EFFECTS OF DIETARY IRON AND OVARIECTOMY ON BONE IN SKELETALLY MATURE OPERATED RATS

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

The following terms are used frequently in the text:

osteoblast- a cell arising from a fibroblast, which, as it matures, is associated with bone production.

osteoclast- a large multinuclear cell associated with the absorption and removal of bone.

trabecular (cancellus) bone- the lattice-like bone.

cortical bone- the compact bone.

fracture force- the measure of force in a three-point bending test at which the femur first fractures.

CHAPTER I

RESEARCH PROBLEM

Introduction to Topic

Osteoporosis is a condition of skeletal fragility defined as a bone mass value greater than 2.5 standard deviations below the young adult mean and characterized by destruction of cancellous bone architecture. It is estimated that one in two women and one in eight men over age 50 will suffer a fracture related to osteoporosis in their lifetime (National Osteoporosis Foundation). Osteoporosis is a major public health concern due to the condition's characteristic low bone mass, the deterioration of trabecular bone, and the associated increased risk and incidence of fracture occurrence.

Bone formation begins before birth and continues until approximately the end of the second decade of life (Matkovic 1996, Gilsanz 1988). During this time, mineral salts are deposited onto a collagen matrix within bone by osteoblasts, and accumulation of bone mass predominates producing peak bone mass. In young adulthood, bone accumulation is replaced by bone remodeling in which osteoclastic cells resorb and osteoblastic cells secrete bone minerals in response to physiological triggers. In older adulthood, bone remodeling changes again and bone resorption exceeds bone formation due to decreased osteoblastic activity (Heaney 1996). This results in a net loss of bone mass. Women experience additional bone loss after menopause as osteoclastic activity increases in response to the decreased production of the bone protective female hormone, estrogen.

Decreased bone mass leads to osteoporosis; therefore, greater bone mass at time

of onset of bone loss in later life should result in lower incidence or delayed onset of osteoporosis and related fracture. Achievement of peak bone mass during growth and maintenance of bone mass in adulthood are major factors affecting bone mass and density during later life. Several factors, including genetics, environmental factors, mechanical loading, and nutrition, influence development and maintenance of peak bone mass. Bone mass has been found to be lower in women with osteoporotic mothers (Matkovic 1996, Johnell 1996). Environmental factors such as alcohol use, disease states, and drug interactions have been shown to result in lower bone mass (Chestnut 1991, Heaney 1996). Mechanical loading or weight bearing exercise can affect bone mass with increased loading leading to increased mass (Chestnut 1991, Heaney 1996). Studies have demonstrated a positive relationship between calcium intake and bone mass (Anderson et al. 1996, Matkovic 1996, Angus et al. 1988). With attainment of peak bone mass during bone growth and maintenance of homeostasis during adulthood, greater mass is present when postmenopausal and/or age-related bone loss begins.

The investigation of the role of nutrition in bone metabolism has been primarily focused on calcium and vitamin D. It is commonly recognized that calcium is necessary for bone health throughout life and that vitamin D is vital in calcium balance. Vitamin C is widely recognized as essential in collagen formation, the connective tissue foundation of bone. Effects of other nutrients on bone metabolism have been less extensively studied. Angus et al. (1988) positively correlated dietary levels of the minerals magnesium, zinc, and iron with forearm bone mineral content in premenopausal women. In a study of connective tissue biosynthesis, O'Dell (1981) reported copper and iron are both required

for post-translational maturation of collagen. To date, few studies have been performed specifically investigating the effects of level of dietary iron on bone metabolism.

Dietary iron intakes vary greatly. Worldwide, iron intakes often fall below recommendations, especially in children, females and the elderly (DeMaeyer and Adiels-Tegman 1985). Conversely, increased iron intakes are more common with individual supplement use and national food fortification policies (Olsson et al. 1997). Therefore, in certain populations iron intake is inadequate, and in others iron intake could be excessive.

Dietary iron absorption also varies due to source, total meal content and physiological state. Heme iron is better absorbed than non-heme iron. Ascorbic acid, organic acids, and animal tissues consumed with non-home iron increases absorption while phytates, polyphenols, calcium and psyllium fiber decrease absorption. Idiopathic or secondary hemachromatosis, iron deficiency and periods of rapid growth such as in childhood and pregnancy increase iron absorption. Decreased hydrochloric acid production associated with aging and secondary achlorhydria reduce iron absorption (Lynch 1997).

As dietary iron intakes are often not optimal and iron plays a critical role in post-translational collagen maturation, dietary iron concentration may affect bone metabolism. Because it has been demonstrated that both inadequate and excessive dietary iron intakes affect bone metabolism (Rothman et al. 1971, Heppenstall and Brighton 1977, Pallares et al. 1996, Campos et al. 1998, Lynch et al. 1970, de Vernejoul et al. 1984, Storey and Greger 1987, Diamond et al. 1989 and 1991, Conte et al. 1989, Schnitzler et al. 1994, Duquenne et al. 1996), it can be hypothesized that both inadequate and excessive dietary

iron intakes may affect connective tissue formation. Therefore, inadequate bone foundation formation and maintenance rates may result. If bone formation and peak mass achievement are hindered during growth and mass is not maintained in early adulthood due to suboptimal iron intake, less mass could be available to be lost in aging and estrogen deficiency.

