

**The Role Of Boron Alone Or In Combination With Estrogen Or PTH  
In The Treatment Of Postmenopausal Osteoporosis In Ovariectomized  
Rats**

by

Matilda H. Sheng

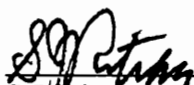
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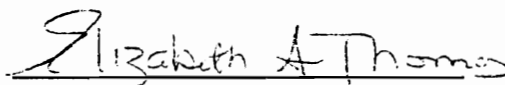
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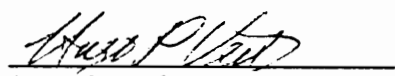
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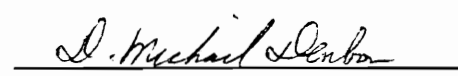
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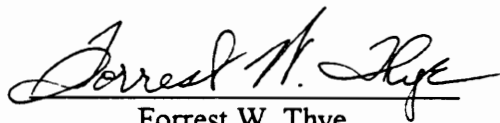
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# **The Role Of Boron Alone Or In Combination With Estrogen Or PTH In The Treatment Of Postmenopausal Osteoporosis In Ovariectomized Rats**

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**(ABSTRACT)**

The effects of boron alone or in combination with estrogen or parathyroid hormone (PTH) on bone quality and Ca, P, and Mg metabolism were investigated in 12-week-old rats. Treatment began on day 43 after ovariectomy and continued for 5 weeks. Treatments included 5 ppm (5 mg per kg diet) boron as boric acid,  $17\beta$  estradiol (30  $\mu\text{g}/\text{kg}/\text{d}$ , s.c.), PTH (rat PTH 1-34 fragment, 60  $\mu\text{g}/\text{kg}/\text{d}$ , s.c.), boron+ $17\beta$  estradiol, and boron+PTH at the same dose. All rats had free access to food (AIN 76) and deionized water. Ovariectomy caused decreases in the ratios of dried bone, bone ash, bone Ca, P, and Mg to body or dried bone weight, wall thickness of the femur, urinary Ca/creatinine excretion, trabecular bone volume, bone surface density, and trabecular plate density, and increases in serum osteocalcin, and trabecular plate separation. However, intermittent PTH injection increased the dried bone weight to body weight ratio, wall thickness of the femur, total bone ash, bone ash to dried bone or body weight ratio, bone Ca, P, and Mg, bone Ca, Mg, and P to dried bone weight or body weight ratios, trabecular bone volume,

bone surface density, trabecular plate density, and thickness, serum osteocalcin concentration, and decreased trabecular plate separation, but had no effects on Ca, P, and Mg metabolism. Treatment with  $17\beta$  estradiol increased the dried bone weight, bone ash, and bone Ca, P, and Mg to body weight ratios, and decreased serum osteocalcin, and trabecular bone separation, but had no effects on Ca, P, and Mg metabolism. However, when combined with boron,  $17\beta$  estradiol not only remained the positive effects as observed in Ovx rats treated with  $17\beta$  estradiol alone, but also improved apparent Ca, Mg, and P absorption, and Ca and Mg retention, trabecular bone volume, bone surface density and trabecular plate density which were not found in Ovx rats treated with  $17\beta$  estradiol alone. In contrast, boron in combination with PTH did not provide an additional benefit for Ca, P, and Mg balance or bone quality when compared with PTH alone, but significantly improved the percentages of apparent Ca absorption and retention compared with Ovx rats without treatment. The improvement of Ca balance by the combined treatment with PTH plus boron was not found in Ovx rats receiving PTH alone. In conclusion,  $17\beta$  estradiol in combination with boron that was initiated 6 weeks after ovariectomy, provided a more beneficial regimen than boron or  $17\beta$  estradiol alone for postmenopausal bone loss in Ovx rats. The importance of the present findings is that boron has the potential to improve estrogen action on bone and Ca balance, in particular when estrogen is initiated at the late state following menopause.

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# CHAPTER 1

## INTRODUCTION

Postmenopausal osteoporosis is a metabolic bone disease in which bone mass is reduced and hip, vertebrae, radius, and humerus fractures are involved. Although the pathogenesis of postmenopausal osteoporosis is related to ovarian hormone deficiency following menopause, the exact mechanism is not clear. Studies in ovariectomized rats and osteoporotic patients indicate a rapid increase in bone turnover with bone resorption exceeding bone formation, leading to a substantial reduction in bone mass or volume, following a loss of ovarian function (Heaney et al., 1978; Miller and Wronski, 1993). Due to the severity of osteoporosis and the high cost for treatment, making progress towards the screening, prevention, and treatment of postmenopausal osteoporosis is critical and necessary.

Therapeutic regimens for postmenopausal osteoporosis must be able to inhibit bone resorption and/or enhance bone formation. Estrogen therapy has been demonstrated to be a good antiresorptive treatment which inhibits bone resorption and improves Ca balance in humans and in animals (Stock et al., 1985; Chow et al., 1992; Turner et al., 1993; Kaplan et al., 1994; ). However, estrogen therapy must be initiated at the onset of menopause, or within 5 years of menopause to exert its greatest efficacy (Han et al., 1992; Lindsay et al., 1993). Intermittent PTH injection at a low dose has pronounced anabolic effects on bone and Ca metabolism (Ibbotson et al., 1992; Reeve et al., 1993; Wronski and

Yen, 1994). PTH increases bone formation but did not affect bone resorption at the early or late state of menopause (Hock et al., 1989; Ibbotson et al., 1992). The disadvantage of PTH treatment is that it must be given by parenteral injection.

Boron, an ultratrace element, has many potential properties related to the prevention and treatment of osteoporosis. Previous studies demonstrated that boron improved bone growth, mineralization, and cartilage maturation in young animals (King et al., 1991a, b, and 1993), and improves Ca and P balance in humans and animals (Elsair et al., 1980; Nielsen et al., 1987a; Nielsen et al., 1990; Hegsted et al., 1991). The effects of boron on Ca and Mg metabolism are associated with the interaction of boron with PTH (Nielsen, 1985; Hunt and Nielsen, 1985; Nielsen, 1986). Boron was reported to have the effect on the prevention of serum  $17\beta$  estradiol from degradation (Nielsen et al., 1990; Beattie and Weersink, 1992). Moreover, current research found that organic boron compounds, such as amine-carboxyborane, appeared to have anti-osteoporotic activity *in vitro* and *in vivo* studies by reducing bone resorption and increasing the incorporation of proline into collagen (Hall et al., 1994; Rajendran et al., 1995).

The present study was designed to assess the role of boron in the treatment of postmenopausal bone loss. To achieve this goal, boron's effects on bone quality and Ca, Mg, and P metabolism were examined. The hypotheses were that, 1) boron alone increases bone quality and improves Ca, Mg, and P balance, 2) boron in combination with  $17\beta$  estradiol improves the antiresorptive effects of estrogen on bone, and Ca balance; 3)

boron in combination with PTH enhances the anabolic effects of PTH on bone mass and Ca, Mg, and P metabolism.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Bone Physiology

##### Bone Matrix

Bone is a highly organized tissue whose metabolic activity is under the control of a large number of hormones and other agents. The bone matrix contains type I collagen, proteoglycans, glycoprotein, phosphoproteins, and minerals. Among these, osteocalcin, also called bone gla protein, is unique to bone. Its serum level has been correlated with active mineralization and bone formation. Osteonectin, however, was initially thought to be unique to bone, but has been found to be present in other connective tissues and in platelets. Bone minerals in normal adult bone is located within and adjacent to the collagen fibrils. A total of 70% of dried weight of the cortical bone is mineral, which is higher than that in trabecular bone. In fully mineralized bone, the calcium content is about  $0.6 \text{ g/cm}^3$  (Nusgens et al., 1972).

The mineral in bone and calcified cartilage is mainly in the form of hydroxyapatite ( $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ ). The initial mineral deposit in bone is octocalcium phosphate (OCP) with the composition of  $[\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}]$ . During bone turnover, OCP transforms spontaneously within a month to apatite minerals, and at the same time small amounts of new OCP are formed. Precipitation of new OCP is a necessary step in the formation of new bone and it occurs when the local pH rises to 7.6. Interestingly, the

body maintains the pH (7.45 to 7.57) of the bone extracellular fluid (BECF) higher than the pH (7.35 to 7.45) of plasma, extracellular fluid, and cerebrospinal fluid. The reason is to keep other body fluids undersaturated with OCP, allowing calcification to occur only in bone (Driessens et al., 1987).

Biological minerals are not pure hydroxyapatite, and the molar ratio of calcium to phosphorus is actually below the theoretical value of 1.67 for hydroxyapatite. The lower ratio of calcium to phosphorus is probably due to ion substitution, the presence of calcium salts other than hydroxyapatite, or surface binding and adsorption of other charged materials. Substitution of calcium by hydrogen is found in calcium-deficient apatites. Other important substitutions are the replacement of phosphate by carbonate and of hydroxyl ions by fluoride. The presence of octocalcium phosphate (OCP), tricalcium phosphate hydrate ( $[\text{Ca}_3(\text{PO}_4)_2\text{H}_2\text{O}]$ ), dicalcium phosphate dihydrate ( $[\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}]$ ), and amorphous calcium phosphate contribute to the low Ca/P ratio. Ions as phosphate, pyrophosphate, diphosphate, and polyphosphate bind to the surface of the crystals, giving a low Ca/P ratio in the crystals (Russell, 1986).

## **Bone Types**

The structure of the bone matrix of rapidly growing embryonic bone is different from that of slowly deposited adult bone. The embryonic bone is rather irregular; the collagen fibers are large and laid down randomly, and the matrix is unevenly calcified. This type of bone is called woven bone (embryonic bone). The woven bone is present

during the formation of the first bone of the embryo or in states of high bone turnover.

The formation of woven bone is also observed in the individuals on a fluoride treatment, in which an initial high rate of bone formation and improper lamellar alignment are observed.

In contrast, adult bone is laid down in layers of lamellae; the collagen fibers are thin, parallel to each other within each lamella and are oriented at right angles in adjacent lamellae. This type of bone called as lamellar bone (mature bone), is found in most of the adult skeleton.

### **Bone Cells**

Osteoblasts *in situ* occur as a contiguous layer of cells in bone. Osteoblasts are responsible for synthesis of the organic bone matrix, or osteoid and subsequently bone mineralization. Active osteoblasts are cuboidal secretory cells that stain strongly for alkaline phosphatase activity (Tam et al., 1989). However, not all osteoblasts have this typical appearance. Some osteoblast-like cells such as intermediate osteoblasts, are thinner and more elongated than active osteoblasts. When the synthesis of organic matrix is terminated, some active osteoblasts become lining cells, which cover quiescent bone surface, and the others become osteocytes, which are buried by the matrix secreted by adjoining osteoblasts or by themselves. Current evidence indicates that osteocytes are mechanosensory cells of bone (Burger et al., 1995). They detect and integrate mechanical loads, and in turn change mechanical loads to a biochemical signal by increasing prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis. Osteoblasts have receptors for parathyroid hormone

(PTH). A two-receptor model for PTH action in osteoblasts is proposed, in which one receptor is coupled to the production of cAMP, whereas the other is involved in the increase of cytosolic calcium. The former involves the activation of already existing osteoclasts, whereas the latter is associated with the recruitment of additional osteoclasts (Lowik et al., 1985).

Osteoclasts are mononucleated or multinucleated cells that stain positive for tartrate-resistant acid phosphatase (TRAP). However, it is found that 29% of osteoclasts have no nucleus by TRAP staining in human bone (Evans et al., 1979). Active osteoclasts have a ruffled border region surrounded by an organelle-free region, actin-containing “clear zone.” They synthesize lysosomal enzymes, in particular TRAP, and cathepsins that are able to degrade type I collagen. Osteoclasts adhere to the mineralized bone surface, release lysosome enzymes, causing mineral dissolution, and subsequently degradation of the organic matrix (Chambers and Fuller, 1985). Current evidence indicates that osteoclasts have no receptors for  $1, 25(\text{OH})_2\text{D}_3$  and parathyroid hormone (PTH) but do have receptors for calcitonin, which inhibits bone resorption. The inhibition due to calcitonin is reached by reducing osteoclast motility, retracting osteoclast cytoplasmic extensions, and decreasing ruffled border size (Price et al., 1994).

## **Bone Tissue**

Cortical bone, constituting 80% of bone mass, is compact, dense bone as seen in the shafts of long bones and in the vertebral endplates. Cortical bone is remodeled

through the activity of bone remodeling units (BRUs), which is about 400 mm in length and 200 mm in width. Cortical bone has the Haversian systems or osteons. Haversian canals are round or oval in cross-section and contain blood vessels and soft connective tissue elements. They are surrounded by lamellae arranged concentrically along the BRU's long axis. The cortical osteon constitutes a bone structural unit (BSU) as the end result of a remodeling cycle in cortical bone. The porosity of cortical bone is frequently used to estimate bone remodeling activity. If remodeling activity increases, the porosity will increase and a reduction in cortical bone mass may be expected. Anatomically, cortical bone is enclosed by periosteal and endosteal surfaces. The periosteal surface is important in appositional growth and fracture repair. Bone formation exceeds resorption so that a net increase in bone on the periosteal surface occur with time. However, the endosteal surface of cortical bone has a higher remodeling activity than the periosteal surface. Bone resorption exceeds formation on the endosteal surface, leading to expansion of the bone marrow space.

Trabecular bone, constituting the remaining 20% of the total bone mass, is made up of an interconnected structure of curved plates and struts, with thickness from 50 to 400 mm. The trabeculae are interconnected, and thus provide maximal mechanical strength. The BRU in the trabeculae is a disk-like, semilunar structure, about 300 mm in width and several times as long. After bone turnover is completed, the trabecular osteon is formed as BSU, representing the end result of a remodeling cycle in trabecular bone. Trabecular bone volume decreases with age and this decrease is due to a loss of the



number of trabecular bone and/or due to a thinning of trabecular bone. A loss of trabecular connection may contribute to the reduction in the mechanical strength of bone. Currently, histomorphometry is used as a method for quantifying three-dimensional structures from two-dimensional sections. It allows the estimation of the trabecular bone volume (BV/TV) and surface areas of trabeculae. With measurement of total perimeter and bone area, mean trabecular plate density (MTPD), mean trabecular plate thickness (MTPT), and mean trabecular plate separation (MTPS) can be derived, and they appear to well relate to bone formation, resorption, strength, and structure.

### **Bone Turnover**

Bone at the tissue level is continuously being resorbed and rebuilt. The terms 'modeling' and 'remodeling' are two different physiological events and are often misused. Modeling means changes in bone size and shape caused by modeling drifts where either bone resorption or formation occurs (Turner, 1991). Bone may widen or change in axis by the removal or addition of bone. In contrast, remodeling means bone turnover in which bone is renewed through the continuous removal and replacement by basic multicellular units (BMUs). BMUs and modeling drifts have different effects on bone mass and structure, respond differently to mechanical and non-mechanical stimuli, react differently with aging, and play different roles in most bone diseases. Most adult-acquired osteoporosis results from malfunctions of remodeling BMUs, rather than deterioration of modeling drifts or osteoblasts or osteoclast (Frost and Jee, 1992).

## **Bone Indices**

There are three approaches to measure bone status. Each approach has advantages and disadvantages. Bone density measurements are nondestructive, site specific, and are sufficiently sensitive, but they are expensive, and unable to monitor current or acute change resulting from therapy. Histomorphometric bone biopsies, gold standards for assessment of bone metabolism, give direct, static, and dynamic, current, and past information of bone activity. However, due to its destructive nature and low reproducibility, histomorphometric bone biopsy is inappropriate for routine monitoring of therapeutic efficacy. Additionally, it is time-consuming and requires special equipment, so its use is greatly limited. Unlike bone biopsies, biochemical assays are noninvasive, generally available, and able to detect acute changes in skeleton metabolism, but these data obtained by this approach vary depending on many factors other than bone metabolism. For example, some biochemical markers such as serum alkaline phosphatase activity are not specific for bone unless they can be defined to tissue by isoenzyme assays. The other markers such as serum osteocalcin exhibit a marked diurnal variation. As a result, their clinical significance often needs to be validated by histomorphometric bone biopsies or calcium kinetics studies.

Bone mineral content (BMC) and bone mineral density (BMD) are essential indices of bone mass. BMD is defined as BMC divided by the area of interest,  $\text{g/cm}^2$ . BMC in the distal part of the forearms has been used as an index of skeletal calcium balance, because the proportion between the cortical (85%) and trabecular (15%) bone is close to

that of the entire skeleton (Christiansen et al., 1982). Because of this, forearm BMC shows a high correlation to whole body skeletal calcium and to vertebral calcium.

Dual-energy X-ray absorptiometry (DEXA) is currently applied to humans and small animals due to high reproducibility, short investigation time, low cost and low radiation exposure. Casez et al. (1994) reported 1.3% and 1.5% error in measuring BMC and BMD by DEXA in rats, respectively. Simple regression analysis of total body calcium which are measured by BMC[DEXA] and by the atomic absorption spectrophotometer reveals high correlation ( $r = 0.996$ ). Other investigators (Hansen et al., 1990; Ammann et al., 1992) support the concept that DEXA is superior to conventional dual photon absorptiometry (DPA).

Serum alkaline phosphatase activity is the most commonly used marker of bone formation, but it lacks sensitivity and specificity, especially in osteoporotic patients. To improve the specificity and sensitivity of the assays, techniques such as the use of activators and inhibitors have been developed to discriminate between the bone and liver isoenzymes. At present, the best method to isolate bone alkaline phosphatase from the total serum alkaline phosphatase is the immunoradiometric assay (IRMA).

Serum osteocalcin is a noncollagenous protein that is specific for bone and dentin. It is synthesized by osteoblasts, and incorporated into the extracellular matrix of bone. A portion of newly synthesized osteocalcin is released into the circulation, where it can be measured by radioimmunoassay. In most cases, serum osteocalcin is a valid marker of bone formation. However, serum osteocalcin can be elevated in patients treated with

1,25(OH)<sub>2</sub> vitamin D without a real increase in bone formation. Unlike alkaline phosphatase, the serum osteocalcin levels vary with time, being high at night, and falling by 50% late morning and early afternoon. Because of this, time of sample collection should be considered when interpreting serum osteocalcin data. Nevertheless, serum osteocalcin is somewhat more accurate than serum alkaline phosphatase activity under various clinical conditions (Delmas, 1993).

Type I collagen, the most abundant protein in bone, is first synthesized as 450 kd procollagen precursor molecules. Before insertion into the extracellular matrix, procollagen precursor molecule is cleaved at the C- and N-terminal ends. These C- and N-terminal peptides circulate in the blood, and their levels in serum reflect bone formation. Serum C-terminal peptide is weakly correlated with histomorphometric bone formation, with *r* value of 0.36 to 0.50, in osteoporotic patients. Ebeling et al. (1992) found that serum C-terminal peptide is not correlated well with serum osteocalcin and alkaline phosphatase activity in metabolic bone diseases. However, serum N-terminal peptide has a strong correlation to serum osteocalcin (*r* = 0.82) and to serum alkaline phosphatase activity (*r* = 0.92) in normal children and healthy adults (Taylor et al., 1994). Further studies are necessary to assess the use of serum C- and N-terminal peptides to detect the increase of bone turnover at menopause and in osteoporotic patients.

Fasting urinary calcium corrected by creatinine excretion is the cheapest assay of bone resorption. However, it lacks sensitivity, being able to detect only when a marked increase of bone resorption occurs. In addition, the ratio of calcium to creatinine is

dramatically different between the day and night urine (Kalu et al., 1991). Hydroxyproline is produced by a posttranslational modification of proline during collagen synthesis and is found almost exclusively in collagen (Nusgens et al., 1972). During bone resorption, the collagen matrix is degraded, and hydroxyproline is released into the blood.

Hydroxyproline is metabolized in the liver and excreted from the body through the kidney.

Urinary hydroxyproline is highly correlated with bone resorption as measured using histomorphometric bone biopsies and calcium kinetics. However, the disadvantage is that hydroxyproline is present in all collagens and not specific for bone tissue. Its urinary level is also influenced following a collagen-containing meal. Bettica and associates (1992) found that urinary hydroxyproline/creatinine failed to indicate the difference in bone resorption between healthy premenopausal and postmenopausal osteoporotic women. Thus, there is an obvious need for a better assay of bone resorption.

Hydroxylysine, similar to hydroxyproline, is produced by posttranslational modifications of lysine during collagen synthesis. Following further modification by glycosylation, galactosyl hydroxylysine and glucosylgalactosyl hydroxylysine are formed. The ratio between the two appears to be specific. A higher ratio is found in bone than in other tissues. Urinary galactosyl hydroxylysine normalized by creatinine excretion has been used to detect bone resorption in osteoporosis. A good negative correlation between urinary galactosyl hydroxylysine and bone density has been found by Moro and associates (1988). Current research indicates that urinary galactosyl hydroxyproline/creatinine is a

more valid marker for bone resorption than urinary hydroxyproline/creatinine (Bettica et al., 1992).

Pyridinoline and deoxypyridinoline are crosslinks of the mature collagen of the bone and cartilage, and released in the blood during bone resorption. Their urinary excretion levels are highly correlated so that either one can be used as a marker for bone resorption. Bettica et al. (1992) reported that urinary pyridinoline and deoxypyridinoline corrected by creatinine excretion were more sensitive biochemical indices for bone resorption occurring in healthy and osteoporotic postmenopausal women than urinary hydroxyproline/creatinine. A disadvantage is that pyridinoline and deoxypyridinoline have a marked diurnal variation, being high at night, and low during the day. A 24-hour urine collection is highly suggested (Taylor et al., 1994).

Histomorphometric bone biopsies are widely used to estimate not only *static* variables that are measured directly but also *dynamic* variables through the use of fluorescent labels. Thus, bone histomorphometry data can represent events that occurred several years ago or recently. Bone histomorphometry analysis requires the use of sections of undecalcified bone. The reasons for undecalcification are to conserve the differences between mineralized and unmineralized osteoid, to make excellent dynamic measurements of the mineralization rate through fluorescent labels, to maintain optimal cell structure, and to minimize shrinkage of the bone marrow (Eriksen, 1994).

