mineral necessary for the structural functions of bone. While the osteoblasts are forming bone they secrete more osteotropic factors into the newly formed bone matrix. Due to this coupling mechanism between osteoclasts and osteoblasts the body is able to maintain both its

structural and metabolic functions. If these processes become uncoupled and bone resorption exceeds bone formation, disorders of bone remodeling, such as osteoporosis, occur. The most common cause of osteoporosis in humans is estrogen deficiency.

Calcitonin is one of the medications used to treat osteoporosis in postmenopausal women. By binding to the calcitonin surface receptors of the osteoclasts calcitonin inhibits osteoclast activity and limits estrogen-deficient bone loss. However, many patients become resistant to the effects of calcitonin, and over time calcitonin appears to become less effective at limiting the increased bone resorption induced by estrogen deficiency. It is possible that oversuppression of bone turnover may be responsible for this resistance to calcitonin. It is our hypothesis that high doses of salmon calcitonin can result in oversuppression of bone turnover.

Description of Bone

The human adult skeleton consists of two types of bones, cortical or compact bone and cancellous or trabecular bone. Cortical bone constitutes approximately 80% of the total skeletal mass. It is characterized by a low surface:volume ratio and is found primarily in the shafts of the long bones of the appendicular skeleton and the outer layer of most bones. The remaining 20% of the skeletal mass is cancellous and is found primarily in the axial skeleton and in the ends of the long bones. Cancellous bone, which is sometimes referred to as spongy, has a much higher surface:volume ratio than cortical bone. Due to this greater surface:volume ratio, cancellous bone is more responsive to the

metabolic needs of the body for calcium and other minerals, and cancellous or trabecular bone mass is lost much earlier than cortical bone mass (Edelson and Kleerekeper 1996).

Proportions of cortical and cancellous bone vary at different sites of the body and may play a role in susceptibility of certain skeletal regions to osteoporotic fracture. After menopause, for instance, there is an increased incidence of Colles fracture in the distal 1/3 of the wrist in subjects between the ages of 40 and 50. The most frequent site of osteoporotic fracture in subjects ranging from 50-60 years of age is the vertebral column, which is predominantly composed of cancellous bone. More than 66% of the total bone of the lumbar spine is cancellous. In contrast, loss of cortical bone is the major predisposing factor for fractures occurring in the hip. Hip fractures occur more commonly in patients older than 60 years of age. Assessment of the femur reveals a ratio of 50% cortical and 50% cancellous bone at the intertrochanteric region, while the neck is 75% cortical and 25% cancellous. The mid radius is calculated to be more than 95% cortical in nature, the distal 1/3 of the radius is primarily trabecular in nature, with the area between these two locations being a mixture of approximately equal portions of cortical and trabecular bone (Melton 1997; Mundy 1999).

These differences are likely due to the differing environments of bone cells in cortical vs. cancellous bone. Cells responsible for bone remodeling on the surfaces of cancellous bone are in close contact with the cells producing potent osteotropic cytokines in the bone marrow cavity. Cells in the cortical bone, however, are located more distant from the influence of these cytokines and are more susceptible to the influence of systemic osteotropic hormones, including parathyroid hormone (PTH) and 1,25-dihydroxyVitamin D_3 (Mundy 1999).

The microstructure of adult compact bone consists primarily of Haversian canals, irregular cylindrical units, containing blood vessels, lymph vessels, nerves, and some loose connective tissue. Thin solid layers of concentric bone, referred to as lamellae, surround these canals. A number of small almond-shaped spaces, called lacunae, are bound between these lamellae. These lacunae, which contain the bone cells or osteocytes, are connected to one another and to the central Haversian canal system by numerous fine radiating channels called canaliculi. Volkmann's canals, which house blood vessels and nerves, extend transversely through compact bone and connect Haversian canals with one another and with the bone surfaces of the marrow cavity (Dawson 1974; and Gray's Anatomy 1980).

Cancellous bone has a fine 3-dimensional lattice composition resulting in many open spaces. These spaces are connected by branching and anastamosing slips of bone called trabeculae or spindles. These are aligned along the lines of stress of the bone, allowing for maximization of the weight-bearing capacity of this type of bone (Paulsen 1993). The trabeculae of cancellous bone consist of superimposed fragments of lamellae with intervening cement lines and sometimes have small islands of calcified cartilage. These trabeculae typically do not have Haversian systems and are not penetrated by blood vessels but are literally surrounded by blood. They receive their nutrients from the blood vessels in the marrow around them (Gray's Anatomy 1980).

Bone contains both organic and inorganic (mineral) constituents. Calcium phosphate and calcium carbonate are the most abundant mineral components, with calcium fluoride and magnesium chloride being present in smaller quantities. These inorganic components account for about 2/3 of the total weight of mature bone and give

the bone its hardness and rigidity (Dawson 1974). Bone mineral contains numerous components making it more soluble than geological hydroxyapatite. This allows bone to act as a reservoir for calcium, phosphate, and magnesium ions (Lian and others 1999).

The remaining 2/3 of the weight of the bone is composed of organic constituents that give the bone its flexibility and elasticity (Dawson 1974). The predominant component of the organic matrix is type I collagen that helps to determine the structural organization of bone. This collagen and the proteins associated with it influence the process of bone mineralization. The constraints of the collagen matrix help to govern the growth of the bone mineral crystals as they are deposited onto the matrix. The size and shape of these mineral crystals are regulated in part by the proteins that bind them. These proteins also play a role in recruitment of osteoclasts to the crystal surface (Lian and others 1999).

Osteoclasts

Osteoclasts are unique and highly specialized cells that are derived from the hematopoietic granulocyte-macrophage colony-forming unit lineage. They are large multinucleated cells formed by the fusion of mononuclear cells, resulting in increased efficiency per cell at removing bone during the bone remodeling process. These cells have indented nuclei, with prominent nucleoli, abundant mitochondria with large cristae, very little rough endoplasmic reticulum, numerous lysosomes, and a large ruffled border surrounded by a clear zone. This clear zone contains actin filaments and tightly attaches to the resorption surface anchoring the ruffled border area to the bone surface undergoing resorption. This produces an enclosed space which functions as an extracellular

lysosomal space and may be critical to the osteoclast becoming polarized when activated just prior to resorption (Rodan and Rodan 1995; Mundy 1999). Another unique feature of mature osteoclasts is their possession of receptors for calcitonin. Exposure to calcitonin causes osteoclasts to retract and loosen from the bone surface (Rubin and Rubin 1996).

Osteoblasts

Osteoblasts, which are derived from pleuripotent mesenchymal stem cells, represent a heterogeneous family of cells that includes mature osteoblasts, osteocytes, and the bone lining cells. Mature osteoblasts are cuboidal in shape and have a single large nucleus with several nucleoli, an abundant amount of rough endoplasmic reticulum, and a well developed Golgi apparatus (Raisz and Rodan 1990). Mature osteoblasts form a dense layer of cells and work in conjunction with one another to synthesize Type I collagen and the proteins of the bone matrix, including osteocalcin, osteopontin, bone sialoproteins, and osteonectin. They are clearly polarized with matrix being produced and secreted only on their basal aspect. Mitotic figures are rarely identified in mature secreting osteoblasts, suggesting that they are nondividing cells (Rodan and Rodan 1995). Functions of mature osteoblasts include production of the proteins of the bone matrix, secretion of growth factors that are stored in the bone matrix, and mineralization of newly formed bone matrix. Through interactions with osteoclasts osteoblasts play a role in allowing normal bone resorption to occur (Mundy 1999).

When osteoblasts are not actively involved in the formation of bone matrix, they are referred to as bone-lining cells. As bone turnover decreases with age the number of

bone-lining cells also appears to decrease. These are likely to be the most common cells found in trabecular bone of adult mammals (Holtrop 1990). Bone-lining cells are flat, thin elongated cells with few organelles and have numerous gaps and spaces between one another.

Osteocytes

Buried within the mineralized matrix of bone are the stellar-shaped osteocytes. These are the most abundant of the bone cells and communicate with one another and with cells on the bone surface through dendritic processes in canaliculi (Mundy 1999). While the precise function of osteocytes remains unknown, they are ideally located to detect changes in bone stress. Possible functions attributed to osteocytes include osteocytic osteolysis, bone formation, and response to mechanical stimuli (Rodan and Rodan 1995). Osteocytes are connected by gap junctions to the osteoblasts allowing for communication between bone cells. Such communication helps explain the coordinated activity of teams of osteoblasts during the remodeling cycle (Rodan and Rodan 1995). When signaled to do so, the protective bone-lining cells retract from the bone surface in order to allow for the attachment of osteoclasts to begin the process of bone resorption (Lian and others 1999).

Functions of Bone

Although cortical and trabecular bone contain the same cells and matrix elements, there are structural and functional differences between these two types of bone. The primary structural difference is due to the amount of calcified substance found in each

type of bone. While 80-90% of the volume of compact bone is calcified, only 15-25% of trabecular bone is calcified. These structural differences predispose each type of bone for their specific functional roles. Functionally, cortical bone fulfills both mechanical and protective roles, while the primary function of trabecular bone is metabolic mineral exchange (Goodman 1998; Baron 1999).

Bone Physiology

The skeletal system's ability to balance structural and metabolic responsibilities is made possible by the complex and tightly regulated processes of bone formation and bone resorption. Bone, blood, skin, and intestinal mucosa are tissues that regenerate continuously and have the ability to accelerate regeneration after trauma (Raisz and Rodan 1990). Bone is under a constant state of flux with remodeling occurring along bone surfaces in focal and discrete packets known as basic multicellular units. Remodeling within these packets is separated from other packets geographically and chronologically. Completion of the remodeling process in each packet takes about 3-4 months, with the process taking longer in cancellous bone than cortical bone (Mundy 1999).

Initiation of this remodeling process occurs when osteoclasts on the bone surface are activated. This activation may occur as a result of microdamage to the bone, following mechanical stress, or at random. Once activated the osteoclasts create a cavity in the bone surface by the production of proteolytic enzymes and hydrogen ions under the ruffled border of the cell. A proton pump transports hydrogen ions across the ruffled border and lysosomal enzymes are released by the osteoclasts. The hydrogen ions

produce an environment that is optimal for degradation of the bone matrix by these lysosomal enzymes (Mundy 1999). This process of bone resorption lasts about 2 weeks at any one spot on the bone surface. Once this activity is completed, osteoclasts signal osteoblasts to travel to the site of bone resorption. The osteoblasts will be activated to produce Type I collagen, which is called an osteoid and begin to fill in the bone cavity. When the osteoid is about 6 microns thick, it will begin to mineralize, the process of which is partially mediated by subcellular particles known as matrix vesicles enriched in alkaline phosphatase. Once the osteoid has mineralized, the new bone formation process is complete. The density of this newly formed bone will continue to increase for months after the cavity has been filled as the crystals of mineral are packed more closely (Ott 03/01/02).

Regulation of Bone Remodeling

Numerous systemic hormones, as depicted in Fig. 1, including parathyroid hormone (PTH), 1,25-dihydroxy Vitamin D, calcitonin, glucocorticoids, estrogens, and androgens, as well as local factors such as Interleukin 1(IL-1), Interleukin 6 (IL-6), Transforming growth factor (TGF), prostaglandins, Tumor necrosis factor (TNF), lymphotoxin, colony stimulating factors, and gamma interferons play a role in regulation of the complex bone remodeling process. PTH, 1,25-dihydroxy Vitamin D, and calcitonin are involved in the process of homeostasis and assist in regulating serum calcium levels by their actions. These systemic hormones are under negative feedback control and are regulated by concentrations of calcium in extracellular fluid. Other

systemic hormones, such as estrogen and androgen, also influence bone cell function but are not under negative feedback control by extracellular fluid calcium (Mundy 1999).

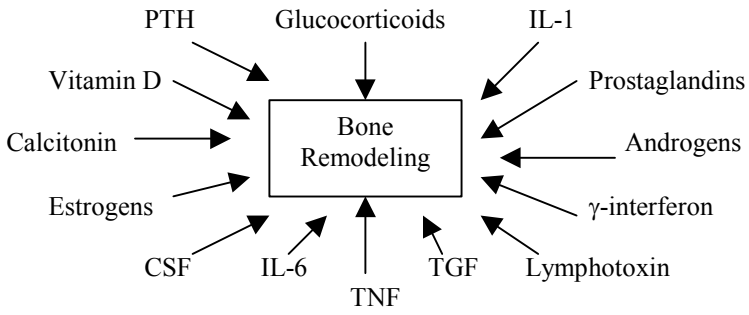


Figure 1 Schematic diagram of select factors that play a role in the bone remodeling process

Role of Systemic Hormones in Regulation of Bone Remodeling

A primary function of PTH is to assist in maintaining serum calcium levels within a narrow physiological range, as demonstrated in Fig. 2. If serum calcium levels tend to drop, the parathyroid gland secretes PTH. Within seconds of being released PTH stimulates bone resorption resulting in release of calcium and phosphate for mineral homeostasis (Rubin and Rubin 1996). Minutes after being released PTH increases renal tubular calcium reabsorption in attempts to elevate serum calcium levels back to normal. A subsequent decrease in serum phosphate concentration occurs as well due to effects of PTH on the kidneys (Juppner and others 1999). If serum PTH levels are low, the kidneys hydroxylate 25-hydroxy-cholecalciferol at either the 23 or 24 position resulting in production of 23,25 $(OH)_2$ Vitamin D or 24,25 $(OH)_2$ Vitamin D, which are both inactive forms. If PTH blood levels increase, renal 1α-hydroxylase is activated to hydroxylate

25-hydroxy-cholecalciferol to create the active 1,25 $(OH)_2$ Vitamin D compound. By this action PTH indirectly regulates the intestinal absorption of calcium. When active Vitamin D is circulating, any calcium in the presence of the gastrointestinal tract is quickly reabsorbed (Glowacki 1996). By each of the above actions PTH will restore serum calcium to appropriate levels. PTH has been shown to have varying effects on bone resorption and bone formation depending on the mode of administration. If administered continuously, PTH acts to increase bone resorption by stimulating osteoclast activity as described above. However, if given in intermittent, low doses, its stimulatory effects on osteoclasts are less significant, while osteoblast activity remains unchanged, or even increases, resulting in a net increase in bone formation (Mundy 1999).

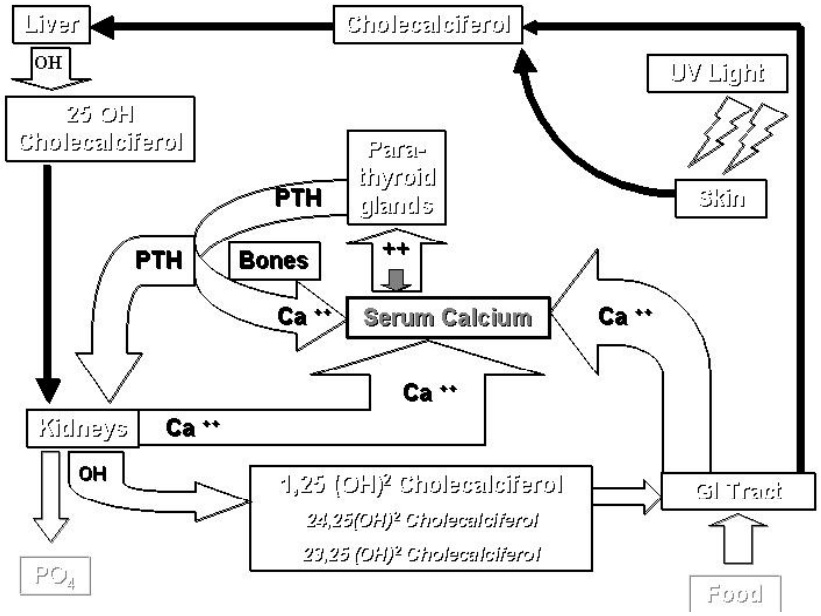


Figure 2 Diagram of the role of PTH to maintain serum calcium levels constant

Vitamin D is another systemic hormone that plays a critical role in bone remodeling and calcium homeostasis. Vitamin D is introduced into the body by diet or through the skin by the action of ultraviolet rays in sunlight. In this form Vitamin D is biologically inert and must undergo successive hydroxylations in both the liver and the kidneys to become the biologically active 1,25-dihydroxy Vitamin D (Holick 1999). The major function of 1,25-dihydroxy Vitamin D is to stimulate renal reabsorption and intestinal absorption of calcium resulting in increased serum calcium concentrations (Rubin and Rubin 1996). Bone cells also possess receptors for this hormone; however, its direct effect on bone remains uncertain. While it acts to promote differentiation of osteoclasts, its effect on osteoblasts is more complex, increasing some osteoblast functions such as osteocalcin and alkaline phosphatase synthesis while decreasing other osteoblast functions such as collagen synthesis (Glowacki 1996). While used in the treatment of bone disorders, it is not clear whether Vitamin D is effective in treating these disorders due to a direct action on bone or by indirect effects via increasing calcium availability (Rodan and Rodan 1995).

Calcitonin is a peptide hormone secreted by the parafollicular cells or "C" cells of the mammalian thyroid gland. Its secretion is regulated by extracellular fluid calcium concentrations so that as serum levels of calcium increase, an increased amount of calcitonin is secreted. Mammalian osteoclasts possess abundant, specific, high-affinity receptors for calcitonin (Nicholson and others 1986). Osteoclasts are the primary, and possibly only, target cells for calcitonin in bone (Goodman 1998).

Calcitonin inhibits bone resorption by inhibiting osteoclast formation and by inhibiting the activity of the mature osteoclast (Mundy 1999). At the cellular level calcitonin causes disappearance of the ruffled border as well as contraction and shrinkage of the osteoclasts (Rodan and Rodan 1995). The kidneys also possess calcitonin receptors, with calcitonin acting to inhibit renal tubular reabsorption of calcium (Glowacki 1996). Osteoclasts have a large number of calcitonin receptors, making calcitonin a powerful inhibitor of osteoclast activity and thus bone resorption. However, the effects of calcitonin on calcium homeostasis are usually short-lived, lasting only 24-48 hours. The physiological role of calcitonin is to regulate the bone resorptive process and thus prevent bone loss, at times of stress on calcium conservation, such as pregnancy, lactation, and growth (Sexton and others 1999).

Glucocorticoids have varying actions depending on the cells involved. The action of glucocorticoids on osteoblasts may be indirect by altering the expression or activity of osteoblastic growth factors, or osteoblasts may be directly affected by activating or repressing osteoblast gene expression (Kessenich and Rosen 1996). Glucocorticoids directly inhibit osteoblast bone formation while osteoclast bone resorption is stimulated indirectly. This indirect effect of increased bone resorption is due to the actions of glucocorticoids to inhibit intestinal calcium absorption and increase urinary calcium excretions resulting in increasing PTH levels and concomitant removal of calcium from bone. The deleterious effects of steroids on bone metabolism appear to be both dose- and duration-dependent (Edelson and Kleerekoper 1996). Glucocorticoids also inhibit the secretion of gonadotropins, estrogen, and testosterone. The absence to these hormones results in further acceleration of glucocorticoid-induced bone loss (Lukert 1999).

The sex hormones, estrogen and androgen, also play a critical regulatory role in bone cell metabolism. These hormones can affect the skeleton both directly and indirectly. Osteoblasts have estrogen receptors, and elevated estrogen levels lead to an increase in osteoblast production (Rubin and Rubin 1996). Estrogen upregulates insulin-like growth factor production and TGF-β resulting in osteoblast stimulation. It also downregulates IL-6 expression resulting in a decrease in osteoclast generation (Rodan and Rodan 1995). Bone mass is associated with levels of bioavailable estrogen in both men and women. Estrogen receptors are found in both males and females, and an increase in osteoblast-like cell number is induced in both sexes by estrogen. Removal of estrogen causes an increase in bone resorption followed by incomplete restitution of the removed bone tissue (Lanyon and others 2004). While estrogen may be important in both men and women, androgen receptors have been found in male osteoblasts and affect these cells in a manner similar to estrogen (Rubin and Rubin 1996).

Aromatase is a critical enzyme involved in the conversion of androgens into estrogen. Inhibition of the aromatization of androgens into estrogen results in increased bone resorption and bone loss. This bone loss attributed to aromatase inhibition is similar to that observed after complete removal of androgens (Vanerschueren and others 1996). Selective aromatase inhibitors can suppress estrogen in men. Also, dual sites of negative feedback for estrogen have been identified at the hypothalamus and the pituitary in human males (Hayes and others 2000). Estrogen is critical to bone metabolism. In fact, study of a man with genetic mutation of the P-450 aromatase gene showed that estrogen plays a crucial role in skeletal maturation. In this case estrogen therapy was more

effective than androgen therapy for skeletal growth and bone maturation (Carani and others 1997).

Role of Local Factors in Regulation of Bone Remodeling

Local factors also play an important role in the regulation of bone homeostasis, as depicted in Fig. 3. Lymphotoxin, TNF, IL-1, and IL-6 are potent stimulators of osteoclast bone resorption (Mundy 1999). TGF along with other growth factors are powerful stimulators of bone formation (Rodan and Rodan 1995; Mundy 1999) and are thus critical for bone formation, remodeling, and repair (Rubin and Rubin 1996). Gamma interferon and IL-4 act to inhibit osteoclast bone resorption (Mundy 1999). Prostaglandins can stimulate both bone formation and resorption (Glowacki 1996; Rubin and Rubin 1996).

Figure 3 Schematic diagram of stimulatory (+) and inhibitory (-) factors that influence osteoclasts (OC) and osteoblasts (OB)

TNF and lymphotoxin are related multifunctional cytokines that stimulate osteoclastic bone resorption. Both stimulate formation of new osteoclasts from precursors as well as indirectly activate mature osteoclasts (Mundy 1999). IL-1 is a powerful and potent stimulator of osteoclastic bone resorption. The effects of IL-1 on

osteoclastic bone resorption occur at multiple sites in the osteoclast lineage. It stimulates osteoclast formation, influences differentiation of osteoclast progenitor cells, and may also activate mature osteoclast cells. IL-1, when administered for short periods of time, stimulates osteoblast cells as well. However, prolonged exposure probably causes inhibition of new bone formation by inhibiting osteoblast differentiation (Raisz and Rodan 1990). IL-6 enhances bone resorption by increasing formation of new osteoclasts from precursors (Mundy 1999).

Growth factors are polypeptides that have multiple effects on their target cells. However, their precise role in bone repair and remodeling remains uncertain. Growth hormone is produced in the pituitary gland and is essential for skeletal longitudinal growth (Rodan and Rodan 1995). Deficiency of growth hormone in children results in impaired skeletal growth, but its role in maintenance of skeletal mass in adults is not well established (Raisz and Rodan 1990). TGF-β is one of the most abundant of the known bone growth regulatory factors. Bone is the major storage site for this growth regulatory factor that is a powerful stimulator of bone formation. TGF-β causes a significant increase in new bone formation, although its stimulatory effects on bone formation are most pronounced when exposure to TGF-β is transient (Mundy 1999).

Gamma interferon is a powerful inhibitor of osteoclastic bone resorption. It acts to inhibit formation and differentiation of osteoclasts from their precursor cells. However, use as an inhibitor in vivo has been limited due to its toxicity. IL-4 also inhibits bone resorption by inhibiting formation of osteoclasts from their precursors (Mundy 1999).

Prostaglandins have multiple effects of bone cells. Studies have shown Prostaglandin E_2 (PGE_2) plays a role in a variety of aspects related to bone metabolism. It has been implicated to react as a modulator in response to mechanical stimuli, fracture repair, inflammatory responses, and possibly in normal remodeling (Rodan and Rodan 1995). Other researchers have assessed that the nature of the effect of prostaglandin may depend on its location. PGE_2 produced in the bone marrow has its greatest effect on osteoclast precursors resulting in increased bone resorption. In contrast, when produced in the periosteum PGE_2 acts to stimulate bone formation due to a greater effect on preosteoblasts (Raisz and Rodan 1990).

Chronological Changes in Bone

Bone is dynamic tissue, being continually broken down and reformed in discrete packets throughout the skeleton by the coordinated actions of osteoblasts and osteoclasts (Mundy 1999). During early skeletal development and throughout adolescence the net activity of osteoblasts is greater than that of osteoclasts allowing for skeletal growth and maturity (Puri 08/13/04). Once peak bone mass is reached in adulthood, the rate of overall activity of bone formation is equal to bone resorption. At this stage of development the processes of bone formation and bone resorption are homeostatically linked resulting in rates of formation and resorption changing in the same direction and bone mass remaining constant. If both processes are accelerated, there is a high remodeling rate, and if both processes are decelerated, there is a low remodeling rate (Greenfield 1986).

This homeostatic linkage is a result of bone resorption, in the process of bone remodeling, being precisely coupled with bone formation, as depicted in Fig. 4. The sequence of events in remodeling typically is initiated when osteoclasts are activated resulting in bone resorption followed by osteoblast activation resulting in bone formation.

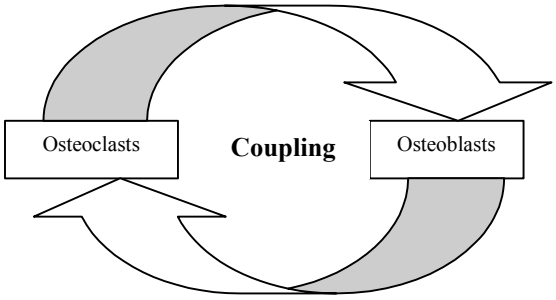


Figure 4 Schematic diagram of the coupling mechanism linking osteoclasts and osteoblasts

Although osteoclasts appear to initiate the process of bone remodeling, they are substantially influenced by the activity of osteoblasts (Lanyon and others 2004). Osteoblasts secrete a variety of osteotropic factors including autocrine and paracrine growth factors during the process of osteogenesis. These osteotropic factors are trapped and stored in the bone matrix and subsequently released by osteoclasts as they resorb the bone matrix. Once these factors are released, they promote differentiation of osteoblasts and activate quiescent osteoblasts (Goodman 1998). Although the precise chemical composition of the agents responsible for coupling is unknown, osteotropic factors that may be involved in the coupling phenomenon of bone remodeling include TGF-β, BMPs, IGF-I and II, platelet-derived growth factor, and heparin-binding fibroblast growth factors (Greenfield 1986; Mundy 1999).

As shown in Fig. 5, at about age 35 bone mass begins to gradually decrease as a result of the rate of bone resorption exceeding the rate of bone formation. This decrease in bone mass is gradual prior to menopause and is accelerated in the postmenopausal state due to estrogen deficiency (Greenfield 1986).

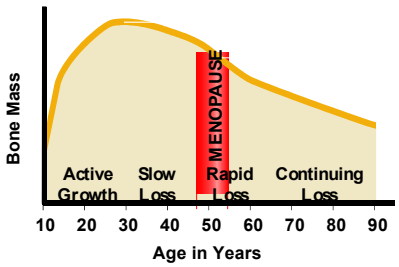


Figure 5 Graph depicting age-associated changes in bone mass

An age-related increase in PTH levels, along with osteoblast senescence, contributes to the slow phase of bone loss seen with aging in both men and women. In some postmenopausal women there is a rapid phase of bone loss lasting about 5 years due to estrogen deficiency. These patients are referred to as "fast losers" and experience up to 5% bone loss per year for about 5 years followed by continued loss of about 1-2% per year thereafter. Other postmenopausal patients undergo a continued, gradual loss of bone at a rate of about 1-2% per year. The major effect of estrogen deficiency is to increase the rate of initiation of new bone remodeling cycles, also known as activation frequency. This increase in activation frequency results in magnification of the remodeling imbalance already produced by the normal aging process (Eastell 1999).

Disorders of Bone Remodeling

During the process of remodeling, if the amount of bone resorbed equals the amount of bone formed, bone mass remains at a stable level. This balance is maintained primarily by the coupling effect between osteoclasts and osteoblasts. As shown in Fig. 6, if the cycle of the remodeling process is uncoupled, because of enhanced recruitment of osteoclasts or impairment of osteoblastic activity, bone resorption exceeds bone formation (Kessenich and Rosen 1996). A variety of disorders including Paget's disease, osteopenia, osteomalacia, osteopetrosis, and osteoporosis can result from such disorders of bone homeostasis.

Paget's disease is an interesting focal disorder affecting predominantly weight-bearing bones and the skull. It is characterized initially by focal excessive bone resorption followed by rapid and excessive bone formation and disordered bone remodeling. The deformity is the result of mechanical stress. The tibia, for instance, bends anteriorly while the femur bends in the lateral direction because the abductor muscles are weaker in comparison to the other muscle groups surrounding the femur. When the skull is affected, bony enlargement may trap the nerves as they cruise through bony foramina. Particularly susceptible are cranial nerves II, V, VII, and VIII (Hamdy 1981). Rates of remodeling can be increased by as much as 100-fold with imperfect coupling between bone resorption and bone formation. This results in focal bony enlargement. The bones, however, are mechanically weak and may fracture readily. Overt fractures are often preceded by stress fractures. The newly formed bone in Paget's disease is disorganized and often described as mosaic in orientation. It has an irregular pattern of immature woven collagen matrix (Singer and Krane 1990; Mundy 1999).

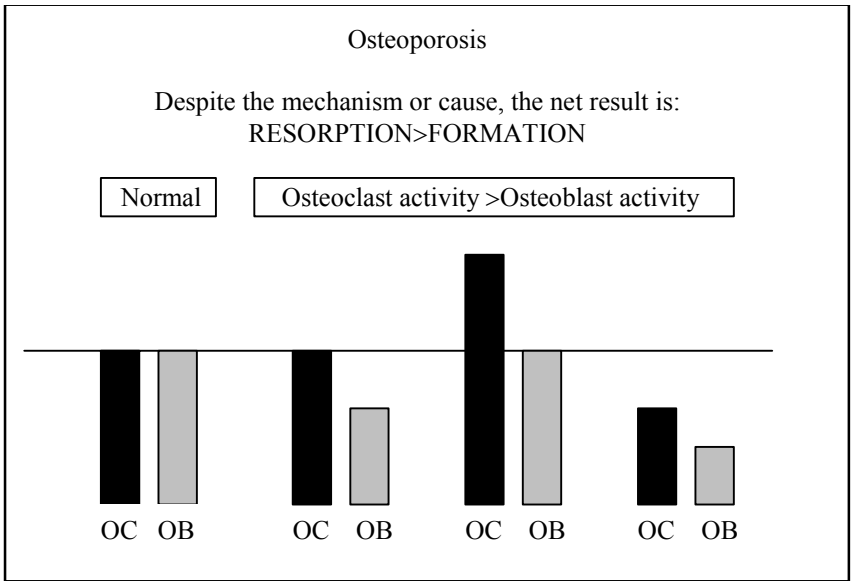


Figure 6 Schematic description of the balance between osteoclast (OC) activity and osteoblast (OB) activity in normal vs. osteoporotic states. When levels of active osteoclasts and osteoblasts are equal, bone remodeling remains homeostatic. However, if osteoclast activity exceeds osteoblast activity, development of osteoporosis occurs.

Osteopenia is a term that denotes a decrease in bone formation relative to bone resorption. Clinically bone densitometry using dual energy x-ray absorptiometry (DXA) is used to measure bone mass and assess effects of such an imbalance between bone formation and bone resorption. T-scores are used to designate the standard deviation below the bone density of a "healthy" young adult of the same sex, body build, and often ethnicity of the tested subject. Similarly, Z-scores are used to designate the standard deviations the tested subject would be below the bone density of an average human of the same age, sex, body mass index, and often ethnic group (Messinger-Rappaport and Thacker 2002). The World Health Organization (WHO) has defined a normal measurement to be a T-score less than 1 standard deviation from a "normal" young adult mean value. Osteopenia is defined as a T-score between 1 and 2.5 standard deviations below a "normal" young adult mean value. Those with a T-score at or below 2.5

standard deviations less than a "normal" young adult mean value are considered osteoporotic (Kanis and others 1997). The measurements referred to in the WHO definition are obtained by DXA of the hips and spine and cannot automatically be applied to other bone sites or other measurement techniques (WHO 1994). Histologically, osteoporosis is characterized by a decrease in number and density of the trabeculae in relation to the total area of the histological section, while the ratio of mineral to organic matrix approximates that of normal bone, and architectural deterioration of the bone tissue is noted. In contrast, osteomalacia results in a low ratio of mineral to organic matrix, while total bone mass may be normal, decreased, or even increased (Avioli and Lindsay 1990).

Osteoporosis

Description of Osteoporosis

The disorder of bone homeostasis known as osteoporosis can occur as a result of calcium deficiency, immobilization, or estrogen deficiency (Hartke 1998). Osteoporosis is defined as 'a progressive systematic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture' (Consensus Development Conference 1993). There is a significant decrease in cortical thickness as well as a decrease in the number and size of the trabeculae with osteoporosis; however, there is no defect in mineralization or abnormality in the organic matrix structure noted (Lavine 1998). In humans osteoporosis occurs most frequently in postmenopausal women due to estrogen

deficiency. Peak bone mass is attained between the ages of 18 & 35 years. Once peak bone mass has been reached, women lose 0.25-1% of bone per year prior to menopause and 2-5% per year after menopause while men lose 0.25-1% per year. Therefore, men will experience a skeletal bone loss of 20-30% over their lifetime, while postmenopausal women suffer approximately a 15% loss every 10 years. The greater surface:volume ratio of cancellous bone causes it to be more susceptible to the deleterious effects of bone remodeling. Therefore, cancellous bone mass is lost much earlier than cortical bone mass (Edelson and Kleerekoper 1996).

In 1947 Albright described two types of osteoporosis; one related to postmenopausal gonadal deficiency and a second type related to a senile decrement of adrenal androgen production (Albright 1947). Riggs and others also described two distinct types of osteoporosis that they prefer to refer to as Type I and Type II osteoporosis. They describe Type I osteoporosis as "high turnover", which occurs in postmenopausal women who are 51-62 years of age, and is due to estrogen deficiency. In this subset of osteoporotic women, bone resorption and bone formation are both occurring rapidly in a coupled fashion; however, there is a greater increase in bone resorption as compared to bone formation. The greatest loss in bone with Type I osteoporosis is seen in cancellous or trabecular bone, and it is characterized by increased incidence of vertebral and wrist fractures (Riggs and Melton 1983).