Females and elderly exhibit the highest incidence of osteoporosis and associated complications and often have dietary iron intakes inconsistent with current recommendations. This study may propose dietary iron intake as one component of osteoporosis prevention.

Postmenopausal osteoporosis is by far the most common cause of age-related bone loss (Heaney 1996). In human females, both natural and surgical menopause are associated with an initial phase of rapid bone loss followed by a phase of continued but slower loss of bone (Cohn et al. 1976). The bone loss is attributed to the imbalance between osteoclastic bone resorption and osteoblastic bone formation secondary to ovarian hormone deficiency. Similar patterns of ovarian hormone deficiency bone loss have been observed in animals. Ovariectomized rats have exhibited increased bone resorption and net bone loss (Wronski et al. 1985). Dick et al. (1996) found in ovariectomized rats as in postmenopausal women negative calcium balance and significant generalized bone loss. Bagi et al. (1996, 1997) found greater resorption rates than formation rates with significantly lower femur bone mineral density in ovariectomized rats compared to sham-operated rats. Because the rat hip and femoral neck were similar in structure and in response to endocrine changes seen in humans, these investigators concluded that the rat

model is suitable for studies of bone metabolism and strength for application to osteoporosis. In addition, Peng et al. (1994) and Li et al. (1997) support the use of the rat femur over the tibia in osteoporosis research. The ovariectomized rat model has been shown to be adequate as long as its limitations are considered (Rodgers et al. 1993, Peng et al. 1997).

Significance of the Problem

Osteoporosis-related fractures result in significant human and economic costs in the United States with increased morbidity, mortality, and utilization of health care resources. Miller (1978) demonstrated only 50% of hip fracture patients regained pre-fracture ambulatory ability within one year of fracture. Schurch et al. (1996) reported within one year of hip fracture 21.5% of men and 35.4% of women followed were deceased. Nationally, medical costs associated with osteoporotic fractures in 1995 have been estimated at $38 million per day or $13.8 billion annually (National Osteoporosis Foundation). These studies illustrate the current human and financial costs of osteoporosis; as the aging of the U.S. population continues into the 21st century, without intervention it seems likely that both the incidence and costs of osteoporosis related fractures will increase in the coming years (Ray et al. 1997).

STUDY PURPOSE AND OBJECTIVES

This study utilizes skeletally mature rats to clarify the association between dietary iron, hormonal status, and bone metabolism. The cumulative effects of dietary iron on bone following ovarian hormone deficiency was the particular focus in this study.

STUDY DESIGN

Experimental design in this study was a 2x4 completely randomized block design. Eighty-four weanling female Sprague-Dawley rats were randomized into four dietary iron regimens (calculated to be 6 ppm, 12 ppm, 35 ppm or 150 ppm as iron citrate) and two surgical treatments (sham-operated or ovariectomized). Animals were pair fed to the 150 ppm sham group and when necessary were fed less to match for weight. Surgery was performed at skeletal maturity after 15 weeks of dietary treatment. Necropsy was performed 12 weeks following surgery with collection of urine, blood, organs and bones for analyses. Analyses of nutrition and bone status were performed. Experimental procedures were approved by the Oklahoma State University Animal Use and Care Board. Statistical analysis of data was performed using SAS (version 7.0, SAS Institute, Cary, NC). Four animals were excluded from analyses secondary to obvious health problems discovered during the experiment.

LIST OF HYPOTHESES

This study advances the following hypotheses:

1) There will be no significant differences by diet or ovarian hormone status (OHS) group and no significant diet x OHS interaction in final body weight.

2) There will be no significant differences by diet or OHS group and no significant diet x OHS interaction in bone metabolism as measured by:

a) insulin-like growth factor I (IGF-I) at necropsy

b) alkaline phosphatase extracted from bone (ALP) at necropsy

c) urinary excretion of calcium (Ca) pre-surgery and at necropsy

d) urinary excretion of magnesium (Mg) pre-surgery and at necropsy

e) urinary excretion of phosphorous (P) pre-surgery and at necropsy

f) urinary excretion of free deoxypyridinoline (DPD) pre-surgery and at necropsy

g) urinary excretion of hydroxyproline pre-surgery and at necropsy.

3) There will be no significant differences by diet or OHS group and no significant diet x OHS interaction in bone size at necropsy as measured by:

a) femur length

b) femur area.

4) There will be no significant differences by diet or OHS group and no significant diet x OHS interaction in bone density at necropsy as measured by:

a) dual energy x-ray absorptiometry (DXA)

b) Archimedes' principle.

5) There will be no significant differences by diet or OHS group and no significant diet x OHS interaction in bone mineral content at necropsy as measured by:

a) dual energy x-ray absorptiometry (DXA).

6) There will be no significant differences by diet or OHS group and no significant diet x OHS interaction in bone strength at necropsy as measured by:

a) femur fracture force.