To obtain good sections of bone, the bone biopsy sample must first be embedded in a hard plastic and sectioned with a heavy-duty microtome. The embedding material

must be as hard as the bone itself to avoid fracture during sectioning and easily dissolved after section, thus allowing the use of any desired staining technique. To visualize bone cells, and distinguish unmineralized osteoid from bone, the sections are stained with Von Kossa or Goldner's Masson Trichrome (Gruber, 1992). To obtain tetracycline-based indices, the sections are left unstained. Quantitative analysis is performed with light, and fluorescent microscopy and computerized digitizing devices.

Indices of histomorphometric bone biopsies are listed in **Table 1**. They include primary parameters that are measured directly on the section, and secondary parameters that are derived from the primary indices. Turner and associates (1993) emphasized that tetracycline double-labeled perimeter was a good parameter to interpret bone formation rate. However, a total of 5% of fluorochrome label incorporated into cancellous bone was resorbed by osteoclasts each day in ovariectomized rats, leading to underestimation of the double-labeled perimeter and thus bone formation rate. To avoid false interpretation of the data, other histomorphometric indices such as the number and surface of osteoclasts and osteoblasts should be also considered.

**Table 1**  
**Indices of histomorphometric bone biopsies**

Indices, units	Abbreviation
Bone Volume, %	BV/TV
Osteoid Volume, %	OV/BV
Osteoid Volume, %	OV/TV
Osteoblast Surface, %	Ob.S/BS
Osteoid Thickness, $\mu\text{m}$	O.Th
Eroded Surface, %	ES/BS
Osteoclast Surface, %	Oc.S/BS
Osteoclast Number, / $\text{mm}^2$	N.Oc/T.Ar
Bone Surface, $\text{mm}^2/\text{mm}^3$	BS/TV
Wall Thickness, $\mu\text{m}$	W.Th
Mineralizing Surface, %	MS/BS
Mineralizing Surface, %	MS/OS
Mineral Apposition Rate, $\mu\text{m}/\text{d}$	MAR
Trabecular Thickness, $\mu\text{m}$	Tb.Th
Trabecular Number, / $\text{mm}$	Tb.N
Trabecular Separation, $\mu\text{m}$	Tb.Sp
Bone Formation Rate, $\mu\text{m}^3/\mu\text{m}^2/\text{year}$	BFR/BS
Bone Formation Rate, %/year	BFR/BV
Mineralization Lag Time*, days	Mlt
Formation Period, days or years	FP

\* MLT= the time from the onset to the completion of organic matrix synthesis before mineralization begins (Parfitt, 1988).



## **2.2 Postmenopausal Osteoporosis**

### **Osteoporosis**

Osteoporosis is defined as a skeletal disorder in which bone mass is reduced and hip, vertebrae, radius, and humerus fractures are involved. In the mid 80s one out of three women and one in every six men developed a hip fracture, making osteoporosis one of the most common diseases of elderly individuals. In the United States, the costs of treating osteoporotic patients are about \$7 billion each year, and with the increase of the population of elderly people, the annual costs for osteoporosis will approach \$60 billion by the next century (Gallagher, 1990). Due to the severity of osteoporosis and due to the dramatic economic load, making progress towards the screening, prevention, and treatment of osteoporosis is tremendously critical.

### **Types of Osteoporosis**

Osteoporosis is classified as primary or secondary depending on cause and/or age of onset. The majority of osteoporotic patients belong to the primary osteoporosis, that includes idiopathic osteoporosis in children and young adults and involutional osteoporosis in adult and aging individuals. Postmenopausal (Type I) and senile osteoporosis (Type II), are the main forms of involutional osteoporosis. Senile osteoporosis results from a gradual loss of the trabecular and cortical bone due to reduced bone cell activity with aging. Inadequate dietary calcium intake and low physical activity in elderly people, in particular over 70 years of age contribute to the etiology. Hip

fractures are the main problem and afflict both genders. Postmenopausal osteoporosis is associated with ovarian hormone deficiency. Its characteristics include an increase in bone turnover with bone resorption exceeding formation. The net result is a rapid bone loss occurring in postmenopausal osteoporotic women. A low bone mass at menopause and a rapid rate of bone loss after menopause are the two major risk factors for postmenopausal osteoporosis (Riis, 1991).

### **Changes in Bone Density**

Bone density is often used to determine bone mass in perimenopausal women. A longitudinal evaluation of BMD was performed in premenopausal women (Gambacciani et al., 1994). Radial BMD was measured with quadruplicate readings (QDR) at the distal forearm by dual photon absorptiometry (DPA). A gradual decrease in radial BMD took place in the premenopausal women suffering from hypoestrogenic oligomenorrhea but not in those with regular menstrual cycles. It was suggested that the assessment of forearm BMD was useful in the identification of perimenopausal women with irregular menstrual cycles and further provided the predication of fracture risk for postmenopausal osteoporosis.

Bone mass can easily be determined by densitometry, but the measurement of the postmenopausal bone loss requires repeated assessments over a certain age-span and in different skeletal areas. Hansen and associates (1995) examined the spontaneous bone loss in different skeletal areas in healthy postmenopausal women. During the 15 years of

follow-up, the bone loss seen 16 years after menopause were 20-25% in all three bone compartments when compared with premenopausal values. The respective percent changes of the initial bone mass levels constituted 21.7% for the distal forearm, 10.6% for the lumbar spine, and 15.3% for the total skeleton. The patterns of bone loss were different for the axial and peripheral skeleton. Two years after menopause, the axial bone loss (BMD for the spine) was 10%, whereas peripheral bone loss (BMC for the forearm) was 5-6%. After 6.5 years and later, the bone loss remained significant in the forearm, and the total skeleton, but insignificant bone loss was observed in the lumbar spine. Thus, spinal bone density may be a good indicator of bone mass in the premenopausal and early postmenopausal (e.g. the first 2 years after menopause) period. It becomes difficult to detect with advancing age following ovarian hormone deficiency.

### **Changes in Bone Calcium Balance**

Postmenopausal osteoporosis is related to ovarian hormone deficiency following menopause, with an initial period of rapid bone loss within the first ten years. Evidence for increased bone turnover with resorption exceeding formation in ovarian hormone deficient women at the age of 35 to 45 years comes from radiocalcium kinetics study (Heaney et al., 1978). Premenopausal women exhibited bone remodeling rates of 0.337 gm calcium per day for mineral accretion and 0.358 for mineral resorption. The difference between the two rates was -0.021 gm calcium per day. However, postmenopausal women exhibited remodeling rates of 0.387 gm calcium per day for accretion and 0.425 for

resorption, with a computed skeletal balance of -0.038 gm calcium per day. The data indicated that bone remodeling was slightly elevated but significantly different following menopause; the bone resorption rises more than the formation (20% vs. 15%).

Hasling et al. (1990) investigated bone metabolism in a group of 85 postmenopausal women with spinal crush fracture osteoporosis. A  $^{47}\text{Ca}$  tracer kinetic turnover study was performed in all patients. The average bone mineralization rate (4 mmole/day) was less than the average resorption rate (4.9 mmol/day), given a negative bone calcium balance of -0.9 mmole/day. It was indicated that annual calcium loss was 13.1 g from bone, or 1% of the total bone calcium. To balance bone calcium loss in postmenopausal osteoporosis, it was suggested that dietary calcium intake should approach to 34-35 mmole per day (1.36 to 1.4 g per day). Net calcium absorption (dietary calcium-fecal calcium) negatively correlated to bone resorption rate ( $r = -0.31$ ,  $P < 0.005$ ), but positively to bone mineralization rate ( $r = 0.29$ ,  $P < 0.01$ ) and to calcium balance ( $r = 0.38$ ,  $P < 0.001$ ). Thus, ovarian hormone deficiency declines intestinal calcium absorption, deteriorates calcium balance, stimulates bone calcium loss, and thus reduces bone mineralization rate.

### **Changes in Ca and Mg Metabolism**

To assess the possibility that a negative calcium balance caused bone loss in postmenopausal women, Devine et al. (1993) used a stable strontium method to measure gut calcium absorption from 196 healthy postmenopausal women 15 years since

menopause. Vertebral bone mass was determined by measuring vertebral BMD in lumbar vertebrae using quadruplicate readings (QDR) bone densitometer. Intestinal calcium absorption was measured by a stable strontium. Intestinal calcium absorption, like vertebral bone mass, fell with years since menopause. There was no correlation between the intestinal calcium absorption and vertebral bone mass. Urine (24-hour) calcium was positively correlated with intestinal calcium absorption ( $r = 0.31$ ,  $P < 0.001$ ). It was suggested that intestinal calcium absorption was regulated in response to urinary calcium excretion, or that the kidney maintained calcium homeostasis by excreting what was absorbed from the intestine.

Morris et al. (1991) studied 300 postmenopausal women who were categorized as 'normal' or 'osteoporosis' by lateral radiographs of the thoracic and lumbar spine. The latter had crushed vertebrae and more than one wedged vertebrae. Hourly fractional absorption of radiocalcium was lower in the osteoporotic than in healthy postmenopausal women. However, plasma alkaline phosphatase activity, fasting urinary calcium, and hydroxyproline were higher in the osteoporotic than in the postmenopausal women. This indicated a higher bone turnover in the former than in the latter. Serum  $1,25(\text{OH})_2$  vitamin  $\text{D}_3$  was not significantly different between the two groups. Thus, poor calcium absorption was not due to the low serum  $1,25(\text{OH})_2$  vitamin  $\text{D}_3$ . It remains possible that other factors regulate intestinal calcium absorption and thus urinary calcium excretion in osteoporotic patients.

Mg metabolism was significantly affected by oophorectomy. Lindsay et al. (1980) investigated serum, urinary, and red cell Mg concentration in groups of 210 premenopausal and postmenopausal women. The postmenopausal women had higher serum and urinary Mg than the premenopausal women, but red cells Mg was similar between the two groups. Serum Mg appeared different between healthy postmenopausal women and osteoporotic patients. Reginster et al. (1989) examined serum, urinary, red cell, and bone Mg in 10 menopausal osteoporotic women and 20 age-matched normal postmenopausal women. The BMC and trabecular bone volume findings indicated that osteoporotic patients had extremely low bone mass and volume when compared with the normal postmenopausal women. The osteoporotic women had a lower serum Mg than the normal women, but no difference was found in red cell, urinary, or bone Mg. It was concluded that serum Mg was a potential parameter used with other biochemical indices to discriminate a group of postmenopausal women with a potentially high risk of developing postmenopausal osteoporosis.

### **Changes in Serum PTH, Calcitonin, and Vitamin D Metabolites**

Serum PTH, calcitonin, 25(OH) vitamin D, and 1,25(OH)<sub>2</sub> vitamin D levels due to postmenopausal osteoporosis are inconsistent. Immunoreactive PTH in serum has been reported to be low (Gallagher et al., 1980), high (Fujita, et al., 1972), and unchanged (Bouillon et al., 1979) in postmenopausal osteoporotic patients when compared with age-matched normal women. Serum 25(OH) vitamin D is constant (Gallagher et al., 1979), or

elevated (Lore et al., 1984) whereas serum 1,25(OH)<sub>2</sub> vitamin D is low (Gallagher et al., 1979; Lore et al., 1984) or unchanged (Gallagher, 1990). Circulating calcitonin levels are low (Milhaud et al., 1978; Stevenson et al., 1981), normal (Chestnut, 1980), or slightly high (Tiegs et al., 1985). The inconsistent data on the hormone levels are probably due to the heterogeneity of postmenopausal osteoporosis, or due to difficulties in measurements of changes in serum hormone levels.

## **2.3 Treatment of Postmenopausal Osteoporosis**

### **Estrogen Replacement Therapy**

To examine the effects of estrogen on biochemical indices of bone activity, Stock et al. (1985) recruited 10 healthy postmenopausal women without fractures. The subjects were injected with 50 µg/day of ethinyl estradiol for 2, 4, or 8 weeks. A substantial reduction in the serum calcium and osteocalcin concentration, and an increase in serum 1,25(OH)<sub>2</sub> vitamin D were found following 2 weeks of estrogen treatment and these alterations were continuous until the end of the study. Serum PTH was not changed until the end of the 8th week. The lack of a rise in serum PTH in the face of elevated serum 1,25(OH)<sub>2</sub> vitamin D during the first 4 weeks indicated that estrogen had a direct effect on 1,25(OH)<sub>2</sub> vitamin D synthesis. After 4 weeks of treatment, serum phosphorus started to decline and was maintained low by the 8th week. Estrogen therapy did not decrease fasting urinary calcium excretion until the end of the 8th week. It was suggested that estrogen replacement slowed down bone turnover during the first 8 weeks of treatment.

Its effects on the serum Ca, P and  $1,25(\text{OH})_2$  vitamin D levels were independent of PTH. The data indicated that short-term estrogen therapy caused a reduction of bone loss and thus a reduction in serum calcium, which in turn caused hyperparathyroidism in postmenopausal women.

The effects of estrogen on indices of calcium balance and bone activity are dose-dependent. A total of 92 healthy women in their early menopause were treated with placebo, low, medium, or high dose of estrogen ( $17\beta$  estradiol/estriol, 1/0.5, 2/1, and 4/2, respectively) for 1 year. Forearm BMC increased by 0%, 0.8%, and 1.5% in postmenopausal women treated with estrogen at a low, medium, and high dose, respectively. A mean 10-20% decrease in serum phosphorus in a dose-dependent manner was found during the first 9 months of estrogen treatment, after which a small rise was observed. In comparison with the premenopausal women, postmenopausal untreated women had higher serum alkaline phosphatase activity and fasting urinary excretion of hydroxyproline and calcium, indicating a higher bone formation and resorption. After 1 year of estrogen treatment, all three indices decreased. Thus, the early phase of postmenopausal bone loss was reversible by estrogen replacement (Christiansen et al., 1982).

Estrogen therapy reduced urinary Ca and Mg excretion. Postmenopausal women 12 to 18 months since menopause received Estraderm TTS 25 (25  $\mu\text{g}$  estradiol/day, twice per week). Following 6 months of estrogen therapy, urinary Ca/creatinine and Mg/creatinine ratios were reduced, and the ratios returned to pretreatment level 6 months



after withdrawal of treatment (Kaplan et al., 1994). The same was found when urinary Mg/creatinine was compared between premenopausal and postmenopausal women (McNair et al., 1984). The 24-hour urinary Mg/creatinine was significantly higher in the postmenopausal women than in the premenopausal women. After estrogen treatment, urinary Mg excretion rate was reduced in the postmenopausal women when compared with the placebo group. The reduction in urinary Mg excretion persisted over a 24-month period. The authors concluded that changes in Mg status were similar to the changes in Ca metabolism. High urinary Mg excretion was associated with an increase in bone turnover, and thus bone loss.

### **Intermittent PTH Treatment**

Reeve and coworkers (1980) examined the anabolic effects of intermittent hPTH 1-34 at a daily dose of 100 µg for 6-24 months on trabecular bone of 21 osteoporotic patients. There were no changes in early-morning fasting plasma calcium and phosphorus. PTH daily treatment raised serum alkaline phosphatase activity, urinary (24-hour) calcium, phosphorus, and hydroxyproline. Substantial increases were observed in the formation rate of new bone as measured by <sup>47</sup>Ca kinetics in the patients receiving PTH treatment. Histomorphometric evidence such as the trabecular bone volume, trabecular osteoid surface, and calcification rate further supported the anabolic effects of PTH on osteoporotic bone. However, calcium, magnesium, and phosphorus balances were not significantly improved by daily PTH injection. The data showed that intermittent PTH

treatment considerably increased bone formation and mineralization of the trabecular bone, and the increases were not through increased intestinal calcium absorption and/or calcium retention but through increased calcium loss from the cortical bone. The authors suggested that PTH daily treatment might best be used in combination with anti-resorptive regimen such as estrogen to limit cortical bone resorption.

In combination with estrogen, PTH holds great promise for the treatment of postmenopausal osteoporosis. A total of 12 patients with two or more fractures of the vertebrae were recruited and received hPTH 1-34 by daily subcutaneous injections for 1 year. The subjects started to receive additional estrogen therapy from the fourth month of PTH treatment. Vertebral BMD increased by 50% above baseline following PTH treatment. The increase in vertebral BMD was particularly evident in the trabecular bone. Combined treatment caused a 42% increase in trabecular bone volume. No significant bone loss was found in the cortical bone. It was concluded that in combination with estrogen, PTH reversed trabecular bone of osteoporotic patients at no expense to the cortical bone (Reeve et al., 1993).

Intermittent PTH at a low dose has pronounced beneficial effects on the trabecular bone formation and calcification. However, the same is not observed when hPTH fragment is continuously delivered to healthy perimenopausal women by a portable pump. Increased serum calcium and magnesium, reduced serum phosphorus and renal phosphorus threshold were observed in response to PTH infusion. Fasting urinary calcium and hydroxyproline were increased, which indicated an acceleration of bone resorption by

24-hour PTH infusion. Indices for bone formation exhibited inconsistent results. PTH infusion decreased serum osteocalcin, and elevated serum alkaline phosphatase activity in premenopausal but did not change in postmenopausal women. Comparison of the biochemical indices with regard to menopause state demonstrated that postmenopausal women displayed greater increases of serum calcium, alkaline phosphatase activity, osteocalcin, urinary calcium, and hydroxyproline. The authors suggested that menopausal states involved alterations in bone sensitivity to PTH. Moreover, PTH exerts its anabolic effects on bone apparently depending on the mode of its administration. Continuous infusion of PTH has been considered to be catabolic rather than anabolic in nature (Joborn et al., 1991).

## **2.4 Postmenopausal Osteopenia**

### **Ovariectomized Rat Model**

To understand the pathogenesis of osteoporosis, it needs an appropriate animal model to study the exact nature of hormone changes. Additionally, by using an animal model, changes in bone activity, turnover, and biomechanical competence can be directly estimated by some invasive techniques such as bone biopsies. Rats are thought to be one of the valuable animals for studying human osteoporosis. The reasons for this are that rats control gains in bone mass, and respond to mechanical stimuli, hormones, drugs, and other agents under the same mechanisms as humans. The only difference between humans and rats is that the latter lack extensive BMU-based remodeling. The lack makes the rat

model useful only for an initial evaluation of the regimen for osteoporosis. Moreover, rats seldom develop bone fractures after ovarian hormone deficiency. The absence of fractures makes rats a real animal model for postmenopausal osteopenia (bone loss) rather than postmenopausal osteoporosis.

Rats have a life span of about 3 to 4 years. Femur length, width, density, and calcium content increase rapidly from 1-3 months of age. After 3 months, the rate of increase is continuous but gradual, and by 6 months of age and on, changes in femur density and calcium content are relatively minor. At the age of 12 months, all the bone parameters in rats remain constant. Thus, 12 months is chosen as the age for the aged rat model to study osteoporosis. The use of the aged rat model ensures that bone changes observed following ovariectomy are primarily due to ovarian hormone deficiency since 12 month-old rats have stable femur characteristics which are independent of rapid longitudinal bone growth.

Although the aged rat model is better to define postmenopausal bone loss in humans, aged rats are more expensive, less available, and prone to infectious and respiratory problems than young rats. Therefore, many researchers have used 3-month-old young rats as a rat model for studying bone loss caused by ovarian function loss. The 3-month-old rats are reproductively mature and have abilities to respond to ovarian hormone deficiency following ovariectomy. Rats at 3 months old are still growing, and the lower bone mass following ovariectomy can be due to impaired bone growth rather than to increased bone turnover. To avoid mistaken interpretation of results, Kalu (1991)

suggested that a group of rats be killed at the beginning to serve as a baseline control.

Any conclusion is made only when the final bone mass of the ovariectomized rats is lower than that in the baseline control group.

It is beyond the scope of this review to completely discuss the impacts of a variety of treatments such as calcitonin, bisphosphate, fluoride, and growth hormone implants on osteoporosis. Therefore, following is a brief review of the current research of the bone quality, and Ca, P, and Mg metabolism following ovariectomy, estrogen replacement, and PTH treatment.

### **Changes in Bone Mass**

Ovariectomy causes a change in bone density in rats as found in humans. Gnudi et al. (1993) used 40 week-old rats; 8 were ovariectomized at the 40th week of age; 8 were ovariectomized at the 52nd week. After 12 weeks, bone mineral density (BMD) of the sixth lumbar vertebra was measured with a single-photon absorptiometry (SPA). The vertebral BMD in the 64-week-old Ovx rats was higher than that of the 52-week-old Ovx rats, although the difference did not reach statistically significant. The slight increase in BMD with aging was also found in the intact control rats. However, in comparison with the intact control groups, Ovx rats had a significantly lower BMD in the sixth lumbar vertebra both at 52 and 64 weeks. The findings were consistent with those reported by Aerssens and associates (1993). Studies with 12-week-old rats demonstrated that BMD of the right femur decreased 27 weeks after ovariectomy. Thus, BMD is a critical

indicator for changes in bone mass of Ovx rats short-term (12 weeks) or long-term (27 weeks) after surgery.

### **Changes in Bone Histomorphometry**

Studies with young rats present similar results as those with aged rats. Wronski et al. (1985) used 75 day-old rats, which were sacrificed at 5 weeks after ovariectomy, to examine the skeletal alterations in Ovx rats. The 110-day old Ovx rats 5 weeks after surgery had enhanced both bone formation and resorption. Trabecular bone volume expressed as the percentage of the total bone section decreased by ovariectomy. The surfaces and numbers of osteoblast and osteoclast were elevated in Ovx rats relative to the control. Tetracycline-based data from the tibial metaphysis indicated a four-fold increase in the percentage of the double-labeled bone surface. The longitudinal growth and calcification rate of the tibia increased by ovariectomy. The authors concluded that 110 day-old Ovx rats 5 weeks after surgery had a marked loss of trabecular bone in the proximal tibial metaphysis. Although both bone resorption and formation increased, the former exceeded the latter, leading to a net reduction in trabecular volume. The findings were similar to the initial phase of postmenopausal bone loss.