Type II osteoporosis is referred to as senile-type osteoporosis and is due to age-related bone changes including impaired bone formation and secondary hyperparathyroidism. Bone loss with Type II osteoporosis is due to a relative deficiency of bone formation while the rate of bone resorption remains normal. While bone loss

with Type II or senile-type osteoporosis is relatively universal, occurring in both cortical and cancellous bone, Riggs and others, found it to be characterized by increased frequency of hip fractures along with fractures of the proximal humerus, proximal tibia, and the pelvis (Riggs and Melton 1983).

Factors Affecting Osteoporosis

Several risk factors associated with development of osteoporosis have been identified and can be categorized as either non-modifiable or modifiable, as indicated in Fig. 7. The modifiable risk factors can be further categorized as lifestyle risks, diseases, and medications that contribute to the development of osteoporosis. The ability of these clinical risk factors to predict fractures due to osteoporosis is good (Cummings and others 1995; Klotzbuecher and others 2000).

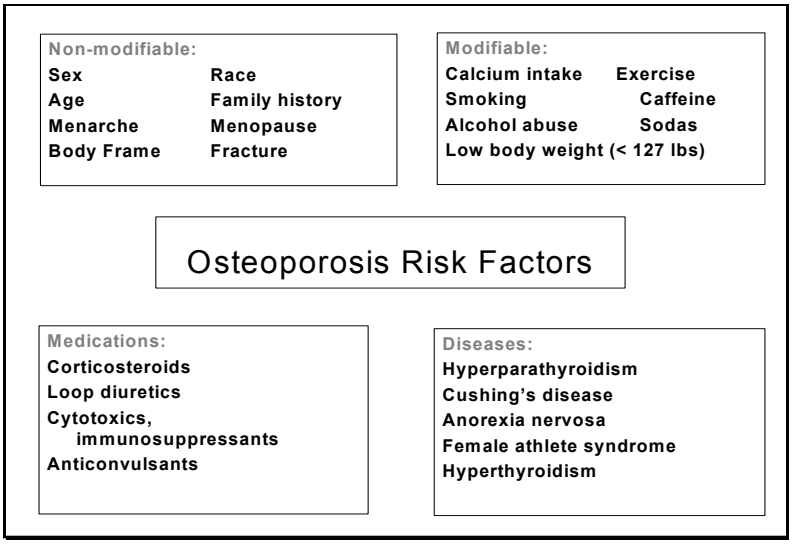


Figure 7 Select common risk factors for osteoporosis

Non-modifiable Factors Affecting Osteoporosis. Several non-modifiable primary factors, including gender, advancing age, Caucasian or Asian race, early or premature menopause, thin/petite body size, low body mass index (height/weight), and family history of a first degree relative with osteoporosis have been associated with an increased risk of osteoporosis. The physical characteristics of the subject's bone, including density (mass), size, geometry, microarchitecture, and composition also impact the relative risk for development of osteoporosis (Wasnich 1999). The ages at which females begin menstruation and menopause are also factors that influence the development of osteoporosis. The shorter the period of time between menarche and menopause, the higher the subject's risk for development of osteoporosis (Kenny and Raisz 2002).

Modifiable Factors Affecting Osteoporosis. Modifiable factors that contribute to development of osteoporosis include calcium deficiency, cigarette smoking, excessive alcohol consumption, and lack or decrease in physical activity (Edelson and Kleerekoper 1996). The relative contribution of these individual risk factors is greatly influenced by the age at which they are expressed. Environmental factors affecting the development or progression of osteoporosis, such as diet and exercise, have been of interest to many researchers. Deficiencies of dietary calcium and Vitamin D are associated with an increased risk of bone loss premenopausally and an increased risk of developing osteoporosis postmenopausally (Edelson and Kleerekoper 1996). Calcium rich dairy products and soy, a rich source of phytoestrogens, have been studied for possible beneficial effects in persons with osteoporosis. However, Muhlbauer and others found a mixture of vegetables to be more effective for inhibition of bone resorption. Rats that

ingested a mixture of vegetables showed less bone resorption than did those ingesting soybeans or even milk powder. They also demonstrated onion to be especially beneficial in inhibiting bone resorption and, therefore, increasing bone mass. The mechanisms responsible for these benefits were not identified by the authors (Muhlbauer 1999).

Several diseases and use of certain medications are correlated with increased risk of developing osteoporosis. Deficiency of endogenous hormones (estrogen or androgen) and chronic diseases such as gastrectomy, cirrhosis, hyperthyroidism, hypercortisolism, and rheumatoid arthritis are considered risk factors for osteoporosis (Wasnich 1999). Other disorders associated with osteoporosis include stroke, hyperparathyroidism, and osteoarthrosis (Johansson and others 2004). A study of patients with alcoholic liver disease revealed a decrease in osteoblast activity in persons consuming 100 grams of ethanol or more per day versus a control group. Their findings showed that ingestion of ethanol causes osteoblastic dysfunction resulting in diminished bone formation and reduced bone mineralization and may, therefore, play a role in the development and/or progression of osteoporosis in alcoholic patients (Diamond and others 1989). Calcium poor diets and long term use of anti-epileptics or corticosteroids are also associated with an increased risk of osteoporosis (Wasnich 1999).

Histological Analysis of Bone Turnover

After processing and sectioning of bone specimens, various cells can be identified by use of histochemical staining. The number of osteoclasts present can be counted in a bone section by identifying the red cytoplasmic staining of cells expressing TRAP. The number of osteoblasts in a processed bone section can be estimated by counting the

number of cells stained with Von Kossa (Amling and others 1999). Histomorphometry can also give insight into the cellular activity associated with bone remodeling. The Osteomeasure histomorphometry system (Osteometrics, Inc., Atlanta, GA) analyzes bone turnover (%), bone volume (%), osteoid volume (%), osteoid surface (%), osteoid width (microns), trabecular width (microns), trabecular number (per mm), trabecular spacing (microns), osteoblast surface per bone surface (%), osteoblast number per bone perimeter (per mm), osteoclast surface per bone surface (%), osteoclasts number per bone perimeter (per mm), and growth plate thickness (microns) in undecalcified specimens embedded in methyl methacrylate (Parfitt and others 1987). Scanning electron microscopy is also beneficial to observe gross histological differences between specimens.

Analysis of Bone Quality

Bone mass and biomechanical strength are gross measurements of changes in bone quality and may provide data to predict risk of fractures. Current means available for measurement of bone mass and strength include geometric measurements of bone length and/or width, bone ash measurements, and three-point bending analysis. Anatomical length of the femur can be measured with a micrometer. To measure bone ash bones are ashed in a muffled furnace, the bone ash weighed, and the weight/length (mg/mm) calculated (Yamamoto and others, 1998). Bone can also be secured into a cantilever bending test device and loaded at a constant rate until failure of the bone is achieved. Bending force to failure and maximum displacement can then be determined by displacement curves (Amling and others 1999; Ducy and others 2000).

Each of these measurement techniques is useful and commonly used to assess bone quality in experimental animals. Other measurement techniques, including biochemical markers and bone densitometry, are also used in studies involving experimental animals as well as studies involving human subjects. Bone biochemical markers help to assess the balance between bone resorption and bone formation. They are not valuable for prediction of bone mass for a given subject. The ability of clinical risk factors to predict bone mass is also poor and risk factor assessment cannot replace measurement of bone density for diagnosis of osteoporosis (Ribot and others 1995; Watts and others 2001). DXA and quantitative computed tomography (qCT) are two methods currently available for generation of data measuring bone density.

DXA is a convenient way to monitor bone mass changes. Accuracy is a reflection of how well a measurement technique predicts the true value. The true value of bone mineral is ash weight, and DXA is able to predict ash weight with >95% accuracy (Genant and others 1996). Precision is a reflection of reproducibility of results. In experienced hands, precision of DXA can be as high as 97%. Knowledge of the precision error is essential for determining if a follow-up measurement shows evidence of a statistically significant change (Blake and Fogelman 2001). Direct measurement of bone mineral density (BMD) by DXA is, therefore, extremely effective for diagnosis of bone disorders and assessment of bone mass (Khosla and Kleerekoper 1999).

Due to its accuracy and cost effectiveness DXA is the most widely used method for bone density measurement (Gertner 1999). DXA can be used to determine whole-body composition (lean body mass, fat, and mineral) as well as the bone mineral content (BMC) of multiple skeletal sites. Analysis of absorption at two different energies and

determination of the ratio of attenuation of the high and low energies allows for calculation of the amount of bone mineral, soft tissue, and fat without need of a constant body-part thickness (Shore and Poznanski 1999).

Use of bone mineral measurements to estimate fracture risk is comparable to use of blood pressure readings to assess risk of stroke or serum cholesterol levels to predict coronary artery disease. Just as a patient is diagnosed as hypertensive if their blood pressure exceeds a cut-off level, so the diagnosis of osteoporosis is based on a bone mineral value that is below a cut-off threshold. Although these thresholds are unable to discriminate absolutely who will suffer a stroke or fracture, they are useful for prediction of risk of these incidents occurring (Kanis and others 1997).

In assessing response to treatment, research studies have typically used densitometry scans of the spine and hips because of the clinical importance of fractures at both of these sites, while some studies have also included total-body BMD to show effects of treatment across the whole skeleton (Blake and Fogelman 2001). Due to the high correlation between changes in BMD and fracture reduction in clinical trials of antiresorptive agents, BMD is widely used to monitor response to therapy (Altkorn and Vokes 2001). Response between skeletal sites varies with antiresorptive therapy with greater changes in bone densitometry measurements being noted at the lumbar spine than the femoral neck or forearm. This difference in response between skeletal sites is partly explained by the greater effect of antiosteoporotic agents on trabecular bone, which has a higher turnover rate, than on cortical bone, which has a lower turnover rate (Kanis and others 1997).

Clinically benefits of DXA include low radiation dose, usefulness in evaluating multiple sites, and ease of use (Kenny and Raisz 2002). One of the major advantages of DXA is its ability to measure skeletal density at sites other than the extremities. The lumbar spine and proximal femur are the most common sites of osteoporotic fractures; therefore, measurement of bone density in these areas is especially beneficial (Shore and Poznanski 1999). DXA scans are beneficial for identifying patients at high risk of fragility fracture as well as providing information necessary for diagnosis of osteoporosis (Blake and Fogelman 2001). BMD is measured by DXA and fracture risk is increased when BMD is decreased (Cefalu 2004). DXA scans are beneficial for monitoring the response of patients undergoing treatment in longitudinal studies (Blake and Fogelman 2001). Finally, DXA is able to measure overall changes in bone mineral status by its ability to assess both the cortical and trabecular components of bone. Due to the differences in bone mineralization of cortical and trabecular bone examination of both types of bone is necessary for adequate evaluation of overall bone mineral status (Shore and Poznanski 1999).

DXA has been shown to provide precise, accurate, and valid measurements of bone mineral by a variety of researchers. The PIXImus Mouse Densitometer is a device that allows for accurate and precise measurement of Bone Mineral Content (BMC) and Bone Mineral Density (BMD) for small animals or bone pieces weighing less than 50 grams. As well as total body analysis, specific regional analysis can be separated out for the spine or femur (Coleman and others 1999; Donahue and others 1999; Nagy and others 1999; Samuels and others 1999). Adaptive computer software is also available for

analysis of bone densitometry scans on small animals when performed on other densitometers.

QCT uses computed tomography (CT) scanning to measure bone mineral. A phantom with known concentrations of hydroxyapatite is scanned simultaneous to the subject, and attenuation numbers for specific regions of interest from the CT scan of the subject is compared to this control. The region of interest of bone mineral measurement with qCT is a well-defined volume and can allow for measurement of trabecular bone without including measurement of cortical bone. QCT measures bone mineral as a true volume density (g/cm^3) and is, therefore, less influenced by the size of the bone (Shore and Poznanski 1999). While qCT gives a true bone density measurement (mass/unit volume), it is more costly than DXA and fewer normative data are available for reference (Gertner 1999).

Measurement of Urinary Calcium Excretion

Dietary calcium intake varies widely among individuals. However, the average diet should include a recommended daily intake of 1000-1200 mg/day of calcium. Only about 1/3 of the calcium ingested is absorbed from the gastrointestinal tract, with the remainder being excreted in the feces. The calcium absorbed by the gastrointestinal tract is eventually eliminated primarily via urine. However, only about 1% of the calcium filtered through the kidneys is actually excreted. The remaining 99% is reabsorbed in the proximal and distal tubules as well as the loop of Henle. This reabsorbed calcium is then returned to the plasma. Regulation of urinary calcium excretion occurs primarily at the distal tubule. PTH is an important regulator of plasma calcium concentration. When

serum calcium levels are too low, PTH stimulates calcium reabsorption in the distal tubules. By stimulating calcium reabsorption PTH lowers urinary calcium concentration (Rhoades and Tanner 1995).

The concentration of calcium in the urine will vary as demands for calcium reabsorption fluctuates during the bone remodeling process. Levels of calcium excreted in the urine can be measured by capillary ion analysis (CIA). CIA is 'an adaptation of capillary ion electrophoresis to a particular electrophoretic analysis of highly mobile, low molecular weight ions.' Improvements in CIA have made it a desirable technique for analysis of minute ion concentrations in biological specimens (Creson and others 1998; Ferslew and others 1998). When performing CIA, an aqueous solution of electrolytes is drawn by hydrostatic sampling into a capillary and voltage is applied resulting in migration of ions toward the oppositely charged electrodes via electroosmotic flow. The mobility or migration of ions in this electrolyte solution is directly proportional to the ion's conductance and is, therefore, related to the ratio of the ion's net charge to ionic size in solution. Due to differences in ionic mobility, ions will be separated as they migrate through the capillary. Preestablished concentrations of an ion with known migration time are added to the solution for use as controls. Ions are identified by their relative migration time and interpolation of the relative peak size for each ion is used for quantitation (Ferslew and Hagardorn 2000).

Measurement of Bone Turnover

Biochemical Analyses of Bone Resorption

Biochemical analysis of bone resorption is possible through various serum and urinary markers. Osteoclasts contain an acid phosphatase, which is resistant to inhibition by tartrate and is released into the circulation. This tartrate resistant acid phosphatase (TRAP), a reflection of bone resorption, can be measured by ELISA in serum samples (Hughes and others 1995; Hughes and others 1996). However, TRAP found in serum is not stable in frozen samples and is not entirely specific for the osteoclast. These limitations have hindered the effectiveness of TRAP as a clinical determinant for assessment of bone resorption. However, recent advancements in development of immunoassays utilizing monoclonal antibodies specific for the bone isoenzyme of TRAP may assist in improving its clinical utility (Khosla and Kleerekoper 1999).

Pyridinoline and deoxypyridinoline are the two major types of amino acids found in the stabilizing crosslinks of collagen fibers in bone. When bone matrix is degraded by osteoclasts, pyridinoline and deoxypyridinoline are released from mature collagen into the circulation and excreted into the urine. The ratio or proportion of free:total crosslinks appears to be constant in healthy individuals as well as those with metabolic bone disorders and arthritis (Gomez and others 1996). Improvements in immunoassay sensitivity have allowed for measurement of free pyridinoline levels in serum and, therefore, provide a method of analyzing collagen degradation in bone and cartilage (Urena and others 1995; Visor and others 1996). A peptide derived from the helical

region of the α1 chain containing residues 620-633 has been isolated from the urine of a patient with Paget's disease. A synthetic version of this peptide has been utilized as an immunogen, allowing for production of monoclonal antibodies. A competitive immunoassay has been developed to measure this helical peptide and is a good marker of bone resorption and a sensitive indication of efficacy of antiresorptive effects of a bisphosphonate (Kamel and others 1995; Ju and others 1997).

Biochemical Analyses of Bone Formation

Osteocalcin is a 50 amino acid peptide and has been identified as the major noncollagenous protein found in bone. It contains three gamma carboxyglutamic acid (GLA) residues and is sometimes referred to as bone gla-protein or BGP. These GLA residues enable osteocalcin to bind with strong affinity to hydroxyapatite and calcium. Osteocalcin is primarily found in osteoblasts and, after synthesis, is partly incorporated into the bone matrix and partly released into the circulatory system. In a study by Ducy and others (1996) using osteocalcin-deficient mice, it was determined that the absence of osteocalcin leads to an increase in bone formation without impairing bone resorption. Their findings support the evidence that measurement of circulating osteocalcin is a determinant and specific marker of bone formation. Although widely accepted as a marker for bone formation one should keep in mind that osteocalcin is incorporated into the bone matrix and released into the circulation during bone resorption. Therefore, at any point in time the serum concentration of osteocalcin contains components of both bone formation and bone resorption (Khosla and Kleerekoper 1999).

Limitations of Biochemical Markers of Bone Turnover

Although biochemical markers are commonly used for measurement of bone formation and bone resorption, there are several major limitations for their use in this capacity. Currently available urinary markers for bone resorption are generally normalized to creatinine excretion. Therefore, variability in creatinine measurements contributes to overall variability in measurement of the urinary markers. Artifactual changes in urinary markers may potentially occur based on alterations in muscle mass. The timing of sampling is important to many bone-turnover markers having circadian rhythms. All urinary bone resorption markers have significant circadian patterns; therefore, it is best to obtain a 24-hour urine collection. Finally, biochemical markers of bone turnover are subject to technical variability due to intra and interassay variability as well as biological variability of individual subjects. Changes in markers of bone resorption occur prior to measurable changes in bone formation markers. Despite these limitations, in postmenopausal women biochemical markers of bone turnover correlate with histomorphometric indices of bone turnover, rates of bone loss, and fracture risk. Currently these markers are used as an adjunct to Dual-energy densitometry (DXA) for estimation of fracture risk, to assist with the evaluation of unexplained osteoporosis, and to monitor the response to therapy. (Khosla and Kleerekoper 1999).

Tetracycline Double Labeling

While a variety of techniques are available for measurement of bone formation and bone resorption, double labeling of bone with fluorochrome labels is a useful technique for assessment of dynamic bone growth. Tetracycline HCl and calcein green

are fluorochromes commonly used for double-fluorochrome labeling. When administered intramuscularly or subcutaneously these labels bind to newly formed bone at the bone/osteoid interface. If these labels are administered at interval time periods, bone biopsies can be used to assess dynamic bone growth. Biopsies are processed and thinly sliced sections analyzed under fluorescent microscopy. In the presence of fluorescent light each marker will appear as a fluorescent line. Measurement of the distance between the two lines will indicate the amount of bone formation that has occurred during the time elapsed between drug administrations (Weinstein and others 1998; Mundy and others 1999; Recker 1999). Tetracyclines are advantageous in that they are less toxic than other fluorochromes and can be safely administered for human analyses (Jowsey 1977).

Treatment Options for Osteoporosis

Prevention of Osteoporosis

While much research has been focused on measurement of bone turnover to improve diagnostic accuracy of osteoporosis, establishing the diagnosis needs to be accompanied by investigation of effective methods of preventing and treating the disease. Many of the previously discussed diagnostic tools are also used to monitor progression of osteoporosis and/or the effectiveness of various treatment techniques. One must consider prevention to be of primary importance in the treatment of osteoporosis. Prevention of osteoporosis must include measures to ensure patients achieve the maximum level of

their genetically determined peak bone mass. Once peak bone mass has been reached, preventive measures must be taken to restore bone loss that will occur throughout the remainder of the patient's life span. Because women reach their maximum BMD in their mid-thirties, it is of vital importance that women maintain appropriate dietary intake of calcium throughout their lives. It is recommended that females ingest 1000-1200 mg/day of calcium either through diet or with additional supplements, such as calcium citrate or calcium carbonate, if needed. Total calcium intake in postmenopausal women should be increased to 1500 mg/day, if not taking estrogen (Rhoades and Tanner 1995).

Avoidance of excessive intake of alcohol or caffeine and cessation of smoking has been shown to have a favorable effect in prevention of osteoporosis (Andrews 1998). Diamond and others (1989) found consumption of ethanol was associated with osteoblast dysfunction resulting in decreased mineralization and decreased bone formation rates. They propose that avoidance of intake of alcohol may result in an improvement in osteoblast function and numbers. Cigarette smoking directly impairs the bone-cell repair process by inhibiting the proliferation of osteoprogenitor cells and differentiation of osteoprogenitor cells toward osteoblast-like cells. An increase in concentration of free radicals, which may be involved in bone resorption, is also associated with cigarette smoking (Notelovitz 2002). Caffeine consumption and smoking are considered secondary causes of generalized osteoporosis. Avoidance of these activities would, therefore, lessen a subject's likelihood of developing osteoporosis (Kanis and others 1997; Jergas and Genant 1999).

Effectiveness of Exercise in the Treatment of Osteoporosis

Lifestyle changes and behavior modification are also beneficial in preventing osteoporosis. Consistent performance of an appropriate exercise program, including weight bearing exercises, is effective in increasing bone mass. Exercise is a key element in adolescence and early adulthood to increase development of peak bone mineral density. Strenuous exercise throughout life is also suggested to be valuable for improvement of bone mineral density (Marcus 1999).

Although exercise has been shown to be beneficial in preventing osteoporosis, the level and intensity of exercise must be appropriate to prevent development of a clinical problem referred to as female athlete triad syndrome. This is a "serious syndrome consisting of disordered eating, amenorrhea, and osteoporosis" found among a small number of young female elite athletes who follow overly vigorous and rigid training programs (ACSM 2000).

In postmenopausal women, fracture risk is associated with history of falls, low physical function, and decreased strength. Therefore, exercise to improve strength and balance are of great importance for this population (Notelovitz 2002). Load bearing exercises such as walking, weight training, and high impact aerobics are more effective in increasing bone mass than other forms of exercise (Delmas 2002). However, osteocytes are only affected by bone loading in the presence of estrogen (Notelovitz 2002). For greatest benefits, exercise should be performed for 30-60 minutes at least 3 times per week (South-Paul 2001).

Medications for Treatment of Osteoporosis

The Food and Drug Administration has approved a limited number of medications for treatment of osteoporosis. Estrogen, hormone replacement therapy (HRT), has been widely used for both prevention and treatment of osteoporosis. If initiated at the onset of menopause, HRT decreases the risk of osteoporotic fractures and increase vertebral bone mass (Messinger-Rapport and Thacker 2002). However, the reduction in fracture risk is lost after withdrawal of HRT regardless of the duration of treatment (Delmas 2002). Side effects of HRT include an increased risk of deep vein thrombosis and pulmonary embolism as well as a significant increase in risk of breast cancer. These effects make its use less desirable for treatment of osteoporosis (South-Paul 2001).

The largest study of risks and benefits associated with HRT was performed by the Women's Health Initiative (WHI). From 1993 to 1998, the WHI studied the effects of HRT on 161,809 postmenopausal women. Although estrogen plus progestin was found to increase BMD and reduce risk of fracture in healthy postmenopausal women as compared to control placebo, it also led to an increased risk of invasive breast cancer. The WHI determined after 5.2 years follow-up that the overall health risks exceeded benefits from use of estrogen plus progestin for postmenopausal women and subsequently discontinued this component of their study (WHI 2002; Cauley and others 2003).

To assess the possible association between postmenopausal estrogen supplementation and lower risk of dementia, the WHI further investigated the effects of conjugated equine estrogens and estrogen plus progestin on dementia and mild cognitive impairment. A randomized, double-blind placebo-controlled study involving 7479

women aged 50-79 years at baseline was conducted from June 1995 to February 2004 in 39 WHI clinical centers. Results of this study showed that estrogen therapy alone did not reduce dementia or the incidence of mild cognitive impairment and increased the risk for both end points combined. When assessing the combined data for estrogen alone and estrogen plus progestin they found an increased risk for both end points. Therefore, they concluded that hormone therapy is not recommended for prevention of dementia or cognitive decline in women 65 years of age or older (Shumaker and others 2004).

Bisphosphonates, including Alendronate, Risedronate, Pamidronate, Ibandronate, and Etidronate, inhibit bone resorption and are effective in treating osteoporosis. A phosphorous-carbon-phosphorous bond characterizes these stable analogues of pyrophosphate. The two phosphoric acids in bisphosphonates cause them to adsorb to the bone surface and the compound is resistant to enzymatic degradation due to the central carbon component. The antiresorptive potency of each bisphosphonate is determined by its side chains (Watts 1999). Bisphosphonates prevent bone resorption by inhibiting osteoclast activity and increasing osteoclast apoptosis (Delmas 2002). Some bisphosphonates inhibit osteoclast activity by limiting the activation of osteoclast precursors, inhibiting precursor cells from differentiating into mature osteoclasts, interfering with chemotaxis, and blocking the attachment of osteoclasts to bone (Watts 1999). Bisphosphonates help to reduce vertebral fracture rates with the greatest benefits occurring in patients with short-term fracture risk. Side effects of Bisphosphonates include gastrointestinal irritation, esophagitis, esophageal ulceritis, and esophageal perforation. These effects can be reduced by taking the medication with a full glass of water after overnight fasting at least 30 minutes prior to ingesting any food or beverage

and maintaining an upright posture for 30-60 minutes after taking the medication (Altkorn and Vokes 2001; Messinger-Rapport and Thacker 2002).

Raloxifene, a selective estrogen-receptor modulator, is also effective for reducing bone loss. Selective estrogen-receptor modulators may act as agonists or antagonists on estrogen depending on the target tissue. Raloxifene is a benzothiaphene that competitively inhibits estrogen action in the breast and uterus while acting as an estrogen agonist on bone and lipid metabolism. Results of the MORE study (Multiple Outcomes of Raloxifene Evaluation), which evaluated 7705 osteoporotic women, indicates a 30-50% reduction in incidence of vertebral fractures. However, side effects of Raloxifene include increased risk of venous thrombosis and pulmonary embolism (Delmas 2002; Messinger-Rapport and Thacker 2002). Raloxifene has not been found to have any beneficial effect on postmenopausal symptoms including hot flashes and may even increase them (Altkorn and Vokes 2001).

Other drugs being studied for effectiveness in treatment of osteoporosis include Calcitriol, a potent form of Vitamin D; PTH; and slow release sodium fluoride. Sodium fluoride and PTH research has been of particular interest because they stimulate bone formation rather than inhibiting bone loss (Kiplinger Washington Editors 1996; Andrews 1998; Mundy 1999). Although not approved by the US FDA, sodium fluoride has been used in other countries for treatment of osteoporosis. Fluoride alters the crystallization sequence of bone leading to formation of hydroxyapatite and replaces hydroxyl groups with a compound that is more resistant to resorption (Watts 1999). One aspect that must be considered with use of sodium fluoride is the ability of the subject to excrete the substance. If creatinine clearance is low, renal excretion may be problematic. Therefore,

patients with renal failure should not be treated with fluoride (Kanis and others 1997). Another disadvantage is that fluoride has a narrow therapeutic window, and administration of toxic doses can lead to production of bone that is histologically abnormal, undermineralized, more dense, but less strong than normal. Therefore, until further studies ensure the safety and efficacy of use, sodium fluoride is not recommended for routine treatment of osteoporosis (Watts 1999).

Calcitonin is a remarkedly safe drug used in slowing osteoporotic bone loss. Produced by the parafollicular ("C") cells of the thyroid gland, calcitonin secretion is dependent on serum calcium levels. As serum calcium levels diminish, the C cells begin to store up calcitonin within their granules. When serum calcium is elevated, these stores are readily discharged. Three classes of calcitonin have been identified: 1) artiodactyl (porcine, bovine, and ovine); 2) human; and 3) teleost (salmon and eel). The teleost calcitonins have been found to be 20 to 50 times more potent than those of other species.

Salmon: H_2N-CYS-***SER***-ASN-LEU-SER-THR-CYS-***VAL***-LEU-GLY-***LYS-LEU-SER***-GLN-***GLU-LEU-HIS***-LYS-***LEU-GLN***-THR-***TYR***-PRO-***ARG***-THR-***ASN-THR***-GLY-***SER***-GLY-***THR***-PRO-NH_2

Human: H_2N-CYS-GLY-ASN-LEU-SER-THR-CYS-MET-LEU-GLY-THR-TYR-THR-GLN-ASP-PHE-ASN-LYS-PHE-HIS-THR-PHE-PRO-GLN-THR-ALA-ILE-GLY-VAL-GLY-ALA-PRO-NH_2

Porcine: H_2N-CYS-***SER***-ASN-LEU-SER-THR-CYS-***VAL***-LEU-***SER-ALA***-TYR-***TRP-ARG-ASN-LEU***-ASN-***ASN***-PHE-HIS-***ARG***-PHE-***SER***-GLY-***MET-GLY-PHE***-GLY-***PRO-GLU-THR***-PRO-NH_2

Figure 8 Amino acid structure of the calcitonins. Adapted from Calcitonin by Deftos and others (Deftos and others 1999). Italicized amino acids in the salmon and porcine classes of calcitonins differ from the amino acids in the human class of calcitonin at the same position.

Calcitonin is quickly degraded by the liver and kidney and has a half-life of only a few minutes in blood. Teleost calcitonins are considered more resistant than mammalian

calcitonin to breakdown by tissue and serum enzymes. The most obvious chemical difference of teleost calcitonin to the other classes is their greater hydrophilicity. Another characteristic that contributes to the higher affinity of teleost calcitonin are the amino acids located in the carboxy-terminal half with critical binding regions found at residues 22-32 (Sexton and others 1999). This higher potency is necessary physiologically for the rapid metabolic changes required for salmon to transition from fresh water to salt water environments. Due to the greater affinity for receptors, salmon calcitonin has a greater biological potency in vivo and is currently the most used class for treatment of osteoporosis (Martin and Moseley 1990).

Calcitonin can be administered intramuscularly, subcutaneously, or intranasally. Calcitonin has been used for nearly 30 years for treatment of bone disorders with limited adverse effects. Nausea and flushing are the most commonly noted side effects with rhinitis and epistaxis occurring occasionally with intranasal administration (The Medical Letter on Drugs and Therapeutics 2000). A variety of clinical uses for calcitonin in humans have been cited including treatment of fracture pain, prevention of fractures, and prevention of bone loss in the early postmenopausal period. The effects of calcitonin in preventing early postmenopausal bone loss in humans have thus far only been assessed for relatively short durations, up to 5 years. Studies suggest that bone density reaches a plateau or may actually decrease after years of calcitonin therapy (Siminoski and Josse 1996).

Inhibition of osteoclastic bone resorption is the main biologic effect of calcitonin. Osteoclasts have receptor sites specific for calcitonin, and a number of direct effects are noted once calcitonin binds to the osteoclast. Deftos reports within minutes of

administering calcitonin, osteoclasts shrink in size, cyclic adenosine phosphate (cAMP) is produced, and there is an increase in cytosolic calcium found in the osteoclast (Deftos and others 1999). However, in support of the above mentioned plateau of the effects of calcitonin, T. J Martin (1999) describes an "escape" phenomenon which has been observed in vitro and in vivo after long-term administration of calcitonin for treatment of patients with excessive bone resorption such as Paget's disease. He defines "escape" as an increase in resorption in bones stimulated by a resorptive agent, despite the continued presence of maximally inhibitory concentrations of calcitonin. He further reports evidence suggesting this "escape" is due to a change in responsiveness to calcitonin as opposed to a decrease in hormone activity.

While the exact biochemical mechanism by which calcitonin induces decreased responsiveness of osteoclasts to calcitonin is not fully known, several theories have been proposed to explain this resistance. The most widely theorized possibility is that long-term administration of calcitonin results in the down-regulation of cell surface receptors on the osteoclasts (Mundy1999). Generation of osteoclasts that are deficient in calcitonin receptor mRNA and protein but yet capable of resorbing bone would lead to a response similar to that described in the "escape" phenomenon (Sexton and others 1999). This theory suggests the resistance to the effects of calcitonin may be due to reduced calcitonin sensitivity of osteoclasts (Martin 1999; Pondel 2000).

A second theory is that after long-term administration calcitonin may begin to influence osteoblast function through cell coupling. Although the main action of calcitonin is to inhibit the activity of osteoclasts, osteoclasts that are quiescent in the presence of calcitonin in vitro regain activity when osteoblasts are added to the culture

(Sexton and others 1999). As calcitonin inhibits the activity of osteoclasts, the osteoclasts are unable to release the osteotropic factors stored in the bone matrix. Because these factors act to promote osteoblast differentiation and to activate osteoblasts by inhibiting osteoclastic release of the factors, calcitonin may indirectly inhibit osteoblastic activity.

Finally, it has also been suggested that a population of calcitonin-resistant osteoclasts emerge following long-term administration of calcitonin. Circulating antibodies to calcitonin develop in about 50% of subjects with long-term use of calcitonin, and it has been postulated that these antibodies may neutralize the effects of calcitonin (Watts 1999). An interesting feature of this theory is that the resistance to the effects of calcitonin in vitro has been prevented by concomitant treatment with glucocorticoids (Martin and Moseley 1990).

Development of calcitonin antibodies can be readily detected by various methods. Immunoassays are a powerful technique available for detection and measurement of antigens and antibodies. In this type of method the antigen (calcitonin) is first bound to a microtiter plate. Next, serum samples proposed to contain the anti-calcitonin antibodies are added to the wells and allowed to bind. A second antibody, specific for anti-calcitonin antibody and labeled for detection, is then added to the well and allowed to bind. The second antibody is enzyme conjugated to catalyze formation of a colored substance which can be quantified to calculate the amount of calcitonin antibody present. While calcitonin antibodies have been detected in >50% of subjects receiving calcitonin for treatment of osteoporosis, the relationship of calcitonin antibody to the escape phenomenon remains unclear. Gruber and others (1994) found no apparent relationship

between the presence of calcitonin antibodies and change in levels of total body calcium. Likewise, Overgaard and others (1990) suggest the formation of calcitonin antibodies does not affect the clinical responsiveness of calcitonin.

As previously mentioned, nasal spray is an effective means of administering appropriate doses of salmon calcitonin for treatment of osteoporosis and has fewer reported side effects than other forms of administration. A dosage of 200 IU/day via nasal spray is an appropriate therapeutic dosage for adults and has been widely used for treatment of osteoporosis in studies with human subjects. In a randomized double blind, placebo-controlled study of postmenopausal women, calcitonin was shown to be effective in increasing bone mineral density. After 2 years of administration 76% of patients receiving 200 IU/day of nasal spray salmon calcitonin demonstrated a 2% to 3.6% increase in bone mineral density while 73% of the placebo group experienced a loss of bone mass. These researchers noted the effects to be more pronounced in women who were more than 5 years postmenopausal (Overgaard and others 1995 as reported in Andrews 1998). Although fewer studies have assessed the effects of calcitonin in men, Trovas and others (2002) found 200 IU/day of salmon calcitonin to be safe and effective in increasing lumbar bone mineral density and in reducing bone turnover in men with idiopathic osteoporosis.