CHAPTER II

REVIEW OF THE LITERATURE

The following sections review literature focusing on parameters of bone metabolism and indicators of bone metabolism. Literature discussing measurements of bone parameters and the rat model are also reviewed.

Bone metabolism has been shown to be affected by multiple factors. While not an all-inclusive list, mechanical strain or load bearing, genetics, hormonal status, nutrition, and disease state are among those factors. In addition, these factors often affect bone in combination.

BONE MASS

Load Bearing. Load bearing has been correlated with bone mass. In a human population-based study of 39 girls and 48 boys with a mean age of 15.1 years (Duppe et al. 1997), total body bone mineral content but not total body bone mineral density at ages 4 and 6 years and in adolescence as measured by DXA was found to be predicted by childhood weight. The authors concluded that these findings suggest skeletal envelope is more influenced by body weight than is skeletal density.

According to Heaney (1996), body weight is the single largest determinant of bone density in adult humans and accounts for approximately one-half of the population level variance. In fact, fracture risk in obese women was cited to be approximately one-third that of normal weight women, and excessively lean women were purported to have high fracture risk. These differences were attributed by Heaney to increased mechanical loading seen with increased body weight as well extra fat padding present in obese women

and unknown factors which cause bone in obese women to behave "as if it had a lower set point for the reference level of bending under load".

In a prospective study of male Sprague-Dawley rats (Mosley et al. 1997) weighing 250 to 300 grams, treatment animals were anesthetized and mechanical load of -0.0005, -0.001, -0.002, -0.003 or -0.004 compressive dynamic strain was applied to the ulnae via padded cups attached to low friction bearings in a servohydraulic material testing machine. Mechanical loading was applied on days one through five and eight through twelve of the experiment. Mechanical loading produced strain-dependent shorter ulnar length in all treatment groups compared to controls. No significant effect was seen in the -0.0005 treatment group and a negative effect on ulnar mass were seen and -0.001 and -0.002 treatment groups versus control rats. Significantly increased ulnar mass was found in the -0.003 and -0.004 treatment rats versus control rats. Mineral apposition rates in these two groups were increased on the lateral bone face, and the medial face was converted from bone resorption to formation. The authors conclude that mechanical loading affects both bone curvature and mass.

Peng et al. (1994) immobilized the right hind leg of 33 (28 male and five female) 10-week-old S.D. rats for three weeks by placing the limb in plantar flexion in a plaster cast. Cast was checked daily and replaced weekly or as needed. On three point bending testing, femora of immobilized animals exhibited lower neck strength and energy absorption versus control rats. Femur and tibia ash weights were significantly lower in immobilized animals compared to control animals. By histological examination, immobilized animals were found to have significantly lower trabecular bone, significantly

lower bone area, and significantly greater marrow cavity versus control animals.

Genetics. It has been shown that genetics may influence the development of peak bone mass. In a study of 31 fourteen-year-old Caucasian girls and their biological parents, Matkovic et al. (1990) investigated hereditary influences on bone by comparing the girls' and parents' bone mass. Positive correlations between mean parent and daughter values were found for metacarpal length ($r = 0.46$) as measured by standardized x-rays and needle-tipped caliper, distal forearm density ($r = 0.72$) and cross-sectional area of the metacarpal ($r = 0.73$) as measured by radiogrammetry and single- and dual-photon absorptiometry. The authors concluded genetics influenced bone growth and consolidation in adolescent girls.

In a study of adult same sex fraternal (mean age 40 years) and identical twins (mean age 47 years), Pocock et al. (1987) investigated hereditary influences on bone. No differences in bone mineral content or bone mineral density of the right proximal femur (femoral neck, Ward's triangle, and trochanter) or lumbar spine as measured by dual photon absorptiometry were seen between participants and age-matched controls. No differences in BMC or BMD of either site were seen within any of the twin pairs. Correlations of lumbar and proximal femur BMD and forearm BMC were all higher for the identical versus fraternal twin pairs. The authors concluded spinal and proximal femoral as well as upper limb bone mass had a significant genetic component and emphasized family history as an important factor in identifying individuals at risk for clinical bone disease.

Estrogen Deficiency. Many studies have demonstrated the deleterious effects of

estrogen deficiency on bone. Clark and Tarttelin (1982) sham-operated or ovariectomized rats at ages two days (D2), four weeks (W4), and seven weeks (W7). One W7 ovx group was injected with estradiol benzoate 2μg daily, and one control group was injected with carrier solvent daily. Animals were given ad libitum access to tap water and pelleted rat diet. Nose-anal lengths were measured with animals anesthetized, and body weights were recorded after sacrifice. Final body weight and nose-anal length were greater in W4 and W7 compared to sham animals. No significant differences in final body weight or nose-anal length were noted between D2 and sham animals. No significant differences in final body weight or nose-anal length were noted between estrogen treated and sham animals. No other bone measurements were performed. The authors concluded that ovariectomy stimulated skeletal growth and estrogen treatment of ovariectomized animals prevented this effect.