Hietala (1993) examined the effect of ovariectomy on periosteal bone formation and endosteal bone resorption in rats. The rats were ovariectomized at the age of 15 weeks and sacrificed within 100 days after surgery. Body weight increased due to hyperphagia in Ovx rats. Ash weight, density, and volume of the femur were lower in Ovx

rats than in the control rats. The length and perimeter of the bones were not affected by ovariectomy. However, the areas of the medullary cavity were 18.8% greater in the Ovx rats, indicating an increase in the activity of osteoclasts in the endosteal surface.

Ovariectomy caused a 28.3% increase in the areas between the tetracycline labeling and the outer perimeter. The increase was due to enhanced periosteal bone formation. The author concluded that ovariectomy increased periosteal bone formation as a response to the endosteal resorption in rats.

The trabecular bone structure was changed in aged female rats following ovariectomy. Female rats were ovariectomized (Ovx) at 100 days of age and sacrificed at 540 days after surgery. As expected, there was a marked decrease in the amount of trabecular bone in the Ovx rats. The reduction in trabecular bone taken from the lumbar vertebral bodies was particularly evident in the central area of the secondary spongiosa. Structural analysis using two-dimensional images taken from the sagittal and cross-sections of the lumbar vertebral bodies demonstrated a loss of trabecular connectivity, an increase in trabecular separation and a loss of plate-like trabecular plates when compared to the age-matched control rats. The authors concluded that changes in bone mass and structure in Ovx rats were likely due to ovarian hormone deficiency (Miller and Wronski, 1993).

The quality of the cortical bone appeared to be changed by long-term ovariectomy. Danielsen and associates (1993) used female rats at 60 days of age to examine the effects of long-term ovariectomy on the cortical bone taken from the femur of rats. Ovariectomy

was performed when the rats were at 6 months of age and sacrificed 3, 6, 9, and 18 months after ovariectomy. In comparison with intact rats, ovariectomy caused an endosteal cortical bone resorption but had no effect on the periosteal bone formation. Ovx rats had 13 to 19% lower the cross-sectional area, wall thickness, ash content (mg of ash/mm of height) of the cortical bone, and percent collagen than the intact rats. Other investigators (Turner et al., 1987; Wronski and Yen, 1994) have reported similar short-term and long-term (19 weeks postovariectomy) effects of ovariectomy on the development of cortical osteopenia.

### **Changes in Bone Biomechanical Competence**

The biomechanical properties of the vertebral bone in mature rats were changed by ovariectomy. Mosekilde and associates (1993) used 3 month-old female Ovx rats, which were sacrificed one, three, and six months after ovariectomy. Following ovariectomy, an initial increase in all biomechanical parameters (maximum load, strain, maximum stress, maximum strength) was found. The increase reached the peak for the four-month-old rats one month after ovariectomy. The biomechanical parameters declined after the age of four months, and maximum load, maximum stress, and maximum normalized load declined to the baseline levels at the age of nine months. No age-related changes were seen in strain of the vertebral body in Ovx rats. In comparison with the age-matched control groups, ovariectomy caused a reduction in maximum load, maximum stress and maximum stiffness. The differences between two groups increased with age. Other investigators



(Danielsen et al., 1993) found that ovariectomy reduced the maximum compressive load of the cortical bone. However, the reduction was not observed when the cross-sectional area, or amount of ash weight or dry weight of the cortical bone was taken into consideration.

### **Changes in Biochemical Indices**

Ovariectomy causes alterations in some but not all biochemical indices. Liu et al. (1991) conducted studies with 95-day-old Ovx rats fed a Teklad diet containing 0.4% calcium, 0.3% phosphorus, and 3 units vitamin D per g. Ovx rats had lower urinary calcium ( $\mu\text{g}/\text{mg}$  creatinine) in the morning urine and higher urinary hydroxyproline ( $\mu\text{g}/\text{mg}$  creatinine) in the evening urine than the intact control. Serum calcium, phosphorus, 25(OH) vitamin D, 1,25(OH)<sub>2</sub> vitamin D, and osteocalcin were not changed following ovariectomy. The reason for lack of increased serum osteocalcin and vitamin D metabolites 40 days after ovariectomy is unclear. *In vivo* studies indicated that vitamin D metabolites increased osteocalcin biosynthesis (Carpenter et al., 1992). The unchanged serum osteocalcin was probably due to unchanged serum vitamin D metabolites. Nevertheless, increased serum osteocalcin was found in 10-month-old Ovx rats 6 months after ovariectomy (Yeh et al., 1994). Thus, it is also possible that the increase in serum osteocalcin after ovariectomy is time dependent.

Aerssens et al. (1993) found that ovariectomy changed other biochemical parameters such as serum insulin-like growth factor-1 (IGF-1). A group of 12-week-old

rats was ovariectomized at the beginning and sacrificed after 27 weeks. As expected, ovariectomy increased serum alkaline phosphatase activity, osteocalcin, and decreased urinary calcium excretion. All of these parameters suggest a rapid bone turnover. Serum IGF-1 was higher in Ovx rats than in the intact control rats. Earlier studies provided evidence that osteoblasts produced IGFs, which stimulates bone cell proliferation and collagen production (Kalu and Liu, 1989; Gallagher, 1990). In contrast to these findings, an *in vitro* study demonstrated that IGF-1 increased the number of murine tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells and the survival rate of osteoclasts (Koike et al., 1987). Studies with human subjects have shown that IGF-1 therapy increased serum bone resorption markers (Ibbotson et al., 1992). Hayden et al. (1995) suggested that IGF production determined subsequent osteoblast proliferation and thus the degree of cavity refill. Other investigators (Finkelman et al., 1992) found that ovariectomy had no effect on the serum IGF-1 level.

## **2.5 Treatment of Postmenopausal Osteopenia**

### **Estrogen Replacement Therapy**

Estrogen exerted its effects on cancellous bone balance in the tibia of Ovx growing rats. Turner and associates (1993) conducted three trials to clarify the effects of estrogen on cancellous bone turnover determined by histomorphometric bone biopsies. Ovx rats at 10 weeks of age were implanted subcutaneously with controlled-release pellets containing synthetic estrogen diethylstilbestrol (DES) 7 days after surgery. Ovx rats were then given

tetracycline either 1 or 8 days after the implantation of the pellets and a second injection of tetracycline 5 days following the first. Estrogen treatment reduced osteoclast number and perimeter in Ovx rats. However, estrogen treatment reduced numerous indices of bone formation, in particular, mineral apposition rate, double-labeled perimeter, and osteoblast number. It was concluded that estrogen prevented osteopenia in Ovx rats, in part by inhibiting both bone resorption and formation. The findings were in agreement with those reported by Wronski et al. (1988).

Chow and associates (1992) found that estrogen maintained trabecular bone volume not only by depression of bone resorption but also by stimulation of bone formation in Ovx rats. Because the increased bone resorption itself increases bone formation, they used 8 week-old Ovx rats in which bone resorption was suppressed by the bisphosphonate 3 amino-1-hydroxypropylidene-1-bisphosphonate (AHPPrBP). AHPPrBP-treated Ovx rats receiving  $17\beta$  estradiol (1, 10, 100  $\mu\text{g}/\text{kg}/\text{d}$ ) for 17 days showed a dose-dependent increase in trabecular bone volume by 2, 26, and 44%, compared with Ovx rats given AHPPrBP alone. Estrogen + AHPPrBP increased fluorochrome-based indices such as bone formation rate, bone apposition rate, and double-labeled surface (%) when compared with Ovx rats treated with AHPPrBP alone. However, the increases did not reach statistical significance when compared with Ovx untreated rats. Furthermore, osteoblast surface (%) was not improved by estrogen + AHPPrBP when compared with either Ovx untreated rats or Ovx rats treated with AHPPrBP alone. Osteoclasts resorb tetracycline double-labeled on the bone by 5% per day as mentioned before. It is possible that the

increases in the tetracycline-based indices were due to a reduction in osteoclast activities rather than a real increase in osteoblast activity since osteoblasts surface was not improved. Therefore, more research is necessary to confirm the suggestion that estrogen stimulates bone formation in Ovx rats.

The effects of estrogen on bone in Ovx rats 10 months after ovariectomy were minor. A group of female Wistar rats were ovariectomized at 6 months of age. Estrogen (2  $\mu\text{g}$  estradiol valerate/rat/week) were not initiated until the end of the 10th month postsurgery. Estrogen treatment for 8 months caused only minimal changes in the real and apparent densities, bone density, ash content (mg/mm length), and the maximum load per mg ash or dry weight of the femur. The same was found in that estrogen initiated 6 years after menopause has less effects on the prevention of bone loss than that initiated immediately or 3 years after menopause (Lindsay, 1993). It was suggested that estrogen treatment exerted the best efficacy in Ovx rats within 2 to 5 weeks after ovariectomy (Han et al., 1992).

To examine whether short-term estrogen treatment during the early stages of ovarian function loss would be sufficient to protect against subsequent bone loss, Wronski et al. (1993) used 3-month-old Ovx rats treated with 10  $\mu\text{g}/\text{kgBW}$  (4 days/week) for a 180-day period. Estrogen treatments were terminated 35, 90, 180, and 360 days prior to sacrifice. After withdrawal of estrogen treatment, rapid cancellous bone loss was observed in the later stages of estrogen depletion. The rate of cancellous bone loss in Ovx rats after estrogen withdrawal was as fast as that found in Ovx untreated rats 180 days

after surgery. The bone loss due to estrogen withdrawal was related to a marked increase in bone turnover. Since the skeletal effect of estrogen deficiency and replacement are very similar in rats and humans, it was suggested that postmenopausal women may be at high risk for subsequent bone loss after estrogen withdrawal.

The skeletal response of Ovx rats to estrogen treatment is dose-dependent. Kalu et al. (1991) gave 3-month-old Ovx rats  $17\beta$  estradiol at a dose of 20, 60, 180, or 6,000 ng/kg for 40 days. The serum  $17\beta$  estradiol level of Ovx rats treated with estrogen at a dose of 6,000 ng/kg was above that of the intact control rats, the remaining Ovx rats had serum estrogen below the normal level. Estrogen treatment depressed ovariectomy-induced increases in biochemical indices such as serum alkaline phosphatase activity, serum tartrate-resistant acid phosphatase and urinary hydroxyproline. Histomorphometric data in which trabecular bone apposition rate decreased by estrogen in a dose-dependent manner further supported the suggestion that the depression of bone turnover is due to estrogen treatment. Serum osteocalcin was not changed by low doses of estradiol, but was decreased in Ovx rats given the highest dose of estradiol. This means that serum osteocalcin, a biochemical marker for bone formation, falls only when serum estradiol is above the normal level. Serum calcium was elevated by estradiol injection, probably because estrogen improved intestinal calcium absorption in Ovx rats.

Serum Ca increases in women at menopause as mentioned above. The increase is reversed by estrogen therapy. To examine the long term effect of estrogen treatment on serum P as well as Ca, Cruess and Hong (1979) used young rats (150 g) receiving 4000

$\mu\text{g } 17\beta$  estradiol/kg BW for 1, 3, 6, 9, and 12 months. In rats, ovariectomy caused a decrease rather than an increase in serum Ca, and the decrease was not temporary but continuous through 12 months. Estrogen therapy significantly prevented the fall in serum Ca over a 12-month period. Serum P was not changed by either ovariectomy or estrogen administration. The data indicated that estrogen corrected the serum Ca level in Ovx rats to normal, and that estrogen effect was prolonged.

### **Intermittent PTH Treatment**

PTH had a pronounced effect on the biochemical properties of the vertebral bone in mature Ovx rats. Mature rats were ovariectomized at 3 months of age. Human PTH (1-34) at a dose of 80  $\mu\text{g/kg}$  BW (6 days/week) began 4 weeks after surgery. After 5 weeks of treatment, PTH increased maximum load and energy absorption capacity; the increases were more pronounced after 15 weeks treatment. When these data were normalized for cross-sectional area or for bone ash (mg ash per mm specimen length), PTH increased all biomechanical parameters except for maximum stiffness that was not affected by PTH. The authors (Mosekilde et al., 1994) concluded that PTH exerted a long-term beneficial effect on the biomechanical competence of the vertebral bone in Ovx rats.

Aged estrogen-deficient animals appeared to respond well to the anabolic effects of PTH. A group of 2-year-old female retired breeder rats were ovariectomized or sham-operated (intact) 1 year previously. Mean initial weights for the Ovx and intact rats were

similar prior to treatment. Human PTH (1-34) was administered daily at a dose of 160  $\mu\text{g}/\text{kg}$  BW for 14 days. Treatment with PTH caused a substantial increase in bone mineral content obtained by single-photon absorptiometry (SPA) of the metaphysis and diaphysis of the femur. The values approached those observed in the age-matched intact rats. PTH increased (63%) the mineralized volume expressed as the percentage of total bone tissue and the thickness of the trabecular bone. Analysis of the lumbar vertebrae showed that PTH dramatically stimulated bone formation on the periosteal, endocortical, and endosteal surfaces. However, PTH injection did not change the indices of bone resorption such as the bone marrow volume, cortical porosity, vertebral trabecular osteoclast-positive eroded surface, and urinary hydroxyproline excretion. It was concluded that PTH treatment initiated 1 year after ovariectomy stimulated bone formation but had no effect on bone resorption in aged rats (Ibbotson et al., 1992).

PTH also exerted its anabolic effects on the cortical bone of mature Ovx rats. Wronski and Yen (1994) examined whether PTH had anabolic effects on cortical bone in Ovx rats as previously observed in cancellous bone. They used 3-month-old rats, gave them 40  $\mu\text{g}/\text{kg}$  BW PTH (6 days/week) 4 weeks after ovariectomy for periods of 5, 10, or 15 weeks. PTH increased total cortical bone tissue area (bone + bone marrow) of Ovx rats. Bone marrow area decreased and cortical width increased with time in Ovx rats treated with PTH. Fluorochrome-based indices of the periosteal and endocortical bone formation increased following 5 to 10 weeks of PTH injection. By 15 weeks, the

elevation of fluorochrome-based indices due to PTH were less pronounced. Thus, PTH improved the cortical bone mass in the tibial diaphysis of Ovx rats.

To examine whether PTH improved trabecular bone mass was at the expense of cortical bone, Hock and associates (1989) gave 4-week-old rats 80  $\mu\text{g}/\text{kgBW}/\text{day}$  hPTH (1-34) alone or in combination with salmon calcitonin (CT), a blocker of bone resorption (experiment 1) or gave dichloromethylene diphosphonate (Cl<sub>2</sub>MDP), another blocker of bone resorption 4 days before hPTH(1-34) injection. The anabolic effects of PTH on bone was not inhibited by CT or Cl<sub>2</sub>MDP. Rats treated with PTH alone or in combination with CT for 3 days increased bone mass (dried femur weight and calcium). Rats injected with PTH following 4 days of pretreatment with Cl<sub>2</sub>MDP had a greater bone mass than rats treated with PTH or Cl<sub>2</sub>MDP alone. Trabecular bone volume, osteoblast surface, osteoid surface, and mineral apposition rate were similar between groups PTH, PTH+CT<sub>3</sub>, and PTH+Cl<sub>2</sub>MDP. The authors suggested that an early stimulation of bone resorption was not a prerequisite to the anabolic effects of PTH on bone. However, whether trabecular bone mineralization is at the expense of cortical bone minerals depends on the availability of calcium from the intestine. If intestinal calcium absorption did not increase appropriately, the calcium demands for trabecular bone formation will be met at the expense of the cortical bone (Slovik et al., 1986).

Shen et al. (1993) used an alternative to the above approach. Instead of the use of CT or Cl<sub>2</sub>MDP, they gave 5-month-old Ovx rats estrogen (30  $\mu\text{g}/\text{kg}/\text{day}$  17 $\beta$  estradiol) to prevent cortical bone loss and PTH (40  $\mu\text{g}/\text{kg}/\text{day}$  rat PTH1-34) to improve trabecular



bone formation 1, 3, 5 weeks after surgery. Treatment with PTH in combination with estrogen caused a dramatic increase in trabecular bone volume in comparison with Ovx untreated rats and the value was even greater than in the intact rats from weeks 1 to 5. The same was found when administered in a curative mode from weeks 5 to 9. Combined estrogen and PTH treatment, starting 3 or 5 weeks after surgery, increased trabecular connectivity, node to node strut length, and bone mineral density of the distal femur, enriched with trabecular bone when compared with either PTH or estrogen alone. The diaphyseal region of the femurs, enriched with cortical bone, showed little change in bone mineral density by treatment during the period of the experiment. The beneficial effects of this combined treatment comes from estrogen's inhibition of bone resorption and PTH's stimulation of bone formation.

## **2.6 Summary**

Bone continuously undergoes resorption and formation over a life span. The balance between bone resorption and formation is under the control of hormones, drugs, and other reagents. Postmenopausal osteoporosis is a metabolic bone disease, which often occurs in women after ovarian hormone deficiency. Because it is preventable, understanding the pathogenesis of postmenopausal osteoporosis is critical and necessary. Rats have been widely used as a model for studying postmenopausal bone loss. Studies with ovariectomized rats and osteoporotic patients indicate that there is a substantial increase in bone turnover with bone resorption exceeding bone formation, leading to a

substantial reduction in bone mass or volume, following a loss of ovarian function. Current research focuses on the development of precise and accurate indices for identifying a subgroup of postmenopausal women with a potentially high risk of postmenopausal osteoporosis. Bone mineral density, serum and urinary biochemical measurements, and bone biopsies are often used for screening and routine monitoring of therapeutic efficacy. A therapeutic regimen for postmenopausal osteoporosis must be able to inhibit bone resorption and/or to enhance bone formation. Estrogen therapy has been demonstrated to be a good antiresorptive reagent to inhibit bone resorption and improve Ca balance. In contrast, PTH as an anabolic reagent stimulates bone formation but does not change bone resorption when it is administered intermittently. With advancing scientific findings and techniques, it is likely that the real mechanism of postmenopausal osteoporosis will be clarified in the near future.

## **2.7 Boron**

### **Chemical Properties**

Boron is next to carbon on the periodic table of elements. In structure, boron and carbon compounds are very similar. Although its concentration in the earth's crust is only 3 ppm, boron compounds are widely used in a range of products, which include glass, fire retardants, detergents, bleaches, and many others. In properties, boron is intermediate between metals and non metals. Its valence shell has three electrons and it forms simple compounds with hydrogen ( $\text{BH}_4^-$ ). In nature, boron almost exclusively exists as three- or

four- coordinate boron oxygen complexes. Thus, it tends to form complexes with hydroxyl groups in biological compounds such as polysaccharides, adenosine-5-phosphate, pyridoxine, riboflavin, dehydroascorbic acid, and pyridine nucleotides (Frost, 1942; Boeseken, 1949). Boron inhibits the activity of serine protease (Berezin et al., 1967; Lindquist and Terry, 1974; Bauer and Pettersson, 1974), xanthine oxidase (Roush and Norris, 1950) and dehydrogenase (Kaneshima et al., 1968; Smith and Johnson, 1976). Weser (1967) found that boron stimulated the synthesis of mRNA in the liver of rats.

The biological activity of inorganic boron in animals is limited when compared with that of organic boron, although the former exerts considerable potential importance in nutrition. Inorganic boron such as boric acid and borate undergo rapid equilibrium reactions with water and hydroxyl and polyhydroxyl compounds. These equilibrium reactions vary with pH. Thus, it is difficult to determine the mechanism of action of inorganic boron at the molecular and cellular level. In contrast, for organic boron, the behavior of the organic boron molecule can be expected to be much more like their organic counterparts. Boron containing peptides such as a dipeptide of borobetaine and phenylalanine can cross cell membranes without hydrolysis of the amide bond as found with the majority of normal peptides. Such stability may be useful in overcoming problems associated with peptide or protein drug delivery. Organic boron analogues have been shown to have potent hypolipidemic, anti-inflammatory, anti-neoplastic, and anti-osteoporotic activities (Hall et al., 1989; Sood et al., 1990; Sood et al., 1991; Hall et al.,

1994; Rajendran et al., 1995). It is possible that many other kinds of biological and pharmacological activity will be discovered in the future.

### **Metabolism**

Boron is rapidly absorbed as shown in early studies with cows (Owen, 1944), dogs (Pfeiffer et al., 1945), rabbits and guinea pigs (Akagi et al., 1962). Nevertheless, the mechanism of boron absorption is still unclear. Reeve and Shive (1944) reported that potassium deficiency influenced boron absorption from synthetic media in plants.

However, this was not confirmed in rat studies (Skinner and McHargue, 1945). Stuttgen and associates (1982) reported that the degree of boron released from digested food stuffs was a limiting factor for boron absorption. Boron as boric acid was completely released from a water-based jelly, while there was a very small amount of boron released from oil-based ointments. Thus, the small intestine was not a regulatory site for boron. Regardless of boron from a water- or oily-based stuffs, once it was released, the majority of boron was absorbed from the intestine of humans.

Boron was shown to be excreted primarily through the kidneys in earlier animal studies (Owen, 1944; Pfeiffer et al., 1945; Akagi et al., 1962; Brown et al., 1989). Pfeiffer found that the majority of boron was excreted during the first 48 hours of urine collection in dogs. Studies (Jansen and Schou, 1984) with males aged 30-58 years (mean 47.3 years) indicated similar results. Urine was collected in 2-hour fractions for up to 12 hours after ingestion and then in 12-hour fractions for a further 84 hours. Regardless of whether boron was ingested from a water-based jelly or oil-based ointments, more than

92% of ingested boron was excreted during the first 96-hour period. Other species such as lactating cows, heifers, and sheep had a different urinary excretion rate depending on the amount of ingested boron (Owen, 1944; Green and Weeth, 1977; Brown et al., 1989). Urinary boron excretion accounted for 23, 46, and 52 percent of total daily boron intake (30, 75, and 200 mg/head/d, respectively) in mature sheep. These values were different from those reported by Owen. He found that urinary boron excretion from heifers on a basal ration (271 mg boron/head/d) and on boron-added rations (2058 and 2602 mg/head/d) were 57 and 71%, respectively. However, complete boron excretion through the kidneys may not occur since a small amount of boron is excreted into feces (1%), sweat (1.54%), and milk (Wiley, 1907).