A large, randomized, double-blind study comparing the effectiveness of different doses of salmon calcitonin in the treatment of postmenopausal women is the PROOF study (Prevention Recurrence of Osteoporotic Fractures Study) published in 2000. In this study the researchers observed 1255 postmenopausal osteoporotic women over a period of 5 years. They found use of a therapeutic dosage of 200 IU/day of nasal spray salmon

calcitonin resulted in a significant reduction in the risk of new vertebral fractures. Reduction in vertebral fractures in patients receiving lower doses of 100 IU/day was not significantly different from patients receiving a placebo. However, patients receiving 400 IU/day of nasal spray salmon calcitonin demonstrated a lower reduction in vertebral fractures than either of the groups receiving lower doses (Chesnut and others 2000).

Bone mineral density of the lumbar spine as measured by DXA increased significantly from baseline in all treatment groups as compared to placebo. However, there was no clinically significant effect on BMD at the femoral neck or trochanter with treatment. Biochemical markers of bone turnover, including measurement of serum levels of C-telopeptide, bone-specific alkaline phosphatase, and osteocalcin, showed only a modest decrease with treatment. Despite these disappointing findings for BMD and biochemical markers, the risk of new vertebral fractures was significantly reduced in postmenopausal patients receiving a dose of 200 IU/day of salmon calcitonin nasal spray (Chesnut and others 2000).

An interesting observation from the PROOF study is the apparent dose discrepancy in the action of salmon calcitonin for reduction of new vertebral fractures. While the treatment group receiving 200 IU/day of nasal spray salmon calcitonin showed a significant decrease in the risk of new vertebral fractures as compared to the placebo, the treatment groups receiving 100 IU/day and 400 IU/day did not show a significant difference in reduction of risk of vertebral fracture as compared to placebo. Surprisingly, the treatment group receiving 400 IU/day was less effective in reducing vertebral fracture risk than either of the groups receiving lower doses (Chesnut and others 2000). This

observation has led to the postulation that higher doses of salmon calcitonin may actually lead to oversuppression of bone turnover.

Some researchers have criticized the PROOF study because of the high dropout rate of subjects (60%), the inconsistent data for biochemical markers of bone turnover, and questionable fracture risk data (Colman and others 2002). Other criticisms include that the trial was only partly blinded due to doctors and patients seeing results of bone density testing during the trial, and that data regarding pain or disability were not included (Cummings and Chapurlat 2000). Chesnut refuted these criticisms by noting that high dropout rates are inherent to most long-term studies. He also notes further investigation is needed for the hypothesis that the effect of antiresorptive osteoporotic treatments to reduce fracture may be exhibited in bone quality involving microarchitecture and may not be evident in bone mineral density. He also concludes calcitonin's effect on reducing vertebral fracture rate has been well confirmed, although its mechanism of action needs further definition (Chesnut 2002).

Animal Models for Study of Osteoporosis

In humans osteoporosis occurs most frequently in postmenopausal women as a result of estrogen deficiency. When assessing animal models of bone disease, Geddes documents that the ovariectomized rat is the most commonly used model to investigate the loss of cancellous bone due to estrogen deficiency (Geddes 1996). Numerous authors have documented the use of ovariectomized rats as models to study postmenopausal bone loss and osteopenic changes. While other ovariectomized animals, including ferrets,

mice, canines, pigs, and sheep, have been used to study osteoporosis, the rat has emerged as the most common estrogen deficiency model used (Geddes 1996; Hartke 1998).

Initial studies by Wronski and others (1991) indicate that ovariectomy of rats induces marked bone loss and accelerated skeletal metabolism. Nakamuta and others (1993) found that ovariectomy of rats induced acute and focal osteopenia within 2 weeks. Similar results were found by Wronski and others (1988) in a study that documented osteopenia and increased indices of bone resorption and formation were detected in ovariectomized rats as early as 14 days after surgery. They found that osteopenia progressively increased with time up to 100 days in these animals and then appeared to plateau. Results of a study by Thompson and others (1995) indicated that the ovariectomized rat model mimics postmenopausal cancellous bone loss when examined over relatively short periods of time, which they defined as less than 12 months. Li and others (1996) found that ovariectomy resulted in statistically significant cancellous bone loss in the femoral neck of rats as early as 30 days post ovariectomy; however, cortical osteopenia was not evident until 12 months post ovariectomy. It is evident from the available research that the ovariectomized rat qualifies as a practical and cost-effective animal model for exploring the aspects of pathogenesis and treatment of postmenopausal bone loss.

Rationale and Aims

Osteoporosis is a metabolic bone disorder that currently affects 25-30 million Americans. The major complication of the disease is fragility fractures, with vertebral and hip fractures being most common. Treatment of these fractures results in substantial

financial burden and they are associated with pronounced morbidity and excess mortality (Delmas 2002). Hip fractures are one of the most devastating manifestations of osteoporosis. Within one year of suffering a hip fracture, 5-24% of patients will die, 50% of those who survive will continue to be incapacitated, and 20% of hip fracture survivors will require long-term nursing home care (Chrischilless and others 1991; Consensus Development Conference 1993; Col and others 1997; Ray and others 1997). Spinal fractures are significantly painful and can also lead to permanent deformity and long-term debility. As many as 1.3 million fractures are reported annually in osteoporotic patients with cost for treatment exceeding 15 billion U.S. dollars (Consensus Development Conference 1993).

Calcitonin is one of a limited number of medications approved for use in the treatment of osteoporosis. When therapeutic dosages of calcitonin are given to humans diagnosed with osteoporosis, there is evidence of an initial increase in bone formation, followed by a plateau in which bone formation equals bone resorption, followed by a period in which bone resorption exceeds bone formation (Gruber and others 1984; Deftos and others 1999; Martin 1999; Cummings and Chapurlat 2000; Colman and others 2002). However, if dosages are given that exceed the therapeutic level, the period of plateau and decline are achieved more rapidly and the level of overall improvement is less than that seen in patients receiving therapeutic dosages (Chesnut and others 2000). Even when administered at therapeutic dosages, when given for extended periods of time the beneficial effects of calcitonin on osteoporosis begin to decline and one is unable to continue to prevent bone loss. The most widely accepted explanation for this phenomenon is down-regulation of calcitonin receptors on osteoclasts. Other possible

explanations for this phenomenon include production of osteoclasts made resistant to calcitonin by antibodies and inhibition of osteoblast functions by coupling (Overgaard and others1990; Martin 1999; Pondel 2000). However, the exact reasons for this phenomenon are unclear at this time. Further information regarding the mechanisms of action of calcitonin would be beneficial in determining the optimum therapeutic use of calcitonin in the treatment of osteoporosis.

Results of a recently completed pilot study have given us pertinent data regarding the dose response curve of salmon calcitonin as well as the most therapeutic dose when administered continuously over time to ovariectomized rats (Owens and others 2003). Sixty female, Sprague-Dawley rats, 12 weeks of age were randomly assigned to one of 5 treatment groups. Groups 1-4 underwent bilateral ovariectomy and received doses of 5, 10, 25, or 50 IU/kg BW/day of salmon calcitonin. The fifth group of animals underwent a sham ovariectomy and received saline. At periods of 4 weeks, 8 weeks, and 16 weeks, four animals from each group were euthanized by CO_2 inhalation. Twenty-four hour urine samples were collected in metabolic cages prior to sacrifice. At the time of sacrifice, serum samples were obtained by cardiac puncture and bilateral femurs were dissected for analyses. Serum and urine samples were used for biochemical assessment of markers of bone turnover including osteocalcin, serum pyridinoline, and urinary helical peptides. The dissected right femur of each subject was used in three-point bending tests to assess biomechanical strength. Computer generated load displacement curves, bone caliper measurements, and photo images of bone cross section were then used to calculate the ultimate stress and elasticity of each specimen. The dissected left

femur of each subject was analyzed by DXA for in vitro assessment of bone mineralization via the PIXImus densitometer.

Due to this pilot study being cross-sectional by design and the limited number of subjects in each group, the information obtained with biochemical analyses and biomechanical strength measurements did not produce significant findings related to our hypothesis. Due to difficulty in applying force at consistent locations in dissected bones, validity of the results obtained with the three-point bending tests was questionable. However, significant and compelling results, as shown in Figure 9, were noted in the measurements of BMD among our subjects.

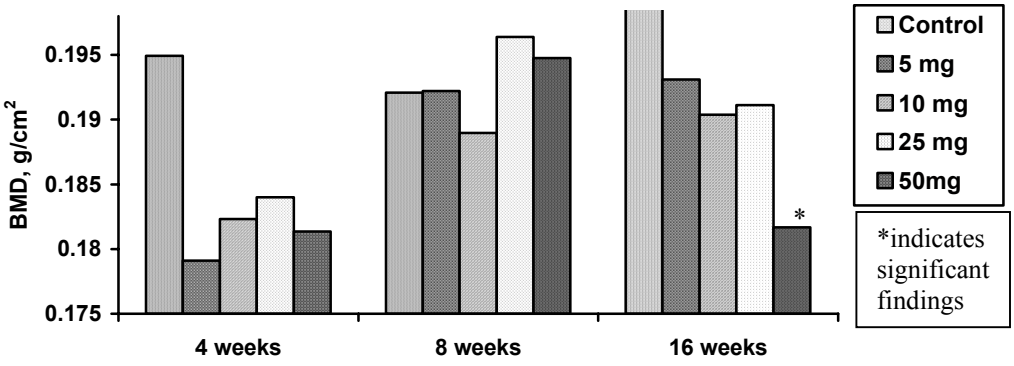


Figure 9 Effects of salmon calcitonin on in vitro bone mineral density in ovariectomized rats vs. saline in sham ovariectomized rats. n=4 in each group at each time point. * = $p<0.05$ for 50 mg vs. control.

Following ovariectomy, animals in our study showed an initial decrease in BMD in each treatment group. Animals receiving lower doses of calcitonin (5, 10 and 25 IU/kg BW/day) were associated with an increase in BMD after 8 weeks of treatment to a level similar to the non-treated control group. This increase in BMD was maintained in these treatment groups until termination of the study at 16 weeks. The animals receiving

higher doses of calcitonin (50 IU/kg BW/day) were associated with a similar increase in BMD at week 8 followed by a statistically significant reduction in BMD at week 16 suggesting a relative increase in resorption compared to formation. These findings were indicative of possible oversuppression of bone turnover with higher doses of calcitonin.

The current study is designed to further examine the effects of calcitonin on bone turnover and help to determine if the decrease in therapeutic effectiveness of calcitonin demonstrated over time with higher doses is due to oversuppression of bone turnover. It is hypothesized that high doses of salmon calcitonin can result in oversuppression of bone turnover in ovariectomized rats. In order to test this hypothesis we will use the ovariectomized rat as an estrogen deficient animal model for study of Type I osteoporosis. Varying doses of calcitonin will be compared to placebo (saline) in ovariectomized and sham-ovariectomized animals. Ovariectomy will be confirmed by pathological tissue evaluation and further verified with analysis of serum estradiol and follicle-stimulating hormone (FSH). The effects of varying doses of salmon calcitonin on bone turnover will be detected by measurement of changes in BMD by DXA scans. The gross anatomical changes in bone quality will be examined by SEM of samples from each group. The level of calcium excreted into the urine will be determined by capillary ion electrophoresis. Furthermore, the activity level of osteoclasts and osteoblasts in each group of subjects will be investigated with ELISA and IRMA for measurement of biochemical markers of bone turnover. Finally, direct ELISA will be used for detection of the development of calcitonin antibodies which will provide information to support (or refute) the theory that antibodies may play a role in the development of resistance to the effects of calcitonin observed with long-term administration.

CHAPTER 2

MATERIALS AND METHODS

Experimental Subjects

Female, Sprague Dawley rats, 12 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). The experimental subjects were housed according to Standard Operating Procedure of the Division of Laboratory Animal Resources at East Tennessee State University (Johnson City, TN) accredited by the American Association for Laboratory Animal Care. The rats were housed in polycarbonate cages (Allentown Caging Equipment Company, Inc., Allentown, NJ) with wire tops and wood chip bedding. Room temperature was maintained at approximately 22°C (72^0F +/- 4^0), and a 12 hour light cycle was used. Experimental subjects were allowed a diet of TEKLAD Rodent Diet #8604 (Harlan Teklad, Madison, WI) and water ad libitum. All procedures and animal protocols used in this study were approved by the Committee of East Tennessee State University on Animal Care as well as the Research and Development, Biosafety and Infection Control Committees of the Veteran's Administration. Once delivered to East Tennessee State University, the experimental subjects were allowed approximately 1 week to acclimate to their new environment prior to initiation of experiments.

Interventions

Interventions for each experimental subject were performed as listed in Table 1 and described below.

Table 1 Timeline of Interventions

Intervention	BL	1 wk	4wk	8wk	12wk	16wk	20wk	23wk	24wk
Ovariectomy	+	-	-	-	-	-	-	-	-
Urine Collection	+	-	+	+	+	+	+	-	+
Serum Collection	+	+	+	+	+	+	+	-	+
DXA scans	+	-	+	+	+	+	+	-	+
Med pump (re)placement	+	-	+	+	+	+	+	-	+
Tetracycline inj.	+	-	-	+	-	-	-	+	-
SEM	-	-	-	-	-	-	-	-	+

Randomization

The experimental subjects were randomly assigned to one of the following groups:

- Group 1 – sham ovariectomy and saline only
- Group 2 – ovariectomized and saline only
- Group 3 – ovariectomized and received 5 IU/kg BW/day of salmon CT
- Group 4 – ovariectomized and received 15 IU/kg BW/day of salmon CT
- Group 5 – ovariectomized and received 50 IU/kg BW/day of salmon CT

Experimental subjects were identified by cage card and ear punch methods.

Urine Collection

Urine samples were collected over a 24-hour period by placing each experimental subject into a metabolic cage 24-48 hours prior to each surgical procedure. While in the metabolic cages, experimental subjects were restricted from food to decrease risk of contamination of urine specimens; however, water remained available ad libitum. Once

collected, urine samples were centrifuged 10 minutes at high speed using an IEC Model HN-S II centrifuge (International Equipment Company, Needham Heights, MA). Two 1-mL aliquots of clean urine were separated off and stored at -70^{0}C until ready for analyses. The remainder of clean urine was stored in sterile 15-mL tubes and stored at –70^{0}C for future study. Following urine collection, subjects were returned to their storage cages. Urine specimens were used for analyses as listed in Table 2.

Table 2 Analyses of Urine Samples

Analysis	BL	4 wk	8 wk	12wk	16 wk	20wk	24wk
Helical Peptides	+	+	+	+	+	+	+
Creatinine	+	+	+	+	+	+	+
Calcium Excretion	+	+	+	+	+	+	+

Transportation of Subjects

The caged experimental subjects were placed into plastic totes, covered with sheets and transported by electric cart to Building 8, Room 126-127 of the Veteran's Administration Medical Center for surgical procedures. Temperature was checked during transport and did not vary > 3^{0}F (-16.1°C) while the experimental subjects were away from the climate-controlled facilities of the Division of Laboratory Animal Resources.

Serum Collection

Each subject was anesthetized by intraperitoneal (IP) injection of 50 mg/kg Ketamine HCl and 5 mg/kg of Xylazine. Blood samples were obtained by clipping the

tip of each subject's tail and milking out 1mL of blood into a sterile vial. Pressure was then applied with a clean cotton ball until bleeding subsided. We found use of non-latex gloves during this procedure to be essential for the prevention of excessive irritation and peeling of the skin on the tail of the animals. Blood samples were centrifuged 10 minutes at 10,000g in a Biofuge A Model 1217 centrifuge (Heraeus Separations Technik, West Germany). Clean serum samples were then pipetted off and stored in sterile vials at -70^{0}C until ready for analysis. Serum samples were utilized for analyses as listed in Table 3.

Table 3 Analyses of Serum Samples

Analysis	BL	1 wk	4 wk	8 wk	12 wk	16wk	20wk	24 wk
Estradiol	-	+	+	-	-	-	-	+
FSH	-	+	+	-	-	-	-	+
Pyridinoline	+	-	+	+	+	+	+	+
Osteocalcin	+	-	+	+	+	+	+	+
Calcitonin Antibodies	+	-	+	+	+	+	+	+

Ovariectomy Procedures

The dorsal surface of each experimental subject was shaved and prepped for surgery by cleansing with betadine and alcohol. Experimental subjects were then secured onto procedure trays in prone position by padded plastic cords attached to each of their extremities. Sterilized instruments and sterile technique were used during all surgical procedures. A small midline incision was made dorsally on each experimental subject and connective tissue was bluntly dissected to the muscle level. A small incision was made through the muscle layer approximately 1 cm lateral to midline and half way

between the caudal most rib and the superior rim of the pelvis to expose each ovary. In those subjects receiving a sham ovariectomy the ovaries were exposed, grossly examined for intactness and adequate blood flow, and then replaced to their original location. The remaining subjects underwent an ovariectomy in which the ovaries were exposed, tied off with suture thread, and surgically excised. The excised ovarian tissue from each experimental subject was placed into a sterile vial with 10% buffered formalin at room temperature until ready for microscopic examination. After completion of ovariectomy or sham-ovariectomy procedure, the muscle tissue was repaired with silk sutures.

Densitometry Scans

While sedated, each experimental subject was placed in a prone position on a procedure tray and secured with padded plastic cords, which bound each of their extremities to the four corners of the procedure tray. Each animal then underwent a Dual X-ray Absorptiometry (DXA) scan using the GE Lunar Prodigy Bone Densitometer (Serial #DF+13539, Lunar Corporation, GE Medical Systems). Each scan was then analyzed with software designed for small animal measurements.

Medication

A sterile 2ML4 Alzet osmotic medication pump (Durect Corporation, Cupertino, CA) containing either saline or appropriate dosage of salmon calcitonin (Cat. # H-2260, Bachem Bioscience Inc., King of Prussia, PA) dissolved in saline was inserted subcutaneously. The skin was then closed with interrupted suture technique using 4-0 silk sutures. The medication or vehicle was delivered at a constant rate of 2.5 μg/hr.

Alzet osmotic pumps were removed and replaced with new, sterile pumps every 4 weeks. The concentration of calcitonin was adjusted for weight at these 4-week intervals.

Euthanasia

Twenty-four weeks after initial surgical procedures each animal was euthanized by CO_2 inhalation and 4-5 mL of blood was collected by cardiac puncture. Blood was processed and serum stored as previously described. Twenty-four to 48 hours prior to euthanasia animals were placed into metabolic cages for collection of 24-hour urine samples. Urine samples were processed and stored as previously described. After termination and blood collection each animal received a final bone densitometry scan. The right femur, 10^{th} rib, and right pelvis were dissected and stored in 4% paraformaldehyde; the left femur, left pelvis, 11^{th} rib, and a tail segment were dissected and stored dry at 4°C; the left tibia was dissected and stored in formalin at room temperature; and the lumbar spine, right tibia, and 12^{th} rib were dissected and frozen at -70°C until ready for further analysis. The remaining parts of each experimental subject were placed into a cold storage freezer (Room 4-50, Building VA 119), as regulated by East Tennessee State University Department of Laboratory Animal Resources, until picked up by a contractor for incineration and disposal.

Estrogen-deficient Animal Model

Pathological Tissue Assessment

Tissue excised during initial ovariectomy surgical procedures was stored in 10% buffered formalin at room temperature. The samples were then dehydrated with graded

alcohol, cleared with xylene, infiltrated with paraffin, and embedded in paraffin. Tissue sections (5μm) were cut, mounted onto slides, and stained with routine H&E stain. Dr. George Youngberg, pathologist, then examined these slides under a light microscope to identify the tissue type.

Serum Estradiol Measurements

Serum samples obtained from 1 week, 4 weeks, and 24 weeks were thawed on ice for measurement of Estradiol-17b with an Estradiol ELISA kit #1920 (Alpha Diagnostics International, San Antonio, TX). Standards, controls, and serum samples were pipetted into anti-mouse IgG coated wells in duplicate. Estradiol enzyme conjugate was then added to each well and allowed to incubate at room temperature for 60 minutes on a plate shaker. The wells were then aspirated and washed three times with wash buffer. Horseradish peroxidase substrate was then added to each well and allowed to incubate for 10 minutes at room temperature. The reaction was stopped by adding a stop solution, and absorbancy was measured at 450nm with an EL312e microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Average absorbancy for each sample was then calculated.

FSH Measurements

Serum samples from 1 week, 4 weeks, and 24 weeks were thawed on ice for analysis with Follicle-stimulating Hormone (FSH) ELISA kit # 0200 (Alpha Diagnostic International, San Antonio, TX). Standards, controls, and serum samples were pipetted into antibody coated wells. Antibody-enzyme conjugate was then added to each well and allowed to incubate 60 minutes at room temperature. Contents were then aspirated and

washed with distilled water. Horseradish peroxidase (HRP) substrate was then added and allowed to incubate at room temperature for 30 minutes. Stop solution was then added and absorbancy read at 450nm with an EL312e microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Samples were tested in duplicate and average absorbancy calculated.

Scanning Electron Microscopy

The dissected left femurs were each cut cross-sectionally at midline. Each of these pieces was then cut longitudinally at midline. Any residual soft tissue was cleared off by placing the bones in 5% KOH for 24 hours followed by rinsing and 24 hour water bath for three cycles. The samples were then dehydrated with graded alcohol (50%, 95%, and 100%; 24 hours in each grade). Each sample was lyophilized with Samdri-PVT-3B critical point dryer (Tousimis Research Corporation, Rockville, MD). The bones were then mounted with colloidal carbon graphite #60790 (Ladd Research Industries, Burlington, VT) onto one-inch circular metal stubs and sputter coated with a thin layer of gold using the Denton Vacuum Desk II Cold Sputter/Etch Unit (Denton Vacuum, Inc., Moorestown, NJ). Samples were then viewed at 20 kV with Digital Scanning Microscope 940 (Zeiis, West Germany) and images were captured digitally for photographs.

Bone Quality

Bone Densitometry

DXA scans were performed using the GE Lunar Prodigy Bone Densitometer and analyzed with software designed for small animal measurements. Values were obtained for total body bone mineral density (BMD). Specific regions of interest, including the spine, right femur, and left femur, were analyzed for BMD, Bone Mineral Content (BMC), and Area.

Precision Study

To ensure that any observed change in bone mass was a true change and not due to poor reproducibility of the technique, a precision study was conducted. Three successive densitometry scans were taken on a representative set of individual subjects. To properly include the effects of positioning errors, the subject was completely removed from the exam table, unstrapped, removed from the procedure tray, then repositioned and scanned again. The values obtained for the bone mass of the spine and both hips were compared for each measurement to determine precision of the examiner. The mean and standard deviation of the repeated measures for each individual were calculated. Precision was then expressed as the % coefficient of variation (%CV), which was determined by calculating the standard deviation as a percentage of the mean.

Urinary Calcium Excretion

Frozen collected urine samples (as previously described) were thawed, vortexed with a Vortex Genie (Serial #29802), and diluted 1:100 with 18 MΩ water. Fifty μL of Barium 2% HNO_3 was added to each 1-mL aliquot of diluted urine samples and vortexed. One hundred μL of this mixture was pipetted into 0.5-mL tubes for analysis. Samples were analyzed with Water's Quanta 4000 Capillary Electrophoresis System with a 745 Data Module (Waters Corporation, Milford, MA). A Water's Accusep Capillary (75 μ X 60 cm) was used for these analyses. Analytical conditions were as follows: hydrostatic sampling time, 10 sec; run time, 18 sec; voltage, 20 kV; autopurge between samples, 1 min; absorbance range 0.02 sec; time constant, 0.3 sec. The 745 Data Module settings were: attenuation 4; chart speed 2 cm min^{-1}; peak width 3; time function now (TFN); and peak marker (PM) ON. Specimen concentrations were determined by their calcium/barium peak area ratios and divided by creatinine values to correct for variations in urine concentration.

Creatinine

Levels of creatinine in frozen collected urine samples were measured by Metra™ Creatinine Assay kit (Cat #8009, Quidel Corporation, Mountain View, CA). Samples were processed according to manufacturer's instructions to determine levels of creatinine. Urine samples were thawed on ice and vortexed for about 5 seconds with a Vortex Genie. Creatinine standards (5, 20, and 40 mmol/L), controls, and samples were diluted 1:40 with deionized water. Fifty μL of the diluted standards, controls, and samples were pipetted into non-coated strip wells along with 150 μL of Working Color Solution

(0.14% Picric acid in sodium borate and SDS; reconstituted with 1N NaOH) and allowed to incubate 30 minutes at room temperature (18-28^{0}C). The absorbancy was then read at 490nm with a TECAN GENios microplate reader #94636 (Phenix Research Products). Results were then analyzed with a linear regression equation. Concentrations of samples and controls were determined from the standard curve. All samples were tested in duplicate and the average concentration determined.

Bone Resorption

Helical Peptides

Levels of helical peptide 620-633α1 of Type I collagen present in each sample were determined by use of an enzyme immunoassay kit (Cat. #8022, Quidel Corporation, Mountain View, CA). Frozen collected urine samples were thawed on ice and vortexed with a Vortex Genie. Twenty μL of standards, controls, and urine samples were added to room temperature strips coated with purified murine monoclonal anti-helical peptide antibody. Reconstituted enzyme conjugate containing lyophilized synthetic helical peptide conjugated to alkaline phosphatase with buffer salts and stabilizers was then added to each well. Strips were then covered and allowed to incubate overnight at 2-8^{0}C. Strips were then emptied and washed three times with diluted wash buffer (non ionic detergent in a buffered solution containing 0.05% sodium azide as a preservative). Working substrate solution (p-Nitrophenyl phosphate dissolved in a diethanolamine and magnesium chloride solution containing 0.05% sodium azide as a preservative) was then added to each well and allowed to incubate 60 minutes at room temperature (18-28^{0}C). The reaction was the stopped by adding 1N NaOH to each well and the absorbancy was

read at 405nm in a TECAN GENios microplate reader #94636 (Phenix Research Products). A 4-parameter calibration curve fitting equation was then used to analyze the helical peptide assay results. A standard curve was plotted using A_{405} values for each helical peptide standard on the Y-axis and the assigned helical peptide concentration for each standard on the X-axis. The concentration of urine samples and controls was then determined from the standard curve. All samples were tested in duplicate and the average concentration of helical peptide determined. Results obtained from the helical peptide assay were then divided by creatinine values to correct for variations in urine concentration.

Serum Pyridinoline

The excretion of pyridinoline crosslinks in serum was measured with Metra™ Serum Pyridinoline enzyme immunoassay kit #8019 (Quidel Corporation, Mountain View, CA). Frozen collected serum samples (as previously described) were thawed on ice, pipetted into 30k MWCO spinfilters, and centrifuged for 30 minutes at 10,000xg with an Eppendorf Centrifuge #5415D (Brinkmann Instruments, Inc., Westbury, NY). Reagent, a glycine solution containing indicator dye and a preservative, was added to each well of the coated strips followed by standards, controls, or filtered samples. Pyridinoline antibody was then dispensed into each well and incubated overnight in the dark at 2-8^{0}C. The strips were then thoroughly washed and an enzyme conjugate containing lyophilized goat anti-rabbit antibody conjugated to alkaline phosphatase was added and allowed to incubate 60 minutes at room temperature. Strips were again washed and working substrate solution (p-Nitrophenyl phosphate) was added and allowed

to incubate 40 minutes at room temperature. Stop solution of 1N NaOH was then dispensed into each well and the Optical Density (OD) was read at 405nm with a TECAN GENios microplate reader #94636 (Phoenix Research Products). The results were then analyzed with a 4-parameter calibration fitting curve equation to determine pyridinoline levels in each sample.

Bone Formation

Osteocalcin

Rat osteocalcin levels in serum were quantitatively determined by immunoradiometric assay (IRMA) using Rat Osteocalcin IRMA kit #50-1500 (Immunotopics, Inc., San Clemente, CA). Serum samples were allowed to thaw on ice and then centrifuged for 5 minutes at 10,000 g to ensure a clean sample. Controls and serum samples were then diluted 1:21 with zero standard. ^{125}I labeled rat osteocalcin antibody was added to standards, diluted controls, and diluted samples and vortexed briefly with a Vortex Genie. One polystyrene bead coated with antibody to rat osteocalcin plus desiccant was added to each specimen. Tubes were then covered and allowed to incubate at room temperature overnight. The content of each tube was aspirated and the beads thoroughly washed with wash solution containing a surfactant in 0.01 M phosphate buffered saline with 0.05% sodium azide. After aspiration of wash solution beads were counted in an LKB 1282 Compugamma Universal Gamma Counter (Wallac Oy, Finland) for 1 minute. The average counts per minute (CPM) for each pair of duplicate assay tubes was calculated. The average CPM of the 0 ng/ml standard was

then subtracted from the average CPM of all other average CPMs to obtain the corrected CPM. A standard curve was then generated by plotting the values obtained for the standards on a log:log linear regression curve. The rat osteocalcin concentration of the diluted controls and diluted samples were then read from the standard curve using their respective corrected CPM. Final rat osteocalcin concentrations were then obtained by multiplying the observed value from the above-mentioned curve by the dilution factor.

Calcitonin Antibodies

Direct ELISA was used for measurement of the production of serum calcitonin antibody titer. Serum samples were thawed on ice, briefly centrifuged, diluted with BSA, and briefly vortexed with a Vortex Genie. Microtiter plates were coated with calcitonin diluted in 20 mM Tris-HCl, pH 8.5 and incubated for 1 hour at room temperature. The wells were thoroughly washed with PBS-T and remaining adsorption sites were blocked by adding BSA/PBS-T to each well. The plates were allowed to incubate for 1 hour at room temperature and washed thoroughly with PBS-T. Calcitonin Antibody F-13 (Cat #sc-9174, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive control while BSA/PBS-T served as a negative control. Controls and diluted serum samples were pipetted into each well and allowed to incubate overnight at 4°C. Plates were then emptied and washed thoroughly. Anti-goat IgG-HRP was added to wells containing the positive control samples while anti-rat IgG-HRP was added to the remaining wells and allowed to incubate for 1 hour at room temperature. Wells were washed and substrate solution of 1-Step™ Ultra TMB-ELISA (Product #34028, Peirce, Rockford, IL) was added. Following a 30-minute incubation period absorbancy was read at 405nm in a

TECAN GENios microplate reader #95636 (Phoenix Research Products). Samples were tested in duplicate and the average amount of antibody was calculated for each subject at each time point.

Material for Future Studies

Due to the magnitude of information available from this study, a select group of experiments were chosen for completion for this dissertation project. Due to the extensive time and resources invested in this study, we did not want to dispose of any materials that might be beneficial for further analyses. Therefore, various materials were preserved for future possible studies.

Tetracycline Labeling

After completion of procedures at baseline and 8 weeks, while experimental subjects were still sedated, each subject received an IP injection of 20 mg/kg BW of Tetracyline HCl (Cat #T-7660, Sigma-Aldrich, St. Louis, MO). A final dosage of Tetracyline HCl was administered for each subject 1 week prior to euthanasia. The right femur, 10^{th} rib, and right pelvis of each animal were stored in 4% paraformaldehyde until ready for further processing and evaluation. The right femurs were then embedded in methyl methacrylate and processed for cutting and further analysis.

Bones Available for Future Studies

The bones listed in Table 4 were dissected and stored as indicated for possible analysis in future studies.

Table 4 Bones Dissected for Possible Analysis

Bone	Storage Condition	Storage Temperature
Right Femur	4% Paraformaldehyde	4°C
Left pelvis	Dry	4°C
11th rib	Dry	4°C
Left tibia	Formalin	Room Temperature
10th rib	4% Paraformaldehyde	4°C
Right pelvis	4% Paraformaldehyde	4°C
Tail segment	Dry	4°C
Lumbar spine	Dry	-70°C
Right tibia	Dry	-70°C
12th rib	Dry	-70°C

Statistical Analysis

One way ANOVA was used to determine statistical differences between groups. This was followed by Tukey's or Fisher's multiple comparison tests if there were significant differences noted between groups. Differences at $p<0.05$ were considered significant. Values are expressed either as mean ± SEM or % change from baseline. Precision to determine significance of results for BMC measurements is expressed as %CV and indicated by double lines within the applicable graphs. GraphPad 4.0 statistical program was used for calculation of area under the curve (AUC).

CHAPTER 3

RESULTS

Experimental Subjects

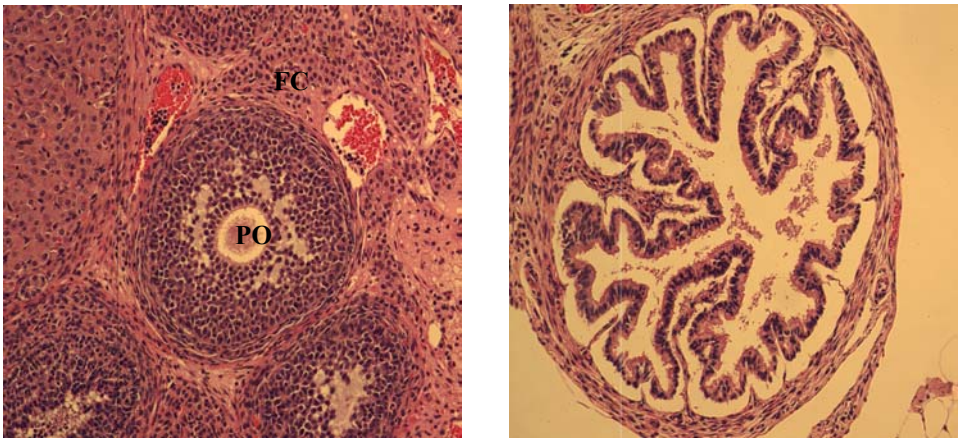
Forty-two female Sprague-Dawley rats were randomized to one of five groups. Group 1 underwent a sham ovariectomy and received saline. Group 2 was ovariectomized and received saline. Group 3 was ovariectomized and received 5 IU/kg BW/day of calcitonin. Group 4 was ovariectomized and received 15 IU/kg BW/day of calcitonin. Group 5 was ovariectomized and received 50 IU/kg BW/day of calcitonin.