In a study of 34 Sprague-Dawley rats randomized into sham or ovx treatment at six months of age, animals were pair fed a natural diet containing 0.1% calcium and 0.3% phosphorous in the amount of 15 grams per day. Plasma dialyzable calcium, plasma complexed calcium, urine calcium excretion, and plasma phosphorous were elevated in the ovx versus sham animals at one, three, and six weeks post-surgery. Bone mineral density and bone mineral content as measured by DXA decreased in both ovariectomized and sham-operated animals during the study, but significantly more so in the ovariectomized group (Dick et al. 1996).

Goulding et al. (1996) deep labeled female albino Wistar rat skeletons with ^{45}Ca, and maintained the animals on a stock diet containing 1.0% calcium for four weeks to

allow freely exchangeable ^{45}Ca to be eliminated. Animals were then transferred to a hydroxyproline-free casein treatment diet providing 0.1% Ca and randomized into surgical treatments of sham-operated, sham-operated plus estradiol, hysterectomized, hysterectomized plus estradiol, ovariectomized, ovariectomized plus estradiol, hysterectomized/ovariectomized, and hysterectomized/ovariectomized plus estradiol. The ovariectomized rats (n=eight, final body weight 293.1 ± 9.4 g) exhibited lower density determined by displacement and DXA bone mineral density, greater femur length, and lower femur Ca and ^{45}Ca content. The ovariectomized group also exhibited greater urinary excretion of ^{45}Ca at weeks one and four post-surgery; greater urinary excretion of hydroxyproline at weeks one, two and four post-surgery; and greater urinary excretion of DPD crosslinks at week four post-surgery. The ovariectomized plus estradiol did not exhibit these effects. Urinary DPD and hydroxyproline excretion showed a high correlation (r = 0.786). The authors concluded that ovariectomized rats lost bone due to resorption exceeding formation, and administration of estradiol prevented this effect.

In two studies (n=40 and n=80) of mature (100 ± 5 days of age) Sprague-Dawley rats, Bagi et al. (1996 and 1997) pair fed ovx to sham animals with standard laboratory diet. The ovx animals were found to have greater total bone area and total bone mineral content and similar femur bone area and mineral content but lower femoral bone mineral density as measured by DXA compared to the sham-operated animals. Ovariectomized animals were also found to have lower trabecular and cortical bone compared to time-matched sham animals. In the second study, nodal analysis of remaining trabecular bone in ovx animals revealed adversely altered architecture compared to sham animals. Femoral

strength as determined by mechanical testing was greater in both sham and ovx groups at study end compared to baseline. However, femoral ultimate strength was significantly greater in sham compared to ovx animals at four and eight weeks post-surgery; and femoral stiffness significantly greater in sham compared to ovx animals at four, eight, and 12 weeks post-surgery. The authors concluded that ovariectomy induced unfavorable changes in rat bone.

In a study by Sims et al. (1996), 126 Sprague-Dawley rats were ovariectomized or sham-operated at 6.5 months of age and fed 15 g/day commercial rat chow containing 0.76% calcium, 0.46% available phosphorous, and 2000 IU/kg vitamin D_3. Ovx rats showed significantly greater urinary excretion of hydroxyproline and serum phosphate compared to sham from six days post-surgery to the end of the study at 42 days post-surgery. On femoral histomorphometry, ovx animals exhibited greater femoral bone resorption and lower trabecular number compared to sham animals.

In the rat tibia, Wronski et al. (1985) demonstrated greater bone formation rates with higher numbers of osteoblasts, percent osteoblast surface, longitudinal bone growth, percent forming bone surface, and calcification rate. Greater bone resorption was also seen with higher numbers of osteoclasts and percent osteoclast surface. Despite the greater bone formation as well as resorption rates, net bone loss with smaller trabecular area was found in Sprague-Dawley rats (n=nine) ovariectomized at 75 days of age compared to control rats. All animals were ad libitum fed rat laboratory chow. Ovariectomized rat weight at study end was 298.4 ± 26.3 g.

Results of Peng et al.'s (1994) study showed lower femoral neck and tibia strength

in mechanical failure load and three-point bending tests, respectively, in Sprague-Dawley rats (n=14) ovariectomized at 12 weeks of age versus sham rats. Energy absorption of the femoral neck and trabecular bone volume were also lower and length was significantly greater in ovx versus sham rats. Rats were killed six weeks post-surgery. Diet components were not listed.

Li et al. (1997) studied ovariectomized Sprague-Dawley rats surgically treated at 90 days of age and weighing 506 ± 19 g at one year post-surgery. Ovx rats were pair fed to sham rats a diet containing 0.95% Ca and 0.67% PO_4. Femoral cancellous bone was lower in ovx and relatively stable in sham animals at 30, 60, 90, 180, 270 and 360 days post-surgery. Percent osteoclast, osteoblast surface, mineralizing surface, mineral apposition rate, and bone formation rate were higher in ovx than sham at the above mentioned time periods. Ovx rats exhibited lower trabecular number beginning at 60 days post-surgery, greater trabecular separation beginning at 30 days post-surgery, cortical thinning beginning at 60 days post-surgery, and negative bone balance at one year post-surgery compared to sham rats. The authors concluded that ovariectomy induced loss of both cortical and trabecular bone.