Although most of the ingested boron was excreted, there is a small amount of boron retained in the body. Ku and coworkers (1991) investigated the tissue deposition of boron in male rats at 60-70 days of age. Boron levels in plasma and the soft tissues were below 4 ppm in the control rats. However, when fed with 1575 ppm boron *ad libitum* for a period of 7 days, the male rats had the greatest accumulation of boron in bone. The findings were consistent with those reported in earlier human studies (Alexander et al., 1951; Forbes et al., 1954). Following boron supplementation, the boron concentration in the epididymis, testis, and accessory sex organs was increased by 8 to 22 fold, but the increases were only reflected in the elevated plasma boron level. The finding of a lack of appreciable accumulation of boron in the soft tissues were consistent with those in young rats (Treinen and Chapin, 1991).

## 2.8 Physiological Function of Boron

### Growth

Accumulated data consistently supported the role of boron on growth. The effects of boron on embryonic growth was first noted by King and associates (1991a, b, 1993). *In ovo* boron administration at a dose of 0.1 or 0.5 mg did not improve the hatchability of chick embryos as determined by  $[(\text{hatched egg}/\text{fertile egg})\times 100]$ , and even reduced the value at a dose of 1.0 mg when compared with their respective controls. The reduction in hatchability of chick embryo might result from the toxicity of boron as demonstrated in earlier studies (Lee and Emmel, 1990; Lee et al., 1990). The relative body weight expressed as  $[(\text{body weight at hatching}/\text{egg weight})\times 100]$  of the boron group (0.5 mg) was higher than its control. Boron at a dose of 0.1 or 1.0 mg had no effect on the relative body weight (King et al., 1991a). In contrast, the hatchability was significantly increased when 0.5 mg boron was injected onto the chorioallantoic membrane of vitamin-D<sub>3</sub> deficient eggs. However, egg weight, body weight at hatch, and relative body weight were similar among the treatments (King et al., 1991b). When it was given at a lower dose, boron exerted its benefit on growth. Studies with 26 day-old turkey embryos indicated that body weight and the relative body weight increased by *in ovo* boron administration at a dose of 75 or 150  $\mu\text{g}$  when compared with the control group (King et al., 1993). The effect of boron on the fetal growth in mammals is not yet clear.

Boron also appeared to have a positive effect on the growth of young animals. Day-old chicks given boron significantly improved growth, and the improvement was

more marked in cholecalciferol-deficient than in cholecalciferol-adequate chicks. It was further found that the growth of day-old chicks was influenced by complex interactions among dietary boron, calcium, magnesium, and cholecalciferol. Boron increased body weight when day-old chicks were fed a magnesium-deficient diet (Hunt, 1989). Boron exacerbated the negative effects on growth induced by marginal cholecalciferol (125 IU/kg), magnesium (300 ppm), and calcium (1%). However, when any one of nutrients (cholecalciferol, calcium, magnesium) was added to this diet, boron supplement reversed the growth depression (Hunt and Nielsen, 1985). The authors suggested that boron might be involved with parathyroid hormone-associated physiological responses in day-old chicks.

The effect of boron on growth in young mammals was even more complicated than that seen in day-old chicks as reported by Nielsen and coworkers (1988a). Body weight in weanling rats was affected by dietary manipulation. When the weanling rats were fed a methionine-deficient diet, the depressed growth due to severe magnesium deficiency (100 ppm) was reversed by boron supplementation (3 ppm). However, when the weanling rats were on a methionine-adequate (2.5 mg/g) diet, boron did not reverse growth depression induced by severe magnesium deficiency. Moreover, it was found that boron supplementation (3 ppm) led to a substantial reduction in the ratios of the spleen and kidney weights to respective body weights, which were previously elevated by severe magnesium deficiency. These changes were more marked when weanling rats were on a methionine-deficient than on a methionine-adequate diet.

Nielsen in 1986 used male weanling rats to test whether or not the influence of boron on major mineral metabolism was via its effect on PTH action. Dietary manipulations which changed PTH action included magnesium deficiency and/or aluminum supplementation. It was found that magnesium deficiency depressed growth and elevated the ratios of spleen and kidney to respective body weights; these changes were more marked in boron-deficient than boron-fortified rats. On the other hand, aluminum supplement depressed growth, which was most marked when the rats were fed a low magnesium diet with added boron (3 ppm). The findings further supported the hypothesis that boron was an essential element, involved in parathyroid hormone function in weanling rats.

Hegsted and associates (1991) conducted a similar experiment using weanling rats. All rats were fed a corn meal based diet fortified with an AIN76A vitamin mix without vitamin D, and a mineral mix with or without a boron supplement (3 ppm). The formulation of the mineral mix essentially followed that of Nielsen et al. (1988a), except for the addition of a higher level of potassium. Furthermore, corn meal was carefully stripped of minerals by washing with 2N hydrochloric acid for three times and rinsing with ultrapure water. All rats had free access to food and ultrapure water for 12 weeks. No differences in food consumption, body weight, and organ weights (spleen, kidney, and liver) were observed between non boron-added and boron-added rats on a vitamin D-deficient diet. The fact that supplemental boron did not lead to greater body weight and organ weight suggested that other nutrient interactions were occurring. Moreover, the



lack of boron's effect on growth was also due to limited time of the experiment. The period of 12 weeks might not have been enough to induce vitamin D-deficiency in weanling rats.

The effects of boron on the growth of mature animals appeared inconsistent. Mature male broiler chickens supplemented with boron appeared to have a heavier body weight and a better feed utilization than those with no boron supplementation (Rossi et al., 1989). Elliot and Edwards (1992) used 240 day-old broiler cockerels fed tibial dyschondroplasia-inducing diet (low calcium and high chloride) from days 1 to 16 of age. During the period of the experiment, the broiler cockerels received a low calcium (0.65%) and vitamin D (110 ICU/kg), and high chloride (0.3%) diet to continuously induce tibial dyschondroplasia. However, boron (40 ppm) alone or in combination with calcium (0.9%) or vitamin D (1,100 ICU/kg) failed to affect feed utilization and weight gain in 240 day-old broiler cockerels with tibial dyschondroplasia.

In summary, the hatchability, body weight, or relative body weight of embryos in the birds was significantly improved by a low dose boron supplement. Boron appeared to have a positive impact on body weight of young animals. Its beneficial effects was influenced by other dietary factors such as calcium, magnesium, aluminum, potassium, cholecalciferol, and methionine. According to the growth findings in animals, it was suggested that boron might be involved in parathyroid hormone function.

## **Bone Health**

Boron was found to have an effect on bone growth, mineralization, and cartilage maturation in animals. King and associates (1993) investigated the role of *in ovo* boron administration in the mineralization of the embryonic and neonatal bone of the turkey. Fertile turkey eggs on day 15 of embryogenesis were first injected with 0, 75, or 150  $\mu\text{g}$  boron. Boron administration increased tibial weight, length, width, percent ash content, and bone zinc of 26 day-old embryos. At hatch, the boron groups had higher bone ash than the control, although there were no differences in the tibial length, width, and calcium, phosphorus, magnesium, potassium, or zinc content. Histologic evidence further supported boron's beneficial effects on bone growth and development. The growth plate of the tibia was shorter in the boron (75  $\mu\text{g}$ ) group than in the control group at 26 d of embryogenesis, indicating a rapid bone forming process. The reserve, proliferative, and hypertrophic zones of the fetal bone were decreased by *in ovo* administration of 75  $\mu\text{g}$  boron. It was found to be the same in the growth plate histo-morphology of the neonatal bone injected with 150  $\mu\text{g}$  boron. The histologic evidence indicated that the demand for boron increased with age or weight gain in the turkey. The authors suggested that a single *in ovo* administration of boron significantly affected embryonic and neonatal bone growth and mineralization in the turkey.

Boron at a high dose demonstrated different results from those in the turkey studies. King and associates (1991a) investigated the effects of *in ovo* boron administration on bone mineralization of day-old chicks. Boron as sodium tetraborate at

0.1, 0.5, and 1.0 mg was injected in 8 day-old embryos. At hatch, boron administration of 0.5 and 1.0 mg decreased dried tibial weight. Boron at 0.1, 0.5, or 1.0 mg, had no effect on bone length, width, ash percentage, calcium, phosphorus, magnesium, sodium, potassium, and copper compared with respective control groups. The heights of the growth-plate, reserve, and hypertrophic zones were not affected by boron administration. However, the proliferation zone was longer in the group injected with 1.0 mg of boron, indicating the inability of chondrocytes to mature. The authors suggested that boron at a high dose may interfere with normal bone calcification. It was speculated that overdosed boron caused either an apparent calcium deficiency or a negative interaction with vitamin D, retarding bone calcification and chondrocyte maturation.

The effect of boron on bone was associated with vitamin D<sub>3</sub>. King and coworkers (1991b) examined whether the existence of an interaction between boron and vitamin D<sub>3</sub>. Vitamin D<sub>3</sub>-deficient chicken embryos at day 8 were injected through the shell with vehicle, boron (0.5 mg), boron plus vitamin D<sub>3</sub> (0.5 mg and 0.3 µg, respectively), or vitamin D<sub>3</sub> (0.3 µg and 0.5 mg). No differences were found in bone dried weight, length, or width among treatments. However, the ash percentage was significantly higher for the boron plus vitamin D<sub>3</sub> group when compared with the vehicle, or boron group. Histologic evidence further confirmed the existence of an interaction between boron and vitamin D<sub>3</sub>. Boron, administrated alone or in combination with vitamin D<sub>3</sub>, reduced the height of the epiphyseal growth plate of day-old vitamin D<sub>3</sub>-deficient chicks. Boron decreased the heights of the proliferative and hypertrophic zones which had been previously enlarged

due to vitamin D<sub>3</sub> deficiency. The authors suggested that vitamin D and boron may have an interaction, positively influencing bone growth and mineralization.

The existence of an interaction between dietary boron and vitamin D<sub>3</sub> was further confirmed in day-old chicks as reported by Hunt (1990). The animals were fed a magnesium-adequate diet (containing 0.18 ppm boron) fortified with 0 or 1.42 ppm boron and 125 or 625 IU/kg vitamin D<sub>3</sub> for 27 days. Boron supplementation reduced the total area of the abnormally enlarged growth plate in vitamin D<sub>3</sub>-deficient chicks but increased that in the vitamin D<sub>3</sub>-supplemented chicks. Within the hypertrophic zone of the growth plate, boron supplementation retarded chondrocyte maturation in the vitamin D<sub>3</sub>-adequate chicks but had no effect in the vitamin D<sub>3</sub>-deficient chicks. It was suggested that boron enhanced or inhibited chondrocyte maturation of the growth plate depending on dietary vitamin D<sub>3</sub> intake, confirming the existence of interaction between boron and vitamin D<sub>3</sub>.

Elliot and Edward (1992) conducted two trials to determine the effects of boron on skeletal development and four trials to confirm whether some interactions existed among boron, vitamin D<sub>3</sub>, and calcium. They used 240 day-old broiler cockerels fed a low calcium and high chloride diet to induce tibial dyschondroplasia from 1 to 16 days of age. It was found that dietary boron (5 to 10 ppm) increased bone ash but had no effect on the incidence and severity of tibial dyschondroplasia in the birds fed a low calcium but adequate vitamin D<sub>3</sub> diet. However, boron supplement (20 to 80 ppm) did not increase bone ash but increased the severity of tibial dyschondroplasia in the birds under the same dietary condition. In addition, regardless of dietary vitamin D<sub>3</sub> level, boron supplement at

doses of greater than 20 ppm did not alleviate a vitamin D<sub>3</sub> deficiency in the birds. However, boron supplement at a dose of 3 ppm improved bone ash; the improvement appeared to be independent of dietary vitamin D<sub>3</sub> level. The authors concluded that an interaction between boron and calcium, or between boron and vitamin D<sub>3</sub> did not exist; the positive effect of boron on bone ash appeared to be independent of the dietary calcium and vitamin D<sub>3</sub>.

Studies with vitamin D<sub>3</sub>-deficient weanling rats showed that the femur length, density (g/ml), dried weight, ash content, percent ash, calcium (mg or mg/g), phosphorus (mg or mg/g), and magnesium (mg) were not enhanced, except for the ratio of femur magnesium to dried femur weight, which was elevated following 12 weeks of boron supplement (Hegsted et al., 1991). The fact that the absence of boron's effects on bone parameters suggested that bone may not show evidence of the interaction between boron and vitamin D<sub>3</sub> unless several nutrients such as magnesium, were deficient in the diet as reported in earlier rat studies (Shuler and Nielsen, 1988). Moreover, the authors suggested that weanling rats needed a longer time to deplete their vitamin D<sub>3</sub> stores than day-old chicks; bone parameters may reflect the interaction between boron and vitamin D<sub>3</sub> only in rats that had been hypocalcemic for several weeks.

Studies with rats supported the hypothesis that the interaction existed between boron and magnesium, and it affected femur calcium, magnesium, iron, and copper contents (Nielsen and Shuler, 1992). The interaction between boron and magnesium became more marked when dietary methionine was marginal. The authors concluded that

the demand for boron was more consistent and evident when weanling rats were fed a low magnesium and methionine diet (Nielsen et al., 1988a). Reduced bone calcium due to both boron and calcium deficiencies was found in rats on a low magnesium and potassium diet. Bone calcium was higher in boron-supplemented than boron-unsupplemented rats on a low magnesium and methionine diet. When dietary magnesium was sufficient, boron did not influence bone calcium. On the other hand, bone boron was affected by dietary manipulation. Calcium deficiency increased bone boron in rats fed a low-potassium but adequate magnesium diet. Once potassium was added to this diet, calcium deficiency decreased bone boron. The authors (Nielsen et al., 1992a) suggested that boron and calcium had similar effects influenced by changes in dietary magnesium and/or potassium.

In summary, boron alone at a dose of 75 or 150  $\mu\text{g}$  had a pronounced effect on bone growth and mineralization in embryos and day-old birds. However, the interaction between boron and vitamin D<sub>3</sub>, between boron and calcium, or between boron and magnesium existed, and it influenced bone growth, mineralization, and chondrocyte maturation in young animals. Other dietary factors such as potassium, arginine, and methionine also affected the degree of the demand for boron. However, the interaction among boron, calcium, and vitamin D<sub>3</sub> was not observed in mature animals. The importance of the relationship between boron and bone mineralization was evident and has drawn attention since bone demineralization occurred often in elderly individuals and aged animals.

## **Ca, Mg, and P Metabolism**

Numerous studies focused on the effects of boron on calcium, phosphorus, and magnesium balance. Elsair and associates (1980) examined the effects of boron on major minerals balance which had been deteriorated by acute fluoride intoxication in rabbits. Fluoride balance was negative 7 to 11 days after fluoride was discontinued, but with boron (5.03 g/l) added into tap water, the negative fluoride balance was more pronounced. Obviously, boron decreased intestinal absorption and increased urinary excretion of fluoride. On day 45 after fluoride was discontinued, the bone fluoride level was reduced by boron, but remained high without boron. Boron corrected calcium and phosphorus balance due to fluoride intoxication. Calcium and phosphorus balance declined during fluoride intoxication by reducing intestinal absorption of calcium and phosphorus, and by slightly reducing renal reabsorption of phosphorus. After fluoride was discontinued, calcium and phosphorus balance tended to return to normal without boron, but hypercalciuria and reduced renal reabsorption of phosphorus were corrected by boron.

Brown and associates (1989) examined the effects of dietary boron on calcium, magnesium, and phosphorus balance in mature sheep on an adequate diet. After 20-day adjustment to the basal diet, an 11-day treatment of boron supplementation improved calcium retention, primarily because of increased apparent absorption of calcium from the intestine over the entire experiment. Urinary calcium excretion was not in a dose-dependent manner as was fecal calcium. Urinary calcium excretion was reduced by a low dose boron supplement (45 mg/head/d) but elevated by a high dose boron supplement

(170 mg/head/d) compared with the control group receiving 30 mg boron per head per day. Boron supplementation at a high dose tended to enhance magnesium retention when compared with the control although the enhancement did not reach statistically significant levels ( $P < 0.10$ ). The apparent absorption and urinary excretion of phosphorus was not affected by dietary boron. It was concluded that supplemental boron enhanced the apparent utilization of calcium by mature sheep.

Hegsted and coworkers (1991) examined apparent calcium, magnesium, and phosphorus balance in vitamin D<sub>3</sub>-deficient rats following 12 weeks of boron supplementation (2.72 ppm). The rats fed a boron-supplemented diet had a higher calcium and phosphorus apparent balance than the rats fed an unsupplemented diet. The improvement of apparent balance was due to an increase in apparent calcium and phosphorus absorption by boron supplementation. Boron supplementation had no effect on magnesium apparent balance. Plasma calcium declined over time for rats on a vitamin D<sub>3</sub>-deficient diet. Boron supplementation had no effect on serum calcium, magnesium, and phosphorus.

Boron supplementation appeared beneficial on major mineral metabolism in humans. Nielsen and associates (1987a) gave a 3 mg per day boron supplement to 12 postmenopausal women who were previously on a low-boron diet (0.25 mg/day) for 119 days. It was found that regardless of dietary aluminum, boron supplementation markedly reduced urinary calcium and magnesium excretion (24 hours). The reduction was more marked when dietary magnesium was low. Urinary phosphorus excretion was reduced by



boron supplementation in postmenopausal women on a low magnesium diet. Thus, this human studies supported that boron was beneficial to postmenopausal women by reducing the urinary excretion of calcium, magnesium, phosphorus, which are the main inorganic components of bone.

Beattie and Peace (1993) recruited 6 volunteers who were healthy, free of steroids, drug, and alcohol abuse, and menopausal for less than 20 years. Their body mass index (weight/height<sup>2</sup>; BMI) was close to the average of the 200 applicants (mean BMI 25). The basal diet contained 900 mg calcium, 300 mg magnesium, and 0.33 ppm boron. After a 3-week boron depletion, the subjects were supplemented with 3 mg boron for additional 3 weeks. Urinary calcium excretion (24 hours) increased rapidly from the beginning to the first 3 weeks of the experiment and then remained high throughout the 6-week study. Urinary magnesium excretion followed a similar pattern to calcium and was not affected by supplemental boron. Urinary phosphorus decreased slightly during the first 3 days and then stabilized until the end of the experiment. Neither urinary calcium, magnesium, nor phosphorus excretion was influenced by boron supplementation.

In summary, boron increased calcium absorption and retention in mature sheep but not in postmenopausal women on a balance diet. However, when animals were under a stress such as fluoride intoxication, vitamin D<sub>3</sub> or magnesium deficiency, boron exerted pronounced effects by enhancing calcium and phosphorus absorption from the intestine or reducing calcium and phosphorus excretion through kidney. Boron had a minor effect on magnesium balance.

## **Steroid Hormone**

Boron enhanced and mimicked some effects of estrogen in human subjects. Nielsen and associates (1987b) conducted a human study to examine the effects of aluminum, magnesium, and boron on mineral metabolism in 12 postmenopausal women. The data indicated that boron supplementation (3 mg/day) elevated serum  $17\beta$  estradiol and testosterone of the subjects who were not on estrogen therapy. The changes in serum  $17\beta$  estradiol and testosterone were abrupt, about 8 days after boron supplement, and independent of dietary magnesium and aluminum. The authors suggested that boron reduced urinary calcium, magnesium, and phosphorus as discussed above through endocrine mechanisms.

To test the hypothesis that boron had a similar effect to estrogen therapy, Nielsen and coworkers (1992a) recruited 4 men, four postmenopausal women, and four postmenopausal women on estrogen therapy. The subjects were fed a three-day menu rotation diet containing 682 mg calcium, 304 mg magnesium, 444 IU cholecalciferol, and 0.25 mg boron per 2,000 kcal. After 63-day boron depletion, and 49-day boron repletion, boron supplementation (3 mg per day) raised serum  $17\beta$  estradiol in the postmenopausal women on estrogen therapy. In contrast, boron supplement had no effect on serum  $17\beta$  estradiol in the men and postmenopausal women not on estrogen therapy. Estrogen therapy elevated serum copper, and the elevation was more marked during boron repletion than depletion. The authors suggested that boron had no effect on the biosynthesis of  $17\beta$  estradiol since it did not elevate serum  $17\beta$  estradiol in the males and females not on

estrogen therapy. The fact that boron raised serum  $17\beta$  estradiol in the postmenopausal women on estrogen therapy indicated that boron may increase absorption, and/or decrease breakdown or excretion of  $17\beta$  estradiol. Moreover, the serum copper findings suggested that boron enhanced some effects of estrogen therapy in postmenopausal women.

Beattie and Peace (1993) recruited 6 volunteers who were healthy, free of steroids, drug, and alcohol abuse, and less than 20 years postmenopausal. Their body mass index (weight/height<sup>2</sup>; BMI) was close to the average of the 200 applicants (mean BMI 25). Fasted blood samples (25 ml ) were collected every other day except for weekends. Plasma estradiol was within the range 1-12 pg/ml, that were obtained by the same commercial kit used by Nielsen and associates. Plasma testosterone varied markedly between subjects. Both steroid hormones in plasma were not affected by boron supplement.