After randomization 8 subjects were included in groups 1, 2, and 4. Nine subjects were included in groups 3 and 5. Five subjects were unfortunately unable to complete the 24-week study. Subject #4 (Group 1) died at week 12, #18 (Group 3) at week 16, and #40 (Group 5) at week 4 after receiving an appropriate dosage of anesthesia during the preparatory process for surgery. Technical error in needle placement during administration of anesthesia may have been a factor in their otherwise unexplained deaths. Subject #19 (Group 3) was euthanized at week 15 at the request of the director of the Department of Laboratory Animal Research of East Tennessee State University due to excessive skin breakdown and resulting complications in the area of the Alzet osmotic pump. Likewise, subject #31 (Group 4) was euthanized at week 16 due to similar problems with skin breakdown and signs of necrosis. All data collected from these animals prior to termination were included in the results. However, due to differing time frames of completion, the bones from these animals were not included in final analyses.

Estrogen-deficient Animal Model

Examination of Ovaries

Ovaries were identified bilaterally in 100% of the subjects used in this study. Both ovaries were completely resected in experimental animals randomized to ovariectomized treatment groups. The tissue samples excised during the initial OVX surgical procedures were analyzed under light microscopy by Dr. George Youngberg, pathologist, and were identified in all ovariectomized instances as ovarian tissue (Fig. 10). Visualization of primary oocytes surrounded by follicular cells confirmed the nature of the excised tissue to be ovarian. Observation of the presence of the oviduct, also referred to as the fallopian or uterine tube, provided evidence of successful excision of the entire ovary.



A B

Figure 10 Light microscopy images of excised ovarian tissues. A. Depicts a primary oocyte (PO) surrounded by follicular cells (FC). B. An oviduct with longitudinal folds of mucosa subdividing the lumen into labyrinthine spaces.

Serum Estradiol Levels

Levels of Estradiol and FSH were measured in each animal by ELISA. While there were no significant differences in levels of FSH between the sham ovariectomized and ovariectomized groups as measured by this kit, levels of estradiol were significantly lower in the ovariectomized group at week 24 as compared to the sham ovariectomized animals. As seen in Fig. 11, at week 24 the ovariectomized treatment groups (n=28) had a mean Estradiol level of 3.645±0.495 pg/ml, which was significantly lower than the control sham group (n=7) which had an Estradiol level of 8.922± 3.543 pg/ml (P<0.05).

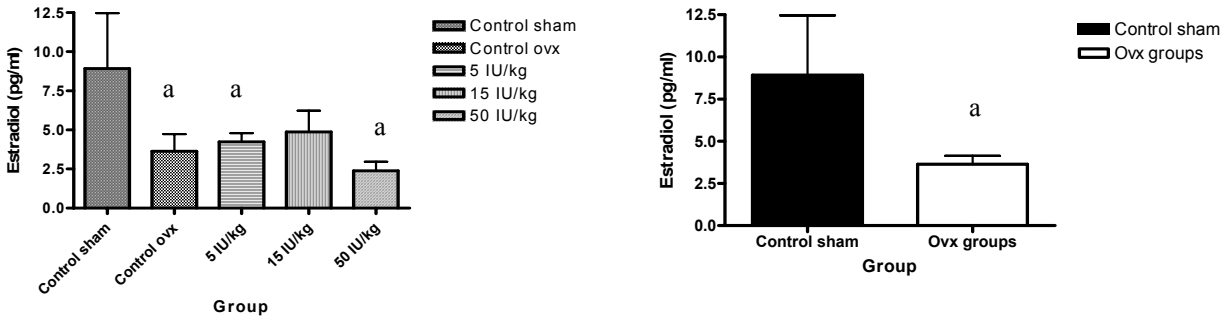


Figure 11 Levels of Estradiol measured in subjects at week 24. [a]P<0.05 vs. control sham subjects. Control sham n=7, control ovx n=8, 5 IU/kg group n=7, 15 IU/kg group n=5, 50 IU/kg group n=8. Ovx groups n=28.

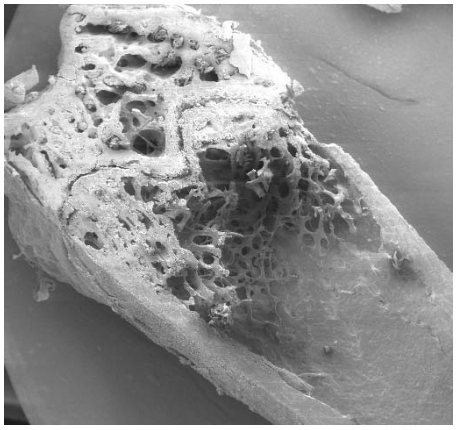
Scanning Electron Microscopy

The SEM images depicted in Fig. 12 illustrate the deleterious effects of ovariectomy on bone tissue. When compared to the bones from the control sham animals, the bones from the control ovariectomized animals have fewer trabeculae. Also, the trabeculae that are present in the control ovariectomized animals are less dense overall than those pictured in the control sham specimen. The cortical walls of the control sham animals are thicker than the corresponding walls depicted in the control ovariectomized specimens.

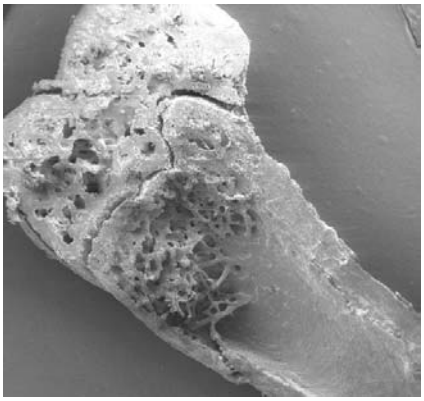
When comparing the ovariectomized subjects, the control ovariectomized subjects have fewer and less dense trabeculae than the groups receiving treatment with calcitonin. Many more trabeculae and more dense trabeculae are visualized in the subjects receiving 5 and 15 IU/kg BW/day of calcitonin as compared to the control ovariectomized subjects. Also, the subjects receiving 50 IU/kg BW/day of calcitonin have a comparable number of, if not fewer, trabeculae than the control ovariectomized subjects.

Differences are also depicted between the groups of subjects receiving varying doses of calcitonin. The subject receiving the smallest dosage of calcitonin (5 IU/kg BW/day) has fewer and less dense trabeculae than the groups receiving higher doses of calcitonin. Decreased trabecular density is seen in the subject receiving 50 IU/kg BW/day as compared to the subject receiving 15 IU/kg BW/day. Thinning of the cortical wall is noted in the subject receiving the highest dose of calcitonin (50 IU/kg BW/day) as compared to the groups receiving lower doses of calcitonin.

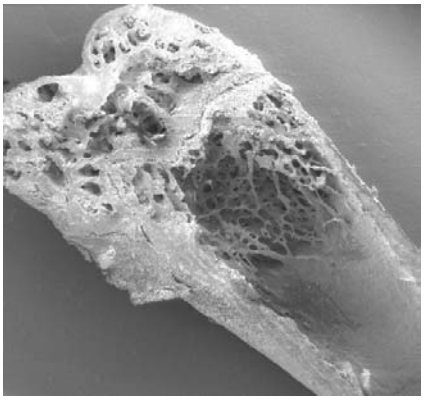
Control Sham



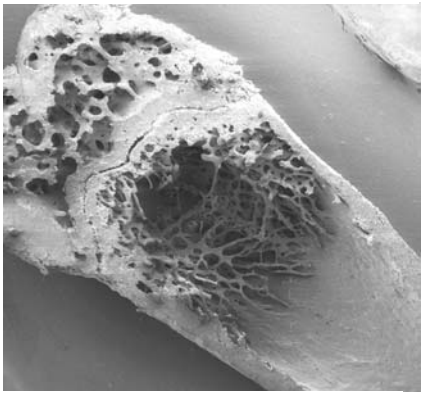
Control Ovx



5 IU/kg/day



15 IU/kg/day



50 IU/kg/day

Figure 12 Scanning Electron Microscopy (SEM) Images

Bone Quality

Bone Densitometry

BMD is a measurement derived from division of the BMC by the area of the bone. Due to the increased weight gain induced by ovariectomy and thus a difference in the overall size of animals in each group, the BMC rather than the BMD of selected regions was used to analyze the amount of bone formation for each subject. Furthermore, these measurements were then divided by the weight of the subject at the time of measurement to adjust for differences in size.

As seen in Fig. 13, there were no significant differences between levels of weight adjusted BMC of the spine between groups at baseline. At week 4 the weight adjusted BMC of the spine for the control sham group was higher than all ovariectomized groups. Initially after the ovariectomy each treatment group has a significant decrease in BMC of the spine as indicated at week 4 ($P<0.05$). At week 8 the groups receiving calcitonin were able to partially compensate for this initial change in BMC in a dose-dependent manner. The spinal BMC of subjects receiving 50 IU/kg BW/day of calcitonin plateaus and even begins to decrease after 8 weeks of treatment. The BMC of the subjects receiving lower doses of calcitonin (5 and 15 IU/kg BW/day) were continuing to gradually increase throughout the 24-week period of our analyses. At week 24 the BMC of the control sham group is significantly higher ($P<0.05$) than both the control ovariectomy group and the group receiving 50 IU/kg BW/day of calcitonin but not significantly different from the subjects receiving 5 and 15 IU/kg BW/day of calcitonin.

The precision of this method, in our study is 5.28%. Two parallel lines are included in the graph in Fig. 13 to represent the precision range.

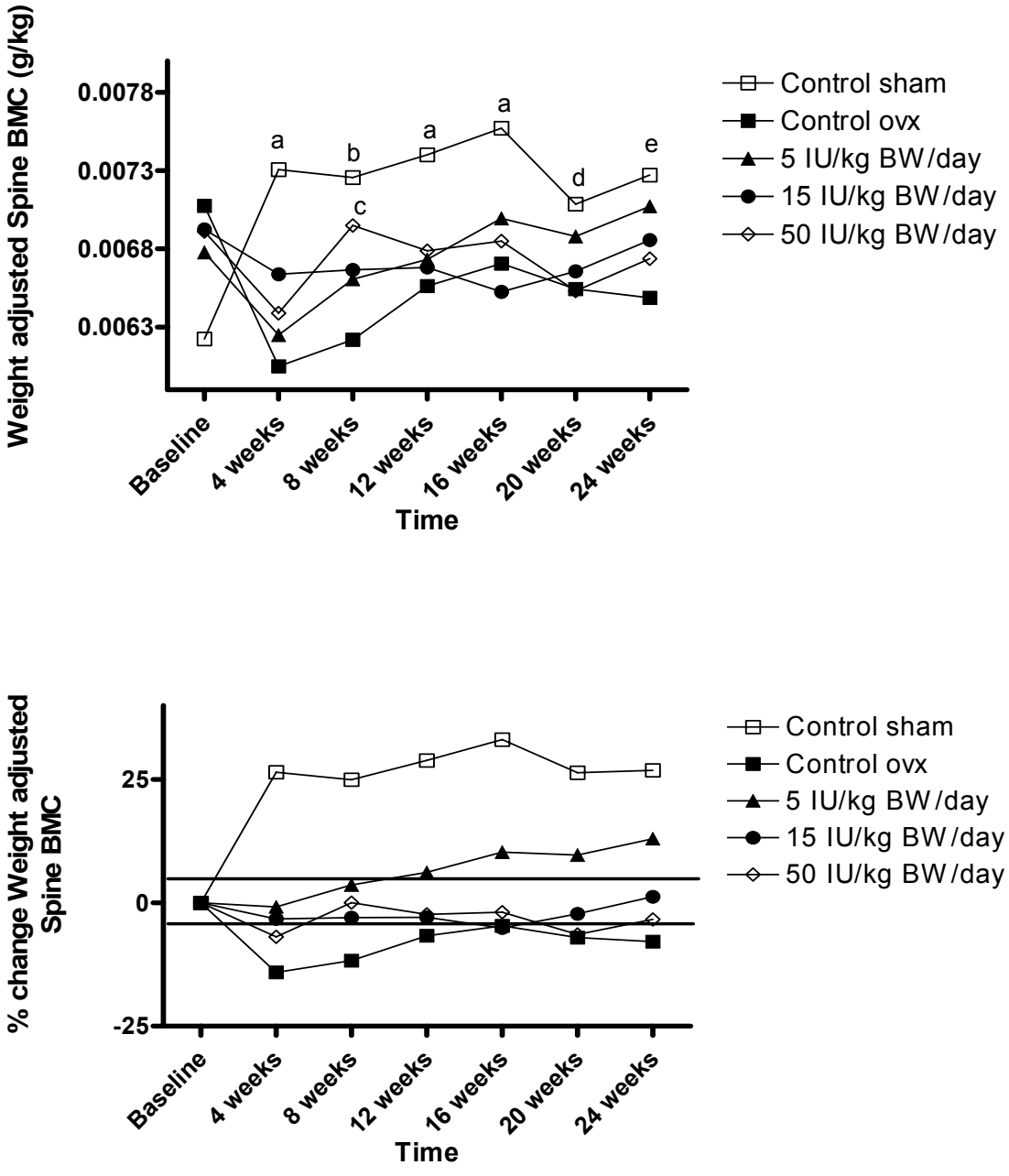


Figure 13 Weight adjusted BMC of the spine. [a]$P<0.05$ vs. Control ovx, 5 IU/kg, 15 IU/kg, and 50 IU/kg groups. [b]$P<0.05$ vs. Control ovx, 5 IU/kg, and 15 IU/kg groups.

[c]$P<0.05$ vs. Control ovx. [d]$P<0.05$ vs. Control ovx, 15 IU/kg, and 50 IU/kg groups. [e]$P<0.05$ vs. Control ovx and 50 IU/kg groups. Control sham BL, 4wks, 8wks, and 12wks n=8, 16wks, 20wks, and 24wks n=7. Control ovx n=8 at each time point. 5IU/kg group BL, 4wks, 8wks, and 12wks n=9, 16wks n=8, 20wks and 24wks n=7. 15 IU/kg group BL, 4wks, 8wks, 12wks, and 16wks n=8, 20wks and 24wks n=7. 50 IU/kg group BL and 4wks n=9, 8wks, 12wks, 16wks, 20wks, and 24wks n=8. Precision error is 5.28% as indicated by lines within the graph.

In the right femur no significant differences were noted in the weight adjusted BMC between control sham and control ovariectomy subjects at baseline (Fig. 14). However, following ovariectomy the weight adjusted BMC of the right femur of the control ovariectomized subjects was significantly lower than the control sham subjects ($P<0.05$) and remains significantly lower throughout the 24-week study. Similarly, the weight adjusted BMC of the right femur of the subjects receiving calcitonin was not significantly different from the control sham subjects at baseline. The weight adjusted BMC of the right femur of the treated groups remained significantly lower than the control sham subjects throughout the 24-week study ($P<0.05$) with the exception of the 5 IU/kg BW/day subjects at week 20. At this time point the weight adjusted BMC of the right femur for subjects receiving 5 IU/kg BW/day remained lower than the control sham subjects but not with significance of $P<0.05$. The precision of this method was 6.64% in our study. Two parallel lines are drawn in the graph of Fig. 14 to show this precision range.

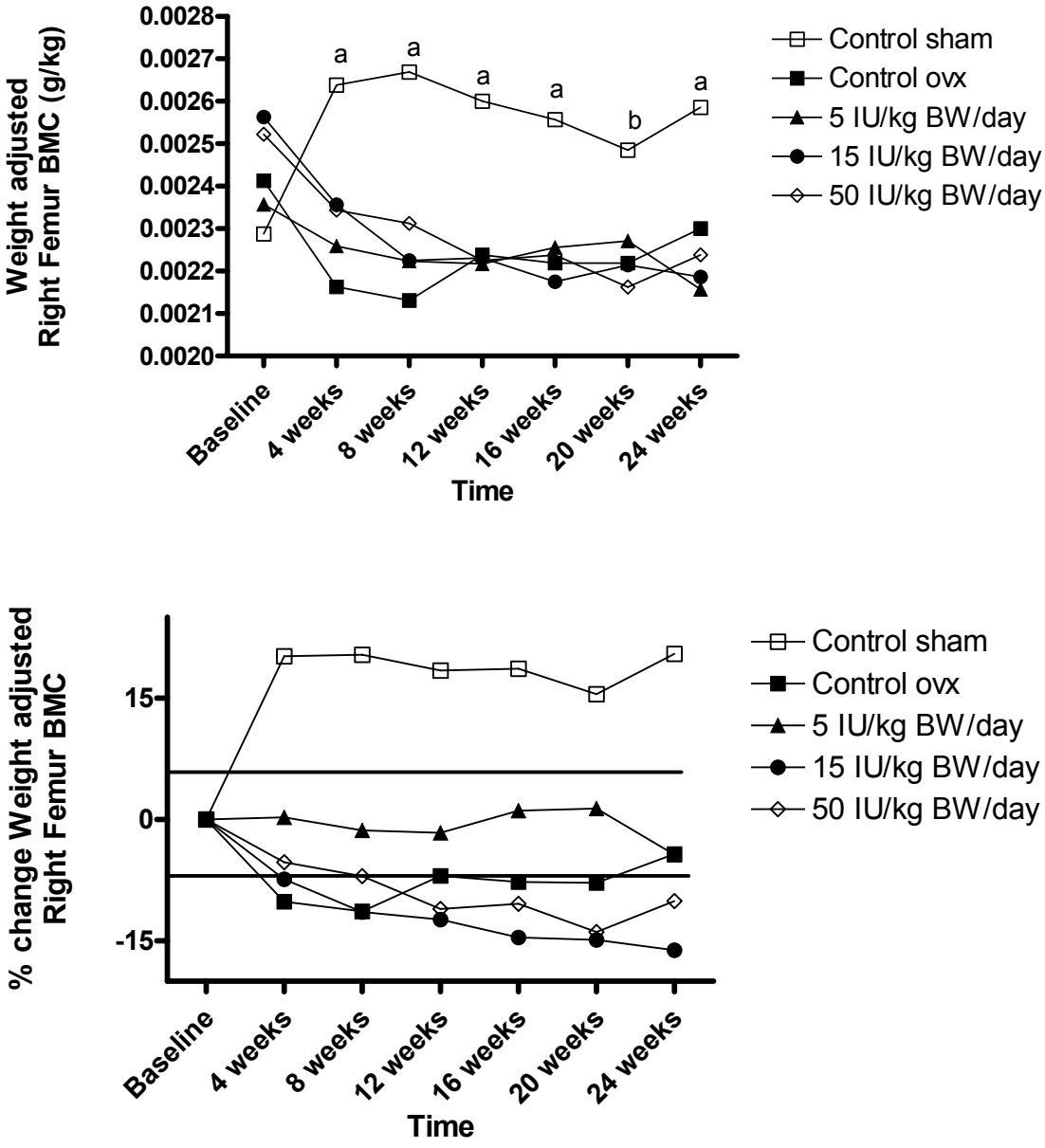


Figure 14 Weight adjusted BMC of right femur. [a]*P*<0.05 vs. Control ovx, 5 IU/kg, 15 IU/kg, and 50 IU/kg groups. [b]*P*<0.05 vs. Control ovx, 15 IU/kg, and 50 IU/kg groups. Control sham BL, 4wks, 8wks, and 12wks n=8, 16wks, 20wks, and 24wks n=7. Control ovx n=8 at each time point. 5IU/kg group BL, 4wks, 8wks, and 12wks n=9, 16wks n=8, 20wks and 24wks n=7. 15 IU/kg group BL, 4wks, 8wks, 12wks, and 16wks n=8, 20wks

and 24wks n=7. 50 IU/kg group BL and 4wks n=9, 8wks, 12wks, 16wks, 20wks and 24wks n=8. Precision error is 6.64% as indicated by the lines within the graph.

In the left femur (Fig. 15) the weight adjusted BMC of the control ovariectomized subjects was significantly higher than the control sham group at baseline ($P<0.05$). This difference is reversed following ovariectomy, as seen at week 4, when the weight adjusted BMC of the left femur of the control ovariectomized subjects is significantly lower than the control sham group and remains significantly lower for 20 weeks of this study ($P<0.05$).

At baseline the weight adjusted BMC of the left femur for the control sham group is significantly lower than the control ovariectomized, 15 IU/kg BW/day, and 50 IU/kg BW/day subjects ($P<0.05$). At 4 weeks the weight adjusted BMC of the left femur of the ovariectomized subjects is significantly lower than the control sham group ($P<0.05$) except for the subjects receiving 5 IU/kg BW/day and 15 IU/kg BW/day. Again, at this time point the weight adjusted BMC of the left femur for subjects receiving 5 IU/kg BW/day and 15 IU/kg BW/day remained lower than the control sham subjects but not with significance of $P<0.05$. A similar response is seen at week 24 for the subjects receiving 5 IU/kg BW/day as compared to the control sham group. Treatment of the ovariectomized groups for 24 weeks did not yield a significant difference between groups for weight adjusted BMC of the right and left femur. The precision of this method, in our study was 7.26%.

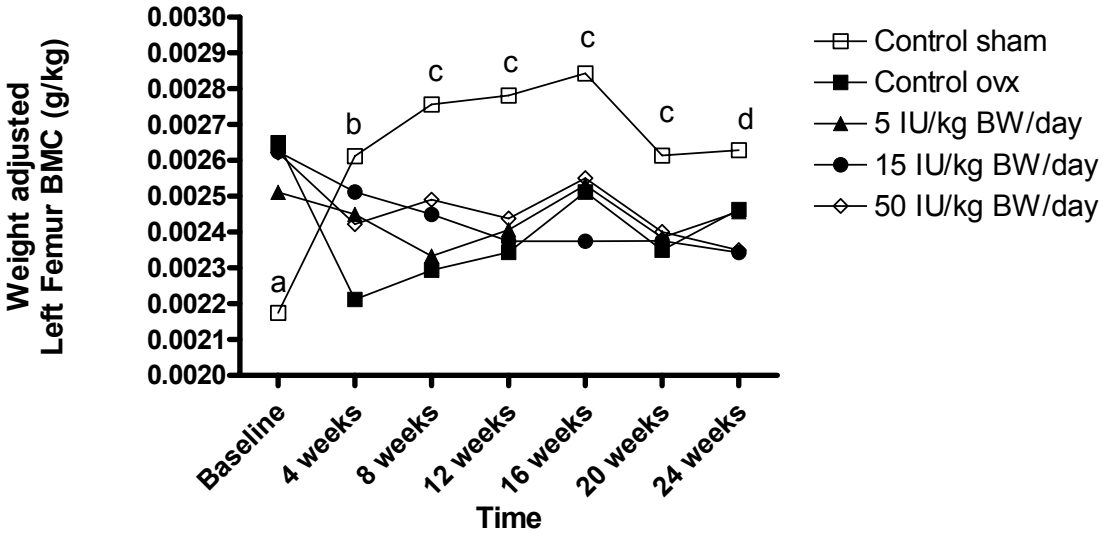


Figure 15 Weight adjusted BMC of left femur. [a]*P*<0.05 vs. Control ovx, 15 IU/kg, and 50 IU/kg groups. [b]*P*<0.05 vs. Control ovx and 50 IU/kg groups. [c]*P*<0.05 vs. Control ovx, 5 IU/kg, 15 IU/kg, and 50 IU/kg groups. [d]*P*<0.05 vs. 15 IU/kg and 50 IU/kg groups. Control sham BL, 4wks, 8wks, and 12wks n=8, 16wks, 20wks, and 24wks n=7. Control ovx n=8 at each time point. 5IU/kg group BL, 4wks, 8wks, and 12wks n=9, 16wks n=8, 20wks and 24wks n=7. 15 IU/kg group BL, 4wks, 8wks, 12wks, and 16wks n=8, 20wks and 24wks n=7. 50 IU/kg group BL and 4wks n=9, 8wks, 12wks, 16wks, 20wks, and 24wks n=8. Precision error is 7.26%.

Urinary Calcium Excretion

Capillary Ion Electrophoresis was used to measure the amount of calcium excreted into the urine. Mean levels of urinary calcium measured for each group were graphed for each time point. The area under the curve of each graph was used to determine the cumulative effects of treatment over the period of 24 weeks on levels of calcium excreted in the urine. Fig. 16 depicts the area under the curve of each treatment group.

The control sham group exhibits a significantly higher level of calcium excreted into the urine than the control ovariectomized subjects (P<0.05). The levels of calcium excreted in the urine for the groups of subject receiving lower doses of calcitonin (5 and 15 IU/kg BW/day) were not significantly different from either of the control groups. However, the experimental subjects who received higher doses of calcitonin (50 IU/kg BW/day) had a significantly higher level of calcium excreted into the urine than the other ovariectomized subjects (P <0.05)

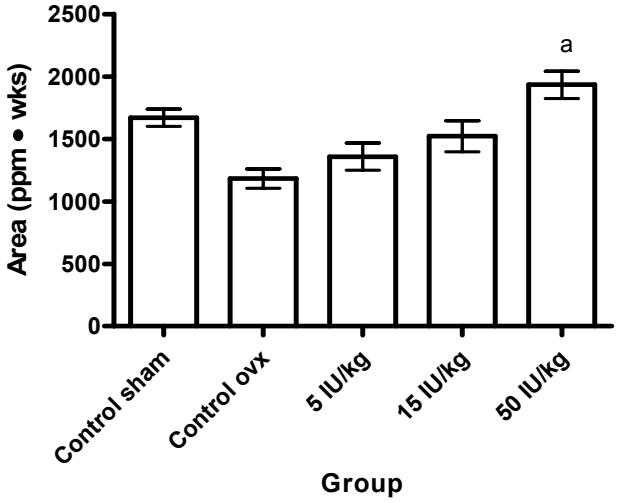


Figure 16 Area under the curve for levels of calcium excreted into the urine. [a]P<0.05 vs. Control ovx, 5 IU/kg/day, and 15 IU/kg/day groups. Control sham n=7, Control ovx n=8, 5 IU/kg/day n=7, 15 IU/kg/day n=8, 50 IU/kg/day n=8.

Bone Resorption

Helical Peptides

The levels of helical peptide 620-633α1, a marker of bone resorption, were measured by ELISA. No consistent significant differences were noted between groups in

the measurement of helical peptides during the 24 weeks observed in this study to support or refute our hypothesis.

Serum Pyridinoline

Levels of pyridinoline, a marker for bone resorption, measured by ELISA in serum samples of each subject at each indicated time point are depicted in Fig. 17. As evidenced in Fig. 17, there is increased bone resorption for the first 16 weeks following ovariectomy. At week 4 the subjects receiving 5 IU/kg BW/day had significantly higher levels of serum pyridinoline than the control sham subjects at $P<0.05$. This difference is not noted at other time points. At week 12 the level of serum pyridinoline measured in the control sham subjects was significantly higher ($P<0.05$) than subjects in the control ovariectomized group and those receiving 50 IU/kg BW/day of salmon calcitonin. At week 16 subjects receiving 50 IU/kg BW/day of salmon calcitonin had significantly lower levels of serum pyridinoline than the subjects receiving 5 IU/kg BW/day ($P<0.05$). Again, these differences were not noted at other time points. Therefore, no consistent significant differences were noted to support or refute our hypothesis.

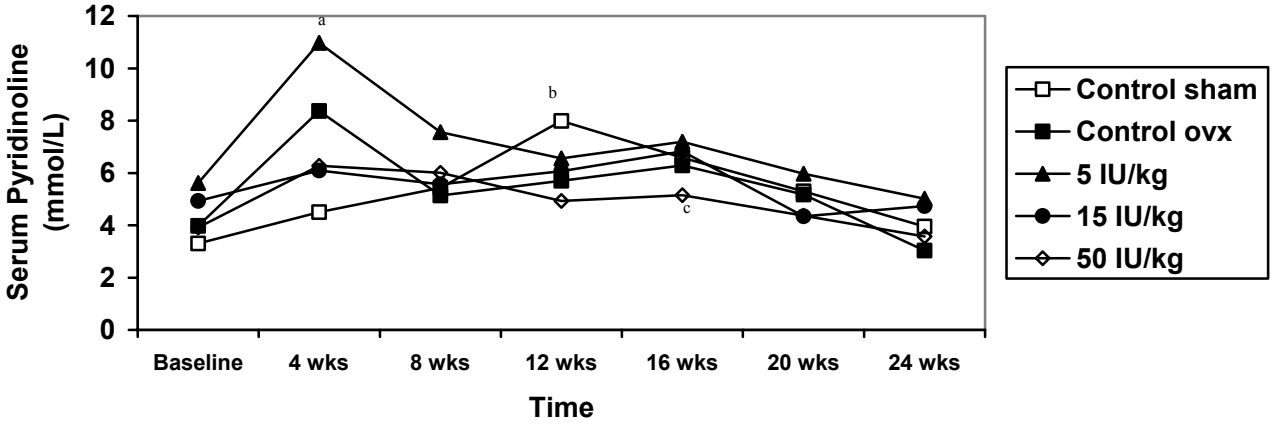


Figure 17 Levels of serum pyridinoline. [a]*P*<0.05 vs. Control sham. [b]*P*<0.05 vs. Control ovx and 50 IU/kg group. [c]*P*<0.05 vs. 5 IU/kg group. Control sham BL, 4wks, 8wks, and 12wks n=8, 16wks, 20wks, and 24wks n=7. Control ovx n=8 at each time point. 5IU/kg group BL, 4wks, 8wks, and 12wks n=9, 16wks n=8, 20wks and 24wks n=7. 15 IU/kg group BL, 4wks, 8wks, 12wks, and 16wks n=8, 20wks and 24wks n=7. 50 IU/kg group BL and 4wks n=9, 8wks, 12wks, 16wks, 20wks, and 24wks n=8.

Bone Formation

Osteocalcin

Mean levels of osteocalcin measured for each group were graphed for each time point. The area under the curve of each graph was used to determine the cumulative effects of treatment over the period of 24 weeks on osteocalcin levels and is depicted in Fig. 18. The group receiving 5 IU/kg BW/day of calcitonin had a significantly higher level of osteocalcin over the 24-week period than the control sham group (*P*<0.05). The group receiving 15 IU/kg BW/day also had a higher level of osteocalcin over the 24-week

period than the control sham, control ovariectomized and 50 IU/kg BW/day groups but not with significant difference. No significant difference in the cumulative level of osteocalcin over the 24-week period was detected between the control sham, control ovariectomized, and 50 IU/kg BW/day groups.

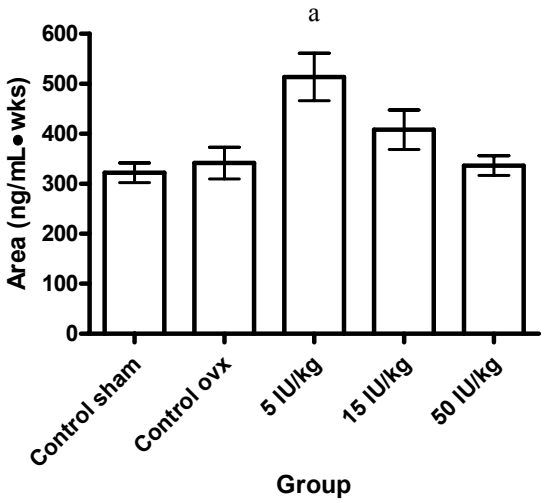


Figure 18 Bar graph of area under the curve measurements for osteocalcin. [a]*P*<0.05 vs. Control sham, Control ovariectomized, and 50 IU/kg BW/day groups. Control sham n=7, Control ovx n=8, 5 IU/kg/day n=7, 15 IU/kg/day n=7, 50 IU/kg/day n=8.

Calcitonin Antibodies

Serum samples were analyzed by Direct ELISA for detection of production of antibody titer to calcitonin. As depicted in Fig. 19, there were no significant differences noted in the level of calcitonin antibodies detected between groups of subjects over a period of 24 weeks.

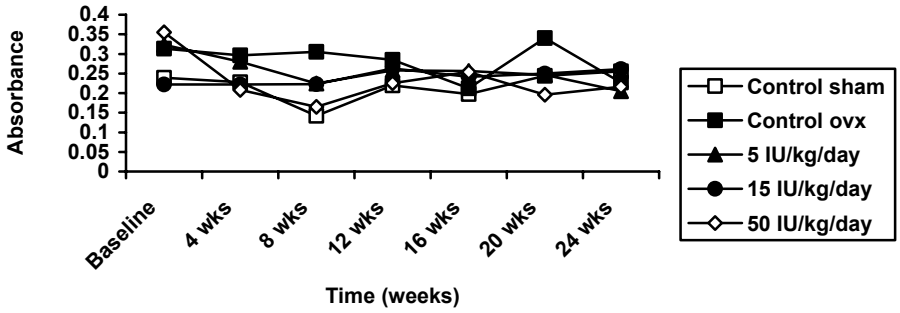


Figure 19 Serum levels of calcitonin antibody as detected by Direct ELISA. No significant differences were noted between groups at any time point. Control sham BL, 4wks, 8wks, and 12wks n=8, 16wks n=6, 20wks and 24wks n=7. Control ovx BL n=6, 4wks n=7, 8wk, 12wks, 16wks, 20wks, and 24wks n=8. 5IU/kg group BL, 8wks, and 12wks n=9, 4wks and 16wks n=8, 20wks n=6, 24wks n=7. 15 IU/kg group BL, 8wks, and 12wks n=8, 4wks, 20wks, and 24wks n=7, 16wk n=6. 50 IU/kg group BL and 4wks n=9, 8wks, 12wks, 16wks, 20wks, and 24wks n=8.

CHAPTER 4

DISCUSSION

Bone is a dynamic tissue that is constantly balancing its structural and metabolic functions. Bone is continuously undergoing turnover to allow these functions to occur. Bone turnover is a result of the combined processes of bone resorption and bone formation. Bone is constantly being resorbed by osteoclasts resulting in release of essential minerals to perform its metabolic functions. Osteoblasts form new bone to restore the bone mineral necessary to provide adequate strength for its structural function.

In adult humans these processes of bone resorption and bone formation are linked together. As osteoclasts resorb bone, osteotropic factors stored in the bone matrix are released. These factors are responsible for activating osteoblasts to begin the process of bone formation. As the osteoblasts proceed with bone formation, they secrete more osteotropic factors into the newly formed bone matrix. Because of this coupling mechanism in adults, the processes of bone resorption and bone formation change in the same direction and the bone mass remains constant. It these processes become uncoupled, disorders of bone remodeling such as osteoporosis occur whenever bone resorption exceeds bone formation.