In a study of 150 Wistar rats randomized into three surgical treatments (sham, ovx, and ovx plus estradiol) at approximately 142 days of age, ovx animals were pair fed to ad libitum fed sham animals a standard diet (1.1 % Ca, 0.8% P and 2 IU vitamin D_3/g) based on AIN-76 recommendations or standard diet modified to contain low calcium (0.2% calcium, 0.8 % P and 2 IU vitamin D_3/g). Beginning at one month post-surgery and continuing until study end at nine months post-surgery, both ovx and ovx + LCD groups

weighed significantly more than sham and sham + LCD groups. First through fourth lumbar vertebrae (L1-L4); femoral neck, femoral mid-shaft, and distal femoral metaphysis DXA BMD; and total femoral, femoral mid-shaft, and distal femoral metaphysis BMC were lower in ovx and ovx plus low calcium diet compared to all other groups at three, six, and nine months post-surgery. DXA total femoral area was greater in ovx and ovx plus low calcium diet compared to all other groups at the same time points. Compressive strength and compressive stiffness of L1 vertebra as measured by a biomechanical testing machine were found to be significantly lower in ovx and ovx plus low calcium diet versus sham animals at the same time points post-surgery. Femoral torsional strength and femoral torsional stiffness were greatest in ovx and ovx plus low calcium diet at six and nine months post-surgery. These effects were not seen in the ovx plus estradiol group. The authors concluded that ovariectomy and ovariectomy in conjunction low calcium diet adversely impacted bone metabolism, and estrogen replacement prevented the adverse effects of ovx. They suggested femoral biomechanical testing was not significantly impacted by ovariectomy as cortical bone provided the majority of long bone stability and cortical bone was not as affected by ovariectomy as was trabecular bone (Jiang et al. 1997).

In a study of 150 Sprague-Dawley rats ovariectomized or sham operated at 12 weeks of age and ad libitum fed commercial laboratory food, Peng et al. (1997) demonstrated greater femur length and marrow area at the distal one-third of the femur in ovx versus sham animals beginning at approximately 5 weeks post-surgery. Animals were injected with $^{45}Ca^{2+}$ three days prior to necropsy, and ovx animals exhibited increased tibial

incorporation of $^{45}Ca^{2+}$ from weeks 4 to 16 post-surgery compared to sham animals. Both groups exhibited decreased percent femur trabecular bone volume over time, and ovx had significantly less volume than sham rats. Osteoclast number in the femur was greater in ovx versus sham animals from weeks two to 28 post-surgery with a dramatic increase at two weeks post-surgery. Maximal femoral load did not increase from baseline in ovx but did in control animals. Humerus torque increased over time in both groups, and was greater in ovx than sham animals. The authors concluded that ovariectomy increased bone metabolism producing increased periosteal apposition and endocortical resorption that sometimes resulted in cortical strength compensating for trabecular loss.

In a study of 110 Sprague-Dawley rats treated surgically at 90 days of age, ovx rats were pair fed a standard diet containing 1% Ca and 0.74% P to sham and ovx plus estradiol rats. Ovx plus estradiol rats were treated at three months post-surgery with 17β-estradiol 10 μg/kg body weight for five days. Double labeling of bone forming sites was performed with intraperitoneal injection of oxytetracycline at six and two days prior to necropsy. On bone histomorphometry, ovx animals had a significantly lower trabecular bone volume at one, three and six months post-surgery versus sham and at three months post-surgery versus estrogen-treated ovx animals. Ovx animals exhibited greater bone formation as measured by percent double labeled surface and bone formation rate. Greater formation was also seen with tibial osteoblastic cells cultured from ovx animals having higher proliferation rates as measured by [^{3}H] thymidine incorporation and cell numbers compared to sham animals. Greater resorption as measured by percent osteoclast surface was also demonstrated compared to estrogen-treated ovariectomized

rats and control rats at the above mentioned time periods. Bone resorption as measured by osteoclast number was significantly greater in ovx versus sham rats at one and six months post-surgery. The authors concluded that ovariectomy induced increased endosteal bone formation was related to the increased proliferation of osteoblast precursor cells (Modrowski et al. 1993).

In a study of 48 SD rats sham-operated or ovariectomized at two to three months of age, animals were ad libitum fed a non-purified diet containing 0.8% calcium and given free access to water containing zero added, two, six or 18 g sodium chloride/liter. Twenty-four hour urine samples were collected at times 0 (10 days post-surgery and before beginning treatment drinking water), two, four, six, 10, and 12 months. At four and six months of treatment and controlling for sodium effect, ovx animals were significantly heavier than sham animals whereas at 10 and 12 months of treatment no significant differences were noted in body weight. Urine calcium excretion was greater at six and 12 months of treatment in ovx versus sham animals when controlled for the sodium effect. Urinary excretion of hydroxyproline controlled for effect of sodium was greater in ovx compared to sham animals at time 0. Femur weight was significantly lower in ovx versus sham animals when controlled for sodium effect. Bone calcium content was affected significantly by sodium intake but more so by ovariectomy. The authors concluded that these results suggested the rapid bone loss seen immediately and increased body weight resulting after ovariectomy in rats did not persist. They also concluded that while sodium exerted a small but significant affect on bone calcium content ovariectomy exerted an overriding affect (Chan and Swarminathan 1998).