Studies with animals did not support the hypothesis proposed by Nielsen's group. Qin and Klandorf (1991) used aged broiler breeder hens to investigate the effect of boron on shell quality and calcium metabolism. Treatment included 1) high calcium (3.5%); 2) high calcium plus 60 ppm boron; 3) low calcium (1.5%) and 4) low calcium plus 60 ppm boron. After 5-week treatment, boron supplement did not affect plasma estradiol regardless of dietary calcium. However, boron significantly depressed plasma estradiol in the morning but not in the evening in laying hens. Plasma estradiol in nonlaying hens was not affected by boron. The findings in hen studies were further confirmed by the administration of radiolabeled estradiol in rats. Beattie (1993) used female rats aged 5

months. The basal diet contained adequate vitamins and minerals but only marginal magnesium (100 ppm), and less than 0.1 ppm of boron. Molybdate and vanadate were excluded since they had similar chemical properties to boron. After a 7-day boron depletion, the animals were given boron supplement at 0 or 40 ppm, ovariectomized, and then infused with [<sup>3</sup>H]17β estradiol using osmotic pump. The data indicated that boron supplementation had no effect on the recovery of tritium from the infused estradiol by measuring urinary conjugated, catechol, and non-catechol estrogen.

In summary, the effects of boron supplementation on estrogen metabolism varied in human and animal subjects. Species, age, time of sample collection, medication, and diet significantly influenced the results.

### **Anti-osteoporosis**

Accumulated data indicated inorganic boron did not consistently exert positive effects on bone growth, mineralization, calcium balance, and serum 17β estradiol. There was no direct and considerable evidence indicating its role in calcium-regulating hormones such as parathyroid hormone (PTH), calcitonin, and 1,25(OH)<sub>2</sub> vitamin D<sub>3</sub>. However, incorporation of boron into a number of heterocyclic amine derivatives has been shown to prevent osteoporosis in rodents. The amine-carboxyborane derivatives listed in **Table 2** were effective because they had a better solubility, bioavailability, and crossed the cell membrane easier than inorganic boron. Rajendran and associates (1995) conducted *in vitro* and *in vivo* studies to test the anti-osteoporotic activity of amine-carboxyborane in

rodents. *In vitro* studies using CF<sub>1</sub> mouse pup calvaria and rat UMR-106 osteosarcoma cells showed that amine-carboxyborane derivatives reduced the loss of intracellular calcium into the growth medium over 48 hours. *In vivo* in CF<sub>1</sub> male mice, amine carboxyboranes at 8 mg/kg/day for 14 days, was more effective than calcitonin to inhibit bone resorption of calcium. The reduction in bone resorption was also reflected by low urinary and serum hydroxyproline levels after drug administration. Incorporation of proline into collagen and non-collagen protein was increased by amine carboxyborane.

To compare the abilities of inorganic and organic boron in preventing osteoporosis, ovariectomized Sprague-Dawley rats were given either amino-carboxyborane at 8 mg/kg/day or borax at 3 mg/kg/day for 14 days. Femur weight, and volume, density, and ash contents were reversed by all amino-carboxyborane compounds tested as shown in **Table 3**. Compound 1 was the most effective in returning bone density to the normal level of the sham-operated rats. The borax salts were ineffective in femur weight and density. The amino-carboxyborane compounds did not change serum 17 $\beta$  estradiol, but slightly elevated serum PTH and 1,25 dihydroxy vitamin D<sub>3</sub>. It was possible that administering these compounds might increase calcium absorption from the intestine.

In conclusion, organic boron, such as amine-carboxyborane compounds, appeared to prevent and treat osteoporosis by modulating bone and calcium metabolism. Treatment with amine-carboxyborane compounds reversed ovariectomy-induced bone parameters to the normal levels.

Table 2  
Amine-carboxyborane derivatives

Compound	
1	$(C_6H_5)_3PBH_2COOH$
2	$C_{16}H_{33}N(CH_3)_2BH_2COOH$
3	$C_{18}H_{37}N(CH_3)_2BH_2COOH$
4	$(CH_3)_2NHBH_2COOCH_3$
5	$(CH_3)_3NBH_2COOCH_3$
6	$CH_3NH_2BH_2COOH$
7	$CH_3CH_2NH_2BH_2COOH$
8	$(CH_3)_2NHBH_2COOH$
9	$CH_3NH_2BH_2COOCH_3$

(Sood et al., 1990)

Table 3  
 Comparison of the effects of organic (amine carboxyboranes) and inorganic (borax) boron in the sham-operated (Sham) and ovariectomized (Ovx) rats

Treatment	Body gain, g	Femur weight, g	Femur volume, ml	Femur density, g/ml	Femur ash, g
Sham Control	50.5 ± 3.9	0.99 ± 0.02**	0.67 ± 0.01	1.49 ± 0.01**	0.38 ± 0.32**
Ovx Control	49.2 ± 5.1	0.87 ± 0.02	0.63 ± 0.02	1.37 ± 0.02	0.26 ± 0.006
Ovx+(C <sub>6</sub> H <sub>5</sub> ) <sub>3</sub> PBH <sub>2</sub> COOH	46.0 ± 12.6	1.04 ± 0.04**	0.71 ± 0.03*	1.46 ± 0.01**	0.35 ± 0.009**
Ovx+C <sub>16</sub> H <sub>33</sub> N(CH <sub>3</sub> ) <sub>2</sub> BH <sub>2</sub> COOH	39.7 ± 7.4	0.98 ± 0.02*	0.71 ± 0.02	1.40 ± 0.04	0.33 ± 0.008**
Ovx+(CH <sub>3</sub> ) <sub>3</sub> NBH <sub>2</sub> COOCH <sub>3</sub>	45.0 ± 10.4	0.98 ± 0.03*	0.71 ± 0.03*	1.39 ± 0.03	0.31 ± 0.007**
Ovx+Borax	45.7 ± 5.43	0.88 ± 0.02	0.61 ± 0.01	1.42 ± 0.01	0.30 ± 0.010

\* P< 0.001; \*\* P<0.005 compared to Ovx Control (Rajendran et al., 1995)

## **CHAPTER 3**

### **The Effects Of Boron Alone Or In Combination With $17\beta$ Estradiol Or Parathyroid Hormone (PTH) On The Bone Quality Of Ovariectomized Rats**

#### **Abstract**

The effects of boron alone or in combination with parathyroid hormone (PTH) or  $17\beta$  estradiol on bone ash, minerals, volume, structure, and mechanical properties were investigated in ovariectomized (Ovx) rats at 84 d of age. Treatments began on day 43 after ovariectomy and continued for 5 weeks. Treatments included 5 ppm boron as boric acid,  $17\beta$  estradiol (30  $\mu\text{g}/\text{kg}/\text{d}$ , s.c.), parathyroid hormone (rat PTH 1-34 fragment, 60  $\mu\text{g}/\text{kg}/\text{d}$ , s.c.), boron+ $17\beta$  estradiol, and boron+PTH at the same dose. All rats had free access to food and d.i. water. Ovariectomy increased body weight, decreased dried bone weight, bone ash, and bone Ca, P, and Mg based on body and/or dried bone weights. Histomorphometric evidence further confirmed that ovariectomy caused significant decreases in trabecular bone volume, bone surface density, trabecular plate density, and a substantial increase in trabecular plate separation. Treatment with PTH appeared to have more anabolic action on bone than  $17\beta$  estradiol. The Ovx rats that received daily PTH injection had significantly increased dried tibia weight/body weight ratio, wall thickness, total bone ash, bone Ca, Mg, and P, trabecular bone volume, bone surface density, trabecular plate density, trabecular plate thickness, and decreased trabecular plate separation. On the contrary, the Ovx rats treated with  $17\beta$  estradiol alone had significantly decreased body weight, increased bone Ca, Mg, and P to body weight ratios,



and reduced trabecular plate separation. Boron alone had no effect on bone and growth. But, combined boron with estrogen significantly improved bone surface density and trabecular plate density when compared with estrogen alone. Additionally, this combined treatment significantly increased trabecular bone volume and decreased trabecular plate separation when compared with Ovx untreated rats. However, boron in combination with PTH did not provide additional benefit compared with PTH alone. In conclusion, boron supplementation had no effect on the secondary spongiosa of the proximal tibia of the Ovx rats. Boron had no effect on PTH action on bone, but it significantly enhanced the action of  $17\beta$  estradiol on trabecular bone volume, density and connectivity in Ovx rats.

(Key words: osteoporosis, ovariectomized rats, boron, parathyroid hormone,  $17\beta$  estradiol)

### **Introduction**

Boron, an ultratrace element, is essential to some types of plants. Its functions in plants are thought to be involved in regulation of plant hormones. However, the essentiality of boron to animals is unclear. Previous studies with weanling rats and young chicks indicated that boron played a role in major minerals (Ca, Mg, K) and bone metabolism (Nielsen and Hunt, 1983; Nielsen and Shuler, 1992; Nielsen et al., 1992b). Its interaction with minerals was presumed to be associated with PTH action (Nielsen, 1985). King et al. (1991a,b) found that boron tended to increase bone weight, bone length, bone width, and the percentage of bone ash in chick embryo. However, studies in broiler chickens indicated that an interaction among boron, cholecalciferol, and calcium did not

exist (Elliot and Edwards, 1992). Moreover, Qin and Klandorf (1991) reported that boron supplementation did not increase the shell quality and calcium retention but the percentage of bone ash in aged broiler breeder hens.

Boron was also found to mimic and enhance estrogen action and thus linked to osteoporosis. Nielsen and associates (1987a, b) reported an increase in plasma  $17\beta$  estradiol level and a decrease in urinary Ca excretion within 8 days of giving boron supplementation (3 mg/day) to postmenopausal women previously on a boron-depleted diet for 119 days. It was suggested that boron might decrease catabolism of  $17\beta$  estradiol by complexing with the vicinal hydroxyl groups of catechol estrogen and thus limiting subsequent methylation (Beattie and Weersink, 1992). However, an increase in  $17\beta$  estradiol by boron supplementation was not found in aged hen and rats (Qin and Klandorf, 1991; Mohammad et al., 1993). A human study conducted in Scotland indicated that a daily 3 mg boron supplement had no effect on bone mineral composition, bone turnover, or plasma  $17\beta$  estradiol level.

Due to inconsistency in the earlier findings and thus an uncertainty of the role of boron in the prevention or treatment of osteoporosis, we used ovariectomized rats as a model to re-evaluate the effects of boron on the quality of the bone. Treatments for postmenopausal osteoporosis must be able to inhibit bone resorption and/or enhance bone formation. Estrogen replacement has been demonstrated to be as a good antiresorptive treatment able to inhibit bone resorption and improve bone Ca balance (Stock et al., 1985; Kaplan et al., 1994). Intermittent PTH injection at a low dose, on the other hand, causes a

dramatic anabolic effects on bone and Ca balance (Ibbotson et al., 1992; Reeve et al., 1993; Wronski and Yen, 1994).

We, therefore, used ovariectomized rats to assess the effects of boron on bone mass, minerals, volume, structure, and mechanical properties. The hypotheses were that : 1) boron alone has a positive impact on bone quality; 2) boron enhances the function of  $17\beta$  estradiol on decreasing bone resorption; 3) boron improves PTH action on increasing bone formation.

### **Materials and Methods**

*Animals and diets.* A total of 84 Sprague-Dawley female rats at 84 d of age were purchased from Harlan Sprague Dawley Inc.(Indianapolis, IN). After 7 d of acclimation, rats were systematically assigned into groups by weight. Seventy six rats were ovariectomized (Ovx) from the dorsal approach, and 8 were sham-operated (sham) where ovaries were exteriorized. A baseline group of 9 Ovx rats was randomly selected and killed 2 weeks after surgery. The remaining rats were housed singly in stainless steel cages at 20-22°C on 12-h light/12-h dark cycles and given free access to an AIN-76 basal diet (**Table 4**) that contained 0.5% Ca, 0.4% P, 0.05% Mg and 0.00004% boron, and d.i.water. Food consumption and body weight were recorded every other day and once per week, respectively. Feed cups and drinking bottles were soaked in 2 N hydrochloric acid overnight to avoid contamination before use. After 42 days, Ovx rats were again systematically assigned into 7 groups based on weight and a pretreatment group of 9 Ovx

rats was randomly chosen and killed. The protocol was approved by the Animal Care Committee in Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

*Treatments.* Treatments began on day 43. There were 6 treatment groups: Ovx+V (vehicle, untreated), Ovx+B (boron, 5 ppm), Ovx+E (17 $\beta$  estradiol, 30 $\mu$ g/kg/d), Ovx+P (PTH, 60  $\mu$ g/kg/d), Ovx+BE (boron + 17 $\beta$  estradiol), and Ovx+BP (boron + PTH). Rats in groups Sham, Ovx+V, Ovx+P and Ovx+E were given an AIN76 basal diet as mentioned above, and rats in groups Ovx+B, Ovx+BP, and Ovx+BE received an AIN 76 basal diet fortified with 5 ppm of boron as boric acid. Rats were allowed free access to food and d.i. water, and weighed on a weekly basis. Groups Sham, Ovx+V, and Ovx+B were injected with vehicle solution (5% ethanol in corn oil and 0.001N HCl). Groups Ovx+P and Ovx+BP were injected s.c. with rat PTH (1-34) fragment (Bachem California Inc., Torrance, CA) at a dose of 60  $\mu$ g/kg/day, and groups Ovx+E and Ovx+BE were injected s.c. with 17 $\beta$  estradiol (Sigma Inc., St Louise, MO), 30  $\mu$ g/kg/day. Rat PTH (1-34) fragment was dissolved in acid saline (0.001N HCl) with 2% heat-inactivated rat serum while 17 $\beta$  estradiol was dissolved in 5% ethanol and 95% corn oil. The volume of injection was based on body weight (1 ml/kg) and adjusted periodically for 50 g increments in weight change. After five weeks of treatments, all rats were sacrificed after an overnight fast.

*Sample collection.* Necropsy was performed between 9 am and 1 pm approximately 20-24 hours after the last injection. Rats were first i.p. injected with pentobarbital sodium at a dose of 25 to 40  $\mu$ g/kg and then exposed to carbon dioxide.

The femurs and tibias were removed and defleshed. The right tibia was longitudinally cut immediately using a handsaw with fine teeth, soaked in 70% ethanol in a glass vial (30 ml) and kept at room temperature in the dark. The femurs and left tibia were frozen at  $-20^{\circ}\text{C}$  in plastic bags for subsequent analysis.

*Analysis.* After thawing, the left tibia was tested immediately to prevent desiccation. We used an Instron Universal Testing Machine (Model 1123, Instron Corp., Canton, MA) at a full loading scale of 500 Newton and at a cross-head speed of 1mm/min. Shear force and energy were calculated and recorded using a established computer software. The crushed left tibia was then dried at  $105^{\circ}\text{C}$  for 48 hours, weighed and ashed in a muffle furnace at  $600^{\circ}\text{C}$  for 24 hours. The ash residue was ground, weighed, and digested with nitric acid/perchloric acid (5/3, v/v) for the measurements of Ca, P and Mg. For Ca and Mg measurements, the wet-ashed samples were diluted with 1% lanthanum oxide by the procedure proposed by Sandel (1959). The diluted sample solution was analyzed by Atomic Absorption Spectroscopy (AAS, Perkin-Elmer Atomic Absorption Spectrophotometer 2100, Rockville, MD). The P content was determined by a colorimetric method (Fiske and Subbarow, 1925) using a microplate reader (Ceres 900 Hdi, Bio-Tek Instruments, Inc., Winooski, VT). For the determination of boron contents, the right femur was first dried at  $105^{\circ}\text{C}$  for 48 hours, weighed and wet-ashed with nitric acid/hydrogen peroxide according to the open-vessel wet-ash, low-temperature, teflon-tube (WALTTT) procedure (Hunt and Shuler, 1989). The wet-ashed bone samples were

measured for boron using an Inductively Coupled Plasma Spectroscopy (ICP, Perkin-Elmer Plasma 400 ICP Emission Spectrometer, Rockville, MD).

Histomorphometric bone biopsies were prepared in Histopathology Laboratory in the College of Veterinary Medicine (Blacksburg, VA). To assess bone structure, the proximal right tibia was sawed off, soaked in Decalcifier II (Surgipath) overnight, dehydrated in graded ethanols, infiltrated with paraffin wax, and embedded in EM400 (Surgipath). The embedded bone samples were then cut longitudinally into 5  $\mu\text{m}$  sections using a Reichert-Jung microtome and stained with hematoxylin and eosin-phyloxin. Each slide was given a random number to blind identity from the observer. All histomorphometric indices were measured in the area of the secondary spongiosa, exactly 1 mm from the lowest point of the growth plate, with a total area of 1.35  $\text{mm}^2$ . Trabecular bone area and perimeter were measured using point and intercept counting using a Mertz eyepiece graticule at X200. Trabecular (Cancellous) bone volume, trabecular bone surface density, trabecular bone surface to volume ratio, trabecular plate thickness, trabecular plate density, and trabecular plate separation were defined and calculated according to Parfitt et al. (1983).

*Statistics.* We first assumed that error distribution was normal. Data were expressed as mean  $\pm$  standard error. Analysis of Variance (ANOVA) was used to test for main effects of ovariectomy and treatment. Tukey's Studentized Range (HSD) test was used to indicate the differences if they existed. We used SAS™ (SAS Institute, Inc., Cary,

NC 27512) to perform these analyses. The Least Square Means (LSMEANS) procedure was used for unequal sample sizes. The significant level was set at 0.05.

## Results

One Sham rat died from surgery, and 4 Ovx rats had serum  $17\beta$  estradiol above the normal range of the intact rats. Thus, these data were excluded.

Ovx rats at 15 to 24 weeks of age were still growing. As shown in **Table 5**, both body and femur weights increased with time ( $P\leq 0.002$ ), although the ratio of femur weight to body weight decreased in Ovx rats with advancing age ( $P=0.054$ ). The alterations in bone quality in Ovx rats with time are listed in **Table 6**. The ash contents of the whole tibia, or the ratios of bone ash to body or dried bone weights were not different between the 19-week-old (pre-treatment) and 24-week-old (post-treatment) untreated Ovx rats. However, the total bone Ca contents, or bone Ca to dried bone weight or to bone ash ratios were slightly higher in 24-week-old Ovx untreated rats than in 19-week-old Ovx rats ( $P\leq 0.042$ ). The changes in bone volume and structure in the secondary spongiosa were found between the two Ovx groups. A decrease in trabecular bone volume (BV/TV), trabecular plate density, thickness, or bone surface density, and an increase in trabecular plate separation were observed in the 24-week-old Ovx untreated rats compared with the 19-week-old Ovx rats, although the tendency did not reach statistically significant.

Body weight was influenced by ovariectomy as shown in Table 7. Body weight was 28% higher in the Ovx+V rats than in the Sham rats ( $P=0.0001$ ). Treatment with estrogen alone or plus boron significantly decreased body weight in comparison with Ovx untreated rats ( $P\leq 0.0006$ ). PTH, boron, or PTH plus boron had no effect on body weight in Ovx rats.

The dried tibia weight was not affected by ovariectomy. Treatment also had no effect on the dried bone weight when compared with the Ovx untreated group. When the dried bone weights were compared between treatments, the Ovx+BP rats had a significantly higher dried bone weight than the Ovx+B or Ovx+E group ( $P\leq 0.027$ ). Ovariectomy caused a 16% decrease in the ratio of the dried bone weight to body weight ( $P=0.0001$ ). Daily treatments with estrogen, PTH, estrogen+boron, or PTH+boron significantly increased the ratio in comparison with the Ovx+V group ( $P\leq 0.007$ ). The dried bone to body weights ratio was not different between groups Ovx+P and Ovx+BP, or between groups Ovx+E and Ovx+BE. Boron alone did not affect the dried bone weight or dried bone weight to body weight ratio in the Ovx rats.

Ovariectomy caused a substantial increase in the wall thickness of the femur ( $P=0.006$ ). Treatment with PTH alone or in combination with boron increased the wall thickness when compared with the Ovx+V group ( $P\leq 0.033$ ). Estrogen, boron, or estrogen plus boron had no effect on the wall thickness of the femur in Ovx rats.

The ash content of the whole tibia was not affected by ovariectomy. However, treatment with PTH alone or plus boron increased total bone ash when compared with the



Ovx+V group ( $P=0.0001$ ). The bone ash contents in both the Ovx+P and Ovx+BP groups were even above that in the Sham group. Treatment with boron or estrogen had no effect on total bone ash. When total bone ash was corrected by body weight or dried bone weight, there was a significant difference between the Ovx+V and Sham groups. The Ovx untreated rats had a lower ratio of bone ash to body weight or dried bone weight ( $P\leq 0.002$ ). Daily treatment with estrogen, PTH, estrogen+boron, or PTH+boron significantly increased the bone ash to body weight ratio when compared with the Ovx rats without treatment ( $P\leq 0.01$ ). The ratio of bone ash to dried bone weight was also increased by PTH alone or with boron ( $P\leq 0.0008$ ), but was not changed by estrogen or estrogen plus boron. This ratio was higher in the Ovx+BP group than in the Ovx+B group ( $P=0.0014$ ). There were no differences in the two ratios between groups P and BP, or between the Ovx+E and Ovx+BE groups. Boron alone did not affect the ratio of bone ash to body weight or dried bone weight in Ovx rats.

Bone Ca, P, and Mg contents were summarized in **Table 8**. Total Ca, P, and Mg contents of the tibia were not changed by ovariectomy. Treatment with PTH alone or with boron increased bone Ca, P, and Mg by more than 11% when compared with the Ovx+V group ( $P\leq 0.045$ ). Total bone Ca, P, and Mg levels in groups Ovx+P and Ovx+BP were even above those in the Sham group and in the Ovx+B group ( $P\leq 0.0037$ ). There were no differences in bone Ca, P, and Mg between the Ovx+P and Ovx+BP groups, or between the Ovx+E and Ovx+BE groups. Boron, estrogen, or boron plus estrogen had no effect on total Ca, P, and Mg contents of the tibia of Ovx rats.