Estrogen deficiency is the most common cause of osteoporosis in humans. Calcitonin is one of the medications used to combat the increase in bone resorption induced by estrogen deficiency in postmenopausal women. Calcitonin binds to calcitonin receptors on the surface of the osteoclast. By inhibiting osteoclast activity, calcitonin is effective in limiting estrogen-deficient bone resorption. However, many patients become

resistant to the effects of calcitonin and over a period of time, calcitonin appears to lose it efficacy. Furthermore, increases in BMD following the administration of calcitonin are transient and continued calcitonin administration is sometimes associated with lower BMD. Oversuppression of bone turnover may be responsible for this resistance to calcitonin. It is our hypothesis that high doses of salmon calcitonin can result in oversuppression of bone turnover. We chose to test this hypothesis in female ovariectomized rats.

The ovariectomized rat has emerged as the most common estrogen-deficient animal model used for study of postmenopausal bone loss. A study by Kalu (1991) found many metabolic similarities between ovariectomized rats and postmenopausal women including: increased rate of bone turnover with resorption exceeding formation, greater loss of cancellous than cortical bone, decreased intestinal absorption of calcium, some protection against bone loss by obesity, and similar skeletal response to therapy. Studies by Wronski and others (1991) support the ovariectomized rat as an appropriate animal model for the preclinical evaluation of prophylactic treatments for postmenopausal bone loss. It is evident from the available research that the ovariectomized rat is an appropriate animal model for study of the pathogenesis and treatment of postmenopausal bone loss.

Estrogen-deficient Animal Model

In this study the ovaries were identified bilaterally in each subject. The ovaries of each subject in the control sham group were identified and noted to have adequate blood supply. For the remaining groups bilateral ovaries were successfully excised in each subject and prepared for examination. Microscopic evaluation of excised tissue samples

by an independent pathologist provided confirmation that ovariectomy surgical procedures were properly performed. Additionally, levels of Estradiol, as measured by ELISA, were significantly lower in subjects who were ovariectomized as compared to sham ovariectomized subjects. The differences in levels of Estradiol between ovariectomized and sham ovariectomized subjects along with the confirmation of tissue type excised during the ovariectomy procedures are evidence of the production of an estrogen-deficient animal model appropriate for the study of Type I osteoporosis.

Scanning Electron Microscopy

Bone strength, and ultimately protection against fracture, is dependent on both bone quantity and bone quality. Density and size of bone determine bone quantity. Together these factors constitute mass and determine the strength of bone. Bone mass is measurable by DXA scans. The association between low BMD as detected by DXA, and increased fracture risk has been established, but there is also an association between decreased cortical thickness and increased fracture risk (Cefalu 2004). Although BMD is an important factor for prediction of fractures, it is not the only factor available for assessment of fracture risk.

There is evidence to show that in humans with osteoporosis changes in BMD induced by medications do not parallel changes in fracture risk reduction. For instance, a review of various vertebral fracture trials, as shown in Table 5, shows a range of 1.2-8.3% increase in spine BMD correlating with a 36-49% decrease in vertebral fractures (Faulkner 2000). In addition to BMD other factors may play an important role in decreasing fracture risk. It is indeed noteworthy that in the PROOF study treatment with

calcitonin resulted in an increase in spinal BMD by only 1.2% but reduced vertebral fracture risk by 36% (Chesnut and others 2000), whereas in the FIT II study increases in BMD of 8.3% were associated with a reduction in vertebral fracture of 44%. This suggests that there is no linear correlation between bone mineral density and bone strength and that factors other than BMD may be responsible for the reduced fracture risk.

Table 5 Data from Vertebral Fracture Trials

Vertebral Fracture Trials		
Drug (Trial)	Increase in Spine BMD	Decrease in Vertebral fx
Alendronate (FIT II)	8.3%	44%
Alendronate (FIT I)	7.9%	47%
Risedronate (RVE)	7.1%	49%
Risedronate (RVN)	5.4%	41%
Raloxifene (MORE)	2.6%	40%
Calcitonin (PROOF)	1.2%	36%

Bone architecture plays an important role in the structural quality of bone and must also be considered. Other factors independent of bone mass that influence bone strength include mineralization density, material properties of the organic matrix, and damage state. Remodeling may alter these factors that contribute to bone quality (Trovas and others 2002). Review of data led Trovas and others (2002) to hypothesize that mechanisms other than those associated with an increase in bone mass attribute to the decrease in fracture risk observed with antiresorptive treatments. With accelerated bone turnover bone resorption by osteoclasts exceeds bone formation by osteoblasts. This results in thinning of the trabeculae and even disappearance of some trabeculae resulting

in increased fracture risk (Cefalu 2004). Thinning of the trabeculae can be assessed by BMD measurements and will be addressed at a later point in this study. The disappearance of trabeculae leads to a structurally weak bone, but there are no means presently available to assess these changes in bone architecture.

Evidence of the deleterious effects of ovariectomy on bone is illustrated in the SEM pictures obtained in this study. A decrease in the thickness of the cortical walls is observed in control ovariectomized subjects when compared to control sham subjects. Disappearance of trabeculae results in formation of cavities in the trabecular lattice of the bone. Visual inspection of the bones of control sham subjects reveals a thick, evenly-spaced lattice of trabecular bone. In comparison, obvious cavities and a decrease in the number and density of trabeculae are noted when observing the same area of bone from the control ovariectomized subjects.

The ability of varying doses of calcitonin to attenuate the effects of estrogen deficiency is also evident. The cortical walls of the subjects receiving 5 & 15 IU/kg BW/day of calcitonin are thicker than the cortical walls of the bone of the subjects receiving 50 IU/kg BW/day of calcitonin. Subjects receiving 50 IU/kg BW/day of calcitonin have bone with sparsely displaced, thinner trabeculae than the subjects receiving 5 & 15 IU/kg BW/day. Large cavities are also noted within the trabecular structure of subjects receiving 50 IU/kg BW/day of calcitonin. These observations indicate that smaller doses of calcitonin are more effective than larger doses at preventing estrogen induced bone loss.

With the naked eye similarities are noted between control ovariectomized subjects and subjects receiving 50 IU/kg BW/day of calcitonin. They have thin cortical walls and

decreased number and density of trabeculae. Cavities indicative of disappearance of trabecular bone are also evident and comparable in control ovariectomized subjects and subjects receiving 50 IU/kg BW/day of calcitonin.

Similarities are also visually evident between control sham subjects and those subjects receiving 5 & 15 IU/kg BW/day of calcitonin. The ovariectomized subjects receiving 5 & 15 IU/kg BW/day of calcitonin do not display thinning of the cortical walls and in comparison to the control sham subjects may even show an increase in thickness of the cortical walls. The number and density of trabeculae, as observed with SEM, in the subjects receiving 5 & 15 IU/kg BW/day of calcitonin are more similar to the control sham subjects than the control ovariectomized subjects.

A discrepancy in the ability to prevent or reduce bone loss induced by estrogen deficiency is noted between doses of calcitonin. Larger doses are less efficient than smaller doses at reducing the increased bone resorption. Similarly, smaller doses appear to induce a larger bone mass than larger doses.

One possible reason for this discrepancy between groups is that calcitonin may indirectly inhibit bone formation when given in larger doses. Although the main effect of calcitonin is inhibition of osteoclasts, osteoblasts may be indirectly inhibited as well with administration of calcitonin due to the coupling of osteoclasts and osteoblasts. While this may not be observed with lower doses of calcitonin, this may take place when larger doses of calcitonin are administered, resulting in a reduced bone mass.

Another possible reason for the difference in response with different doses of calcitonin is increased calcium loss induced by the direct physiological effects of calcitonin on the renal tubules as depicted in Fig. 20. By increasing the urinary calcium loss calcitonin may induce a negative calcium balance thus activating the parathyroid glands to produce more PTH. PTH then directly stimulates the recruitment of osteoclasts and their activity thus increasing the rate of bone resorption and mobilization of calcium from the bones to the circulation to compensate for the negative calcium balance. The activation of the renal-parathyroid-bone axis may be responsible for the reduced efficacy of large doses of calcitonin to protect bones from the estrogen deficiency induced increased rate of bone resorption by directly increasing resorption.

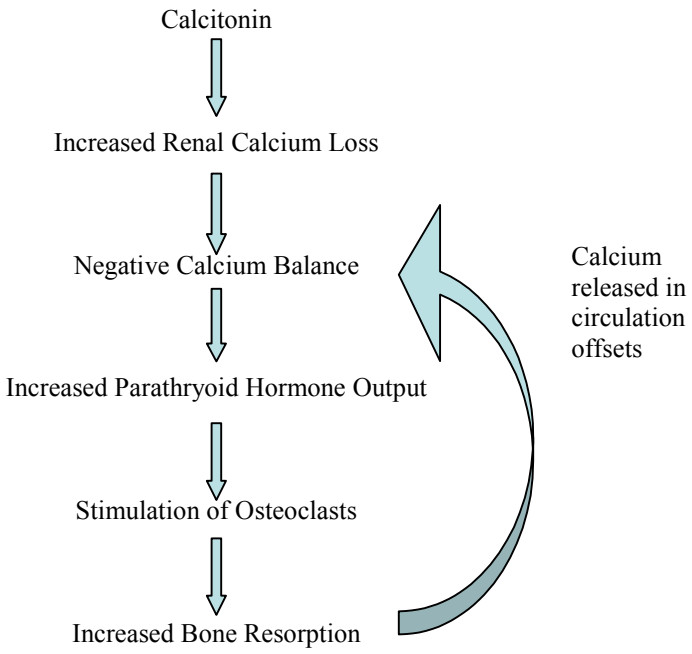


Figure 20 The renal-parathyroid-bone-axis

The direct renal effect of calcitonin is very important in some species of animals. Although in mammals this action is of marginal importance, compared to other factors, in the salmon it is crucial to survival. In the salmon, calcium filters from the water to the blood stream as seawater passes through the gills. When migrating from fresh water to salt water, salmon experience an excessive calcium load. One mechanism salmon use to rid the system of this excessive calcium is calcitonin, which increases the renal calcium loss and, therefore, prevents the accumulation of calcium and the increase in serum calcium.

When administered pharmacologically calcitonin stimulates increased renal calcium excretion probably leading to a negative calcium balance, especially if given in larger doses for long periods of time. In response to this negative calcium balance PTH stimulates osteoclastic bone resorption to restore normal levels of serum calcium. The details of this process will be further discussed in the section regarding urinary calcium excretion. Bone resorption is more evident in the SEM images of subjects receiving 50 IU/kg BW/day of calcitonin than the subjects receiving the lower 5 & 15 IU/kg BW/day doses.

The discrepancy between doses of calcitonin may also be due to production of antibodies to calcitonin. A final possibility for the discrepancy between different doses of calcitonin and the resulting bone mass is that calcitonin may directly stimulate bone formation when given in low doses. The mechanism for this possible action is unknown at this time because osteoblasts do not have calcitonin receptors.

The SEM photographs nevertheless provide proof that larger doses (50 IU/kg BW/day) of calcitonin administered continuously over a 24-week period to

ovariectomized rats are associated with a bone mass not dissimilar from that of ovariectomized subjects receiving placebo. Conversely, smaller doses (5 & 15 IU/kg BW/day) are associated with a bone mass not that dissimilar from sham ovariectomized subjects receiving placebo.

Bone Quality

Bone Densitometry

BMD is a derived measurement calculated by dividing the BMC of the bone scanned by the surface area of the bone. In clinical practice the BMD is the preferred measurement because it adjusts for the size of the bone and makes it possible to compare an individual patient with a cohort reference population of the same sex or same age and sex. While monitoring the patient's response to therapy the BMD also is taken into account rather than the BMC because very little to no change is expected in the bone surface area. In humans there is no further bone growth once the peak bone mass is reached in adulthood.

The experimental subjects used in this study do not reach peak bone mass but continue to grow throughout life provided they remain healthy and receive adequate nourishment. Indeed, they showed continual skeletal growth and weight gain throughout the 24-week study. This increase in weight gain and overall size of subjects may impact on the significance of observed bone mineral density changes. Therefore, the weight adjusted BMC rather than the BMD was chosen for analysis between subjects thus eliminating any variation differences in the area and size of subjects may have produced.

As expected, following 24 weeks of treatment with saline the weight adjusted spinal BMC of the control sham group is significantly higher than the control ovariectomized subjects ($P<0.05$). At baseline no significant differences were noted in weight adjusted spinal BMC measurements between control sham and control ovariectomized subjects. However, at 4 weeks the weight adjusted spinal BMC of control ovariectomized subjects is significantly lower than the control sham subjects ($P<0.05$) and remains significantly lower throughout the 24 weeks of this study. This is expected because of the reduced inhibitory activity of osteoclasts and subsequent increase in bone resorption that occurs as a result of estrogen deficiency following ovariectomy.

The weight adjusted spinal BMC of the subjects receiving lower doses of calcitonin (5 and 15 IU/kg BW/day) showed an initial decrease following ovariectomy. These groups then showed a gradual increase in weight adjusted spinal BMC which was not significantly different from the control sham group after 24 weeks of treatment. This response is indicative of the ability of salmon calcitonin in doses of 5 and 15 IU/kg BW/day to reduce bone loss caused by estrogen deficiency.

In contrast, the weight adjusted spinal BMC of the subjects receiving 50 IU/kg BW/day of calcitonin shows a similar initial decrease at 4 weeks following ovariectomy with a smaller gradual increase at 8 weeks of treatment. The weight adjusted spinal BMC of this group plateaus and even begins to decrease after 8 weeks of treatment. At week 24 the weight adjusted spinal BMC of the subjects receiving 50 IU/kg BW/day of salmon calcitonin remains significantly lower than the control sham group and the 5 IU/kg group but not significantly different from either the 15 IU/kg subjects or the control ovariectomized subjects ($P<0.05$).

The higher doses of calcitonin (50 IU/kg BW/day) unlike the lower ones (5 & 15 IU/kg BW/day) were not able to compensate for the increased bone loss induced by estrogen deficiency. As a result, instead of the bone mass gradually increasing in the subjects receiving higher doses of calcitonin over the 24-weeks observation period, as happened with the lower doses, it was not significantly different from the control ovariectomized subjects. Thus, after an initial increase in week 8, the BMC then gradually decreased in the subjects receiving higher doses of calcitonin. This may be due to oversuppression of bone turnover; increased renal calcium excretion resulting in a negative calcium balance, and subsequent stimulation of PTH secretion and bone resorption; or production of neutralizing antibodies to calcitonin.

In analysis of BMC in this study, the femurs responded differently than the spine. Following ovariectomy experimental subjects also showed a significant decrease in weight adjusted BMC for the femurs as compared to the control sham subjects. After baseline measurements the weight adjusted BMC of the right and left femur of the control ovariectomized subjects remains significantly lower than the subjects in the control sham group ($P<0.05$). The ovariectomized groups receiving varying doses of calcitonin showed a gradual decrease and subsequent plateau in weight adjusted BMC of the right and left femur after ovariectomy. Treatment of the ovariectomized subjects in this study with calcitonin did not yield a significant difference in weight adjusted BMC of the right or left femur between treatment groups during the 24 weeks these subjects were observed.

The differences in response between bone mass in the vertebrae and the femurs are most probably due to the different nature of the bones in both anatomical sites. The

spine is composed primarily of trabecular or cancellous bone that is lost much earlier and more rapidly than cortical bone. This occurs due to trabecular bone being more vascular and more responsive to the body's metabolic needs than cortical bone. Trabecular bone is also more responsive to various therapeutic modalities than cortical bone. Therefore, it is not surprising that in this study the largest differences in BMC between treatment groups is noted in the spine, while the differences found in the right and left femur were less notable.

This data are in agreement with clinical findings. Trovas and others (2002) found treatment with nasal spray salmon calcitonin of men with idiopathic osteoporosis over 12 months led to an increase in lumbar BMD in the calcitonin treatment groups that was significantly greater than that of the group receiving placebo treatment. However, no significant changes were noted in BMD of the femoral neck or greater trochanter between groups after 12 months of treatment. Clinical experience shows that in virtually all studies the vertebral BMD changes are faster and of larger magnitude than femoral BMD changes.

Similarly, data published by Chesnut and others (2000) showed significant increases in BMD of the lumbar spine from baseline in all treatment groups as compared to placebo, but they did not find any clinically significant effect on BMD at the femoral neck or trochanter with treatment over 5 years. In their study involving treatment of postmenopausal women with nasal spray salmon calcitonin, these researchers found a significant decrease in risk of new vertebral fractures in patients receiving 200-IU dose of salmon calcitonin nasal spray as compared to placebo but no difference in hip fractures. Also, no significant difference in vertebral fracture risk was found in patients receiving

the larger 400-IU dose, thus indicating that the lower dose of 200-IU of calcitonin was more effective than the larger 400-IU dose for prevention of new vertebral fractures (Chesnut and others 2000). However, as mentioned earlier, BMD is not the only factor related to increased fracture risk. The architectural structure of the bone also plays an important role in fracture risk.

Other clinical studies have documented that the effect of calcitonin is transient and when continued over a period of time the bone mass may actually decrease. However, in these studies the dose effect of calcitonin was not studied. Furthermore, in many studies there was a high withdrawal rate of patients. For instance, the withdrawal rate of subjects in the PROOF study was 59%. Also, in clinical practice, researchers are unable to control for other variables that may have contributed to the results such as dietary calcium intake, exercise, cigarette smoking, excessive alcohol consumption, and genetics (Chesnut and others 2000). Due to the controlled environment and experimental subjects used, many of these external variables were eliminated in our study.

The subjects receiving 50 IU/kg BW/day of calcitonin were less effective at diminishing the bone resorption effects of post-ovariectomy estrogen deficiency than the subjects receiving 5 & 15 IU/kg BW/day. This is in agreement with SEM findings already discussed and provides further proof that differing doses of calcitonin result in different responses. These significant dose differences noted in this study could be due to several factors including: oversuppression of bone turnover; increased renal loss of calcium leading to a negative calcium balance; or production of neutralizing antibodies to calcitonin.

Urinary Calcium Excretion

Our results show a dose-dependent effect of calcitonin on urinary calcium excretion with the amount of calcium excreted increasing with increased doses of calcitonin. While the ovariectomized treatment groups receiving lower doses of calcitonin did not show a significant difference in total calcium excretion measured in the 24-hour urine samples, the subjects receiving higher doses of calcitonin had a significantly higher total calcium excretion than those receiving lower doses and than the control ovariectomized subjects. This increase in calcium excreted into the urine may be an indicator of increased bone resorption in this group or a direct effect on the renal tubules.

Although calcitonin has a direct inhibitory effect on the osteoclasts, its renal effect of increasing urinary calcium excretion can result in increased PTH mediated bone resorption. As depicted in Fig. 21, along with inhibiting osteoclasts, calcitonin also has a direct effect on the renal tubules to increase urinary calcium excretion. Therefore, continuous calcitonin administration may lead to a negative calcium balance. This negative calcium balance may then lead to an excessive rate of bone resorption in order to maintain the serum calcium level constant. This increased bone resorption may be mediated by the action of PTH released in response to the negative calcium balance. PTH is able to activate osteoclasts and recruit precursors to osteoclasts in order to increase bone resorption and ensure that the serum calcium levels remain within the normal range necessary physiological functions.

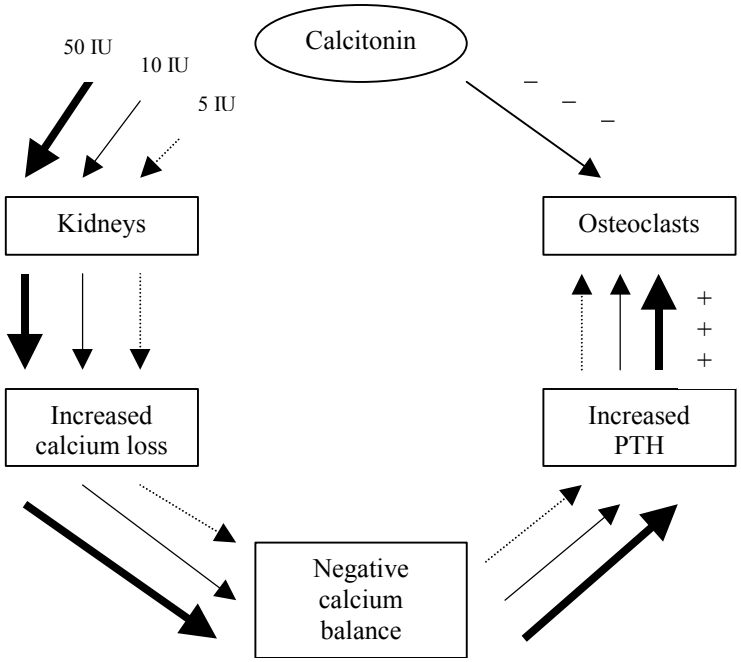


Figure 21 Diagram of multiple dose effects of calcitonin. Calcitonin directly inhibits osteoclast functions and directly stimulates the kidneys to excrete excess calcium. By this direct effect on the kidneys, calcitonin may also cause indirect stimulation of osteoclasts.

The direct effect of calcitonin on urinary calcium excretion could also be responsible for the less effective outcomes seen with higher doses of calcitonin. Indeed, in this study there is evidence of smaller bone mass in the BMC measurements and SEM images of the subjects receiving 50 IU/kg BW/day of calcitonin than the subjects receiving 5 & 15 IU/kg BW/day of calcitonin.

Bone Resorption

Estrogen deficiency is a contributing factor to the development of osteoporosis after menopause (Hartke 1998). With osteoporosis the rate of bone resorption exceeds the rate of bone formation. Use of biochemical markers of bone resorption and bone

formation is a good means to monitor response to antiresorptive therapies such as calcitonin (Delmas 2002). Biochemical markers of bone resorption found in blood and urine are products of collagen breakdown, which reflect bone resorption (Cefalu 2004).

Helical Peptides & Serum Pyridinoline

Amino acids from the stabilizing crosslinks of collagen are released into the blood and urine as bone matrix is degraded (Ott 03/01/02). Thus, levels of helical peptides were measured to assess the amount of bone resorption occurring in the experimental subjects in this study. No consistent significant differences were noted between groups in the measurement of urinary helical peptides. No evidence was obtained from these measurements to support or refute our hypothesis that higher doses of calcitonin resorb bone to a different degree than smaller doses. In fact, no significant change could be observed in any of the groups studied, including 2 control groups and 3 treatment groups.

Pyridinoline is one of the amino acids released from mature collagen into the circulation as bone is degraded and thus serves as a marker for bone resorption (Gomez and others 1996). Results of this study showed fluctuations in serum pyridinoline levels between subjects indicating a slight increase in bone resorption for the first 16 weeks after ovariectomy. However, similar to the findings seen with measurement of urinary helical peptides, no consistent significant differences were noted between groups for measurement of levels of serum pyridinoline during the 24 weeks observed in this study.

In our study, this lack of significant differences between subjects for levels of biochemical markers of bone resorption is not due to the subjects studied. The rat has been established as an appropriate model for evaluation of treatments for postmenopausal

bone loss and has shown many metabolic similarities to postmenopausal women (Kalu 1991; Wronski 1999). It is possible that the quantities of these markers released in the smaller rat subjects were too minute to accurately assess changes. It is evident from the changes in BMC that bone has been resorbed in these subjects, but this resorption was not detected by the assays used for biochemical markers of bone resorption.

Temporary fluctuations in levels of markers of bone turnover can occur without resulting in an overall anatomical change in bone quality or strength. Measurement of biochemical markers of bone turnover to monitor response to therapy is controversial because of high variability (Altkorn and Vokes 2001). Artifactual changes in urinary markers, circadian patterns, and biological variability of individual subjects are limitations associated with measurement of biochemical markers of bone turnover (Khosla and Kleerekoper 1999). Despite collection of a 24-hour urine specimen, normalization for creatinine excretion, and repeated serum collections at 4-week intervals, the possibility of temporary fluctuations in these biochemical markers cannot be eliminated.

Levels of urinary helical peptides and serum pyridinoline are biochemical markers of bone resorption and have been used successfully for assessment of bone resorption in previous studies involving human subjects (Kamel and others 1995; Urena and others 1995; Gomez and others 1996; Visor and others 1996; Ju and others 1997). These markers have been shown to have questionable clinical relevance due to high variability. Chesnut and others (2000) documented only a modest decrease in biochemical markers of bone turnover in the PROOF study despite their findings of relevant changes in spinal BMD and fracture risk between treatment groups. Currently, these markers are most

beneficial as an adjunct to DXA for estimation of fracture risk and should not be used alone for diagnosing osteoporosis or monitoring the long-term response to therapy (Khosla and Kleerekoper 1999). Indeed, these markers are mainly used in large population studies and are not very useful for individual patients. Due to the high variability of these markers, any changes <40% in human subjects are not considered clinically significant. Also, the precision of the measurement of these markers is unknown for this study.

Bone Formation

Osteocalcin

Serum levels of osteocalcin, a product of osteoblasts and a biochemical marker of bone formation, as measured by radioimmunoassay, were not significantly different when comparing area under the curve for control sham and control ovariectomized subjects. However, levels of osteocalcin in this study were significantly higher in subjects receiving 5 IU/kg BW/day of calcitonin as compared to control sham subjects and control ovariectomized subjects ($P<0.05$). Subjects receiving 5 IU/kg BW/day of calcitonin had osteocalcin levels that were significantly higher than the subjects receiving 50 IU/kg BW/day ($P<0.05$) with subjects receiving 15 IU/kg BW/day showing osteocalcin levels between these two groups of subjects but not significantly different from either. This is indicative of an inverse dose-dependent response.

From these findings we conclude that lower doses of calcitonin (5 IU/kg BW/day) may be able to increase the rate of bone formation in ovariectomized subjects after 24

weeks of treatment. The experimental subjects receiving the higher dose of calcitonin, 50 IU/kg BW/day, on the other hand, had lower levels of osteocalcin as compared to treatment groups receiving 5 & 15 IU/kg BW/day, indicating less bone formation in this group of subjects. These subjects had a cumulative measurement similar to and not significantly different from that of the ovariectomized subjects receiving saline.

Previous studies involving use of calcitonin for treatment of osteoporosis have shown decreases in indices of both bone resorption and bone formation. Trovas and others (2002) observed treatment with nasal spray salmon calcitonin over a 12-month period of time resulted in a significant suppression of bone resorption markers and to a lesser extent of bone formation markers in men with idiopathic osteoporosis. A study using human calcitonin for treatment of bone loss in ovariectomized dogs also found lower levels of osteocalcin, a biochemical marker of bone formation, in subjects receiving calcitonin as compared to placebo after 4 months of treatment (Monier-Faugere and others 1996). Chesnut and others (2000) measured significant decreases in serum osteocalcin levels in active treatment groups as compared to baseline but found no significant differences as compared to placebo after 3 years of treatment with salmon calcitonin in postmenopausal women.

Our findings are not in agreement with these results. The differences between our results and the results of the studies by Monier-Faugere and others (1996) and Trovas and others (2002) mentioned above may be attributed to the fact that these studies did not incorporate a dose-response method for analysis of effects of calcitonin. Secondly, the subjects in our study received calcitonin continuously throughout the 24-week study, while the subjects in the above mentioned studies were administered calcitonin

intermittently. Also, whereas most clinical trials involving calcitonin are of short duration, we observed changes in levels of osteocalcin for about 1/6 of the expected lifespan of the subjects utilized in our study. This is equivalent to about 12-13 years in humans. In comparison, the above mentioned studies observed their subjects for a much smaller portion of the expected normal lifespan. Changes in biochemical markers are detected earlier in markers of bone resorption than markers of bone formation. This may explain the lower levels of osteocalcin noted by these researchers after intermediate periods of time.

The cumulative measurements for serum levels of osteocalcin in subjects of this study correlate well with the BMC measurements and SEM observations. The subjects in this study receiving lower doses of calcitonin (5 & 15 IU/kg BW/day) had higher levels of osteocalcin indicating increased bone formation as compared to the subjects receiving 50 IU/kg BW/day of calcitonin. Temporary fluctuations in levels of markers of bone turnover can occur without resulting in an overall anatomical change in bone quality or strength. However, the increase in bone formation found in the subjects receiving 5 and to a lesser extent 15 IU/kg BW/day of calcitonin corresponds with our findings of higher spinal BMC in these subjects as compared to the subjects receiving 50 IU/kg BW/day of calcitonin. This also corresponds with the thicker cortical walls and less trabecular thinning noted with SEM of the subjects receiving 5 and 15 IU/kg BW/day of calcitonin as compared to the subjects receiving the higher 50 IU/kg BW/day dose. This is an indication that the changes in bone formation detected by osteocalcin in this study are related to changes in bone mass.

Our study evaluated only the effects of salmon calcitonin. Other types of calcitonin, including human, porcine, and eel calcitonin, may affect bone turnover differently and produce results different from this study. The magnitude of the renal calciuric effect of calcitonin may be different in other types of calcitonin. Unlike the salmon, which is dependent for its survival on the ability to quickly dispose of larger doses of sodium and calcium, mammals do not have to face this challenge. The calciuric effect of different types of calcitonin may therefore be different.

Another major difference between the methodology of our study and that of studies involving humans is the method of calcitonin administration. The most common use of calcitonin for treatment in human subjects is by nasal spray or injection. Due to the short half-life of calcitonin, these administrations would be considered intermittent. The main action of calcitonin is to inhibit osteoclasts. However, due to the short half-life of calcitonin, intermittent dosing in humans results in short spikes of osteoclastic inhibition. Conversely, the subjects used in our study were administered calcitonin continuously via medication pumps. Continuous administration of calcitonin results in a constant level of calcitonin being present in the bloodstream and continuous inhibition of osteoclasts. In this study, ovariectomized treated subjects received continuous calcitonin for a period of 24 weeks. This could be a further explanation of the differences in response seen with our subjects from other published data involving human subjects.

There is other evidence to support the differences between continuous and intermittent administration of medications. A differential effect is seen with PTH depending on whether administration is continuous or intermittent. If given continuously PTH acts to stimulate osteoclasts resulting in an increase in bone resorption. Indeed,

osteoporosis is a main manifestation of hyperparathyroidism. In comparison, if given intermittently the stimulatory effects on osteoclasts are reduced, while osteoblast activity is increased resulting in a net increase in bone formation and increased bone mass. This paradoxical action has in fact been translated into a therapeutic indication for use of PTH in the management of osteoporosis. It is possible that the paradoxical effects of calcitonin on bone turnover may differ depending on the method of administration and whether the blood levels are continuously or intermittently elevated.

Calcitonin Antibodies

Production of antibodies to calcitonin may play a role in the observed resistance seen with long-term administration of calcitonin for treatment of disorders of bone remodeling (Watts 1999). No significant differences were noted in this study in the level of calcitonin antibodies measured in subjects receiving calcitonin as compared to subjects receiving saline over a period of 24 weeks. Similarly, there was no significant difference in the level of calcitonin antibodies measured between groups regardless of the dosage of calcitonin administered or the length of time calcitonin was administered. This would suggest that in our study production of antibodies to calcitonin does not play a significant role in the reduced ability of larger doses of calcitonin to lessen the impact of estrogen deprived increased bone resorption.

These findings are in agreement with other researchers who found no apparent relationship between the presence of calcitonin antibodies and changes in levels of total body calcium or clinical responsiveness to calcitonin (Gruber and others 1984; Overgaard and others 1990). Similarly, Chesnut and others (2000) detected high titers of antibodies

to calcitonin among treatment groups receiving calcitonin but the presence of these antibodies did not influence the effect of salmon calcitonin on risk reduction of new vertebral fractures.

The formation of antibodies against heterologous calcitonin occurs frequently with use of calcitonin for treatment of osteoclast-mediated bone resorption. However, not all subjects expressing antibodies to calcitonin develop a secondary resistance to calcitonin. Therefore, the clinical significance of antibodies to salmon calcitonin is controversial (Grauer and others 1995). Antibodies to salmon calcitonin have been detected in human subjects but did not interfere with the efficacy of long-term therapy for treatment of Paget's disease (Singer and Krane 1990). In review of the clinical course of nine patients with Paget's disease treated with salmon calcitonin, Grauer and others (1990) documented 3 patients with ^{125}I-salmon calcitonin binding antibodies but no neutralizing antibodies and no clinical resistance.

Similar results were found by Hosking and others (1979) who observed development of antibodies to salmon calcitonin in 8 of 20 patients treated for 6 months with salmon calcitonin. Despite the presence of these antibodies, the acute hypocalcemic response to salmon calcitonin was not diminished in relation to bone turnover. These findings indicate that antibodies that develop in response to salmon calcitonin therapy do not necessarily have a neutralizing action that is functionally effective. Similarly, findings from this study indicate that production of antibodies to calcitonin does not play a significant role in the development of resistance to calcitonin.

Conclusions

In this study lower doses of calcitonin were more effective at reducing the impact of estrogen deficiency on bone mass as evidenced by SEM and DXA. SEM provided evidence that lower doses of salmon calcitonin are more effective than higher doses in attenuating the architectural changes in bone structure induced by estrogen deficiency. At the end of the study weight adjusted spinal BMC of subjects receiving lower doses of calcitonin were not significantly different from control sham subjects. However, subjects receiving higher doses of calcitonin had significantly lower weight adjusted spinal BMC measurements than control sham subjects and not dissimilar from the ovariectomized nontreated subjects, indicating that lower doses are more effective than higher doses for reduction of bone loss caused by estrogen deficiency.

Results of our study do not support the hypothesis that higher doses of calcitonin oversuppress bone turnover. There were no significant differences noted in the markers of bone resorption in any of the studied groups, and larger doses of calcitonin did not have a different effect than lower doses on bone resorption. Calcitonin, therefore, did not oversuppress bone resorption.

There is also no evidence to support that higher doses of calcitonin reduce bone formation. Indeed, subjects receiving higher doses of calcitonin had similar levels of markers of bone formation as sham operated subjects. Calcitonin, therefore, did not oversuppress bone formation.

The decrease in therapeutic effectiveness, therefore, cannot be due to oversuppression of bone resorption, bone formation, or bone turnover. The lack of

significant increase in antibodies mitigates against these antibodies being responsible for the reduced effectiveness of higher doses of calcitonin or of prolonged treatment in humans.