To date, results of studies seeking to determine if load bearing offsets estrogen deficiency induced bone loss have been conflicting. Wronksi et al. (1987) found in Sprague-Dawley rats ovariectomized or sham operated at 78 days of age decreased tibial trabecular bone volume both in obese and weight-matched animals compared to control animals. Additionally, tibial trabecular bone volume was significantly less in weight-matched versus obese ovariectomized rats. Westerlind et al. (1997) found in ovariectomized Sprague-Dawley rats treated surgically at 90 days of age that the more load bearing portion of the femur (epiphysis) shows less cancellous bone loss than the metaphysis which bears less mechanical strain. In addition, load bearing bones versus non-load bearing bones showed decreased loss. Estrogen treatment of ovariectomized rats decreased loss in unloaded bones and prevented loss in loaded bones. In contrast, in a study of Sprague-Dawley rats (weight 225-250g at surgery), Roudebush et al. (1993) found significantly lower femur bone mineral density in ovariectomized compared to sham animals even when ovariectomized animals were significantly heavier than sham animals. Protection from osteopenia was demonstrated only with estrogen replacement.

ROLE OF NUTRIENTS IN BONE FORMATION

Background. Information on the beneficial role of dietary intake of calcium and vitamin D is widely available (Anderson 1991, Henry and Norman 1984). A review of all such information and studies is beyond the scope of this thesis. Adequate ascorbic acid, required in the hydroxylation of proline into hydroxyproline in the manufacture of bone matrix collagen, intake is also accepted as necessary for proper bone metabolism (Englard and Seifter 1986). Dietary intake of other vitamins and minerals and their impact on bone

have been less extensively researched.

Collagen. Collagen is one of the major proteins of connective tissues and is composed of three polypeptide units containing multiple amino acids. The hydroxy amino acids present in the polypeptide chains, hydroxyproline and hydroxylysine, are found almost exclusively in collagen. It has been determined that iron and copper are integral in post-translational modification of type I collagen, the connective tissue foundation of bone. Iron plays a role in the hydroxylation of specific prolyl and lysyl residues of collagen, and copper functions in collagen crosslink formation. Copper, but not iron, deficiency has been demonstrated to impair crosslink formation (O'Dell 1981) and impair mechanical strength of bone (Jonas et al. 1993).

Copper. In a study of 14 weanling Wistar rats, animals randomized to a copper deficient (0.4 mg Cu/kg) diet were pair fed to animals fed a copper sufficient (11.0 mg Cu/kg) diet. Animal weights were not significantly different at necropsy. Femora did not differ in length, external mid-shaft diameter, or mid-shaft cortical thickness. On torsional loading, copper deficient animals had significantly lower maximal femoral sustained torque, ultimate angular deformation and toughness in torsional loading until fracture versus copper sufficient animals. No difference in femoral torsional stiffness was seen. Femoral calcium content and percent bone ash did not differ between the two groups. The authors concluded the above femoral changes were likely related to changes in the collagen matrix resulting from the copper deficiency (Jonas et al. 1993).

Iron. Iron, the nutrient of focus in this research project, has several known biological roles. Its functions in oxygen transport, cell growth and oxidative metabolism

are well established. Its potential role in bone metabolism has not been fully explored.

Angus et al. (1988) performed a study of 160 (89 pre-menopausal and 71 post-menopausal) Caucasian Australian women aged 23 to 75 years. Menopausal status was determined by serum measurement of follicle stimulating hormone, luteinizing hormone, and estradiol. A Lunar DP3 dual photon absorptiometer was used to measure bone mineral density (g/cm^2) of the right proximal femur and second through fourth lumbar vertebrae (L2-L4). A single photon densitometer was used to measure forearm BMC (U/cm). Participants completed a four day food record including a weekend as well as semi-quantitative food frequency questionnaire to determine usual nutrient intake. Food records were analyzed using a computerized dietary analysis program based on the British tables of food composition. Calcium intake in childhood was estimated with a retrospective questionnaire. Current mean calcium intake (738 ± 32 mg/day in premenopausal and 698 ± 37 mg/day in postmenopausal women) as analyzed from four day food records fell below recommendations for both pre- and postmenopausal women. Current mean iron intake (10.9 ± 0.4 mg/day in premenopausal and 9.9 ± 0.4 mg/day in postmenopausal women) as analyzed from four day food records fell below recommendations for premenopausal and exceeded recommendations for postmenopausal women. The authors found no correlation between current calcium or calcium plus vitamin D intake and bone mass. However, results suggested a relationship between early calcium intake and forearm BMC. A positive correlation between forearm bone mineral content and current iron, zinc, and magnesium intake as determined by food record analysis was demonstrated in pre-menopausal women. Current iron intake as determined

by food record analysis was also found to be an independent predictor of femoral neck bone mass in pre-menopausal women. The authors concluded that prospective studies are required to further assess the relationship between bone mass and mineral deficiencies.