Ovariectomy decreased the ratios of bone Ca, P, and Mg to body weight or dried bone weight ( $P \leq 0.004$ ). Treatments also had an impact on the ratios. The ratios were elevated by treatment with PTH alone or with boron compared with the Ovx untreated group ( $P \leq 0.035$ ). The ratios were also higher in the Ovx+BP than in the Ovx+B ( $P \leq 0.049$ ). Estrogen treatment elevated the ratios of bone Ca, P, and Mg to body weight but did not affect bone Ca, P, and Mg to dried bone weight ratios ( $P \leq 0.031$ ). There were no differences in the two ratios between groups Ovx+E and Ovx+BE, or between groups Ovx+P and Ovx+BP. Boron alone had no effect on the ratios. Moreover, when bone Ca, P, or Mg was normalized by total bone ash, there was no difference between Ovx+V and any of other treatment groups.

Bone boron reflected the amount of dietary boron intake. Groups (Ovx+B, BE, and BP) that received a boron-fortified diet had a 55% greater bone boron content in comparison with non boron-added Ovx groups (Ovx+V, E, and P). The Ovx+V rats had a significantly lower ratio of bone boron:body weight than the Sham rats.

As shown in **Table 9**, ovariectomy caused a 70% decrease in the trabecular bone volume expressed as the percentage of the total bone section ( $P < 0.0001$ ). Daily treatments with PTH, PTH+ boron, and estrogen + boron significantly improved the trabecular bone volume by 285%, 128%, and 107% in comparison with group Ovx+V ( $P \leq 0.05$ ). The trabecular bone volume was even higher in the Ovx+P group than in the Sham group. When compared between treatments, the trabecular bone volume was lower in the Ovx+B rats than in the Ovx+BP group and in the Ovx+BE group ( $P \leq 0.026$ ).

Estrogen alone caused a nonsignificant increase in the trabecular bone volume of the Ovx rats ( $P=0.154$ ). There were no differences in the trabecular bone volume between groups Ovx+E and Ovx+BE, or between groups Ovx+P and Ovx+BP. Boron alone had no effect on the trabecular bone volume.

The bone surface density was defined as the bone surface area in  $\text{mm}^2$  per  $\text{mm}^3$  of tissue (Parfitt et al., 1983). Ovariectomy decreased the bone surface density by 39% ( $P=0.0001$ ). Treatments with either PTH, PTH + boron, or estrogen + boron increased the bone surface density when compared with the Ovx+V group ( $P\leq 0.022$ ). Estrogen slightly and nonsignificantly increased the bone surface density in Ovx rats ( $P=0.086$ ). The Ovx+BE rats had higher bone surface density than the Ovx+E rats ( $P=0.040$ ). There was no difference in bone surface density between groups Ovx+P and Ovx+BP. Boron alone did not enhance bone surface density in the Ovx rats.

The trabecular plate thickness was not affected by ovariectomy but was elevated by 78% in the Ovx+P group compared with the Ovx+V group ( $P=0.048$ ). There was no difference in the trabecular plate thickness among the Ovx+V, Ovx+B, Ovx+E, Ovx+BE, and Ovx+BP groups.

Ovariectomy decreased the trabecular plate density by 71% ( $P=0.0001$ ). Treatments with either PTH, estrogen+boron, or PTH+boron elevated the trabecular plate density by 108%, 124% and 133% when compared with respective Ovx+V groups ( $P<0.02$ ). The trabecular plate density was slightly higher in the Ovx+E rats than in the Ovx+V rats ( $P=0.086$ ). The Ovx+BE had a significantly higher the trabecular plate

density than the Ovx+E group ( $P=0.040$ ). There was no significant difference in the trabecular plate density between groups Ovx+P and Ovx+BP. Boron had no effect on the trabecular plate density in Ovx rats.

Ovariectomy caused a 375% increase in the trabecular plate separation ( $P=0.0001$ ). However, daily treatments with either PTH, PTH+boron, estrogen, or estrogen+boron significantly decreased the trabecular plate separation in comparison with the Ovx+V rats ( $P\leq 0.031$ ). The trabecular plate separation was nonsignificantly lower in the Ovx+BE group than in the Ovx+E group ( $P=0.086$ ). There was no difference in the trabecular plate separation between groups Ovx+P and Ovx+BP. Boron alone had no effect on the trabecular bone separation in Ovx rats.

Bone strength of the tibia and femur is summarized in **Table 10**. Shear force and energy of the tibia and femur were not affected by ovariectomy. In fact, no treatment changed bone strength except estrogen that significantly decreased shear force of the femur, compared with Ovx untreated group ( $P=0.045$ ). Shear force and energy of the femur were higher by 13% and 25% in group Ovx+P than in group Ovx+V, but the tendency did not reach statistical significance. In comparison with the effects between treatments, PTH caused significantly higher shear energy of the femur than estrogen alone ( $P=0.033$ ). Treatment with boron alone had no effect on bone strength of the tibia and femur of Ovx rats.

## **Discussion**

Rats have a life span of about 3 to 4 years. Bone length, width, density, and calcium content increase rapidly from 1 to 3 months of age, and the increases are continuous but gradual by 6 months of age (Kalu, 1991). Our Ovx rats at 15 to 24 weeks of age still grew with age. However, bone histomorphometric indices in the present study demonstrated that the bone quality in the secondary spongiosa of the proximal tibia were decreased with age in Ovx untreated rats.

Following ovariectomy in rats, there is a rapid decrease in bone turnover with increases in both bone resorption and formation, but with a greater bone resorption than bone formation, leading to a net outcome of bone loss, similar to the postmenopausal bone loss in humans (Heaney et al., 1978). A rapid bone loss occurring in the initial phase after surgery is transitory. With time, bone turnover is slowed down, but bone loss continuously occurs due to a reduction in bone formation (Wronski et al., 1985; Wronski et al., 1986; Kalu, 1991; Mosekilde et al., 1993).

We and others have shown that ovariectomy increases body weight, decreases trabecular bone volume, decreases bone ash and minerals (Ca, P, and Mg) based on body weight and/or dried bone weight, decreases bone surface density and trabecular plate density, and increases trabecular plate separation in rats 11 weeks after surgery. Since our animal model was selected to represent postmenopausal women, an unlimited diet seemed to be a more realistic approach. Shear force and shear energy in the present study were insensitive to the alterations in bone competence as reported in previous studies (Turner,

1991; Frost, 1992). It has been suggested that the trabecular bone mass and its degree of connectivity may be more important in determining bone mechanical competence than shear force and energy (Shen et al., 1993).

Continuous PTH infusion enhances bone resorption and inhibits bone formation as reported in previous studies (Joborn et al., 1991). However, PTH given intermittently at a low dose shows anabolic action on bone by increasing bone mass in normal intact rats, Ovx rats, and in osteoporotic patients (Gunnesh-Hey and Hock, 1984; Hock et al., 1988; Reeve et al., 1993; Mosekilde et al., 1994; Wronski and Yen, 1994). Its anabolic action on bone is associated with the stimulation of adenyl cyclase located on osteoblasts (Rixon et al., 1994; Watson et al., 1995; Whitfield et al., 1995). PTH activates this enzyme, leading to the production of cAMP which in turn increases gene expression of anabolic growth factors such as IGF-1 and TGF- $\beta$ . These two growth factors stimulate osteoblast proliferation and subsequently bone growth.

In the present study PTH was found to markedly relieve negative effects due to ovariectomy by increasing trabecular bone volume, bone surface density, trabecular plate thickness, trabecular plate density, and decreasing trabecular plate separation in the secondary spongiosa of the proximal tibia of the Ovx rats. The anabolic action of PTH according to histomorphometric findings was further confirmed by increasing total bone minerals (Ca, P, and Mg) and total bone ash content, indicating the enhancement of bone mineralization.

Estrogen significantly prevents or treats postmenopausal bone loss by slowing down bone turnover, through decreases in both bone resorption and formation in both animals and humans (Christiansen et al., 1982; Stock et al., 1985; Shen et al., 1992; Chow et al., 1992; Turner et al., 1993). However, slight and nonsignificant increases in trabecular bone volume and trabecular plate density of Ovx rats treated with estrogen were found in the present study. The same were found that estrogen therapy had no effect on bone ash or bone Ca unless both of the bone parameters were normalized by body weight. The inability of estrogen to improve bone quality in the present study might result from three possibilities. First, human studies indicated that estrogen exerts its efficiency within the first 5 years after oophorectomy (Lindsay, 1993). Han et al. (1992) suggested that estrogen should be initiated in Ovx rats within 2 to 5 weeks after surgery. Our Ovx rats began receiving estrogen replacement at 6 weeks after surgery. It may have been too late to allow complete recovery from trabecular bone loss. Secondly, histomorphometric data were collected in the present study from the area of the proximal rather than the distal tibia, which is more sensitive to estrogen action than the former. Thirdly, we gave all Ovx rats an unlimited diet, leading to a greater difference in body weight between the Ovx+V and Ovx+E groups compared with that in other studies in which all Ovx rats were given a restricted diet. Wronski et al. (1987) reported that heavier body weights in Ovx rats on an unlimited diet caused the mechanical strain within bone and provided partial protection from bone loss in comparison with Ovx rats on a restricted diet. Thus, the greater increase in body weight of the Ovx rats without estrogen

treatment due to an unlimited diet might have made estrogen replacement less efficient than in previous studies using a restricted diet .

Boron as boric acid, given at a dose of 5 ppm, starting at 6 weeks after surgery, had no effect on bone quality of Ovx rats. The lack of any effect of boron supplement could not be due to a poor absorption of boron from the intestine since there was a 55% increase in the bone boron content compared with non boron-fortified groups. Our data were consistent with human studies as boron supplementation had no effect on bone minerals and bone turnover in postmenopausal women (Beattie and Peace, 1993).

Several recent reports (Hunt and Nielsen, 1983; Nielsen et al., 1988a, b) support the concept that boron deficiency is induced when other dietary nutrients such as Ca, Mg, Vitamin D, and K are inadequate. However, the hypothesis has not been systematically studied. Current knowledge is not adequate to determine the minimum boron level which will affect bone health and thus identify a real boron depletion in humans and animals. Boron as boric acid acts as a Lewis acid accepting hydroxyl ions, and thus leaving an excess of protons which may cause changes in pH values in the lumen of the intestine and in the circulation of Ovx rats. We do not have enough information about whether such a change in pH might affect the absorption of other nutrients from the intestine and might speed dissolution of bone minerals. In the present study, estrogen alone had no effect on bone surface density or trabecular plate density, but when combined with boron, estrogen therapy significantly increased bone surface density and trabecular plate density in Ovx rats. In contrast, boron in combination with PTH did not provide additional benefit on



bone quality when compared with PTH alone. Spielvogel et al. (1994) developed boron compounds such as amine-carboxyboranes. This organic compound of boron has showed a strong anti-osteoporotic ability by reducing bone Ca loss and by increasing Ca and proline incorporation. Therefore, studies in clarifying the physiologic and cellular roles of boron and the mechanisms involving major minerals and hormones are needed.

## **Conclusion**

Boron as boric acid at a dose of 5 ppm had no effect on body weight, dried bone weight, total bone ash, bone mineral contents, bone strength, wall thickness of femur, trabecular bone volume, trabecular plate density, thickness, and separation in Ovx rats. Boron in combination with  $17\beta$  estradiol elevated bone surface density and trabecular plate density in comparison with  $17\beta$  estradiol alone, and increased trabecular bone volume and decreased trabecular plate separation when compared with Ovx untreated rats. Boron plus PTH did not provide additional benefit on bone quality compared with PTH alone, although this combined treatment remained the positive effects of PTH in Ovx rats.

Table 4  
Composition of the basal (AIN 76) diet

Ingredient	Amount (%)
Dextrose	54
Corn Starch	17
Casein, vitamin-free, 90%	14
Corn oil	5
Alphacel	5
AIN 76 minerals	3.5
AIN 76 vitamin	1
DL-methionine	0.3
Choline bitartrate	0.2
 Calculated composition	
Calcium, %	0.5
Phosphorus, %	0.4
Magnesium, %	0.05
Vitamin D, ICU/100g	100
Boron, mg/kg	0.4

Table 5  
 Growth in the 15-week-old (baseline), 19-week-old  
 (pre-treatment) and 24-week-old (post-treatment) ovariectomized (Ovx) rats

	15-week-old Ovx (n=9)	19-week-old Ovx (n=9)	24-week-old Ovx (n=9)
Body weight, g	255±6 <sup>a</sup>	308±7 <sup>b</sup>	343±7 <sup>c</sup>
Bone weight, g	0.48±0.01 <sup>a</sup>	0.56±0.02 <sup>b</sup>	0.59±0.02 <sup>b</sup>
Bone/BW, g/g	0.190±0.004 <sup>a</sup>	0.181±0.005	0.173±0.005 <sup>b</sup>

a, b, c P<0.05 within a row

Table 6  
 Bone quality in the 19-week-old (pre-treatment)  
 and 24-week-old (post-treatment) ovariectomized (Ovx) rats

	19-week-old Ovx (n=9)	24-week-old Ovx (n=9)
Total ash, g	0.27±0.01	0.30±0.01*
Ash/BW, g/kg	0.87±0.04	0.87±0.03
Ash/bone, g/kg	0.58±0.01	0.59±0.01
Total Ca, mg	92±4	104±3*
Ca/BW, g/kg	0.30±0.01	0.31±0.01
Ca/bone, g/g	0.197±0.003	0.208±0.002*
Ca/ash, g/g	0.342±0.004	0.353±0.003*
Bone volume (BV/TV), %	11.2±3.4	7.7±3.4
Surface density, mm <sup>2</sup> /mm <sup>3</sup>	4.97±0.86	3.30±0.86
Tb. plate density, mm <sup>-1</sup>	2.49±0.43	1.65±0.43
Tb. plate thickness, μm	43.14±8.06	45.74±8.06
Tb. plate separation, μm	413±72	646±72*

\* P<0.05 compared to 19-week-old Ovx rats

Table 7

Alterations of body weight (BW) and bone physical properties in the sham-operated (Sham) and ovariectomized (Ovx) rats after 5 weeks of treatment

Sham (n=7)	Ovx+V (n=8)	Ovx+B (n=10)	Ovx+E (n=9)	Ovx+P (n=8)	Ovx+BE (n=10)	Ovx+BP (n=7)
Body Weight, g	269±13 <sup>†</sup>	318±11	283±11 <sup>†</sup>	328±12	283±11 <sup>†</sup>	338±13
Bone Weight, g	0.47±0.02	0.50±0.02	0.47±0.01	0.53±0.02	0.49±0.01	0.54±0.02 <sup>*</sup>
Bone/BW, g/kg	1.75±0.05 <sup>†</sup>	1.47±0.05	1.67±0.05 <sup>†</sup>	1.63±0.05 <sup>†</sup>	1.73±0.05 <sup>†</sup>	1.60±0.05 <sup>†</sup>
Wall Thickness, mm	0.45±0.01 <sup>†</sup>	0.48±0.01	0.47±0.01	0.51±0.01 <sup>†</sup>	0.46±0.01	0.50±0.02 <sup>†</sup>
Total Ash, g	0.29±0.01	0.30±0.01	0.28±0.01	0.33±0.01 <sup>†</sup>	0.29±0.01	0.33±0.01 <sup>†*</sup>
Ash/BW, g/kg	1.08±0.03 <sup>†</sup>	0.87±0.03	1.00±0.03 <sup>†</sup>	1.01±0.03 <sup>†</sup>	1.02±0.03 <sup>†*</sup>	0.99±0.03 <sup>†</sup>
Ash/Bone, g/g	0.62±0.01 <sup>†</sup>	0.59±0.01	0.59±0.00	0.62±0.01 <sup>†</sup>	0.59±0.01	0.62±0.01 <sup>†*</sup>

<sup>†</sup> P<0.05 compared to the Ovx+V group

\* P<0.05 compared to Ovx+B group

V=Vehicle

B=Boron, 5 ppm

E=17β estradiol, 30 µg/kg/d

P=PTH, 60 µg/kg/d

BE=Boron+17β estradiol

BP=Boron+PTH

Table 8  
Bone Ca, P, Mg, and B contents in the sham-operated (Sham) and ovariectomized (Ovx) rats  
after 5 weeks of treatment

	Sham (n=7)	Ovx+V (n=8)	Ovx+B (n=10)	Ovx+E (n=9)	Ovx+P (n=8)	Ovx+BE (n=10)	Ovx+BP (n=7)
Total Ca, mg	100±3	104±3	99±3	99±3	115±3 <sup>†</sup>	100±3	117±3 <sup>†</sup>
Ca/BW, g/kg	0.38±0.01 <sup>†</sup>	0.31±0.01	0.31±0.01	0.35±0.01 <sup>†</sup>	0.35±0.01 <sup>†</sup>	0.36±0.01 <sup>†</sup>	0.35±0.01 <sup>†</sup>
Ca/Bone, g/g	0.215±0.002 <sup>†</sup>	0.208±0.002	0.205±0.002	0.211±0.002	0.217±0.002 <sup>†</sup>	0.206±0.002	0.217±0.002 <sup>†</sup>
Ca/Ash, g/g	0.348±0.003	0.353±0.003	0.348±0.003	0.355±0.003	0.351±0.003	0.348±0.003	0.351±0.003
Total P, mg	53±2	53±2	51±2	50±2	59±2 <sup>†</sup>	48±2	60±2 <sup>†</sup>
P/BW, g/kg	0.20±0.01 <sup>†</sup>	0.16±0.01	0.16±0.01	0.18±0.01 <sup>†</sup>	0.18±0.01 <sup>†</sup>	0.17±0.01	0.18±0.01 <sup>†</sup>
P/Bone, g/g	0.113±0.003 <sup>†</sup>	0.107±0.002	0.105±0.002	0.107±0.002	0.111±0.002 <sup>†</sup>	0.101±0.002	0.112±0.002 <sup>†</sup>
P/Ash, g/g	0.183±0.004	0.181±0.003	0.179±0.003	0.180±0.004	0.180±0.004	0.170±0.004	0.181±0.003
Total Mg, mg	2.01±0.07	1.99±0.06	1.86±0.06	1.86±0.06	2.22±0.06 <sup>†</sup>	1.81±0.06	2.20±0.06 <sup>†</sup>
Mg/BW, µg/kg	7.50±0.30 <sup>†</sup>	5.84±0.27	5.95±0.24	6.62±0.25 <sup>†</sup>	6.80±0.27 <sup>†</sup>	6.50±0.25	6.54±0.29 <sup>†</sup>
Mg/Bone, g/kg	4.28±0.11 <sup>†</sup>	3.97±0.11	3.87±0.10	3.95±0.11	4.17±0.11 <sup>†</sup>	3.78±0.11	4.09±0.12 <sup>†</sup>
Mg/Ash, g/kg	6.94±0.16	6.75±0.13	6.57±0.13	6.65±0.14	6.74±0.14	6.39±0.14	6.62±0.13
Total B, µg	0.40±0.04	0.39±0.03	0.61±0.02 <sup>†</sup>	0.39±0.02	0.43±0.03	0.59±0.02 <sup>†</sup>	0.65±0.03 <sup>††</sup>
B/BW, µg/kg	1.48±0.19 <sup>†</sup>	1.13±0.10	1.95±0.08 <sup>†</sup>	1.37±0.09	1.31±0.10	2.09±0.08 <sup>†</sup>	1.94±0.16 <sup>††</sup>
B/Bone, µg/g	0.71±0.08	0.66±0.04	1.08±0.04 <sup>†</sup>	0.66±0.04	0.67±0.04	1.02±0.04 <sup>†</sup>	1.02±0.04 <sup>††</sup>

<sup>†</sup> P<0.05 compared to Ovx+V group; <sup>††</sup> P<0.05 compared to Ovx+B group; <sup>‡</sup> P<0.05 compared to Ovx+E group; <sup>†††</sup> P<0.05 compared to Ovx+P group  
B=Boron, 5 ppm; E=17β estradiol, 30 µg/kg/d; P=PTH, 60 µg/kg/d; BE=Boron + 17β estradiol; BP=Boron +PTH

Table 9  
 Histomorphometric indices of the trabecular (Tb) bone in the sham-operated (Sham) and ovariectomized (Ovx) rats after 5 weeks of treatment

	Sham (n=6)	Ovx+V (n=5)	Ovx+B (n=6)	Ovx+E (n=6)	Ovx+P (n=5)	Ovx+BE (n=6)	Ovx+BP (n=4)
Tb. Bone Volume, %	25.4±2.8 <sup>†</sup>	7.7±3.4	6.5±3.4	12.2±2.8	29.4±3.4 <sup>†</sup>	15.8±2.8 <sup>†*</sup>	17.5±3.4 <sup>†*</sup>
Surface Density, mm <sup>2</sup> /mm <sup>3</sup>	11.37±0.70 <sup>†</sup>	3.30±0.86	3.17±0.86	5.12±0.70	6.87±0.86 <sup>†</sup>	7.39±0.70 <sup>†*</sup>	7.69±0.86 <sup>†*</sup>
Tb. Plate Thickness, μm	44.97±6.58	45.74±8.06	42.45±8.06	49.08±6.58	81.52±8.06 <sup>†</sup>	43.52±6.58	47.63±8.06
Tb. Plate Density, mm <sup>-1</sup>	5.68±0.35 <sup>†</sup>	1.65±0.43	1.59±0.43	2.56±0.35	3.43±0.43 <sup>†</sup>	3.69±0.35 <sup>†*</sup>	3.85±0.43 <sup>†*</sup>
Tb. Plate Separation, μm	136±58 <sup>†</sup>	646±72	637±72	363±59 <sup>†</sup>	219±72 <sup>†</sup>	251±59 <sup>†*</sup>	248±72 <sup>†*</sup>

<sup>†</sup> P<0.05 compared to Ovx+V group

\* P<0.05 compared to Ovx+B group

<sup>†</sup> P<0.05 compared to Ovx+E group

V=Vehicle

B=Boron, 5 ppm

E=17β estradiol, 30 μg/kg/d

P=PTH, 60 μg/kg/d

BE=Boron + 17β estradiol

BP=Boron + PTH



Table 10  
 Bone shear force and energy in the sham-operated (Sham) and ovariectomized (Ovx) rats after 5 weeks of treatment

	Sham (n=7)	Ovx+V (n=8)	Ovx+B (n=10)	Ovx+E (n=9)	Ovx+P (n=8)	Ovx+BE (n=10)	Ovx+BP (n=7)
<b>Femur</b>							
Shear Force, N	213	212	209	185 <sup>†</sup>	240	195	223
Shear Energy, N.mm	32.0	41.8	37.8	30.6	52.2 <sup>†</sup>	33.3	37.7
<b>Tibia</b>							
Shear Force, N	212	218	218	219	247	214	243
Shear Energy, N.mm	44.0	48.9	43.4	46.3	43.3	43.7	55.7

<sup>†</sup> P<0.05 compared to Ovx+V group

<sup>†</sup> P<0.05 compared to Ovx+E group

## CHAPTER 4

### **The Effect Of Boron Alone Or In Combination With $17\beta$ Estradiol Or Parathyroid Hormone On Ca, P, And Mg Metabolism In Ovariectomized Rats**

#### **Abstract**

The effects of boron alone or in combination with  $17\beta$  estradiol or parathyroid hormone (PTH) on Ca, P, and Mg metabolism were investigated in 3-month-old ovariectomized (Ovx) rats. Treatments began on day 43 after surgery and continued for 5 weeks. Treatments included 5 ppm boron as boric acid,  $17\beta$  estradiol (30  $\mu\text{g}/\text{kg}/\text{d}$ , s.c.), parathyroid hormone (PTH, 60  $\mu\text{g}/\text{kg}/\text{d}$ , s.c.), boron+ $17\beta$  estradiol, and boron+PTH at the same dose. All rats had free access to food and d.i. water. Ovariectomy caused an increase in food consumption, weight gain, and serum osteocalcin, and a decrease in serum Mg and urinary Ca excretion ( $P < 0.05$ ). Treatment with  $17\beta$  estradiol alone elevated serum P and Mg, decreased serum osteocalcin concentration, but did not change the percentages of apparent Ca absorption and Ca retention in Ovx rats. However, in combination with boron,  $17\beta$  estradiol improved apparent Ca, P, and Mg absorption, and Ca, Mg retention in Ovx rats. PTH alone caused an increase in serum P, Mg and osteocalcin concentration but did not significantly change apparent Ca, P, and Mg absorption and retention in Ovx rats. PTH combined with boron significantly enhanced apparent Ca absorption and Ca retention, elevated serum Ca, P, and osteocalcin

concentration when compared with Ovx untreated rats. In conclusion, boron or estrogen treatment, initiating 6 weeks after ovariectomy, had no significant impact on Ca, P, and Mg balance in Ovx rats. However, when boron was combined with estrogen, Ca and Mg balance, and apparent P absorption were significantly improved. In contrast, boron in combination with intermittent PTH injection did not provide additional benefit in Ca, Mg, and P balance compared with PTH alone, but significantly improved Ca balance when compared with Ovx untreated rats.