This study on the other hand has documented a well-known effect of calcitonin to increase renal calcium excretion. Larger doses of calcitonin resulted in an increase in urinary calcium excretion in a dose-dependent manner. This increase in urinary calcium excretion results in a negative calcium balance. In response, PTH is increased resulting in increased osteoclast recruitment and activity. It is probable that this excessive recruitment, mobilization, and activity of osteoclasts cannot be counteracted by calcitonin. In these subjects not only are the osteoclasts released from the inhibitory activity of estrogen, but they are actively recruited and energized by the increase in PTH secretion. It is likely in these subjects that the life-preserving importance of maintaining calcium homeostasis takes precedence over the relatively less important function of maintaining bone mass.

Future Directions

This study has shown that higher doses of calcitonin are less efficient at protecting ovariectomized rats from the deleterious effects of estrogen deprivation and increased rate of bone resorption. Indicators of bone quality, including weight adjusted BMC and SEM images, provide evidence that although lower doses of calcitonin are effective in attenuating the effects of estrogen deficiency, higher doses of calcitonin are detrimental to bone mass and quality. Our results show that in ovariectomized rats salmon calcitonin affects renal calcium excretion in a dose-dependent manner. It is,

nevertheless, not known whether other classes of calcitonin, such as human, porcine, and eel, have a similar effect. Different types of calcitonin may affect the renal calcium excretion in differing magnitudes resulting in different impacts on calcium homeostasis. Each class of calcitonin would need to be assessed to determine if the effects of calcitonin on renal calcium excretion are similar or if the renal-parathyroid-bone axis responds differently with different types of calcitonin. Therefore, it may be worthwhile to further explore the differences that would occur with different species of calcitonin.

To complement these findings further examination of the effects of intermittent vs. continuous administration of calcitonin may yield beneficial clinical information. Due to calcitonin being administered intermittently for clinical use further investigation of the differences between continuous and intermittent administration of calcitonin would be important. It is also not known whether there are differences in renal calcium excretion with continuous vs. intermittent administration of calcitonin. Indeed, it is possible that when administration is intermittent the renal calcium excretion is more limited resulting in lesser ability to activate the renal-parathyroid-bone axis. We need further studies to clarify if the renal calcium excretion response elicits a differential effect depending on whether calcitonin administration is continuous or intermittent.

To further assess the renal-parathyroid-bone axis levels of ionized serum calcium need to be monitored. Further assessment of the renal-parathyroid-bone axis response to calcitonin would include monitoring of serum PTH levels and visual examination of the parathyroid glands to determine response to treatment. Due to the limitations with biochemical markers of bone turnover measurement of bone resorption was difficult.

The use of different markers of bone resorption would be useful to determine a more appropriate marker for use with experimental animals.

Further measurement of bone formation can be achieved by analysis of tetracycline double-labeled bones. The right femur of each subject that completed this 24-week study has been processed for analysis of dynamic bone growth by measurement of double tetracycline labels. Results of these measurements may provide further evidence of the effects of calcitonin on osteoblastic activity. We plan to perform these studies in the future.

Bone strength is the ultimate proof of the efficacy of therapeutic interventions in treatment of osteoporotic bone loss. Initially, we had planned to assess bone strength with three-point bending analysis. However, difficulty achieving consistent placement of stress during three-point bending tests in long bones during the pilot study preceding this study led us not to include biomechanical strength testing in the current study. Due to prevention of fracture being one of the primary benefits of calcitonin in the treatment of osteoporosis patients investigation of more consistent techniques and apparatus for these measurements would be beneficial. Establishment of equipment that would allow for consistent placement of stress for three-point bending tests is essential. Ability to center the samples precisely for placement of central force exactly at midline would improve the reliability of the results of this type of testing. Assessment of the amount of compressive force required to produce vertebral fractures would also lead to probable beneficial clinical information. Equipment that would apply measurable forces along consistent areas of the bone until compression resulted in fracture would yield valid results of bone strength. Bones of the lumbar spine, the right tibia, and the 12^{th} rib of subjects

completing this 24-week study have been stored to allow for performance of these tests in the future.

CHAPTER 5

SUMMARY AND CONCLUSIONS

1. Successful bilateral ovariectomy produces an estrogen deficient animal model appropriate for study of Type I osteoporosis.
2. Changes in bone densitometry measurements with treatment after ovariectomy are evident more rapidly in the spine than the femurs.
3. Weight adjusted increase in spinal BMC of control ovariectomized subjects and ovariectomized subjects receiving higher doses (50 IU/kg BW/day) of calcitonin were significantly lower that of control sham subjects.
4. Subjects receiving lower doses (5 & 15 IU/kg BW/day) of calcitonin were not significantly different from the control sham subjects after 24 weeks of treatment. These findings suggest that lower doses of calcitonin are more effective for treatment of estrogen deficient bone loss than higher doses.
5. Salmon calcitonin resulted in increased bone formation when administered over 24 weeks in ovariectomized rats with this response occurring in an inverse dose-dependent manner.
6. Biochemical markers of bone resorption used in this study (urinary helical peptides and serum pyridinoline) are inconclusive in experimental rats.
7. Production of antibodies to calcitonin does not correlate with changes in bone turnover or bone density, and, therefore, increased production of calcitonin antibodies is not responsible for the blunting effect of larger doses seen with administration of calcitonin over 24 weeks in rats.

8. Anatomical changes in bone quality are evident with SEM following ovariectomy. Differing doses of calcitonin are able to attenuate these changes in varying degrees. Subjects receiving higher doses of calcitonin display bones with fewer and less dense trabeculae as well as thinner cortical walls than subjects receiving lower doses of calcitonin. This is also indicative of lower doses of calcitonin being more effective than higher doses in treatment of bone loss due to estrogen deficiency.
9. Calcitonin appears to affect calcium homeostasis in a dose-dependent manner. The direct effect of calcitonin on urinary calcium excretion may be responsible for the less effective outcomes seen with higher doses of calcitonin, probably through a PTH-mediated increase in renal calcium excretion.
10. Calcitonin does not oversuppress bone turnover.

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APPENDICES

APPENDIX A

Data Tables

Appendix A-1: Estradiol (pg/ml)

Control sham			
Subject #	1 wk	4 wks	24 wks
1	27.725	20.50	0.088
2	57.77	6.91	17.555
3	19.21	19.86	2.97
4	73.275	50.59	*
5	24.515	36.95	5.96
6	19.245	16.39	26.315
7	62.52	11.41	4.02
8	32.92	19.24	4.755
Mean	39.6475	22.73	8.809
SD	21.4899	14.25416	9.4920
SEM	7.59786	5.03961	3.5876
Control ovx			
Subject #			
9	77.49	21.585	2.165
10	49.35	5.04	0.89
11	66.29	6.315	1.57
12	36.66	11.24	4.14
13	59.32	107.755	0.2
14	49.535	26.79	4.22
15	57.76	121.29	6.495
16	11.965	23.52	9.345
Mean	51.0463	40.44188	3.6281
SD	19.9451	46.54716	3.0970
SEM	7.05165	16.45691	1.095
5 IU/kgBW/day			
Subject #			
17	89.86	7.48	6.36
18	35.205	20.055 *	
19	47.625	32.84 *	
20	104.915	13.235	3.015
21	120.895	19.565	5.525
22	48.33	21.37	3.825
23	86.445	66.495	2.8
24	27.805	15.09	2.905
42	15.805	131	5.22
Mean	64.0983	36.34778	4.2357
SD	37.1806	39.48274	1.4511
SEM	12.3935	13.16091	0.5485
15 IU/kgBW/day			
Subject #			
25	94.2	33.33	1.97
26	16.9	25.3	0.965
27	59.09	37.53	9.695
28	35.975	17.54 *	
29	108.955	15.505 *	
30	21.36	47.28	8.64
31	113.375	8.275 *	
32	33.53	17.33	3.055
Mean	60.4231	25.26125	4.865
SD	39.7187	13.12954	4.0139
SEM	11.8547	6.12708	1.3662

50 IU/kgBW/day			
Subject #	1 wk	4 wks	24 wks
33	83.32	24.04	1.495
34	15.005	27.975	2.165
35	126.75	100.28	1.13
36	71.025	13.54	2.42
37	73.23	56.835	1.165
38	46.535	15.795	2.22
39	54.26	14.93	2.245
40	54.345	113.13	*
41	36.055	12.38	6.22
Mean	62.2856	42.1	2.3825
SD	31.8067	39.213	1.6327
SEM	10.6022	13.071	0.5772

Appendix A-2: FSH (mIU/ml)

Control sham	1 weeks	4 weeks	24 weeks
1	5.315	6.11	4.815
2	4.86	9.335	4.32
3	4.08	5.06	4.24
4	4.37	5.24	*
5	4.08	5.24	4.155
6	4.03	4.775	4.365
7	4.17	5.94	4.565
8	4.37	6.065	4.24
Mean	4.409375	5.970625	4.385714
SD	0.454269	1.447889	0.229808
SEM	0.160608	0.511906	0.086859
Control ovx			
9	4.485	5.23	4.205
10	4.36	5.525	4.605
11	4.975	6.53	4.565
12	4.405	6.065	4.525
13	4.25	3.955	4.775
14	4.48	4.565	5.065
15	4.77	4.44	4.485
16	4.94	4.69	5.065
Mean	4.583125	5.125	4.66125
SD	0.274798	0.875834	0.295003
SEM	0.097156	0.309654	0.104299
5 IU/kgBW/day			
17	5.73	4.775	4.485
18	4.975	5.52	*
19	4.71	5.785	*
20	4.28	4.77	4.85
21	4.275	6.32	10.295
22	4.61	5.445	4.525
23	4.32	6.57	4.36
24	4.98	5.025	3.95
42	4.605	6.53	4.155
Mean	4.720556	5.637778	5.231429
SD	0.464996	0.713806	2.250984
SEM	0.154999	0.237935	0.850792
15 IU/kgBW/day			
25	4.07	6.87	4.24
26	4.855	5.27	4.195
27	5.15	6.52	4.695
28	4.735	4.935	4.155
29	4.445	5.905	4.61
30	4.235	5.68	4.61
31	4.605	5.52	*
32	4.855	5.695	4.08
Mean	4.61875	5.799375	4.369286
SD	0.355796	0.633276	0.25777
SEM	0.125793	0.223897	0.097428
50 IU/kgBW/day			
33	4.66	8.14	4.075
34	5.275	6.28	4.4
35	4.48	4.65	4.61
36	4.11	5.36	4.565
37	4.155	5.985	4.69
38	4.9	5.655	4.57
39	4.61	5.1	4.855
40	4.44	6.74	*
41	4.815	5.27	4.405
Mean	4.605	5.908889	4.52125
SD	0.366308	1.050306	0.232805
SEM	0.122103	0.350102	0.082309

Appendix A-3: Total body BMD (g/cm^2)

Control sham	Baseline	4 wks	8wks	12wks	16wks	20 wks	24wks
1	0.195	0.181	0.187	0.205	0.202	0.214	0.212
2	0.225	0.191	0.199	0.184	0.2	0.219	0.191
3	0.163	0.192	0.194	0.203	0.202	0.22	0.203
4	0.176	0.186	0.193	0.189	*	*	*
5	0.162	0.19	0.189	0.202	0.2	0.221	0.217
6	0.194	0.185	0.21	0.196	0.194	0.206	0.215
7	0.196	0.179	0.202	0.2	0.197	0.195	0.219
8	0.192	0.2	0.19	0.182	0.192	0.192	0.201
Mean	0.187875	0.188	0.1955	0.195125	0.198143	0.209571	0.208286
SD	0.020629	0.006719	0.007728	0.008983	0.003934	0.012122	0.010242
SEM	0.007293	0.002375	0.002732	0.003176	0.001487	0.004582	0.003871
Control ovx							
9	0.191	0.193	0.197	0.21	0.202	0.212	0.219
10	0.208	0.177	0.204	0.214	0.209	0.202	0.212
11	0.16	0.194	0.181	0.208	0.21	0.2	0.201
12	0.174	0.186	0.189	0.223	0.208	0.22	0.218
13	0.166	0.192	0.179	0.199	0.199	0.231	0.22
14	0.196	0.203	0.209	0.208	0.219	0.216	0.206
15	0.195	0.2	0.182	0.201	0.206	0.213	0.216
16	0.186	0.182	0.197	0.195	0.216	0.231	0.211
Mean	0.1845	0.190875	0.19225	0.20725	0.208625	0.215625	0.212875
SD	0.01644	0.00879	0.011222	0.00894	0.006632	0.0116	0.006728
SEM	0.005813	0.003108	0.003968	0.003379	0.002345	0.004101	0.002379
5 IU/kgBW/day							
17	0.174	0.201	0.202	0.188	0.201	0.197	0.202
18	0.189	0.192	0.19	0.2	0.202	*	*
19	0.174	0.195	0.204	0.192	*	*	*
20	0.195	0.191	0.195	0.214	0.201	0.202	0.219
21	0.195	0.195	0.21	0.207	0.196	0.211	0.206
22	0.197	0.208	0.2	0.209	0.207	0.201	0.223
23	0.172	0.188	0.197	0.2	0.201	0.201	0.206
24	0.271	0.179	0.192	0.214	0.2	0.203	0.21
42	0.178	0.196	0.191	0.199	0.207	0.196	0.205
Mean	0.193889	0.193889	0.197889	0.202556	0.201875	0.201571	0.210143
SD	0.030629	0.0081	0.006698	0.009167	0.003643	0.004894	0.007862
SEM	0.01021	0.0027	0.002233	0.003056	0.001288	0.00185	0.002972
15 IU/kgBW/day							
25	0.173	0.182	0.191	0.195	0.19	0.199	0.206
26	0.188	0.2	0.197	0.227	0.226	0.202	0.208
27	0.178	0.192	0.193	0.197	0.198	0.208	0.215
28	0.18	0.19	0.191	0.197	0.205	0.201	0.206
29	0.19	0.207	0.195	0.21	0.211	0.219	0.22
30	0.182	0.199	0.195	0.2	0.216	0.197	0.21
31	0.201	0.182	0.204	0.193	0.201	*	*
32	0.198	0.182	0.191	0.196	0.202	0.209	0.217
Mean	0.188143	0.193143	0.195143	0.202857	0.208429	0.206	0.212667
SD	0.008877	0.009424	0.004488	0.011936	0.009914	0.007797	0.005502
SEM	0.003139	0.003332	0.001587	0.00422	0.003505	0.002947	0.002079
50 IU/kgBW/day							
33	0.186	0.204	0.194	0.185	0.211	0.218	0.211
34	0.203	0.18	0.186	0.207	0.2	0.212	0.215
35	0.194	0.2	0.193	0.207	0.204	0.201	0.201
36	0.187	0.199	0.193	0.199	0.212	0.193	0.208
37	0.195	0.202	0.2	0.189	0.2	0.195	0.205
38	0.18	0.189	0.178	0.187	0.2	0.192	0.206
39	0.191	0.188	0.188	0.191	0.2	0.195	0.193
40	0.189	0.192	*	*	*	*	*
41	0.186	0.205	0.188	0.2	0.193	0.202	0.207
Mean	0.190625	0.194375	0.189429	0.197143	0.201286	0.198571	0.205
SD	0.006906	0.008501	0.006876	0.008295	0.005736	0.007044	0.006758
SEM	0.002302	0.002834	0.002431	0.002933	0.002028	0.00249	0.002389

Appendix A-4 Total body BMD % change from baseline

Control sham		4 wks	8 wks	12 wks	16 wks	20 wks	24 wks
	1	-7.17949	-4.10256	4.878049	3.589744	9.74359	8.717949
	2	-15.1111	-11.5556	-22.2826	-11.1111	-2.66667	-15.1111
	3	17.79141	19.0184	19.70443	23.92638	34.96933	24.53988
	4	5.681818	9.659091	6.878307	*	*	*
	5	17.28395	16.66667	19.80198	23.45679	36.41975	33.95062
	6	-4.63918	8.247423	1.020408	0	6.185567	10.82474
	7	-8.67347	3.061224	2	0.510204	-0.5102	11.73469
	8	4.166667	-1.04167	-5.49451	0	0	4.6875
Mean		1.165075	4.994128	3.313258	5.76743	12.02019	11.3349
SD		12.13434	10.43403	13.60364	13.07959	16.72536	15.47412
SEM		4.290138	3.688987	4.809614	4.943619	6.321593	5.848667
Control ovx							
	9	1.04712	3.141361	9.047619	5.759162	10.99476	14.65969
	10	-14.9038	-1.92308	2.803738	0.480769	-2.88462	1.923077
	11	21.25	13.125	23.07692	31.25	25	25.625
	12	6.896552	8.62069	21.97309	19.54023	26.43678	25.28736
	13	15.66265	7.831325	16.58291	19.87952	39.15663	32.53012
	14	3.571429	6.632653	5.769231	11.73469	10.20408	5.102041
	15	2.564103	-6.66667	2.985075	5.641026	9.230769	10.76923
	16	-2.15054	5.913978	4.615385	16.12903	24.19355	13.44086
Mean		4.242184	4.584408	10.85675	13.8018	17.79149	16.16717
SD		11.00422	6.287803	8.456776	9.947929	13.24878	10.7208
SEM		3.89058	2.223074	3.196361	3.517124	4.68415	3.790375
5 IU/kgBW/day							
	17	15.51724	16.09195	7.446809	15.51724	13.21839	16.09195
	18	1.587302	0.529101	5.5	6.878307	*	*
	19	12.06897	17.24138	9.375	*	*	*
	20	-2.05128	0	8.878505	3.076923	3.589744	12.30769
	21	0	7.692308	5.797101	0.512821	8.205128	5.641026
	22	5.583756	1.522843	5.741627	5.076142	2.030457	13.19797
	23	9.302326	14.53488	14	16.86047	16.86047	19.76744
	24	-33.9483	-29.1513	-26.6355	-26.1993	-25.0923	-22.5092
	42	10.11236	7.303371	10.55276	16.29213	10.11236	15.16854
Mean		2.019148	3.973839	4.517366	4.751846	4.132042	8.523628
SD		14.70094	14.09904	12.00077	14.03599	13.87863	14.34906
SEM		4.900313	4.699681	4.000257	4.962472	5.24563	5.423436
15 IU/kgBW/day							
	25	5.202312	10.40462	12.71676	9.82659	15.0289	19.07514
	26	6.382979	4.787234	20.74468	20.21277	7.446809	10.6383
	27	7.865169	8.426966	10.67416	11.23596	16.85393	20.78652
	28	5.555556	6.111111	9.444444	13.88889	11.66667	14.44444
	29	8.947368	2.631579	10.52632	11.05263	15.26316	15.78947
	30	9.340659	7.142857	9.89011	18.68132	8.241758	15.38462
	31	-9.45274	1.492537	-3.9801	0	*	*
	32	-8.08081	-3.53535	-1.0101	2.020202	5.555556	9.59596
Mean		3.220062	4.682694	8.625784	10.86479	11.43668	15.10206
SD		7.554924	4.420476	7.79596	7.122893	4.428313	4.068693
SEM		2.671069	1.562874	2.756288	2.518323	1.673745	1.537822
50 IU/kgBW/day							
	33	9.677419	4.301075	-0.54054	13.44086	17.2043	13.44086
	34	-11.33	-8.37438	1.932367	-1.47783	4.433498	5.91133
	35	3.092784	-0.51546	6.280193	5.154639	3.608247	3.608247
	36	6.417112	3.208556	6.030151	13.36898	3.208556	11.22995
	37	3.589744	2.564103	-3.1746	2.564103	0	5.128205
	38	5	-1.11111	3.743316	11.11111	6.666667	14.44444
	39	-1.57068	-1.57068	0	4.712042	2.094241	1.04712
	40	1.587302	*	*	*	*	*
	41	10.21505	1.075269	7	3.763441	8.602151	11.29032
Mean		2.964298	-0.05283	2.65886	6.579668	5.727208	8.26256
SD		6.52786	3.974925	3.710294	5.453737	5.336909	4.958123
SEM		2.175953	1.405348	1.311787	1.928187	1.886882	1.752961

Appendix A-5: Weight adjusted spinal BMC (g/kg)

Control sham	Baseline	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks
1	0.0063	0.0069	0.0064	0.00715	0.0074	0.00675	0.0082
2	0.0029	0.0068	0.0066	0.0074	0.0072	0.00725	0.0065
3	0.0073	0.0075	0.00755	0.00805	0.00715	0.0072	0.0071
4	0.0064	0.00705	0.00825	0.0076	*	*	*
5	0.0073	0.0079	0.0077	0.0082	0.0081	0.007	0.0074
6	0.0076	0.00765	0.008	0.00775	0.00855	0.0074	0.0079
7	0.0065	0.0073	0.0068	0.0065	0.0075	0.00675	0.007
8	0.0055	0.00735	0.00675	0.00655	0.0071	0.00725	0.0068
Mean	0.006225	0.007306	0.007256	0.0074	0.007571	0.007085714	0.007271
SD	0.001507	0.000377	0.000702	0.000635	0.000549	0.000257737	0.000605
SEM	0.000533	0.000133	0.000248	0.000224	0.000208	9.74156E-05	0.000229
Control ovx							
9	0.0064	0.00625	0.0062	0.00635	0.00645	0.0069	0.0064
10	0.0068	0.0064	0.00675	0.00695	0.00775	0.007	0.0063
11	0.0072	0.0067	0.00605	0.0063	0.0065	0.00675	0.0064
12	0.0075	0.0059	0.0063	0.0062	0.006	0.0062	0.0064
13	0.0079	0.0063	0.006	0.0062	0.0065	0.0064	0.0066
14	0.0072	0.00475	0.00625	0.00645	0.00675	0.0064	0.0058
15	0.0066	0.006	0.00595	0.0074	0.0069	0.00605	0.0071
16	0.007	0.0061	0.00625	0.00665	0.0068	0.00665	0.0069
Mean	0.007075	0.00605	0.006219	0.006563	0.006706	0.00654375	0.006488
SD	0.000486	0.000581	0.000251	0.000422	0.000505	0.000336407	0.000394
SEM	0.000172	0.000206	8.86E-05	0.000149	0.000179	0.000118938	0.000139
5 IU/kgBW/day							
17	0.0074	0.0066	0.0062	0.00605	0.00635	0.00635	0.0063
18	0.0066	0.006	0.00655	0.0068	0.0068	*	*
19	0.0065	0.00585	0.0069	0.00645	*	*	*
20	0.0076	0.0066	0.0069	0.007	0.0068	0.00705	0.0071
21	0.0069	0.00655	0.00635	0.0063	0.00725	0.00695	0.0072
22	0.0075	0.0063	0.00675	0.0077	0.0079	0.00755	0.0077
23	0.0078	0.0051	0.0064	0.0066	0.0069	0.0067	0.0067
24	0.0034	0.007	0.0067	0.0072	0.00725	0.0074	0.0077
42	0.0073	0.00625	0.0067	0.0065	0.0067	0.00615	0.0068
Mean	0.006778	0.00625	0.006606	0.006733	0.006994	0.006878571	0.007071
SD	0.005111	0.004522	0.004321	0.004172	0.004364	0.004406026	0.004356
SEM	0.001704	0.001507	0.00144	0.001391	0.001543	0.001665321	0.001646
15 IU/kgBW/day							
25	0.007	0.0066	0.0063	0.0064	0.00575	0.00655	0.0066
26	0.0072	0.0066	0.0063	0.0069	0.00705	0.00665	0.0061
27	0.007	0.00655	0.0068	0.007	0.00625	0.00675	0.0074
28	0.0075	0.0067	0.0065	0.0062	0.0068	0.0069	0.0069
29	0.0068	0.00655	0.00635	0.00635	0.0065	0.0065	0.0066
30	0.0071	0.00665	0.0069	0.007	0.0068	0.00695	0.0072
31	0.0074	0.0068	0.00715	0.0074	0.0068	*	*
32	0.0054	0.00665	0.0069	0.0062	0.00625	0.0063	0.0072
Mean	0.006925	0.006638	0.00665	0.006681	0.006525	0.006657143	0.006857
SD	0.000656	8.35E-05	0.000328	0.00045	0.000423	0.000229907	0.000454
SEM	0.000232	2.95E-05	0.000116	0.000159	0.00015	8.68966E-05	0.000172
50 IU/kgBW/day							
33	0.0061	0.00665	0.0074	0.0071	0.0069	0.00665	0.007
34	0.0073	0.0064	0.0073	0.00625	0.00715	0.0062	0.0072
35	0.0068	0.00635	0.0072	0.0071	0.00625	0.0065	0.0066
36	0.0066	0.0061	0.00725	0.0073	0.0078	0.0065	0.0068
37	0.0082	0.00695	0.00675	0.00685	0.00755	0.00655	0.0064
38	0.0065	0.00625	0.0073	0.0071	0.0063	0.00615	0.0061
39	0.0074	0.0058	0.00605	0.0064	0.0064	0.0066	0.0067
40	0.006	0.0061	*	*	*	*	*
41	0.0073	0.0069	0.00635	0.0062	0.00645	0.0071	0.0071
Mean	0.006911	0.006389	0.00695	0.006788	0.00685	0.00653125	0.006738
SD	0.000704	0.000384	0.000508	0.000438	0.000599	0.000292694	0.00037
SEM	0.000235	0.000128	0.00018	0.000155	0.000212	0.000103483	0.000131

Appendix A-6: Weight adjusted spinal BMC % change from baseline

Control sham		%chg4wk	%chg 8wk	%chg12wk	%chg16wk	%chg20wk	%chg24wk
	1	9.52381	1.587302	13.49206	17.46032	7.142857	30.15873
	2	134.4828	127.5862	155.1724	148.2759	150	124.1379
	3	2.739726	3.424658	10.27397	-2.05479	-1.36986	-2.73973
	4	10.15625	28.90625	18.75	*	*	*
	5	8.219178	5.479452	12.32877	10.9589	-4.10959	1.369863
	6	0.657895	5.263158	1.973684	12.5	-2.63158	3.947368
	7	12.30769	4.615385	0	15.38462	3.846154	7.692308
	8	33.63636	22.72727	19.09091	29.09091	31.81818	23.63636
Mean		26.46546	24.94871	28.88523	33.08797	26.38517	26.88612
SD		44.77639	42.66834	51.49638	51.62434	55.87286	44.53939
SEM		15.83085	15.08554	18.20672	19.51217	21.11796	16.83431
Control ovx							
	9	-2.34375	-3.125	-0.78125	0.78125	7.8125	0
	10	-5.88235	-0.73529	2.205882	13.97059	2.941176	-7.35294
	11	-6.94444	-15.9722	-12.5	-9.72222	-6.25	-11.1111
	12	-21.3333	-16	-17.3333	-20	-17.3333	-14.6667
	13	-20.2532	-24.0506	-21.519	-17.7215	-18.9873	-16.4557
	14	-34.0278	-13.1944	-10.4167	-6.25	-11.1111	-19.4444
	15	-9.09091	-9.84848	12.12121	4.545455	-8.33333	7.575758
	16	-12.8571	-10.7143	-5	-2.85714	-5	-1.42857
Mean		-14.0916	-11.705	-6.65289	-4.6567	-7.03268	-7.86046
SD		10.49405	7.461026	11.0297	11.33118	9.19584	9.309353
SEM		3.710206	2.637871	3.899589	4.006178	3.25122	3.291353
5 IU/kgBW/day							
	17	-10.8108	-16.2162	-18.2432	-14.1892	-14.1892	-14.8649
	18	-9.09091	-0.75758	3.030303	3.030303	*	*
	19	-10	6.153846	-0.76923	*	*	*
	20	-13.1579	-9.21053	-7.89474	-10.5263	-7.23684	-6.57895
	21	-5.07246	-7.97101	-8.69565	5.072464	0.724638	4.347826
	22	-16	-10	2.666667	5.333333	0.666667	2.666667
	23	-34.6154	-17.9487	-15.3846	-11.5385	-14.1026	-14.1026
	24	105.8824	97.05882	111.7647	113.2353	117.6471	126.4706
	42	-14.3836	-8.21918	-10.9589	-8.21918	-15.7534	-6.84932
Mean		-0.80541	3.654382	6.168366	10.27478	9.679478	13.01277
SD		10.26791	13.33185	15.65735	12.86607	12.33223	12.83838
SEM		3.422636	4.443951	5.219116	4.548842	4.661145	4.852453
15 IU/kgBW/day							
	25	-5.71429	-10	-8.57143	-17.8571	-6.42857	-5.71429
	26	-8.33333	-12.5	-4.16667	-2.08333	-7.63889	-15.2778
	27	-6.42857	-2.85714	0	-10.7143	-3.57143	5.714286
	28	-10.6667	-13.3333	-17.3333	-9.33333	-8	-8
	29	-3.67647	-6.61765	-6.61765	-4.41176	-4.41176	-2.94118
	30	-6.33803	-2.8169	-1.40845	-4.22535	-2.11268	1.408451
	31	-8.10811	-3.37838	0	-8.10811	*	*
	32	23.14815	27.77778	14.81481	15.74074	16.66667	33.33333
Mean		-3.26466	-2.9657	-2.91034	-5.12407	-2.21381	1.217547
SD		10.87069	13.12889	9.171151	9.753428	8.600702	15.67421
SEM		3.843368	4.641764	3.242492	3.448358	3.25076	5.924294
50 IU/kgBW/day							
	33	9.016393	21.31148	16.39344	13.11475	9.016393	14.7541
	34	-12.3288	0	-14.3836	-2.05479	-15.0685	-1.36986
	35	-6.61765	5.882353	4.411765	-8.08824	-4.41176	-2.94118
	36	-7.57576	9.848485	10.60606	18.18182	-1.51515	3.030303
	37	-15.2439	-17.6829	-16.4634	-7.92683	-20.122	-21.9512
	38	-3.84615	12.30769	9.230769	-3.07692	-5.38462	-6.15385
	39	-21.6216	-18.2432	-13.5135	-13.5135	-10.8108	-9.45946
	40	1.666667	*	*	*	*	*
	41	-5.47945	-13.0137	-15.0685	-11.6438	-2.73973	-2.73973
Mean		-6.89225	0.051267	-2.34837	-1.87594	-6.37951	-3.35386
SD		9.044456	14.89051	13.78089	11.55459	8.966567	10.44796
SEM		3.014819	5.264589	4.872281	4.085164	3.17016	3.693913

Appendix A-7: Weight adjusted right femur BMC (g/kg)

Control sham	Baseline	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks
1	0.0018	0.0024	0.00245	0.0027	0.00245	0.00255	0.0028
2	0.0014	0.0026	0.0023	0.0024	0.0024	0.0023	0.0024
3	0.0027	0.0028	0.003	0.00275	0.0025	0.0027	0.0025
4	0.0025	0.0028	0.00295	0.0028	*	*	*
5	0.0029	0.0028	0.0027	0.0027	0.0025	0.00235	0.0026
6	0.0024	0.0026	0.00295	0.00275	0.0032	0.0026	0.0027
7	0.0023	0.0028	0.0024	0.0023	0.0024	0.0026	0.0025
8	0.0023	0.0023	0.0026	0.0024	0.00245	0.0023	0.0026
Mean	0.002288	0.002638	0.00266875	0.0026	0.00255714	0.002485714	0.002586
SD	0.000482	0.0002	0.000275081	0.000198	0.0002864	0.000165112	0.000135
SEM	0.000171	7.06E-05	9.72559E-05	7.01E-05	0.00010825	6.24064E-05	5.08E-05
Control ovx							
9	0.0026	0.00225	0.0023	0.00245	0.0023	0.0026	0.0024
10	0.0023	0.0023	0.0023	0.0021	0.0023	0.0022	0.0025
11	0.0024	0.00235	0.00215	0.0023	0.00215	0.0022	0.0021
12	0.0022	0.0021	0.002	0.0022	0.00205	0.00195	0.0021
13	0.0023	0.0019	0.0019	0.00215	0.0023	0.0024	0.0021
14	0.0027	0.0022	0.00205	0.0021	0.0023	0.0021	0.0022
15	0.0025	0.0023	0.00215	0.0024	0.0021	0.0022	0.0024
16	0.0023	0.0019	0.0022	0.0022	0.00225	0.0021	0.0026
Mean	0.002413	0.002163	0.00213125	0.002238	0.00221875	0.00221875	0.0023
SD	0.000173	0.000179	0.000141263	0.000133	0.00010329	0.000199888	0.0002
SEM	6.11E-05	6.32E-05	4.99442E-05	4.7E-05	3.652E-05	7.06712E-05	7.07E-05
5 IU/kgBW/day							
17	0.00262	0.002303	0.002125	0.001882	0.00205	0.00195	0.0019
18	0.00203	0.00212	0.002273	0.002254	0.0022	*	*
19	0.002597	0.002181	0.002108	0.002102	*	*	*
20	0.002521	0.002572	0.002457	0.002285	0.00215	0.0026	0.0021
21	0.002304	0.002128	0.002065	0.002055	0.00205	0.0021	0.0024
22	0.002358	0.002219	0.002246	0.002367	0.0026	0.0025	0.0025
23	0.002427	0.001761	0.00219	0.002143	0.00245	0.002	0.002
24	0.001442	0.002623	0.002424	0.002457	0.0023	0.00255	0.0022
42	0.002913	0.002422	0.002128	0.002408	0.00225	0.0022	0.002
Mean	0.002357	0.002259	0.002224	0.002217	0.00225625	0.002271429	0.002157
SD	0.000421	0.000262	0.00013968	0.000187	0.00019168	0.000273644	0.000223
SEM	0.00014	8.75E-05	4.656E-05	6.23E-05	6.7769E-05	0.000103428	8.41E-05
15 IU/kgBW/day							
25	0.0023	0.0021	0.00225	0.002	0.002	0.00205	0.0019
26	0.0027	0.0022	0.0022	0.0023	0.0023	0.0022	0.0019
27	0.0026	0.00265	0.00205	0.00225	0.0022	0.0022	0.0024
28	0.0026	0.00265	0.00225	0.00205	0.00205	0.0023	0.0021
29	0.0027	0.00205	0.00205	0.002	0.0022	0.00195	0.002
30	0.0029	0.0024	0.00235	0.00245	0.0022	0.0022	0.0024
31	0.0022	0.0025	0.00235	0.00235	0.0023	*	*
32	0.0025	0.0023	0.0025	0.00245	0.00215	0.0026	0.0026
Mean	0.002563	0.002356	0.00225	0.002231	0.002175	0.002214286	0.002186
SD	0.000226	0.000234	0.00015353	0.000191	0.0001069	0.000205577	0.000279
SEM	8E-05	8.26E-05	5.4281E-05	6.74E-05	3.7796E-05	7.77008E-05	0.000106
50 IU/kgBW/day							
33	0.0022	0.0027	0.0026	0.0025	0.00245	0.0023	0.0023
34	0.0019	0.0022	0.00235	0.00205	0.0021	0.0018	0.0025
35	0.0029	0.002	0.00225	0.00225	0.00205	0.0019	0.0023
36	0.0024	0.0024	0.0024	0.0022	0.0022	0.0022	0.0021
37	0.0026	0.00245	0.0023	0.0023	0.00245	0.00245	0.0021
38	0.0028	0.0025	0.0024	0.0023	0.0021	0.0021	0.0022
39	0.0029	0.00225	0.0021	0.0021	0.00215	0.00215	0.0023
40	0.0023	0.0021	*	*	*	*	*
41	0.0027	0.0025	0.0021	0.0021	0.0024	0.0024	0.0021
Mean	0.002522	0.002344	0.0023125	0.002225	0.0022375	0.0021625	0.002238
SD	0.000346	0.000223	0.000166369	0.000146	0.0001685	0.000227957	0.000141
SEM	0.000115	7.43E-05	5.88202E-05	5.18E-05	5.9574E-05	8.05949E-05	4.98E-05