Iron Deficiency. Iron deficiency has been associated with altered metabolism of other nutrients. In a study of recently weaned (four weeks of age) 84 male albino Wistar rats, Pallares et al. (1996) found decreased serum hemoglobin and serum iron and decreased iron concentration in liver and femur in iron deficient versus control animals. Decreased calcium concentration in the femur, sternum, and liver; increased phosphorous concentration in the liver and femur; and increased magnesium concentration in the liver were also found in iron deficient versus control animals. No significant differences were seen between iron deficient and control animals in serum calcium, phosphorous or magnesium concentrations. With iron replacement with ferrous ascorbate and ferrous sulfate, tissue concentrations of iron, calcium, phosphorous and magnesium approached normal. Animals given iron replacement in the form ferric citrate continued to exhibit reduced magnesium absorption and low femoral calcium and phosphorous concentrations, suggesting the ferrous ascorbate and ferrous sulfate were more effectively absorbed than the ferric citrate. No bone measures besides mineral content were performed. The authors concluded that iron deficiency altered the status of calcium, phosphorous and magnesium and that iron was involved in bone mineralization.

In a study of 94 weanling male albino Wistar rats, animals were fed a diet adhering to AIN-76 recommendations for all nutrients except iron. The treatment diet was altered to contain no added iron and was analyzed as containing 4.4 mg kg^{-1} iron . Liver, spleen,

sternum, and femur iron was lower in treatment diet animals versus control diet animals for all time points measured (10, 20, 30 and 40 days of treatment diet consumption). Treatment diet animals showed increased intestinal calcium, phosphorous, and magnesium absorption with decreased or unchanged balance. Liver calcium, phosphorous, and magnesium concentrations were significantly greater at 40 days of treatment diet consumption in iron deficient compared to control animals. Spleen calcium, phosphorous, and magnesium concentrations were all greater in treatment versus control animals after 40 days of diet consumption, calcium and phosphorous significantly so. Sternum calcium, phosphorous, and magnesium concentrations showed trends of being lower after 40 days of diet consumption in treatment compared to control rats. Femur calcium, phosphorous, and magnesium content and serum calcium concentration were lower at all time periods measured compared to control rats. No bone measures besides mineral content were performed. Serum cortisol and parathyroid hormone (PTH) were significantly greater after 40 days of dietary treatment in iron deficient compared to control animals. The authors concluded iron deficiency anemia altered the metabolism of calcium, phosphorous, and magnesium with increased intestinal absorption and changed metabolic utilization arising from hormonal changes associated with the anemia (Campos et al 1998).

Yokoi et al. (1990) fed fifteen young male Wistar rats (initial weight ~ 100g) a control diet (analyzed to contain 128 μg/g iron citrate) or iron deficient diet (analyzed to contain 5.9 μg/g iron citrate). Iron concentration in blood, brain, lung, heart, liver, spleen, kidney, testis, femoral muscle, and tibia were significantly lower in treatment compared to control rats. Blood calcium and copper were significantly higher while

magnesium and zinc were significantly lower in treatment versus control animals. Liver calcium and copper were significantly greater in treatment rats compared to control rats. Manganese concentrations were significantly higher in iron deficient animals versus controls in the brain, heart, spleen, kidney, testis, femoral muscle and tibia. No other bone measures were performed. Ratio of heart to body weight was significantly greater in treatment animals compared to control animals.

Iron deficiency has been associated with decreased bone fracture healing. In a study of 120 Sprague-Dawley rats (weight 300g), Rothman et al. (1971) randomized the animals into a control group fed a diet of powdered milk with a multiple vitamin supplement and distilled water or a treatment group fed the same diet plus multiple vitamin supplement without iron. No mineral mix was listed. The treatment group was phlebotomized weekly for four weeks to produce anemia. One week following the fourth phlebotomy, both groups underwent a surgical fracture of the mid-shaft of the right fibula. Hematocrit and serum iron measures demonstrated anemia in the treatment group. No difference in serum calcium, phosphorous, alkaline phosphatase (ALP), albumin, uric acid, and serum glutamic-oxaloacetic transaminase (currently termed aspartate aminotransferase or AST) was seen between anemic and control rats. The treatment group was found to have lower fibular tensile strength as well as fracture non-union and retardation of fracture healing versus control rats at three, six, and eight weeks post-fracture.

In a study of 30 male white New Zealand rabbits (weight two to three kg), a surgical fibular fracture was produced. Animals were than randomized into three groups: control group (phlebotomized and whole blood re-injected), normovolemic treatment

(phlebotimized, red cells discarded, and equal volume plasma re-injected), hypovolemic treatment (phlebotimized). Serum hematocrit was lower in both treatment groups compared to control group. No other serum biochemical tests were performed. Lower fibular strength as measured by modulus of elasticity and delayed fracture healing as seen on roentgenogram and in histological testing was reported in the hypovolemic treatment versus normovolemic treatment and control animals. The authors concluded that hypovolemic iron deficiency anemia was associated with delayed fracture healing and possibly decreased tissue oxygen delivery whereas normovolemic iron deficiency anemia was not associated with delayed fracture healing possibly due to compensation mechanisms, i.e. increased cardiac output, normalizing tissue oxygen delivery (Heppenstall and Brighton 1977).