(Key words: osteoporosis, rats, Ca, Mg, P, boron,  $17\beta$  estradiol, parathyroid hormone)

### **Introduction**

Calcium (Ca), phosphorus (P), and magnesium (Mg) doubtless are the most important minerals for bone development and integrity. Ca and P are the major components of hydroxyapatite, which is the primary mineral structure in bone and calcified cartilage. In elderly individuals, low Ca intake and malabsorption aggravate negative Ca balance and contribute to the bone loss seen in postmenopausal osteoporosis (Gennari et al., 1990; Morris et al., 1991; Devine et al., 1993). The role of P in development of osteoporosis is less well defined than Ca. However, P deficiency may limit the formation of hydroxyapatite, making a high Ca diet useless. Mg is important to maintain a normal pH in bone extracellular fluid and thus influences metabolism of both organic and inorganic components of bone (Driessens et al., 1987). The fact that subnormal bone and erythrocyte Mg contents were found in osteoporotic patients indicated low total body Mg

stores in osteoporosis (Alfrey et al., 1974; Reginster et al., 1989; Eisinger et al., 1987). Further evidence indicated that low Mg intake and Mg malabsorption existed in postmenopausal osteoporosis (Cohen and Kitzes, 1983).

A therapeutic regimen for osteoporosis must be able to inhibit bone resorption and/or to enhance bone formation. Estrogen replacement has been demonstrated to be a good antiresorptive reagent to inhibit bone resorption and improve Ca balance (Christiansen et al., 1982; Stock et al., 1985; Kaplan et al., 1994). Intermittent PTH daily injection, on the other hand, causes a pronounced anabolic effects on bone and Ca metabolism (Ibbotson, et al., 1992; Reeve et al., 1993; Wronski and Yen, 1994). Boron was reported to improve Ca and P absorption and balance in humans (Nielsen et al., 1987a; Nielsen et al., 1990) and in animals (Elsair et al., 1980; Hegsted et al., 1991). It has been speculated that boron prevents serum  $17\beta$  estradiol from degradation (Nielsen et al., 1990; Beattie and Weersink, 1992) and interacts with PTH action on mineral metabolism in young rats (Nielsen, 1985).

The present study was designed to assess the role of boron in the treatment of postmenopausal bone loss. To achieve this goal, boron's effects on Ca, Mg, and P metabolism were examined. The hypotheses were that 1) boron alone improves Ca, Mg, and P balance; 2) boron enhances the positive impact of estrogen on Ca balance; 3) boron expands the anabolic role of PTH on Ca, Mg, and P metabolism.

## Materials and Methods

*Animals and diets.* A total of 84 Sprague-Dawley female rats at 84 d of age were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). After 7 d of acclimation, rats were systematically assigned into groups by weight. Seventy six rats were ovariectomized (Ovx) from the dorsal approach, and 8 were sham-operated (Sham) where ovaries were taken out and put back immediately. A baseline group of 9 Ovx rats was randomly selected and killed. The remaining rats were housed singly in stainless steel cages at 22°C on 12-h light/12-h dark cycles and given free access to an AIN-76 basal diet that contained 0.5% Ca, 0.4% P, 0.05% Mg and 0.00004% boron, and d.i. water. Food consumption and body weight were recorded every other day and once per week, respectively. Feed cups and drinking bottles were soaked in 2 N hydrochloric acid overnight to avoid contamination before use. On day 43, Ovx rats were again systematically assigned into 7 groups based on weight and a pretreatment group of 9 Ovx rats was randomly chosen and killed. The protocol was approved by the Animal Care Committee in Virginia Polytechnic Institute and State University (Blacksburg, VA).

*Treatments.* Treatments started on day 43. There were 6 treatment groups (9-10 rats per group): Ovx+V (vehicle, untreated), Ovx+B (boron, 5 ppm), Ovx+E (17 $\beta$  estradiol, 30 $\mu$ g/kg/d), Ovx+P (PTH, 60  $\mu$ g/kg/d), Ovx+BE (boron, 5 ppm + 17 $\beta$  estradiol, 30  $\mu$ g/kg/d), Ovx+BP (boron, 5 ppm + PTH, 60  $\mu$ g/kg/d), and Sham (intact, n=7) groups. Rats in groups Sham, Ovx+V, Ovx+P and Ovx+E were given with an AIN76 basal diet as mentioned above, and rats in groups Ovx+B, Ovx+BP, and Ovx+BE

received an AIN 76 basal diet fortified with 5 ppm of boron as boric acid. Rats were allowed free access to food and d.i. water, and were weighed on a weekly base. Groups Sham, Ovx+V, and Ovx+B were injected with vehicle solution (5% ethanol in corn oil and 0.001N HCl). Groups Ovx+P and Ovx+BP were injected s.c. with rat PTH (1-34) fragment (Bachem California Inc., Torrance, CA) at a dose of 60 µg/kg/d, and groups Ovx+E and Ovx+BE were injected s.c. with 17β estradiol (Sigma Inc., St Louise, MO), 30 µg/kg/d. Rat PTH (1-34) fragment was dissolved in acid saline (0.001N HCl) with 2% heat-inactivated rat serum while 17β estradiol was dissolved in 5% ethanol and 95% corn oil. The volume of injection was based on body weight (1 ml/kg) and adjusted periodically for 50 g increments in weight change.

*Sample collection.* Approximately 1 week before their sacrifice, five rats were randomly selected from each group and placed in metabolic cages. After 3 d of acclimation, 3-day urine and feces were collected into acid-washed plastic containers and stored at -20°C. Necropsy was performed between 9 am and 1 pm approximately 20-24 hours after the last injection. Rats were first i.p. injected with pentobarbital sodium at a dose of 25 to 40 µg/kg BW and then exposed to carbon dioxide. Blood was collected through a heart puncture into ice-chilled tubes and spun in a refrigerated centrifuge to separate serum which was stored at -80°C.

*Analysis.* Food and feces were dried at 105°C for 48 hours, weighed, and digested with nitric acid/perchloric acid (5/3, v/v) for mineral measurements. For Ca or Mg measurements, the wet-ashed samples were diluted with 1% lanthanum oxide (Sandel,

1959), and the diluted samples were measured using Atomic Absorption Spectroscopy (AAS, Perkin-Elmer Atomic Absorption Spectrophotometer 2100, Rockville, MD). The P contents were determined by a colorimetric method (Fiske and Subbarow, 1925) using a microplate reader (Ceres 900 Hdi, Bio-Tek Instruments, Inc., Winooski, VT). For boron measurement, food and feces were dried at 105°C for 48 hours, weighed, and wet-ashed with 16.1 N nitric acid and 30% hydrogen peroxide according to the open-vessel wet-ash, low-temperature, teflon-tube (WALTTT) procedure (Hunt and Shuler, 1989). The estimation of boron was done using an Inductively Coupled Plasma Spectroscopy (ICP, Perkin-Elmer Plasma 400 ICP Emission Spectrometer, Rockville, MD). Serum 17 $\beta$  estradiol and osteocalcin were measured using a commercial radioimmunoassay kit purchased from Nichols Institute Diagnostics (San Juan Capistrano, CA) and from Biomedical Technologies Inc. (Stoughton, MA), respectively. Urine creatinine was colorimetrically determined by a modified Jaffe reaction.

*Statistics.* We first assumed that error distribution was normal. Data were expressed as mean  $\pm$  standard error. Analysis of Variance (ANOVA) was used to test for main effects of ovariectomy and treatment. Tukey's Studentized Range (HSD) test was used to indicate the differences if they existed. We used SAS<sup>TM</sup>(SAS Institute Inc., Cary, NC 27512) to perform these analyses. The Least Square Means (LSMEANS) procedure was used for unequal sample sizes. The significance level was set at 0.05.

## Results

One Sham rat died from surgery, and 4 Ovx rats had serum  $17\beta$  estradiol above the normal range of the intact rats. Thus, these data were excluded. Serum  $17\beta$  estradiol was  $30\pm 6$  pg/ml in the Sham group. As expected, Ovx rats with no estrogen replacement had a lower serum  $17\beta$  estradiol level than Ovx rats treated with estrogen ( $16\pm 3$  vs.  $57\pm 4$  pg/ml,  $P=0.0001$ ). Administration of boron had no effect on serum  $17\beta$  estradiol in comparison with Ovx+V rats ( $21\pm 3$  vs.  $12\pm 3$  pg/ml). Urinary creatinine excretion was not different between groups Sham and Ovx+V, or between any two of the treatment groups ( $P>0.05$ ).

Initial weight was similar between groups Sham and Ovx ( $224\pm 4$  vs.  $221\pm 2$  g) at baseline. As summarized in **Table 11**, no difference in food consumption, weight gain, or food efficiency was observed 2 weeks after surgery between these two groups. However, food consumption, weight gain, and food efficiency were higher in all of the Ovx rats than in the Sham rats, starting 3 weeks after surgery ( $P<0.05$ ). The increases continued and led to a heavier body weight in all Ovx groups ( $304\pm 5$  g) than Sham ( $253\pm 13$  g) 6 weeks after surgery ( $P=0.0005$ ). Overall comparison, the Ovx rats consumed more food, gained weight faster, and had better feed utilization than the Sham rats during the first 6 weeks after surgery.

To assess the effect of treatment on weight gain, we systematically assigned Ovx rats into groups to allow a similar initial weight among Ovx groups on day 43 as shown in **Table 12**. However, following 5 weeks of treatment, Ovx rats treated with either  $17\beta$



estradiol alone or plus boron lost weight ( $P=0.0007$ ) due to a reduction of food consumption ( $P=0.0016$ ). Administration of boron, PTH, or boron + PTH had no effect on weight gain, food consumption or efficiency compared to the Ovx untreated rats. Less food intake and weight gain were observed in Sham than in Ovx untreated rats. Food efficiency was similar between Ovx+V and Sham rats at the end of the experiment.

Essentially, all of the boron consumed was absorbed from the intestine of the rats as shown in **Table 13**. Boron supplementation caused slight but significant increases in the amount and percentage of boron apparent absorption ( $P=0.0001$ ). Boron retention was dependent on the amount of boron intake. Ovx rats treated with boron supplement retained more boron or boron per g intake than Ovx rats with no boron supplement ( $P=0.0001$ ). Higher urinary boron per mg creatinine was observed in Ovx rats treated with boron supplement than Ovx rats with no boron supplement ( $5.96\pm 0.17$  vs.  $0.74\pm 0.17$   $\mu\text{g/ml}$ ,  $P=0.0001$ ). Ovariectomy,  $17\beta$  estradiol, or PTH alone had no effect on boron metabolism in mature female rats.

Ca, P, and Mg metabolism in Ovx rats is summarized in **Table 14**. Ovariectomy did not significantly change the percentages of Ca apparent absorption and retention. However, urinary Ca excretion per mg creatinine was reduced by ovariectomy ( $P=0.054$ ). The percentages of Ca apparent absorption and retention were greater in both Ovx+BE and Ovx+BP rats when compared with Ovx untreated rats ( $P\leq 0.032$ ). Moreover, Ovx+BE rats had higher percentages of Ca apparent absorption and retention and lower urinary Ca excretion per mg creatinine than Ovx+E rats ( $P\leq 0.022$ ). No differences in Ca

apparent absorption, retention, and excretion were found between groups Ovx+B and Ovx+BE, between groups Ovx+B and Ovx+BP, or between groups Ovx+P and Ovx+BP ( $P>0.05$ ). Boron or PTH alone caused slight but nonsignificant increases in the percentages of Ca apparent absorption and retention in Ovx rats ( $P\leq 0.135$ ).

Ovariectomy did not change the percentages of P apparent absorption and retention, and urinary P excretion in comparison with Sham rats. Treatment with boron plus  $17\beta$  estradiol significantly enhanced the percentage of P apparent absorption when compared with respective Ovx+V ( $P=0.037$ ), Ovx+B ( $P=0.026$ ), or Ovx+E ( $P=0.058$ ) rats. Treatment with PTH alone or in combination with boron slightly and nonsignificantly increased the percentages of P apparent absorption and retention compared with Ovx+V rats ( $P\leq 0.150$ ). There were no differences in P apparent absorption, retention, and urinary excretion between groups Ovx+B and Ovx+BP, or between groups Ovx+P and Ovx+BP. Boron alone had no effect on P metabolism in Ovx rats.

Mg apparent absorption and retention were not changed by ovariectomy. Urinary Mg excretion or the urinary Mg level corrected by creatinine was nonsignificantly lower in Ovx untreated than in Sham rats ( $P\leq 0.115$ ). Treatment with  $17\beta$  estradiol in combination with boron increased the percentages of Mg apparent absorption and retention compared with respective Ovx+V ( $P\leq 0.031$ ). Mg apparent absorption was higher in Ovx+BE than in Ovx+B rats ( $P=0.005$ ) and in Ovx+E ( $P=0.086$ ). Ovx+BE rats had greater the percentage of Mg retention ( $P=0.031$ ), and less urinary Mg excretion per mg creatinine

( $P=0.067$ ) than Ovx+E rats. Boron,  $17\beta$  estradiol, PTH, or PTH plus boron did not significantly affect the percentages of Mg absorption and retention of Ovx rats.

Serum Ca, P, Mg, and osteocalcin concentrations are listed in **Table 15**. Serum Ca was not affected by ovariectomy. Treatment with boron, estrogen, and PTH alone had no effect on serum Ca. However, serum Ca was higher in groups Ovx+BE, and Ovx+BP compared with the Ovx untreated rats ( $P\leq 0.022$ ). Ovx +BE rats had higher serum Ca than Ovx+E rats ( $P=0.049$ ). There was no difference in serum Ca between groups Ovx+B and Ovx+BE, between groups Ovx+B and Ovx+BP, or between groups Ovx+P and Ovx+BP.

No difference was observed in serum P between groups Sham and Ovx+V. Administration of boron alone or with  $17\beta$  estradiol had no effect on serum P. Ovx+E, Ovx+P, and Ovx+BP rats had higher serum P than respective Ovx untreated rats ( $P\leq 0.040$ ). Serum P was similar between groups Ovx+E and Ovx+BE, between groups Ovx+P and Ovx+BP, between groups Ovx+B and Ovx+BE, or between groups Ovx+B and Ovx+BP.

Serum Mg was significantly decreased by ovariectomy ( $P=0.028$ ). Treatment with PTH,  $17\beta$  estradiol, or  $17\beta$  estradiol plus boron elevated serum Mg in Ovx rats ( $P\leq 0.025$ ). Ovx rats treated with PTH alone had higher serum Mg than Ovx rats treated with PTH plus boron ( $P=0.042$ ). There was no difference in serum Mg between groups Ovx+E and Ovx+BE, between groups Ovx+B and Ovx+BE, or between groups Ovx+B and Ovx+BP. Boron alone had no effect on serum Mg in Ovx rats.

Serum osteocalcin as a biochemical marker for bone formation was higher in Ovx untreated rats than in Sham rats ( $P=0.0001$ ). Treatment with either PTH alone or with boron increased serum osteocalcin in comparison with Ovx untreated rats ( $P\leq 0.008$ ). Treatment with  $17\beta$  estradiol alone or plus boron reduced serum osteocalcin in the Ovx rats ( $P=0.0001$ ). Ovx+B rats had higher and lower serum osteocalcin than Ovx+BE ( $P=0.0001$ ) and Ovx+BP ( $P=0.0001$ ) rats, respectively. Among all of the treatment groups, serum osteocalcin was highest in groups Ovx+P and Ovx+BP and lowest in groups Ovx+E and Ovx+BE ( $P=0.0001$ ). No difference in serum osteocalcin was observed between groups Ovx+E and Ovx+BE, or between groups Ovx+P and Ovx+BP. Boron alone had no effect on serum osteocalcin and thus bone formation in Ovx rats.

## Discussion

Ovx rats in the present study gained more weight than the age-matched Sham rats from 3 weeks after surgery until the end of the experiment, confirming earlier findings (Aerssens et al., 1993). Feed utilization (= weight change/food intake) was better in Ovx rats than in Sham rats prior to treatment, probably because the former had lower physical activity than the latter. Ovx rats treated with  $17\beta$  estradiol decreased ovariectomy-induced hyperphagia in the present study. This was consistent with earlier findings (Cruess and Hong, 1979).

Osteocalcin, also called bone gla protein, is synthesized by osteoblasts, and its level in serum is used as a marker for bone formation particularly when bone formation and resorption are uncoupled. Ovarian hormone deficiency elevates serum osteocalcin concentration in Ovx rats (Liu et al., 1991; Aerssens et al., 1993; Gnudi et al., 1993; Yeh et al., 1994). However, estrogen therapy slows down bone turnover and thus bone formation. As a result, estrogen treatment declines serum osteocalcin (Kalu et al., 1991; Stock et al., 1985). In contrast, intermittent PTH administration at a low dose to Ovx rats dramatically increases bone formation, that was reflected by elevating the serum osteocalcin level (Shen et al., 1993). Ovx rats in the present study had a higher serum osteocalcin than Sham rats, indicating an initial increase in bone turnover, and thus bone formation after ovariectomy. Estrogen administration to Ovx rats declined serum osteocalcin as reported in earlier findings (Stock et al., 1985; Kalu et al., 1991). The decline demonstrated that estrogen therapy had slowed down bone turnover, and thus

bone formation. On the contrary, PTH treatment with PTH dramatically elevated serum osteocalcin in the Ovx rats.

Intestinal Ca absorption declines with age and the decline is further deteriorated by ovarian hormone deficiency as reported in earlier human studies (Morris et al., 1991; Devine et al., 1993; Minisola et al., 1993). The depression of the intestinal Ca absorption due to ovariectomy is associated with a reduction in the number of estrogen receptor of the intestinal mucosal cells. These receptors interact with  $17\beta$  estradiol with enhancing Ca uptake by the mucosal cells (Arjmandi et al., 1993). However, the same was not found in the present study in which ovariectomy did not reduce apparent absorption of Ca from the intestine. It is likely that endogenous Ca confounds real Ca absorption rate.

Ovariectomy in the present study significantly reduced urinary Ca/creatinine ratio, confirming previous findings in rats (Liu et al., 1991; Aerssens et al., 1993). However, postmenopausal women and osteoporotic patients had higher urinary Ca excretion than premenopausal women (Christiansen et al., 1982; Morris et al., 1991; Devine et al., 1993). It indicates that rats have a different physiological response to ovarian hormone deficiency from humans.