Appendix A-8: Weight adjusted right femur BMC % change from baseline

Control sham	4wks	8wks	12wks	16wks	20wks	24wks
1	33.33333	36.11111	50	36.11111	41.66667	55.55556
2	85.71429	64.28571	71.42857	71.42857	64.28571	71.42857
3	3.703704	11.11111	1.851852	-7.40741	0	-7.40741
4	12	18	12	*	*	*
5	-3.44828	-6.89655	-6.89655	-13.7931	-18.9655	-10.3448
6	8.333333	22.91667	14.58333	33.33333	8.333333	12.5
7	21.73913	4.347826	0	4.347826	13.04348	8.695652
8	0	13.04348	4.347826	6.521739	0	13.04348
Mean	20.17194	20.36492	18.41438	18.64887	15.48053	20.49586
SD	29.0675	21.8045	27.55746	29.97015	28.25628	31.12282
SEM	10.27691	7.709056	9.743033	11.32765	10.67987	11.76332
Control ovx						
9	-13.4615	-11.5385	-5.76923	-11.5385	0	-7.69231
10	0	0	-8.69565	0	-4.34783	8.695652
11	-2.08333	-10.4167	-4.16667	-10.4167	-8.33333	-12.5
12	-4.54545	-9.09091	0	-6.81818	-11.3636	-4.54545
13	-17.3913	-17.3913	-6.52174	0	4.347826	-8.69565
14	-18.5185	-24.0741	-22.2222	-14.8148	-22.2222	-18.5185
15	-8	-14	-4	-16	-12	-4
16	-17.3913	-4.34783	-4.34783	-2.17391	-8.69565	13.04348
Mean	-10.1739	-11.3574	-6.96542	-7.72025	-7.82686	-4.2766
SD	7.464141	7.457351	6.648169	6.453312	8.104215	10.48853
SEM	2.638972	2.636572	2.350483	2.28159	2.865273	3.708255
5 IU/kgBW/day						
17	-12.0992	-18.8931	-28.1679	-21.7557	-25.5725	-27.4809
18	4.433498	11.97044	11.03448	8.374384	*	*
19	-16.0185	-18.8294	-19.0605	*	*	*
20	2.023007	-2.53868	-9.36136	-14.7164	3.133677	-16.6997
21	-7.63889	-10.3733	-10.8073	-11.0243	-8.85417	4.166667
22	-5.89483	-4.74979	0.381679	10.26293	6.022053	6.022053
23	-27.4413	-9.76514	-11.7017	0.947672	-17.5937	-17.5937
24	81.90014	68.09986	70.38835	59.50069	76.83773	52.56588
42	-16.8555	-26.9482	-17.3361	-22.76	-24.4765	-31.3423
Mean	0.267605	-1.33636	-1.62559	1.103654	1.35665	-4.33743
SD	32.14023	28.36036	29.26448	26.82318	35.53924	28.89943
SEM	10.71341	9.453452	9.754826	9.483428	13.43257	10.92296
15 IU/kgBW/day						
25	-8.69565	-2.17391	-13.0435	-13.0435	-10.8696	-17.3913
26	-18.5185	-18.5185	-14.8148	-14.8148	-18.5185	-29.6296
27	1.923077	-21.1538	-13.4615	-15.3846	-15.3846	-7.69231
28	1.923077	-13.4615	-21.1538	-21.1538	-11.5385	-19.2308
29	-24.0741	-24.0741	-25.9259	-18.5185	-27.7778	-25.9259
30	-17.2414	-18.9655	-15.5172	-24.1379	-24.1379	-17.2414
31	13.63636	6.818182	6.818182	4.545455	*	*
32	-8	0	-2	-14	4	4
Mean	-7.38089	-11.4412	-12.3873	-14.5635	-14.8896	-16.1588
SD	12.63161	11.43241	10.3797	8.612799	10.40239	11.30668
SEM	4.465948	4.041966	3.669778	3.045084	3.931735	4.273525
50 IU/kgBW/day						
33	22.72727	18.18182	13.63636	11.36364	4.545455	4.545455
34	15.78947	23.68421	7.894737	10.52632	-5.26316	31.57895
35	-31.0345	-22.4138	-22.4138	-29.3103	-34.4828	-20.6897
36	0	0	-8.33333	-8.33333	-8.33333	-12.5
37	-5.76923	-11.5385	-11.5385	-5.76923	-5.76923	-19.2308
38	-10.7143	-14.2857	-17.8571	-25	-25	-21.4286
39	-22.4138	-27.5862	-27.5862	-25.8621	-25.8621	-20.6897
40	-8.69565	*	*	*	*	*
41	-7.40741	-22.2222	-22.2222	-11.1111	-11.1111	-22.2222
Mean	-5.27979	-7.02255	-11.0525	-10.437	-13.9095	-10.0796
SD	16.80414	19.24063	14.87892	15.8006	13.14564	19.05
SEM	5.601379	6.80259	5.260493	5.586356	4.647686	6.735193

Appendix A-9: Weight adjusted left femur BMC (g/kg)

Control sham	Baseline	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks
1	0.0023	0.0026	0.0024	0.003	0.0029	0.00255	0.0025
2	0.001	0.0024	0.0027	0.00295	0.0029	0.0026	0.0024
3	0.0027	0.0026	0.00285	0.00275	0.0025	0.00255	0.0028
4	0.002	0.0028	0.0029	0.0028	*	*	*
5	0.0024	0.0026	0.0029	0.0027	0.003	0.00265	0.0026
6	0.0024	0.0026	0.0031	0.0029	0.0031	0.00275	0.0027
7	0.0023	0.0026	0.0026	0.00245	0.0029	0.0026	0.0025
8	0.0023	0.0027	0.0026	0.0027	0.0026	0.0026	0.0029
Mean	0.002175	0.0026125	0.00275625	0.002781	0.002842857	0.002614286	0.002629
SD	0.000512	0.000112599	0.000222707	0.000175	0.00021492	6.90066E-05	0.00018
SEM	0.000181	3.98098E-05	7.87387E-05	6.19E-05	8.1232E-05	2.6082E-05	6.8E-05
Control ovx							
9	0.0026	0.00225	0.0024	0.00245	0.00265	0.0026	0.0026
10	0.0027	0.0023	0.00245	0.00265	0.00295	0.0025	0.0025
11	0.0029	0.0022	0.0023	0.00245	0.0026	0.0022	0.0024
12	0.0022	0.00195	0.00215	0.0022	0.0022	0.0023	0.0024
13	0.0028	0.00235	0.0022	0.0023	0.0025	0.00225	0.0024
14	0.0027	0.002	0.0022	0.0023	0.0023	0.002	0.0022
15	0.0025	0.00235	0.0023	0.0021	0.0024	0.00255	0.0026
16	0.0028	0.0023	0.00235	0.0023	0.0025	0.0024	0.0026
Mean	0.00265	0.0022125	0.00229375	0.002344	0.0025125	0.00235	0.002463
SD	0.00022	0.000155265	0.000105009	0.00017	0.000231069	0.000201778	0.000141
SEM	7.79E-05	5.48944E-05	3.71261E-05	6.01E-05	8.16952E-05	7.13392E-05	4.98E-05
5 IU/kgBW/day							
17	0.0026	0.00245	0.00215	0.0022	0.0023	0.0022	0.0022
18	0.0026	0.0025	0.00255	0.0024	0.0025	*	*
19	0.0026	0.00215	0.00225	0.0024	*	*	*
20	0.0025	0.0026	0.00245	0.00255	0.0026	0.00245	0.0024
21	0.0023	0.0025	0.0024	0.0022	0.0026	0.0025	0.0024
22	0.0028	0.0027	0.00255	0.00285	0.0029	0.00265	0.0028
23	0.0029	0.00215	0.00215	0.0023	0.00245	0.0023	0.0026
24	0.0014	0.0026	0.00225	0.00245	0.0027	0.0024	0.0025
42	0.0029	0.0024	0.00225	0.0023	0.0022	0.0022	0.0023
Mean	0.002511	0.00245	0.002333333	0.002406	0.00253125	0.002385714	0.002457
SD	0.000459	0.000192029	0.000158114	0.000202	0.000221903	0.000165112	0.000199
SEM	0.000153	6.40095E-05	5.27046E-05	6.74E-05	7.84547E-05	6.24064E-05	7.51E-05
15 IU/kgBW/day							
25	0.0028	0.0024	0.0024	0.0023	0.0022	0.0025	0.0022
26	0.0027	0.00255	0.00235	0.0024	0.00255	0.0024	0.0025
27	0.0026	0.0025	0.0025	0.0025	0.00235	0.0022	0.0024
28	0.0026	0.0025	0.0024	0.0022	0.0024	0.0024	0.0024
29	0.0027	0.0024	0.0023	0.0022	0.0025	0.0021	0.0022
30	0.0029	0.0028	0.0025	0.00245	0.0024	0.0025	0.0021
31	0.0026	0.00265	0.0025	0.0025	0.0023	*	*
32	0.0021	0.0023	0.00265	0.00245	0.0023	0.00253	0.0026
Mean	0.002625	0.0025125	0.00245	0.002375	0.002375	0.002375714	0.002343
SD	0.000238	0.000157548	0.000110195	0.000125	0.000113389	0.000164708	0.000181
SEM	8.4E-05	5.57017E-05	3.89597E-05	4.43E-05	4.00892E-05	6.22536E-05	6.85E-05
50 IU/kgBW/day							
33	0.0026	0.00275	0.0029	0.0025	0.0026	0.0023	0.0026
34	0.0024	0.0024	0.0025	0.0022	0.0025	0.0022	0.0028
35	0.0029	0.0023	0.0026	0.0024	0.0025	0.0023	0.002
36	0.0024	0.0024	0.0027	0.0025	0.0028	0.0025	0.0024
37	0.0026	0.0026	0.0023	0.0025	0.0026	0.0021	0.0021
38	0.0028	0.0025	0.0024	0.0026	0.00255	0.00235	0.0022
39	0.0029	0.00225	0.0021	0.0024	0.0023	0.00275	0.0023
40	0.0023	0.0021	*	*	*	*	*
41	0.0027	0.0025	0.00245	0.0024	0.00255	0.0027	0.0024
Mean	0.002622	0.002422222	0.00249375	0.002438	0.00255	0.0024	0.00235
SD	0.000222	0.000193828	0.000245586	0.000119	0.000138873	0.000231455	0.000262
SEM	7.41E-05	6.46095E-05	8.68278E-05	4.2E-05	4.9099E-05	8.18317E-05	9.26E-05

Appendix A-10: Weight adjusted left femur BMC % change from baseline

Control sham	4wks	8wks	12wks	16wks	20wks	24wks
1	13.04348	4.347826	30.43478	26.08696	10.86957	8.695652
2	140	170	195	190	160	140
3	-3.7037	5.555556	1.851852	-7.40741	-5.55556	3.703704
4	40	45	40	*	*	*
5	8.333333	20.83333	12.5	25	10.41667	8.333333
6	8.333333	29.16667	20.83333	29.16667	14.58333	12.5
7	13.04348	13.04348	6.521739	26.08696	13.04348	8.695652
8	17.3913	13.04348	17.3913	13.04348	13.04348	26.08696
Mean	29.55515	37.62379	40.56663	43.13952	30.91442	29.71647
SD	46.30222	55.13018	63.61128	65.99827	57.33025	49.14212
SEM	16.37031	19.49146	22.48998	24.945	21.6688	18.57398
Control ovx						
9	-13.4615	-7.69231	-5.76923	1.923077	0	0
10	-14.8148	-9.25926	-1.85185	9.259259	-7.40741	-7.40741
11	-24.1379	-20.6897	-15.5172	-10.3448	-24.1379	-17.2414
12	-11.3636	-2.27273	0	0	4.545455	9.090909
13	-16.0714	-21.4286	-17.8571	-10.7143	-19.6429	-14.2857
14	-25.9259	-18.5185	-14.8148	-14.8148	-25.9259	-18.5185
15	-6	-8	-16	-4	2	4
16	-17.8571	-16.0714	-17.8571	-10.7143	-14.2857	-7.14286
Mean	-16.2041	-12.9916	-11.2084	-4.92573	-10.6068	-6.43812
SD	6.516082	7.094242	7.422948	8.171512	12.09613	10.12227
SEM	2.303783	2.508193	2.624409	2.889066	4.276628	3.578761
5 IU/kgBW/day						
17	-5.76923	-17.3077	-15.3846	-11.5385	-15.3846	-15.3846
18	-3.84615	-1.92308	-7.69231	-3.84615	*	*
19	-17.3077	-13.4615	-7.69231	*	*	*
20	4	-2	2	4	-2	-4
21	8.695652	4.347826	-4.34783	13.04348	8.695652	4.347826
22	-3.57143	-8.92857	1.785714	3.571429	-5.35714	0
23	-25.8621	-25.8621	-20.6897	-15.5172	-20.6897	-10.3448
24	85.71429	60.71429	75	92.85714	71.42857	78.57143
42	-17.2414	-22.4138	-20.6897	-24.1379	-24.1379	-20.6897
Mean	2.756887	-2.98163	0.254372	7.304033	1.793554	4.64288
SD	32.97091	25.89529	29.28665	36.57848	32.74962	33.73732
SEM	10.9903	8.631764	9.762217	12.93245	12.37819	12.75151
15 IU/kgBW/day						
25	-14.2857	-14.2857	-17.8571	-21.4286	-10.7143	-21.4286
26	-5.55556	-12.963	-11.1111	-5.55556	-11.1111	-7.40741
27	-3.84615	-3.84615	-3.84615	-9.61538	-15.3846	-7.69231
28	-3.84615	-7.69231	-15.3846	-7.69231	-7.69231	-7.69231
29	-11.1111	-14.8148	-18.5185	-7.40741	-22.2222	-18.5185
30	-3.44828	-13.7931	-15.5172	-17.2414	-13.7931	-27.5862
31	1.923077	-3.84615	-3.84615	-11.5385	*	*
32	9.52381	26.19048	16.66667	9.52381	20.47619	23.80952
Mean	-3.83076	-5.63134	-8.67678	-8.86941	-8.63449	-9.50226
SD	7.337598	13.64264	11.75835	9.167629	13.63562	16.68919
SEM	2.594233	4.823401	4.157205	3.241246	5.153779	6.30792
50 IU/kgBW/day						
33	5.769231	11.53846	-3.84615	0	-11.5385	0
34	0	4.166667	-8.33333	4.166667	-8.33333	16.66667
35	-20.6897	-10.3448	-17.2414	-13.7931	-20.6897	-31.0345
36	0	12.5	4.166667	16.66667	4.166667	0
37	0	-11.5385	-3.84615	0	-19.2308	-19.2308
38	-10.7143	-14.2857	-7.14286	-8.92857	-16.0714	-21.4286
39	-22.4138	-27.5862	-17.2414	-20.6897	-5.17241	-20.6897
40	-8.69565	*	*	*	*	*
41	-7.40741	-9.25926	-11.1111	-5.55556	0	-11.1111
Mean	-7.12795	-5.60117	-8.07446	-3.51669	-9.60867	-10.8535
SD	9.693649	13.87046	7.209352	11.49594	8.978209	15.48758
SEM	3.231216	4.90395	2.548891	4.06443	3.174276	5.475686

Appendix A-11: Urinary calcium excretion (ppm)

Control sham	Baseline	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks
1	24.7	21.5	62	63.1	116	81.2	53.4
2	17.9	15.4	26.7	74.9	203.1	46.8	62.5
3	24.1	32.8	73.9	77.8	146.3	89.8	61.3
4	20.7	32.9	62.5	26.7	*	*	*
5	14	35.7	29	56.1	116	108.5	73.04
6	36.4	20.7	62.6	41.9	72.03	109.3	73.02
7	24.5	68.8	108.5	64.3	42.95	134.3	114.9
8	30.6	35.2	75.4	57.8	116	94.9	73
Mean	24.1125	32.875	62.575	57.825	116.0543	94.97143	73.02286
SD	7.031244	16.41861	26.23105	16.84236	51.27401	27.30065	19.94538
SEM	2.48592	5.804855	9.274076	5.954673	19.37975	10.31868	7.538644
Control ovx							
9	17.3	35.1	22.8	61.5	64.7	30.2	35.3
10	19.7	10.2	17.1	53.3	34.6	64	26.3
11	23	58.4	70.6	61.6	45.9	67.9	53.3
12	12.6	23.6	28.3	72.9	81.9	90.9	83.2
13	26	28.5	70.5	60.4	77.1	33.4	119.2
14	46.7	21	31.1	67.1	58.8	63	50.8
15	66.7	28.5	23.4	78.2	49	103.4	28.1
16	40.8	22.9	52.9	37.6	58.9	37.9	82.2
Mean	31.6	28.525	39.5875	61.575	58.8625	61.3375	59.8
SD	18.35009	14.06147	21.85527	12.42023	15.81762	26.71404	32.57589
SEM	6.487736	4.971481	7.727004	4.391215	5.592372	9.444839	11.51732
5 IU/kgBW/day							
17	36.05	45.4	49.3	77.7	46.6	29.2	28.8
18	24.6	47.9	63.6	60.6	100.4	*	*
19	45.2	16.1	92	16.1	*	*	*
20	39.9	21.2	48	66.2	92	35.3	58.8
21	40.4	78.6	46.9	47.2	91	85	52.8
22	47.7	24.5	36.8	38.9	28	51.4	45.9
23	27.5	44.8	29.3	56.5	91.6	96.3	31
24	27.9	13.4	54.1	55.3	132	87.3	41.6
42	35.2	75.5	23.5	50.1	151	98.4	43.1
Mean	36.05	40.82222	49.27778	52.06667	91.575	68.98571	43.14286
SD	8.097376	24.31871	20.24894	17.51521	40.19317	29.52408	10.81324
SEM	2.699125	8.106235	6.749646	5.838403	14.21043	11.15905	4.087021
15 IU/kgBW/day							
25	15	30.5	76	18	83.2	61	35.8
26	15	45.9	21.4	63.4	110.2	57.8	39.95
27	26.9	60.8	23.9	50.9	41.7	75.96	40
28	49.1	39.8	106.2	63	110	100	39.95
29	21.3	58.2	66.9	43.4	123.8	54	20.4
30	30	42.6	63.2	54.3	141.6	107	38.2
31	47.4	18.3	63.2	43	110.4	*	*
32	14.1	71.7	84.8	48	160.7	75.96	65.4
Mean	27.35	45.975	63.2	48	110.2	75.96	39.95714
SD	14.13961	17.26232	28.74722	14.42429	36.13961	20.72531	13.22997
SEM	4.999107	6.103153	10.16368	5.099755	12.77728	7.833433	5.000457
50 IU/kgBW/day							
33	23.2	86	83.4	65.9	99.1	80.16	60
34	9.4	33.4	64.3	59.5	103.2	125.9	30.9
35	47.6	29	47.6	51.6	277.1	90.9	74.6
36	36.3	100.2	44.4	63.2	266.7	74.79	59.6
37	24.3	64.1	99	57.9	78.1	58.2	78.7
38	29.64	84.1	105.2	45.2	136.6	22.8	40.1
39	41.8	21.1	60.6	72.3	235.9	132.3	47.3
40	24.9	50.9	*	*	*	*	*
41	29.64	61.3	64.7	74.2	189.9	13.3	55.89
Mean	29.64222	58.9	71.15	61.225	173.325	74.79375	55.88625
SD	11.2744	27.71516	22.53594	9.857811	79.75131	43.0402	16.25084
SEM	3.758134	9.238386	7.967658	3.485262	28.19634	15.21701	5.74554

Appendix A-12: Urinary calcium excretion % change from baseline

Control sham	%chg 4wk	%chg 8wk	%chg12wk	%chg16wk	%chg20wks	%chg24wk
1	-12.9555	151.0121	155.4656	369.6356	228.7449	116.1943
2	-13.9665	49.16201	318.4358	1034.637	161.4525	249.162
3	36.09959	206.639	222.8216	507.0539	272.6141	154.3568
4	58.9372	201.9324	28.98551	*	*	*
5	155	107.1429	300.7143	728.5714	675	421.7143
6	-43.1319	71.97802	15.10989	97.88462	200.2747	100.6044
7	180.8163	342.8571	162.449	75.30612	448.1633	368.9796
8	15.03268	146.4052	88.88889	279.085	210.1307	138.5621
Mean	46.979	159.6411	161.6088	381.3034	293.868	202.8423
SD	81.31891	92.93748	114.6856	346.9464	184.524	128.9011
SEM	28.75058	32.85836	40.54749	131.1334	69.74351	48.72004
Control ovx						
9	102.8902	31.79191	255.4913	273.9884	74.56647	104.0462
10	-48.2234	-13.198	170.5584	75.63452	224.8731	33.50254
11	153.913	206.9565	167.8261	99.56522	195.2174	131.7391
12	87.30159	124.6032	478.5714	550	621.4286	560.3175
13	9.615385	171.1538	132.3077	196.5385	28.46154	358.4615
14	-55.0321	-33.4047	43.68308	25.91006	34.90364	8.779443
15	-57.2714	-64.9175	17.24138	-26.5367	55.02249	-57.8711
16	-43.8725	29.65686	-7.84314	44.36275	-7.10784	101.4706
Mean	18.6651	56.58026	157.2295	154.9328	153.4207	155.0557
SD	84.31352	99.59532	157.4645	186.3717	206.0563	204.6383
SEM	29.80933	35.21226	55.67211	65.89236	72.85191	72.35055
5 IU/kgBW/day						
17	25.9362	36.75451	115.534	29.26491	-19.0014	-20.111
18	94.71545	158.5366	146.3415	308.1301	*	*
19	-64.3805	103.5398	-64.3805	*	*	*
20	-46.8672	20.30075	65.91479	130.5764	-11.5288	47.36842
21	94.55446	16.08911	16.83168	125.2475	110.396	30.69307
22	-48.6373	-22.8512	-18.4486	-41.2998	7.756813	-3.77358
23	62.90909	6.545455	105.4545	233.0909	250.1818	12.72727
24	-51.9713	93.90681	98.20789	373.1183	212.9032	49.10394
42	114.4886	-33.2386	42.32955	328.9773	179.5455	22.44318
Mean	20.08305	42.17592	56.42052	185.8882	104.3219	19.77876
SD	73.67791	63.59566	68.72495	149.0842	113.0747	25.63527
SEM	24.5593	21.19855	22.90832	52.70922	42.73822	9.689222
15 IU/kgBW/day						
25	103.3333	406.6667	20	454.6667	306.6667	138.6667
26	206	42.66667	322.6667	634.6667	285.3333	166.3333
27	126.0223	-11.1524	89.21933	55.01859	182.3792	48.69888
28	-18.9409	116.2933	28.30957	124.0326	103.666	-18.6354
29	173.2394	214.0845	103.7559	481.2207	153.5211	-4.22535
30	42	110.6667	81	372	256.6667	27.33333
31	-61.3924	33.33333	-9.2827	132.9114	*	*
32	408.5106	501.4184	240.4255	1039.716	438.7234	363.8298
Mean	122.3465	176.7471	109.5118	411.7791	246.7081	103.143
SD	147.3725	185.7576	114.9842	325.153	112.0558	134.339
SEM	52.10405	65.67522	40.65305	114.9589	42.35312	50.77536
50 IU/kgBW/day						
33	270.6897	259.4828	184.0517	327.1552	245.5172	158.6207
34	255.3191	584.0426	532.9787	997.8723	1239.362	228.7234
35	-39.0756	0	8.403361	482.1429	90.96639	56.72269
36	176.0331	22.31405	74.10468	634.7107	106.0331	64.18733
37	163.786	307.4074	138.2716	221.3992	139.5062	223.8683
38	183.7382	254.9258	52.49663	360.8637	-23.0769	35.29015
39	-49.5215	44.97608	72.96651	464.3541	216.5072	13.15789
40	104.4177	*	*	*	*	*
41	106.8151	118.2861	150.3374	540.6883	-55.1282	88.56275
Mean	130.2446	198.9293	151.7013	503.6483	244.9608	108.6417
SD	113.8271	195.6136	164.4026	237.852	415.0175	84.31847
SEM	37.94235	69.15985	58.1251	84.09339	146.7308	29.81108

Appendix A-13: Urinary helical peptides (μg/L)

Control sham	Baseline	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks
1	188	91	44	80.5	198.3	31	37
2	219	59.5	28.5	79.5	213.5	90.5	102.25
3	147.5	90.5	37	80.5	251	54	126
4	170	85.6	42.5	97.5	*	*	*
5	103	48.5	46	54	151	61	57
6	215	111	47	30.5	222.5	33.5	51.5
7	166.5	80	46	98.5	212	51.5	131.5
8	172.7	118.5	105.5	115.5	140	101	122.5
Mean	172.7125	85.575	49.5625	79.5625	198.3286	60.35714	89.67857
SD	37.23656	23.51205	23.43142	26.81742	39.61867	26.65476	40.00852
SEM	13.16511	8.312764	8.284257	9.48139	14.97445	10.07455	15.1218
Control ovx							
9	121	95.5	23.5	35.5	70.5	20	28
10	120.5	75.5	61.5	34.5	57.5	37.5	48.5
11	118	111	71	78.5	204.5	101	115.5
12	162.5	117	74	63	85.5	43.5	35.5
13	171	193	65	84.5	255.5	81.5	60
14	120	129	51.5	62.5	91	74.5	104.5
15	121.5	108	35.5	52.5	143.5	29	62.5
16	153	86.5	74	90	221.5	29	65
Mean	135.9375	114.4375	57	62.625	141.1875	52	64.9375
SD	22.27016	36.05397	18.7959	21.07597	76.67321	29.62142	30.82953
SEM	7.873689	12.747	6.645353	7.45148	27.10807	10.47275	10.89988
5 IU/kgBW/day							
17	177	106	91	110	109	51.5	75
18	180.5	51	96.5	67.5	189	*	*
19	83.5	143	77	203	*	*	*
20	186.5	50	57	94.5	129.6	55.5	88.5
21	96	89.5	70	78.5	130	45.5	68
22	192	169.5	56.5	43.5	59	36.5	218.5
23	139.5	99	96.5	87	129	60.5	131.5
24	236.5	125	72.5	67.5	135	72.5	81
42	95	238	147.5	101	156	109	188.5
Mean	154.0556	119	84.94444	94.72222	129.575	61.57143	121.5714
SD	53.10746	59.31642	27.94687	45.31219	37.15549	23.788	60.21035
SEM	17.70249	19.77214	9.315625	15.10406	13.13645	8.99102	22.75737
15 IU/kgBW/day							
25	114	122.5	86	78.5	93	32.5	71
26	109	60.5	90	61.5	133.5	58	155
27	117	69	86.5	104.5	164	46	161
28	155.5	90.5	74.5	72.5	204.4	61.5	87
29	197.5	179	125.5	92.5	177.5	78	111
30	211.5	196.5	119	105	235	59.5	72
31	315	299	170.5	141	358	*	*
32	259.5	210.5	110.5	115.5	270	57.5	93
Mean	184.875	153.4375	107.8125	96.375	204.425	56.14286	107.1429
SD	75.23285	82.49478	31.01145	25.68595	83.34542	14.05262	37.31143
SEM	26.59883	29.16631	10.9642	9.081354	29.46706	5.311392	14.1024
50 IU/kgBW/day							
33	175.5	131.5	73.5	93.5	166	143	125
34	161.5	82	44.5	100.5	210.5	67.5	45
35	91.5	165	59	60	166.4	63.5	89.5
36	100.5	140	45	50.5	179	67	63.5
37	122.1	151	87	59	183.5	77.5	89
38	90	128.8	66.5	64	146	111	87
39	125.5	119	71.5	61.5	188	100	106
40	120.5	120	*	*	*	*	*
41	111.5	122	72	78	90.5	45	86.4
Mean	122.0667	128.8111	64.875	70.875	166.2375	84.3125	86.425
SD	29.50364	23.30764	14.66714	17.92793	35.93538	31.64529	24.28761
SEM	9.834548	7.769214	5.185617	6.338481	12.70507	11.1883	8.586966

Appendix A-14: Urinary helical peptides % change from baseline

Control sham		%chg 4wk	%chg 8wk	%chg12wk	%chg16wk	%chg20wk	%chg 24wk
	1	-51.5957	-76.5957	-57.1809	5.478723	-83.5106	-80.3191
	2	-72.8311	-86.9863	-63.6986	-2.51142	-58.6758	-53.3105
	3	-38.6441	-74.9153	-45.4237	70.16949	-63.3898	-14.5763
	4	-49.6471	-75	-42.6471	*	*	*
	5	-52.9126	-55.3398	-47.5728	46.60194	-40.7767	-44.6602
	6	-48.3721	-78.1395	-85.814	3.488372	-84.4186	-76.0465
	7	-51.952	-72.3724	-40.8408	27.32733	-69.0691	-21.021
	8	-31.3839	-38.9114	-33.121	-18.9346	-41.5171	-29.0677
Mean		-49.6673	-69.7826	-52.0374	18.80284	-63.0511	-45.5716
SD		12.03271	15.27894	16.64048	31.02275	17.75059	25.92506
SEM		4.254204	5.401921	5.883298	11.7255	6.709091	9.798751
Control ovx							
	9	-21.0744	-80.5785	-70.6612	-41.7355	-83.4711	-76.8595
	10	-37.3444	-48.9627	-71.3693	-52.2822	-68.8797	-59.751
	11	-5.9322	-39.8305	-33.4746	73.30508	-14.4068	-2.11864
	12	-28	-54.4615	-61.2308	-47.3846	-73.2308	-78.1538
	13	12.8655	-61.9883	-50.5848	49.4152	-52.3392	-64.9123
	14	7.5	-57.0833	-47.9167	-24.1667	-37.9167	-12.9167
	15	-11.1111	-70.7819	-56.7901	18.107	-76.1317	-48.5597
	16	-43.4641	-51.634	-41.1765	44.77124	-81.0458	-57.5163
Mean		-15.8201	-58.1651	-54.1505	2.503694	-60.9277	-50.0985
SD		20.31916	12.84271	13.48852	49.86021	24.27864	28.17755
SEM		7.183906	4.540582	4.768911	17.62825	8.583796	9.962269
5 IU/kgBW/day							
	17	-40.113	-48.5876	-37.8531	-38.4181	-70.904	-57.6271
	18	-71.7452	-46.5374	-62.6039	4.709141	*	*
	19	71.25749	-7.78443	143.1138	*	*	*
	20	-73.1903	-69.437	-49.3298	*	-70.2413	-52.5469
	21	-6.77083	-27.0833	-18.2292	35.41667	-52.6042	-29.1667
	22	-11.7188	-70.5729	-77.3438	-69.2708	-80.9896	13.80208
	23	-29.0323	-30.8244	-37.6344	*	-56.6308	-5.73477
	24	-47.1459	-69.3446	-71.4588	-42.9175	-69.3446	-65.7505
	42	150.5263	55.26316	6.315789	64.21053	14.73684	98.42105
Mean		-6.43693	-34.9898	-22.7804	-7.71169	-55.1397	-14.0861
SD		73.07951	40.18218	67.52178	51.30107	32.24373	57.41232
SEM		24.35984	13.39406	22.50726	20.94358	12.18698	21.69982
15 IU/kgBW/day							
	25	7.45614	-24.5614	-31.1404	-18.4211	-71.4912	-37.7193
	26	-44.4954	-17.4312	-43.578	22.47706	-46.789	42.20183
	27	-41.0256	-26.0684	-10.6838	40.17094	-60.6838	37.60684
	28	-41.8006	-52.09	-53.3762	31.44695	-60.4502	-44.0514
	29	-9.36709	-36.4557	-53.1646	-10.1266	-60.5063	-43.7975
	30	-7.0922	-43.7352	-50.3546	11.11111	-71.8676	-65.9574
	31	-5.07937	-45.873	-55.2381	13.65079	*	*
	32	-18.8825	-57.4181	-55.4913	4.046243	-77.842	-64.1618
Mean		-20.0358	-37.9541	-44.1284	11.79443	-64.2329	-25.1255
SD		19.9096	14.23714	15.79339	19.86749	10.33814	45.68887
SEM		7.039106	5.033588	5.583806	7.024218	3.907449	17.26877
50 IU/kgBW/day							
	33	-25.0712	-58.1197	-46.7236	-5.41311	-18.5185	-28.7749
	34	-49.226	-72.4458	-37.7709	30.34056	-58.2043	-72.1362
	35	80.32787	-35.5191	-34.4262	81.85792	-30.6011	-2.18579
	36	39.30348	-55.2239	-49.7512	78.10945	-33.3333	-36.8159
	37	23.66912	-28.7469	-51.679	50.28665	-36.5274	-27.1089
	38	43.11111	-26.1111	-28.8889	62.22222	23.33333	-3.33333
	39	-5.17928	-43.0279	-50.996	49.8008	-20.3187	-15.5378
	40	-0.41494	*	*	*	*	*
	41	9.41704	-35.426	-30.0448	-18.8341	-59.6413	*
Mean		12.88191	-44.3276	-41.2851	41.0463	-29.2264	-26.5561
SD		38.87492	16.18067	9.584143	36.85582	26.16807	23.9536
SEM		12.95831	5.720731	3.388506	13.0305	9.251812	9.053611

Appendix A-15: Serum pyridinoline (nmol/L)