Estrogen replacement therapy increases intestinal Ca absorption and decreases Ca excretion, leading to the improvement of bone mineral density in human subjects (Christiansen et al., 1982; Stock et al., 1985; Kaplan et al., 1994). However, the findings from human studies are not in agreement with those in animal studies. Estrogen treatment at a high dose ( $40\mu\text{g}/\text{kg}/\text{d}$ ) increased urinary Ca excretion as well as intestinal Ca

absorption in rats (Arjmandi et al., 1994). The same was found that Ovx rats receiving 5  $\mu\text{g}/\text{kg}/\text{d}$  estrogen elevated urinary Ca/creatinine (Kalu et al., 1991). Obviously, the increase in urinary Ca excretion in estrogen-treated rats are associated with the increased intestinal Ca absorption rate. The present study indicated that treatment with 30  $\mu\text{g}/\text{kg}/\text{d}$   $17\beta$  estradiol had no effect on apparent Ca absorption and urinary Ca excretion in Ovx rats. The inability of estrogen to improve Ca retention or balance is unlikely due to the dosage but likely due to time of initiating treatment, since Ovx rats had serum  $17\beta$  estradiol above that in Sham rats by 250%. Human studies indicated estrogen therapy was less effective when given 6 years after menopause (Lindsay, 1993). Han et al. (1992) suggested estrogen treatment should be given to Ovx rats within 2 to 5 weeks after ovariectomy. Our Ovx rats began to receive estrogen treatment 6 weeks after surgery. It may be too late to exert the positive impact of estrogen on Ca metabolism.

Numerous studies have reported the effects of PTH on Ca absorption after ovarian hormone deficiency. However, PTH administration to osteoporotic patients did not affect true Ca absorption but increased 24-hour urinary Ca excretion (Reeve et al., 1980). Studies with Ovx rats indicated that intermittent PTH injection decreased urinary Ca/creatinine ratio (Liu et al., 1991). Following daily treatment with PTH, serum Ca increased in Ovx rats (Liu et al., 1991), or did not change in Ovx rats (Hock et al., 1988), and in osteoporotic patients (Reeve et al., 1980 and 1993). The present study demonstrated that Ovx rats treated with intermittent PTH had no effects on apparent Ca

absorption, urinary Ca excretion and serum Ca. It indicated that intermittent PTH injection at a dose of 60 µg/kg/d did not affect Ca metabolism in Ovx rats.

The present study indicated that boron alone caused slight increases in the percentages of apparent Ca absorption and Ca retention in Ovx rats. However, when combined boron with estrogen administration to Ovx rats, the percentages of apparent Ca absorption and Ca retention were dramatically improved with no increase in urinary Ca excretion. It is suggested that in combination with 5 ppm boron, estrogen treatment, initiating 6 weeks after ovariectomy, significantly enhances Ca balance in Ovx rats. The present data indicated that Ovx+BP rats had better apparent Ca absorption and Ca retention rates than Ovx untreated rats. The same was not found in Ovx+P rats.

Mg metabolism is affected by oophoectomy. Osteoporotic patients had lower Mg absorption and serum Mg than normal postmenopausal women (Cohen et al., 1983; Cohen, 1988; Reginster et al., 1989). Urinary Mg/creatinine was higher in postmenopausal women than in premenopausal women (Lindsay et al., 1980; McNair et al., 1984). Serum Mg were similar (McNair et al., 1984) or elevated (Lindsay et al., 1980) in postmenopausal women compared with premenopausal women. However, estrogen administration to early postmenopausal women reversed urinary Mg/creatinine to normal (Kaplan et al., 1994). The same was not found in the present study in which ovariectomy reduced serum Mg which was corrected by estrogen treatment. The percentages of apparent Mg absorption and Mg retention were slightly improved following estrogen treatment. However, in combination with boron supplementation,



estrogen significantly improved the percentages of apparent Mg absorption and retention in Ovx rats.

The relationship of P metabolism and the development of osteoporosis has been not extensively studied. Earlier findings indicated that serum P or P balance was not affected by ovariectomy, estrogen therapy, or intermittent PTH injection (Cruess and Hong, 1979; Reeve et al., 1980; Carpenter et al., 1992; Shen et al., 1993). Other investigators reported PTH or estrogen decreased serum P (Christiansen et al., 1982; Reeve et al., 1993). The present study demonstrated that serum P was elevated by estrogen or PTH. Daily treatment with estrogen, PTH, or boron had no effect on the percentages of apparent P absorption and P retention. However, combined treatment with boron and estrogen significantly improved the percentage of apparent P absorption in Ovx rats.

The present findings demonstrated that the majority of ingested boron was absorbed from the intestine in Ovx rats as earlier reports in animals (Pfeiffer et al., 1945; Akagi et al., 1962; Brown et al., 1989) and in humans (Jansen and Schou, 1984). It indicates that the intestine is not the main regulating site for boron metabolism. However, urinary boron/creatinine ratio differed depending on the amount of boron intake. Ovx rats supplemented with boron had higher urinary boron/creatinine excretion than those without supplement, indicating the kidney as a regulating site for boron. The present data demonstrated that ovariectomy, PTH, or estrogen had no effect on boron metabolism.

## **Conclusion**

Boron as boric acid at a dose of 5 ppm tended to improve Ca balance by increasing apparent Ca absorption from the intestine without an increase in urinary Ca/creatinine excretion in Ovx rats. Estrogen treatment, initiating 6 weeks after ovariectomy had a minor effect on Ca balance. However, in combination with boron, estrogen (30 µg/kg/day) significantly improved the percentages of apparent Ca, Mg, P absorption and Ca, Mg retention in Ovx rats. The finding was important particularly when estrogen therapy was effective only in the early state of ovarian hormone deficiency in animals and humans. Boron in combination with PTH (rat PTH 1-34, 60 µg/kg/d) did not provide additional benefit for Ca, Mg, P balance when compared with PTH alone, but significantly improved the percentages of apparent Ca absorption and Ca retention compared with Ovx untreated rats. We, therefore, concluded that boron in combination with estrogen treatment, initiating 6 weeks after ovariectomy, provided more beneficial regimen than boron or estrogen alone for the treatment of postmenopausal bone loss. However, due to a small sample size, the present findings needed to be further confirmed with a larger sample size over a longer period.

Table 11  
 Food intake, weight gain, and food efficiency in the sham-operated  
 (Sham) and ovariectomized (Ovx) rats before treatment

	Sham (n=7)	Ovx (n=63)
Week 1-2		
Food intake, g/d	16.0±0.7	17.1±0.2
Weight gain, g/d	1.41±0.49	2.47±0.12
Food efficiency, g/g	0.086±0.027	0.142±0.006
Week 3-4		
Food intake, g/d	14.1±0.8 <sup>†</sup>	18.4±0.2
Weight gain, g/d	0.06±0.48 <sup>†</sup>	2.06±0.12
Food efficiency, g/g	0.005±0.027 <sup>†</sup>	0.106±0.007
Week 5-6		
Food intake, g/d	14.4±0.9 <sup>†</sup>	17.9±0.2
Weight gain, g/d	0.56±0.30 <sup>†</sup>	1.39±0.07
Food efficiency, g/g	0.038±0.021	0.076±0.005
Week 1-6		
Food intake, g/d	14.9±0.8 <sup>†</sup>	17.8±0.2
Weight gain, g/d	0.68±0.19 <sup>†</sup>	1.97±0.05
Food efficiency, g/g	0.044±0.010 <sup>†</sup>	0.108±0.002

<sup>†</sup> P<0.05 compared to Ovx+V group

Table 12  
 Body weight, food consumption, and food efficiency in the sham-operated (Sham)  
 and ovariectomized (Ovx) rats after 5 weeks of treatment

	N	Initial weight g	Final weight g	Weight gain g/day	Food intake g/day	Food efficiency g/g
Sham	7	253±13 <sup>†</sup>	269±13 <sup>†</sup>	0.44±0.14 <sup>†</sup>	14.9±0.7 <sup>†</sup>	0.029±0.008
Ovx+V	9	314±12	343±12	0.81±0.13	18.2±0.6	0.044±0.008
Ovx+B	10	298±11	318±11	0.57±0.12	17.0±0.6	0.033±0.007
Ovx+E	9	298±12	283±11 <sup>†</sup>	-0.42±0.12 <sup>†</sup>	15.5±0.6 <sup>†</sup>	-0.028±0.007 <sup>†</sup>
Ovx+P	8	306±12	328±12	0.64±0.13	17.5±0.6	0.037±0.008
Ovx+BE	10	298±11	283±11 <sup>†</sup>	-0.43±0.12 <sup>†*</sup>	15.1±0.6 <sup>†*</sup>	-0.029±0.007 <sup>†*</sup>
Ovx+BP	7	319±13	338±13	0.52±0.14	17.8±0.7	0.029±0.008

<sup>†</sup> P<0.05 compared to the Ovx+V group

\* P<0.05 compared to the Ovx+B group

V=Vehicle

B=Boron, 5 ppm

E=17β Estradiol, 30 μg/kg/d

P=Parathyroid hormone (PTH), 60 μg/kg/d

BE=Boron, 5 ppm + 17β Estradiol, 30 μg/kg/d

BP=Boron, 5 ppm + PTH, 60 μg/kg/d

**Table 13**  
**Apparent boron absorption, retention, and urinary excretion on a daily base in the**  
**sham-operated (Sham) and the ovariectomized (Ovx) rats after 5 weeks of treatment (n=5)**

	Absorption $\mu\text{g}$	Absorption %	Retention $\mu\text{g}$	Retention %	Excretion <sup>†</sup> $\mu\text{g}/\text{mg Cr}$
Sham	12.9 $\pm$ 6.0	99.8 $\pm$ 0.1	7.6 $\pm$ 5.6	58.9 $\pm$ 3.6	0.75 $\pm$ 0.25
Ovx+V	13.9 $\pm$ 6.0	99.5 $\pm$ 0.1	8.9 $\pm$ 5.6	64.9 $\pm$ 3.6	0.77 $\pm$ 0.25
Ovx+B	214.8 $\pm$ 6.0 <sup>†</sup>	99.9 $\pm$ 0.1 <sup>†</sup>	175.8 $\pm$ 5.6 <sup>†</sup>	81.6 $\pm$ 3.6 <sup>†</sup>	5.30 $\pm$ 0.25 <sup>†</sup>
Ovx+E	12.6 $\pm$ 6.7	99.7 $\pm$ 0.1	7.7 $\pm$ 6.3	61.6 $\pm$ 4.0	0.72 $\pm$ 0.25
Ovx+P	13.6 $\pm$ 6.7	99.5 $\pm$ 0.1	8.3 $\pm$ 6.3	60.6 $\pm$ 4.0	0.72 $\pm$ 0.25
Ovx+BE	183.8 $\pm$ 6.0 <sup>†*</sup>	99.9 $\pm$ 0.1 <sup>†*</sup>	140.0 $\pm$ 5.6 <sup>†*</sup>	76.0 $\pm$ 3.6 <sup>†*</sup>	6.52 $\pm$ 0.25 <sup>†*</sup>
Ovx+BP	205.7 $\pm$ 6.0 <sup>†‡</sup>	99.9 $\pm$ 0.1 <sup>†‡</sup>	159.7 $\pm$ 5.6 <sup>†‡</sup>	77.7 $\pm$ 3.6 <sup>†‡</sup>	6.06 $\pm$ 0.25 <sup>†‡</sup>

<sup>†</sup> P<0.05 compared to the Ovx+V group

<sup>\*</sup> P<0.05 compared to the Ovx+B group

<sup>†</sup> P<0.05 compared to the Ovx+E group

<sup>‡</sup> P<0.05 compared to the Ovx+P group

<sup>†</sup> Urinary excretion normalized by creatinine

V=Vehicle

B=Boron, 5 ppm

E=17 $\beta$  Estradiol, 30  $\mu\text{g}/\text{kg}/\text{d}$

P=Parathyroid hormone (PTH), 60  $\mu\text{g}/\text{kg}/\text{d}$

BE=Boron, 5 ppm + 17 $\beta$  Estradiol, 30  $\mu\text{g}/\text{kg}/\text{d}$

BP=Boron, 5 ppm + PTH, 60  $\mu\text{g}/\text{kg}/\text{d}$

Table 14  
Ca, P, and Mg apparent absorption, retention, and urinary excretion in the sham-operated (Sham) and ovariectomized (Ovx) rats after 5 weeks of treatment (n=5)

	Ca			P			Mg		
	Absorption %	Retention %	Excretion <sup>†</sup> mg/mg Cr	Absorption %	Retention %	Excretion <sup>†</sup> mg/mg Cr	Absorption %	Retention %	Excretion <sup>†</sup> µg/mg Cr
Sham	17.8±4.9	17.3±4.9	0.047±0.007 <sup>†</sup>	52.2±3.2	25.8±3.8	2.44±0.10	49.5±3.7	49.4±3.7	1.22±0.24
Ovx+V	12.7±4.4	12.6±4.4	0.011±0.006	47.6±2.8	27.3±3.4	2.31±0.09	42.7±3.3	42.6±3.5	0.25±0.11
Ovx+B	23.5±4.4	23.4±4.4	0.010±0.006	49.6±2.8	27.3±3.4	2.18±0.09	48.1±3.3	48.0±3.5	0.16±0.11
Ovx+E	15.4±4.9	15.3±4.9	0.014±0.007	52.7±3.2	28.4±3.8	2.29±0.10	54.9±3.7	54.9±3.9	0.40±0.12
Ovx+P	26.9±4.9	26.8±4.9	0.009±0.007	57.3±3.2	32.8±3.8	2.40±0.10	49.3±3.7	49.3±3.9	0.43±0.12
Ovx+BE	34.3±4.9 <sup>††</sup>	34.2±4.9 <sup>††</sup>	0.009±0.007 <sup>††</sup>	60.6±3.2 <sup>†††</sup>	32.3±3.8	2.44±0.10	61.9±3.7 <sup>††</sup>	61.9±3.9 <sup>††</sup>	0.15±0.12
Ovx+BP	29.8±4.4 <sup>†</sup>	29.7±4.4 <sup>†</sup>	0.008±0.006	54.4±2.8	28.2±3.4	2.33±0.09	50.9±3.3	50.8±3.5	0.11±0.11

<sup>†</sup> P<0.05 compared to the Ovx+V group

<sup>\*</sup> P<0.05 compared to the Ovx+B group

<sup>††</sup> P<0.05 compared to the Ovx+E group

<sup>†††</sup> Urinary mineral modified by Creatinine

V=Vehicle

B=Boron, 5 ppm

E=17β Estradiol, 30 µg/kg/d

P=Parathyroid hormone (PTH), 60 µg/kg/d

BE=Boron, 5 ppm + 17β Estradiol, 30 µg/kg/d

BP=Boron, 5 ppm + PTH, 60 µg/kg/d

Table 15  
Serum calcium, phosphorus, magnesium and osteocalcin in the sham-operated (Sham) and ovariectomized (Ovx) rats after 5 weeks of treatment

	N	Calcium (mg/dl)	Phosphorus (mg/dl)	Magnesium (mg/dl)	Osteocalcin (ng/ml)
Sham	7	9.6±0.2	6.7±0.4	2.45±0.10 <sup>†</sup>	33.5±3.2 <sup>†</sup>
Ovx+V	9	9.4±0.1	6.3±0.4	2.24±0.10	47.6±3.0
Ovx+B	10	9.6±0.1	7.1±0.4	2.34±0.09	42.7±2.7
Ovx+E	9	9.5±0.1	7.4±0.4 <sup>†</sup>	2.54±0.09 <sup>†</sup>	25.1±2.9 <sup>†</sup>
Ovx+P	8	9.9±0.1 <sup>†</sup>	7.6±0.4 <sup>†</sup>	2.66±0.10 <sup>†</sup>	67.7±3.0 <sup>†</sup>
Ovx+BE	10	9.8±0.1 <sup>††</sup>	7.1±0.4	2.52±0.09 <sup>†</sup>	28.6±2.7 <sup>†*</sup>
Ovx+BP	7	9.9±0.2 <sup>†</sup>	7.3±0.4 <sup>†</sup>	2.24±0.10 <sup>‡</sup>	64.9±3.2 <sup>†*</sup>

<sup>†</sup> P<0.05 compared to the Ovx+V group

<sup>\*</sup> P<0.05 compared to the Ovx+B group

<sup>†</sup> P<0.05 compared to the Ovx+E group

<sup>‡</sup> P<0.05 compared to the Ovx+P group

V=Vehicle

B=Boron, 5 ppm

E=17β Estradiol, 30 μg/kg/d

P=Parathyroid hormone (PTH), 60 μg/kg/d

BE=Boron, 5 ppm + 17β Estradiol, 30 μg/kg/d

BP=Boron, 5 ppm + PTH, 60 μg/kg/d

## CHAPTER 5

### General Discussion and Suggestion for Future Study

Ovariectomized rats are a good animal model for studying bone status following ovarian hormone deficiency. According to measurements of bone physical, biochemical mechanical, and histomorphometric indices as well as blood and urine biochemical parameters, postmenopausal osteoporosis is associated with a rapid increase in bone turnover with bone resorption exceeding formation, leading to bone loss and fractures. The regulation of bone mass and structure is governed by the mechanical strain, hormones and drugs. Changes in the threshold levels of the mechanical strain, or in the set point for normal bone mass and structure by hormones or drugs initiate bone adaptation and as a result, turn on the modeling /remodeling systems. Estrogen, parathyroid hormone (PTH), insulin-like growth factors (IGFs), calcitonin, and prostaglandin are possible set point altering hormones or peptides. Some of them have been used to prevent or treatment postmenopausal osteoporosis.

Estrogen replacement therapy reduced bone turnover in humans and animals following ovarian hormone deficiency. The antiresorptive effects of estrogen on bone are mediated through decreased bone responsiveness to parathyroid hormone (PTH). Also, estrogen suppresses gene expression of tartrate-resistant acid phosphatase (TRAP) and carbonic anhydrase II which are the main two enzymes responsible for bone demineralization.



There is no consistent evidence indicating direct effects of estrogen on bone formation. Our data indicates that estrogen treatment that was initiated 6 weeks after ovariectomy had minor effects on Ca balance, bone volume, density, and thickness. The inability of estrogen to enhance bone mass in the late state of postmenopause is probably because bone cells have lost the capacity to rebuild bone with time following ovarian hormone deficiency. Since estrogen appears to inhibit bone turnover, including resorption and formation, estrogen therapy should be initiated as early as possible following ovarian hormone deficiency. However, another possibility is that to cause net bone gain, subjects need higher estrogen than to cause bone resorption. Estrogen replacement therapy delivers enough serum estradiol levels (50 to 100 pg/ml) to prevent bone loss in humans, but not enough to cause net bone gain. It is true that young women who become amenorrheic lose bone mass, and gain bone mass when normal menses are resumed. Perhaps serum estrogen peaks (600 pg/ml) within the natural cycle are necessary to increase bone mass.

PTH often exerts resorptive effects on bone tissue. Continuous treatment with PTH stimulates osteoclast activity and inhibits organic matrix synthesis. However, PTH given intermittently appears to have anabolic effects on the bone of rats and humans. Bone resorption and formation are thought to be closely coupled. It is not clear so far whether the increase in bone formation is either due to previous activation of osteoclasts or due to a direct action of the hormone. Our data indicated that intermittent PTH treatment caused hypercalcemia in ovariectomized (Ovx) rats. The presence of

hypercalcemia is not due to bone demineralization or resorption but due to a slight increase in Ca apparent absorption and a small reduction in Ca excretion. Intermittent PTH in combination with boron significantly improve Ca apparent absorption and retention in Ovx rats. It is unclear whether boron in combination with PTH significantly increases the biosynthesis of 1,25(OH)<sub>2</sub> vitamin D, leading to an increase in Ca apparent absorption. The anabolic effects of intermittent PTH on bone is further confirmed by histomorphometric bone biopsies. The volume, density, thickness, and connectivity of the trabecular bone were enhanced by intermittent PTH treatment. The ability of intermittent PTH to lay down new bone is thought to be mediated by IGF-1 that stimulates osteoblast proliferation and organic matrix synthesis, and regulates the degree of cavity refill. Another possibility is that intermittent PTH injection increases calcitonin secretion. Intermittent PTH injection elevates extracellular Ca (i.e. hypercalcemia) and activates the adenylate cyclase-cAMP system. Either of the two events influences calcitonin synthesis and secretion.

Boron seems to be important in bone metabolism, mainly through its interaction with other minerals, in particular magnesium, calcium, and vitamin D. Boron as boric acid tends to form complexes with hydroxyl groups in biological compounds. However, the present data indicated that boron as boric acid had no direct effects on bone and Ca, P, and Mg metabolism. But, when combined with estrogen, boron exerts physiological response similar to that induced by PTH. Boron in combination with estrogen elevated serum Ca, improved Ca and P absorption, and increased the volume, density, and

connectivity of the trabecular bone in Ovx rats. There are two possibilities for the findings. First, it is possible that boron forms covalent bonds with the hydroxyl groups of estrogen and thus influence the binding of estrogen to the receptors, leading to a reduction in the inhibitory effect of estrogen on skeletal response to PTH. Secondly, boron and estrogen might involve in the biosynthesis of  $1,25(\text{OH})_2$  vitamin  $\text{D}_3$  by stimulating the activity of  $25\alpha$  hydroxylase in the liver or  $1\alpha$  hydroxylase in the kidney.

Therefore, for the future studies, it is suggested:

1. Investigating whether boron has the ability to complex with estrogen and whether boron has effects on the affinity of estrogen to the receptors on bone cells and on the mucosal cells of the intestine.
2. Investigating whether boron influences  $1,25(\text{OH})_2$  vitamin  $\text{D}_3$  synthesis by measuring the activity of  $25\alpha$  hydroxylase or  $1\alpha$  hydroxylase or by directly measuring serum  $25(\text{OH})$  vitamin D and  $1,25(\text{OH})_2$  vitamin D levels following boron supplementation.
3. If boron does have the ability in 1 or 2, boron should be supplemented to diet, particularly when estrogen replacement therapy is initiated in the late state after menopause. Therefore, future research should focus on the study of the optimal dosages of combined treatment with boron and estrogen.
4. Investigating whether combined treatment with boron plus estrogen has effects in the early state following menopause and whether the combined treatment remained its positive effects over a long period of time.

5. Investigating the mechanism of the action of intermittent PTH and how organic boron alone can inhibit bone resorption in molecular and cellular levels.

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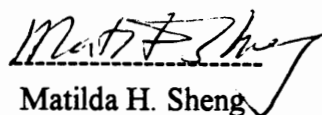
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