Control sham	Baseline	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks
1	3.466	3.5	4.45	9.37	4.18	4.37	1.975
2	2.8705	5.465	6.605	11.365	9.945	8.895	6.165
3	2.8415	5.865	6.89	10.575	8.53	4.745	6.055
4	4.1155	4.36	5.765	9.89	*	*	*
5	2.858	3.18	2.53	5.47	5.455	4.53	3.275
6	3.6	3.185	3.475	6.205	5.71	2.65	2.77
7	2.5595	4.795	6.695	5.56	6.595	7.56	3.53
8	4.0975	5.7	7.05	5.44	5.68	4.315	3.875
Mean	3.301063	4.50625	5.4325	7.984375	6.585	5.295	3.949286
SD	0.604388	1.122013	1.733759	2.550379	1.988228	2.152131	1.595197
SEM	0.213683	0.396691	0.612976	0.901695	0.751479	0.813429	0.602928
Control ovx							
9	7.201	8.52	3.18	2.965	6.14	5.21	2.345
10	2.798	10.235	5.165	4.315	3.405	3.725	1.73
11	4.4185	6.08	3.08	5.11	6.855	8.87	3.86
12	4.4305	6.49	6.485	6.87	7.415	5.095	1.94
13	3.151	7.72	7.025	6.07	4.185	4.62	3.41
14	3.592	8.305	5.295	4.7	5.375	4.47	3.655
15	2.8855	11.635	5.305	6.64	7.66	5.695	3.275
16	3.3425	7.93	5.59	8.965	9.31	3.72	4.015
Mean	3.977375	8.364375	5.140625	5.704375	6.293125	5.175625	3.02875
SD	1.444658	1.83457	1.399072	1.843755	1.934501	1.645584	0.894546
SEM	0.510764	0.648618	0.494647	0.651866	0.683949	0.581802	0.31627
5 IU/kgBW/day							
17	2.7545	3.065	4.555	5.03	4.775	3.805	5.97
18	4.5865	5.52	5.455	6.885	7.435	*	*
19	8.647	8.79	7.07	3.84	*	*	*
20	7.8435	7.79	8.84	10.35	7.38	9.91	3.59
21	8.8735	6.19	6.425	8.575	9.525	5.35	2.28
22	4.0935	7.87	5.255	9.855	8.085	3.905	4.555
23	2.864	5.75	4.995	5.375	6.17	5.055	5.43
24	4.59	5.12	9.69	5.13	7.48	7.9	7.105
42	6.2545	5.62	5.54	3.945	6.71	5.82	6.19
Mean	5.611889	6.190556	6.425	6.553889	7.195	5.963571	5.017143
SD	2.381171	1.732784	1.786872	2.485289	1.390596	2.215242	1.661094
SEM	0.793724	0.577595	0.595624	0.82843	0.49165	0.837283	0.627834
15 IU/kgBW/day							
25	3.863	5.885	3.605	6.23	6.635	3.675	3.465
26	3.52	6.685	4.065	7.635	5.51	3.505	6.145
27	2.737	6.24	5.83	5.2	6.435	4.56	6.1
28	7.6835	8.105	7.555	8.62	6.465	4.685	4.18
29	8.349	6.635	6.345	5.685	5.245	4.435	5.366
30	3.507	4.505	6.05	4.02	9.12	6.63	7.685
31	2.729	3.755	4.935	5.57	7.53	*	*
32	5.0815	6.97	6.165	5.57	7.695	2.92	4
Mean	4.68375	6.0975	5.56875	6.06625	6.829375	4.344286	5.277286
SD	2.191357	1.38946	1.294071	1.44345	1.25722	1.195566	1.491901
SEM	0.774762	0.491248	0.457523	0.510337	0.444495	0.451882	0.563886
50 IU/kgBW/day							
33	4.7935	5.09	4.255	5.795	7.795	5.79	5.115
34	4.402	5.025	3.255	9.205	5.41	6.34	3.01
35	3.363	3.885	2.61	3.14	3.845	3.51	3.525
36	4.092	7.04	2.965	3.09	2.65	3.87	2.91
37	3.67	4.79	5.33	3.295	3.115	3.75	3.365
38	3.203	5.915	4.45	3.35	5.335	4.145	4.3
39	4.675	8.175	8.44	5.17	5.77	3.905	2.35
40	3.537	8.115	*	*	*	*	*
41	3.502	8.455	16.745	6.36	7.275	3.615	4.04
Mean	3.915278	6.276667	6.00625	4.925625	5.149375	4.365625	3.576875
SD	0.591923	1.70832	4.713856	2.167471	1.85519	1.076238	0.880105
SEM	0.197308	0.56944	1.6666	0.766317	0.655909	0.380508	0.311164

Appendix A-16: Serum pyridinoline % change from baseline

Control sham	%chg 4wk	%chg 8wk	%chg 12wk	%chg16wk	%chg20wk	%chg24wk
1	0.980958	28.39008	170.3405	20.60012	26.08194	-43.0179
2	90.38495	130.0993	295.9241	246.4553	209.8763	114.7709
3	106.4051	142.4776	272.1626	200.1936	66.98927	113.0917
4	5.940955	40.08018	140.311	*	*	*
5	11.26662	-11.4766	91.39258	90.86774	58.50245	14.59062
6	-11.5278	-3.47222	72.36111	58.61111	-26.3889	-23.0556
7	87.34128	161.5745	117.2299	157.6675	195.3702	37.91756
8	39.10921	72.05613	32.76388	38.62111	5.308115	-5.43014
Mean	41.23766	69.96612	149.0607	116.1452	76.5342	29.83817
SD	46.81458	67.52588	93.34949	86.46476	91.76974	62.97101
SEM	16.55145	23.874	33.00403	32.68061	34.6857	23.80081
Control ovx						
9	18.3169	-55.8395	-58.8252	-14.7341	-27.6489	-67.4351
10	265.797	84.59614	54.2173	21.69407	33.13081	-38.1701
11	37.60326	-30.2931	15.65011	55.14315	100.7469	-12.64
12	46.4846	46.37174	55.06151	67.3626	14.99831	-56.2126
13	145.0016	122.9451	92.63726	32.81498	46.62012	8.219613
14	131.2082	47.41091	30.84633	49.63808	24.44321	1.753898
15	303.223	83.85029	130.1161	165.4653	97.36614	13.49853
16	137.2476	67.24009	168.2124	178.534	11.29394	20.11967
Mean	135.6103	45.78521	60.98948	69.48976	37.61881	-16.3583
SD	104.512	60.34149	70.3843	68.06458	43.57952	33.4685
SEM	36.95059	21.33394	24.88461	24.06446	15.40769	11.8329
5 IU/kgBW/day						
17	11.27246	65.36577	82.61027	73.3527	38.13759	116.7362
18	20.35321	18.93601	50.11447	62.10618	*	*
19	1.653753	-18.2375	-55.5915	*	*	*
20	-0.68209	12.70479	31.9564	-5.90935	26.34666	-54.2296
21	-30.2417	-27.5934	-3.36395	7.342086	-39.7081	-74.3055
22	92.25602	28.37425	140.7475	97.50824	-4.60486	11.27397
23	100.7682	74.40642	87.67458	115.433	76.5014	89.59497
24	11.54684	111.1111	11.76471	62.96296	72.11329	54.79303
42	-10.1447	-11.4238	-36.9254	7.282756	-6.947	-1.03126
Mean	21.86466	28.18263	34.33189	52.50982	23.11985	20.40455
SD	44.80256	46.84539	63.0965	44.91003	43.0231	71.1018
SEM	14.93419	15.61513	21.03217	15.87809	16.2612	26.87395
15 IU/kgBW/day						
25	52.34274	-6.67875	61.27362	71.7577	-4.86668	-10.3029
26	89.91477	15.48295	116.9034	56.53409	-0.42614	74.57386
27	127.9868	113.0069	89.98904	135.1114	66.60577	122.8718
28	5.485781	-1.67241	12.18846	-15.8587	-39.0252	-45.5977
29	-20.5294	-24.0029	-31.908	-37.1781	-46.8799	-35.7288
30	28.45737	72.51212	14.62789	160.0513	89.05047	119.1332
31	37.59619	80.83547	104.1041	175.9252	*	*
32	37.16422	21.32244	9.613303	51.43166	-42.5367	-21.2831
Mean	44.80231	33.85074	47.09897	74.72184	3.131674	29.09518
SD	46.60335	48.86407	53.5632	78.17434	54.54598	73.9826
SEM	16.47677	17.27606	18.93745	27.6388	20.61644	27.96279
50 IU/kgBW/day						
33	6.185459	-11.234	20.89288	62.61604	20.78857	6.706999
34	14.15266	-26.0563	109.1095	22.89868	44.02544	-31.622
35	15.52186	-22.3907	-6.63098	14.33244	4.371097	4.817128
36	72.04301	-27.5415	-24.4868	-35.2395	-5.42522	-28.8856
37	30.51771	45.23161	-10.218	-15.1226	2.179837	-8.31063
38	84.67062	38.93225	4.589447	66.5626	29.40993	34.24914
39	74.86631	80.53476	10.58824	23.42246	-16.4706	-49.7326
40	129.4317	*	*	*	*	*
41	141.4335	378.1553	81.61051	107.7384	3.226728	15.36265
Mean	63.20253	56.95392	23.18185	30.90107	10.26322	-7.17687
SD	50.22455	135.8381	47.18299	46.39705	19.73996	27.88208
SEM	16.74152	48.02602	16.6817	16.40384	6.979129	9.857805

Appendix A-17 Osteocalcin (ng/mL)

Control sham	Baseline	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks
1	34.385	3.485	3.7	13.015	11.87	10.18	9.275
2	54.675	2.42	3.2	16.605	19.41	11.125	18.06
3	49.955	2.73	3.79	14.79	12.25	10.47	11.085
4	53.667	5.89	3.15	5.105	*	*	*
5	51.705	5.385	4.125	14.245	11.08	4.375	13.445
6	50.68	5.935	3.18	10.535	10.155	9.885	13.61
7	52.08	5.82	15.595	17.37	12.09	10.48	10.265
8	60.68	2.385	15.85	17.34	12.965	14.745	12.005
Mean	50.97838	4.25625	6.57375	13.62563	12.83143	10.18	12.535
SD	7.495077	1.647699	5.657582	4.156903	3.036948	3.046621	2.906121
SEM	2.64991	0.58255	2.000257	1.469687	1.147859	1.151515	1.098411
Control ovx							
9	55.23	4.69	19.765	13.76	3.595	7.22	17.685
10	9.4	4.425	2.63	5.065	2.945	7.78	13.51
11	53.08	5.025	14.47	17.725	12.845	14.07	18.875
12	44.15	3.555	2.55	14.915	9.405	10.305	15.54
13	10.9	20.04	19.16	17.69	14.935	14.845	11.285
14	17.28	13.6	34.21	14.2	16.99	15.58	17.245
15	36.455	16.225	18.46	14.645	13.44	13.095	15.045
16	26.05	16.575	16.835	13.185	5.765	10.305	11.265
Mean	31.56813	10.51688	16.01	13.89813	9.99	11.65	15.05625
SD	18.35378	6.75247	10.17359	3.950732	5.374908	3.203061	2.866176
SEM	6.48904	2.387359	3.596907	1.396795	1.900317	1.132453	1.013346
5 IU/kgBW/day							
17	46.12	12.44	12.74	37.775	15.335	18.795	18.47
18	32.67	28.8	16.37	27.515	16.635	*	*
19	12.51	16.375	15.675	12.53	*	*	*
20	14.575	27.3	26.12	17.68	13.01	14.795	15.1
21	13.83	19.315	17.4	18.29	14.165	11.02	13.53
22	32.78	5.925	17.5	7.55	15.24	13.485	18.04
23	19.09	42.01	27.78	26.275	11.48	11.945	19.785
24	51.415	44.785	35.16	35.62	18.06	18.115	19.79
42	17.26	48.35	16.875	28.205	17.985	17.74	18.41
Mean	26.69444	27.25556	20.62444	23.49333	15.23875	15.12786	17.58929
SD	13.83076	14.25276	6.925515	9.617042	2.171077	2.903424	2.203602
SEM	4.610253	4.750919	2.308505	3.205681	0.767591	1.097391	0.832883
15 IU/kgBW/day							
25	35.64	41.475	19.125	27.155	12.145	15.655	14.18
26	15.04	5.305	15.445	17.415	13.25	12.975	17.56
27	16.895	10.47	10.655	16.04	14.395	11.3	16.07
28	16.71	3.665	12.745	15.09	11.005	5.085	17.605
29	15.32	3.905	14.56	20.725	13.375	14.75	10.915
30	15.405	39.325	15.565	11.225	34.14	15.28	18.125
31	44.46	5.26	12.82	19.1	16.56	*	*
32	31.565	44.655	15.585	16.785	16.83	3.085	16.345
Mean	23.87938	19.2575	14.5625	17.94188	16.4625	11.16143	15.82857
SD	11.61487	18.85282	2.542606	4.666773	7.418038	5.09072	2.536832
SEM	4.106478	6.66548	0.898947	1.649953	2.622673	1.924111	0.958832
50 IU/kgBW/day							
33	37.805	5.275	13.055	19.49	3.235	15.385	17.38
34	10.4	10.945	3.15	19.43	18.615	20.225	15.25
35	12.325	39.92	2.87	11.44	7.82	17.95	20.645
36	12.705	5.71	4.335	14.73	5.975	17.83	35.36
37	16.105	48.395	20.64	11.185	10.51	5.23	20.515
38	15.9	28.72	12.36	16.42	20.545	14.245	16.965
39	18.105	7.775	18.725	16.655	16.78	12.25	15.97
40	19.515	49.445	*	*	*	*	*
41	36.32	3.935	13.03	20.51	11.825	7.39	18.48
Mean	19.90889	22.23556	11.02063	16.2325	11.91313	13.81313	20.07063
SD	10.14505	19.40266	6.917235	3.586658	6.238272	5.269703	6.478099
SEM	3.381682	6.467555	2.445612	1.268075	2.205562	1.863121	2.290354

Appendix A-18: Osteocalcin % change from baseline

Control sham		%chg 4wk	%chg 8wk	%chg 12wk	%chg 16wk	%chg 20wk	%chg24wk
	1	-89.8648	-89.2395	-62.1492	-65.4791	-70.3941	-73.026
	2	-95.5738	-94.1472	-69.6296	-64.4993	-79.6525	-66.9684
	3	-94.5351	-92.4132	-70.3934	-75.4779	-79.0411	-77.81
	4	-89.0249	-94.1305	-90.4876	*	*	*
	5	-89.5851	-92.022	-72.4495	-78.5707	-91.5385	-73.9967
	6	-88.2893	-93.7253	-79.2127	-79.9625	-80.4953	-73.1452
	7	-88.8249	-70.0557	-66.6475	-76.7857	-79.8771	-80.2899
	8	-96.0695	-73.8794	-71.4239	-78.6338	-75.7004	-80.2159
Mean		-91.4709	-87.4516	-72.7992	-74.2013	-79.5284	-75.0646
SD		3.308294	9.741845	8.644804	6.460707	6.372366	4.759291
SEM		1.169659	3.444262	3.0564	2.441918	2.408528	1.798843
Control ovx							
	9	-91.5082	-64.2133	-75.086	-93.4909	-86.9274	-67.9794
	10	-52.9255	-72.0213	-46.117	-68.6702	-17.234	43.7234
	11	-90.5332	-72.7393	-66.607	-75.8007	-73.4928	-64.4405
	12	-91.9479	-94.2242	-66.2174	-78.6976	-76.6591	-64.8018
	13	83.85321	75.77982	62.29358	37.01835	36.19266	3.53211
	14	-21.2963	97.97454	-17.8241	-1.67824	-9.83796	-0.20255
	15	-55.4931	-49.3622	-59.8272	-63.1326	-64.079	-58.7299
	16	-36.3724	-35.3743	-49.3858	-77.8695	-60.4415	-56.7562
Mean		-44.5279	-26.7725	-39.8464	-52.7902	-44.0599	-33.2069
SD		58.37193	72.48156	44.88159	45.54956	42.70662	42.67185
SEM		20.63759	25.6261	15.86804	16.1042	15.09907	15.08678
5 IU/kgBW/day							
	17	-73.0269	-72.3764	-18.0941	-66.7498	-59.2476	-59.9523
	18	-11.8457	-49.8929	-15.779	-49.0817	*	*
	19	30.89528	25.29976	0.159872	*	*	*
	20	87.30703	79.21098	21.3036	-10.7376	1.509434	3.602058
	21	39.66016	25.81345	32.24873	2.42227	-20.3181	-2.1692
	22	-81.925	-46.6138	-76.9677	-53.5082	-58.8621	-44.9664
	23	120.0629	45.52122	37.63751	-39.8638	-37.428	3.64065
	24	-12.8951	-31.6153	-30.7206	-64.8741	-64.7671	-61.5093
	42	180.1275	-2.23059	63.41251	4.200463	2.780997	6.662804
Mean		30.92891	-2.98706	1.466762	-34.7741	-33.7618	-22.0988
SD		82.16028	47.70006	39.93935	27.36415	26.76482	29.41446
SEM		27.38676	15.90002	13.31312	9.674689	10.11615	11.11762
15 IU/kgBW/day							
	25	16.37205	-46.3384	-23.8075	-65.9231	-56.0746	-60.2132
	26	-64.7274	2.692819	15.79122	-11.9016	-13.7301	16.75532
	27	-38.029	-36.934	-5.06067	-14.7973	-33.1163	-4.8831
	28	-78.067	-23.7283	-9.69479	-34.1412	-69.5691	5.356074
	29	-74.5104	-4.96084	35.28068	-12.6958	-3.72063	-28.7533
	30	155.2743	1.038624	-27.134	121.6164	-0.81142	17.6566
	31	-88.1691	-71.1651	-57.04	-62.753	*	*
	32	41.46998	-50.6257	-46.824	-46.6815	-90.2265	-48.218
Mean		-16.2983	-28.7526	-14.8111	-15.9096	-38.1784	-14.6142
SD		83.69052	27.04776	30.7242	59.66092	34.69302	31.43076
SEM		29.58907	9.562828	10.86265	21.09332	13.11273	11.87971
50 IU/kgBW/day							
	33	-86.0468	-65.4675	-48.446	-91.4429	-59.3043	-54.0272
	34	5.240385	-69.7115	86.82692	78.99038	94.47115	46.63462
	35	223.8945	-76.714	-7.18053	-36.5517	45.63895	67.50507
	36	-55.0571	-65.8796	15.93861	-52.9713	40.33845	178.3156
	37	200.4967	28.15896	-30.5495	-34.7408	-67.5256	27.3828
	38	80.62893	-22.2642	3.27044	29.21384	-10.4088	6.698113
	39	-57.0561	3.424468	-8.00884	-7.31842	-32.3391	-11.7923
	40	153.3692	*	*	*	*	*
	41	-89.1657	-64.1244	-43.5297	-67.4422	-79.6531	-49.1189
Mean		41.81157	-41.5722	-3.95983	-22.7829	-8.5978	26.44971
SD		125.6412	39.56395	43.04322	55.10143	62.8138	74.83013
SEM		41.8804	13.98797	15.21808	19.4813	22.20803	26.45645

Appendix A-19: Calcitonin antibodies (absorbance at 450nm)

Control sham	Baseline	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks
1	0.44725	0.2736	0.17085	0.1754	0.18425	0.252	0.1695
2	0.1913	0.2214	0.1226	0.5884	*	0.38475	0.60685
3	0.26515	0.33335	0.2254	0.2035	0.26985	0.18055	0.2276
4	0.25615	0.22005	0.1251	0.14335	*	*	*
5	0.20135	0.17625	0.137	0.165	0.1273	0.2181	0.2354
6	0.1851	0.1832	0.11735	0.1286	0.133	0.1555	0.1526
7	0.1488	0.1905	0.1265	0.20345	0.15765	0.19405	0.20245
8	0.22055	0.2207	0.11325	0.14885	0.3145	0.3234	0.1959
Mean	0.239456	0.227381	0.142256	0.219569	0.197758	0.24405	0.255757
SD	0.092133	0.05268	0.038077	0.151454	0.077169	0.082868	0.157575
SEM	0.032574	0.018625	0.013462	0.053547	0.029167	0.031321	0.059558
Control ovx							
9	0.21745	0.1853	0.12775	0.1376	0.1369	0.3494	0.187
10	*	0.2187	0.12095	0.12345	0.16055	0.14585	0.2195
11	0.2075	0.22665	0.1354	0.14965	0.14455	0.67435	0.24155
12	*	*	0.1471	0.18645	0.12045	0.1687	0.1813
13	0.2131	0.15985	0.14485	0.19705	0.1985	0.20185	0.19975
14	0.72605	0.89115	1.4923	1.1152	0.5583	0.83325	0.2328
15	0.28265	0.18555	0.14915	0.196	0.1642	0.1772	0.3343
16	0.2361	0.2049	0.1264	0.17235	0.2133	0.16985	0.2215
Mean	0.313808	0.296014	0.305488	0.284719	0.212094	0.340056	0.227213
SD	0.203804	0.2634	0.479658	0.336672	0.143233	0.266392	0.048219
SEM	0.083203	0.099556	0.169585	0.119031	0.050641	0.094184	0.017048
5 IU/kgBW/day							
17	0.382	0.25895	0.6064	0.2503	0.23415	0.40845	0.19
18	0.44645	0.2687	0.1812	0.2279	0.1897	*	*
19	0.1694	0.22085	0.17575	0.1873	*	*	*
20	0.24045	0.22835	0.1364	0.1613	0.1683	0.13575	0.1544
21	0.1969	0.26335	0.1605	0.20555	0.3039	0.14615	0.2174
22	0.87995	0.60905	0.1598	0.38455	0.3126	*	0.3158
23	0.1951	0.16225	0.12075	0.137	0.15825	0.1378	0.16335
24	0.2721	0.22975	0.12295	0.1473	0.1531	0.1301	0.1702
42	0.12805	*	0.3539	0.62385	0.52855	0.52205	0.2239
Mean	0.323378	0.280156	0.224183	0.258339	0.256069	0.246717	0.205007
SD	0.232426	0.137154	0.159673	0.156053	0.126767	0.173122	0.055556
SEM	0.077475	0.048491	0.053224	0.052018	0.044819	0.070677	0.020998
15 IU/kgBW/day							
25	0.19675	0.185	0.1251	0.14745	0.1225	0.21155	0.2086
26	0.2096	0.1562	0.10935	0.1394	*	0.3309	0.48605
27	0.26715	0.32555	0.1282	0.13195	0.1566	0.2072	0.22395
28	0.2255	0.1823	0.3107	0.5164	*	0.2706	0.23595
29	0.18655	0.1717	0.1182	0.16845	0.45455	0.2995	0.1919
30	0.15175	0.19175	0.25165	0.1955	0.19125	0.16055	0.2534
31	0.35865	0.3401	0.18005	0.4024	0.1406	*	*
32	0.18345	*	0.5611	0.4033	0.38615	0.2663	0.22675
Mean	0.222425	0.2218	0.223044	0.263106	0.241942	0.249514	0.260943
SD	0.064577	0.0768	0.154522	0.152422	0.141695	0.059154	0.101159
SEM	0.022832	0.029028	0.054632	0.053889	0.057847	0.022358	0.038235
50 IU/kgBW/day							
33	0.16905	0.15445	0.1209	0.1633	0.34675	0.2846	0.34235
34	0.4693	0.18255	0.1043	0.12795	0.1772	0.1601	0.1492
35	0.56935	0.32605	0.204	0.41995	0.17845	0.17075	0.1887
36	0.56525	0.3367	0.17355	0.1251	0.31235	0.21275	0.20715
37	0.1427	0.13935	0.13625	0.30045	0.15215	0.1295	0.16065
38	0.6238	0.18485	0.17385	0.16285	0.16555	0.14105	0.18585
39	0.29995	0.22555	0.292	0.33325	0.466	0.3282	0.31545
40	0.1305	0.1398	*	*	*	*	*
41	0.226	0.1817	0.114	0.1723	0.23145	0.13935	0.1823
Mean	0.3551	0.207889	0.164856	0.225644	0.253738	0.195788	0.216456
SD	0.201615	0.074929	0.061943	0.110362	0.111651	0.073857	0.071959
SEM	0.067205	0.024976	0.0219	0.039019	0.039475	0.026113	0.025442

Appendix A-20: Calcitonin antibodies % change from baseline

Control sham	%chg 4wk	%chg 8wk	%chg 12wk	%chg 16wk	%chg 20wks	%chg24wk
1	-38.8262	-61.7999	-60.7826	-58.8038	-43.6557	-62.1017
2	15.73445	-35.9122	207.5797	*	101.1239	217.2243
3	25.72129	-14.9915	-23.251	1.772582	-31.9065	-14.1618
4	-14.0933	-51.1614	-44.0367	*	*	*
5	-12.4659	-31.9593	-18.0531	-36.7768	8.318848	16.91085
6	-1.02647	-36.6018	-30.524	-28.1469	-15.9914	-17.5581
7	28.02419	-14.9866	36.72715	5.947581	30.40995	36.05511
8	0.068012	-48.6511	-32.5096	42.59805	46.63342	-11.1766
Mean	0.392019	-37.008	4.393725	-12.2349	13.5618	23.59886
SD	22.53832	16.6778	86.82713	36.21498	50.47572	90.70499
SEM	7.9685	5.896494	30.69803	14.7847	19.07803	34.28326
Control ovx						
9	-14.785	-41.2509	-36.7211	-37.043	60.68062	-14.0032
10	*	*	*	*	*	*
11	9.228916	-34.747	-27.8795	-30.3373	224.988	16.40964
12	*	*	*	*	*	*
13	-24.9883	-32.0272	-7.53168	-6.85124	-5.27921	-6.26466
14	22.73948	105.5368	53.59824	-23.1045	14.76482	-67.9361
15	-34.3534	-47.2316	-30.6563	-41.907	-37.3076	18.27348
16	-13.2147	-46.4634	-27.0013	-9.65693	-28.0601	-6.18382
Mean	-9.22884	-16.0305	-12.6986	-24.8167	38.29774	-9.95078
SD	21.39638	59.86743	33.93097	14.33558	97.90456	31.29849
SEM	8.735036	24.44077	13.85226	5.852478	39.96937	12.77755
5 IU/kgBW/day						
17	-32.212	58.74346	-34.4764	-38.7042	6.924084	-50.2618
18	-39.8141	-59.4131	-48.9529	-57.5092	*	*
19	30.3719	3.748524	10.56671	*	*	*
20	-5.03223	-43.273	-32.9174	-30.0062	-43.5434	-35.7871
21	33.7481	-18.4865	4.393093	54.34231	-25.7745	10.41138
22	-30.7858	-81.8399	-56.2987	-64.4753	*	-64.1116
23	-16.8375	-38.1087	-29.7796	-18.8877	-29.3696	-16.2737
24	-15.5641	-54.8144	-45.8655	-43.7339	-52.1867	-37.4495
42	*	176.3764	387.1925	312.7684	307.6923	74.85357
Mean	-9.51573	-6.34081	17.09576	14.22427	27.29038	-16.9455
SD	27.92491	79.93006	140.6446	126.0899	138.8526	47.05662
SEM	9.872948	26.64335	46.88153	44.57953	56.68635	17.78573
15 IU/kgBW/day						
25	-5.97205	-36.4168	-25.0572	-37.7382	7.522236	6.022872
26	-25.4771	-47.8292	-33.4924	*	57.87214	131.8941
27	21.86038	-52.012	-50.6083	-41.3812	-22.4406	-16.1707
28	-19.1574	37.78271	129.0022	*	20	4.634146
29	-7.96033	-36.639	-9.70249	143.6612	60.54677	2.867864
30	26.35914	65.83196	28.83031	26.02965	5.799012	66.98517
31	-5.17217	-49.7979	12.19852	-60.7974	*	*
32	*	205.8599	119.8419	110.4933	45.16217	23.60316
Mean	-2.21708	10.84747	21.37658	23.37788	24.92311	31.40523
SD	19.49421	90.50623	68.38224	86.1146	30.8231	51.41922
SEM	7.368119	31.99878	24.17677	35.15614	11.65004	19.43464
50 IU/kgBW/day						
33	-8.6365	-28.4827	-3.40136	105.1168	68.35256	102.514
34	-61.1016	-77.7754	-72.736	-62.2416	-65.8854	-68.208
35	-42.7329	-64.1697	-26.2404	-68.6572	-70.0097	-66.8569
36	-40.4334	-69.2968	-77.8682	-44.7413	-62.3618	-63.3525
37	-2.34758	-4.51997	110.5466	6.622285	-9.25018	12.57884
38	-70.3671	-72.1305	-73.8939	-73.461	-77.3886	-70.2068
39	-24.8041	-2.65044	11.10185	55.35923	9.418236	5.167528
40	7.126437	*	*	*	*	*
41	-19.6018	-49.5575	-23.7611	2.411504	-38.3407	-19.3363
Mean	-29.211	-46.0729	-19.5316	-9.94892	-30.6832	-20.9625
SD	26.44982	30.43635	62.53108	64.76127	50.57513	60.49469
SEM	8.816607	10.76088	22.10808	22.89657	17.88101	21.3881

Appendix A-21: Area under the curve measurements

Subject #	BMC Sp	BMC RF	BMC LF	OC	HP	PYD	Uca
1	0.1674	0.0594	0.0634	256.3	2229	114.4	1531
2	0.161	0.0556	0.061	356.5	2529	187.2	1628
3	0.1786	0.0654	0.064	298.2	2599	164.2	1853
4	*	*	*	*	*	*	*
5	0.185	0.0632	0.0654	287.1	1762	96.93	1555
6	0.1884	0.0666	0.068	287.3	2311	97.64	1445
7	0.1664	0.0596	0.0622	370.1	2548	137	1954
8	0.1646	0.058	0.0632	398.5	2912	128.7	1724
Mean	0.173057	0.061114	0.063886	322	2412.857	132.2957	1670
SD	0.010816	0.004045	0.002277	52.69589	361.326	33.76249	183.483
SEM	0.003824	0.00143	0.000805	19.91717	136.5684	12.76102	69.35004
9	0.1542	0.0576	0.0598	342	1278	123.2	962.4
10	0.1656	0.0544	0.0618	137.2	1404	116.4	808.8
11	0.1564	0.0536	0.0576	400.5	2731	136.5	1370
12	0.1502	0.0498	0.0524	282.3	1928	142.2	1382
13	0.1534	0.0514	0.0568	391.1	3180	131.6	1370
14	0.1484	0.0528	0.053	447.4	2083	127.1	1159
15	0.1566	0.0544	0.057	406.5	1842	160.1	1320
16	0.1576	0.0524	0.0582	325.3	2440	156.8	1087
MEAN	0.1553	0.0533	0.057075	341.5375	2110.75	136.7375	1182.4
SD	0.005247	0.002327	0.003159	97.75098	647.6081	15.558	216.0645
SEM	0.001855	0.000823	0.001117	34.56019	228.964	5.500582	76.39034
17	0.1536	0.05028	0.0548	517.5	2374	102.4	1123
18	*	*	*	*	*	*	*
19	*	*	*	*	*	*	*
20	0.1668	0.0575	0.0604	455	2054	199.9	1248
21	0.1618	0.051	0.0582	375.5	1982	166.6	1581
22	0.1752	0.05744	0.0658	340.4	2281	157.2	905.6
23	0.1558	0.05103	0.0564	555.7	2430	126	1391
24	0.164	0.0567	0.0574	763.1	2525	164.7	1507
42	0.1574	0.05546	0.0558	588	3573	135.4	1751
MEAN	0.162086	0.054201	0.0584	513.6	2459.857	150.3143	1358.086
SD	0.007432	0.003288	0.003731	142.64	528.6226	31.85312	288.5188
SEM	0.002477	0.001096	0.001244	47.54667	199.8005	12.03935	109.0499
25	0.1536	0.05	0.0572	561.9	2020	118.8	1176
26	0.1606	0.054	0.0594	322.8	2142	128.9	1305
27	0.1622	0.0554	0.0582	317.4	2436	130.7	1147
28	0.1612	0.0546	0.0576	321.7	2499	165.4	1854
29	0.1558	0.0504	0.0558	321.7	3227	140.8	1469
30	0.1658	0.057	0.0606	529.2	3427	143.7	1771
31	*	*	*	*	*	*	*
32	0.1544	0.0582	0.05832	483.6	3761	135.4	1924
MEAN	0.159086	0.054229	0.05816	408.3286	2787.429	137.6714	1520.857
SD	0.004553	0.003097	0.001545	111.3942	678.5197	14.72998	327.6326
SEM	0.00161	0.001095	0.000546	39.3838	256.4563	5.56741	123.8335
33	0.165	0.0592	0.0626	336.1	3031	134.7	1825
34	0.1622	0.0508	0.0576	340.8	2433	131.8	1626
35	0.1604	0.0522	0.0582	385.9	2418	81.74	2229
36	0.1666	0.0546	0.0612	290.5	2254	92.46	2389
37	0.1678	0.0572	0.0578	457.1	2654	95.19	1635
38	0.1576	0.0556	0.0596	434.9	2419	107.8	1715
39	0.1532	0.0534	0.0576	356.9	2623	139.9	2267
40	*	*	*	*	*	*	*
41	0.1604	0.0556	0.0606	336.4	2026	141.9	1785
MEAN	0.16165	0.054825	0.0594	367.325	2482.25	115.6863	1933.875
SD	0.004852	0.002703	0.001906	55.57062	297.9097	24.10909	309.665
SEM	0.001617	0.000901	0.000635	19.64718	105.327	8.523851	109.4831

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Presentations: "Moving with arthritis". Community informational presentation for the Health Resources Center at the Johnson City Mall. June 2004.
"Osteoarthritis of the wrist and hands". Community informational presentations for the Health Resources Center at the Johnson City Mall. February 2004, March 2004, and April 2004.
"Dose-dependent effects of salmon calcitonin in ovariectomized rats – a pilot study". Poster presentation for East Tennessee State University Research Forum. April 2003.

"Carpal Tunnel Syndrome Prevention". System-wide employee informational seminars for Mountain States Health Alliance. September 2002, April 2003, October 2003, March 2004.

"Dose-dependent effects of salmon calcitonin in ovariectomized rats-a pilot study". Abstract submitted and accepted for poster presentation to the International Society for Clinical Densitometry. November 2002.

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