In a study of weanling female and male wild-type BALB/cj mice, mice heterozygotic for hypotransferrinemia, and mice homozygotic for hypotransferrinemia, animals were ad libitum fed for five weeks purified diets based on AIN-93 recommendations containing either adequate (35mg/kg) iron as ferric citrate or no added iron (Fe-). Five groups resulted: wild-type iron adequate (+/+ Fe+), wild-type iron deficient (+/+ Fe-), heterozygote iron adequate (+/hpx Fe+), heterozygote iron deficient (+/hpx Fe-) and homozygote iron adequate (hpx/hpx Fe+). A homozygotic iron deficient group was not implemented as the researchers felt the animals would not survive. The hpx/hpx animals were given weekly intraperitoneal injections of human apo-transferrin until 2 weeks prior to kill. Animals were given free access to distilled water. The hpx/hpx group was anemic with significantly lower serum transferrin, plasma iron and

percent hematocrit than +/+ Fe+ and +/hpx Fe+ groups. Calculated cross-section femoral area and moment of inertia as measured by three-point bending were significantly lower in female hpx/hpx Fe+ versus +/hpx Fe+ and in male hpx/hpx Fe+ group as compared to +/+ Fe+. Femoral failure load and extrinsic stiffness were significantly lower whereas ultimate stress and modulus of elasticity were higher in hpx/hpx mice compared with +/+ mice. The higher ultimate stress and modulus of elasticity in the hpx/hpx group was felt to be due to small femur size. The hpx/hpx mice had the highest total tibial iron and calcium content as well as the greatest tibial wet weight. No dietary iron main effects were seen in measures of bone properties. The authors concluded their results suggested an association between transferrin and bone mineralization and raised questions about iron deficiency affecting bone mineralization [Malecki et al. 2000].

Iron Excess. Excess dietary iron has also been associated with altered metabolism of nutrients. In a study of 96 male Sprague-Dawley rats (no age or weight given), animals maintained on excess iron diets chronically had decreased tibial zinc retention compared to animals fed excess iron in only one meal. Rats fed 3042 μg iron/g diet had significantly lower final body weight, daily food intake, and higher ratio of liver to body weight. Kidney and liver iron levels were significantly higher in the 3042 μg iron/g diet group than in all other groups. Tibia copper levels were significantly greater while liver copper levels were significantly lower in the excess iron group compared to the control group. Kidney and tibia zinc levels tended to be lower but not significantly so (Storey and Greger 1987).

In a study of 53 African males diagnosed with skeletal disorders (vertebral osteoporosis, femoral neck fracture, osteonecrosis of the femoral head), participants were

divided into two groups based on number of hemosiderin-laden macrophages per mm^2 seen on iliac crest bone biopsy: iron overloaded (+Fe, n=38, median age 44 years) and non-iron overloaded (-Fe, n=15, median age 37). Dietary iron overload in this population was caused by consumption of traditional home-brewed beer prepared in metal drums or iron pots; iron from the vessel leaches into the brew as the pH of the beer decreases during fermentation. Control values for structural and static bone turnover were obtained from 43 age-matched black males (34 cadavers of individuals who had died suddenly but previously had been healthy and 9 limb surgery patients). Control values for tetracycline-based variables were obtained from 8 of the limb surgery patients. Alcohol consumption was significantly higher in the -Fe group versus the +Fe and control groups. The +Fe group had significantly higher serum ferritin and transferrin than the -Fe and control groups, and significantly higher serum iron but lower leukocyte ascorbic acid than the controls. Utilizing a tetracycline double labeled iliac crest bone biopsy, it was found that both the +Fe and -Fe groups had significantly smaller trabecular thickness and greater trabecular separation while +Fe also had significantly lower trabecular number (number of trabeculae intersected per mm straight line) compared to controls. Erosion depth was significantly greater in +Fe versus -Fe and control groups, and significantly greater in the +Fe group versus -Fe group. Iron granules in iliac crest bone correlated negatively with trabecular number and positively with trabecular spacing and erosion depth. The authors concluded that osteoporosis was associated with dietary iron overload and resulted from a combination of alcohol abuse, iron overload, and hypovitaminosis C. They also concluded that further research is necessary to separate the effects of iron overload and

hypovitaminosis C on bone metabolism (Schnitzler et al. 1994).

Excess iron has been associated with adverse bone results. In Storey and Greger's (1987) study, rats fed 3042 μg iron/g diet had lower tibia weights and higher tibia iron concentration than controls. In female long white pigs 76 to 91 days of age given 300 mg dextran iron per day intramuscularly for 36 days, plasma iron was greater in treated versus control pigs at days 21 and 36. Liver weight and liver iron were significantly greater in treated animals compared to control animals. SGOT (AST) and SGPT (ALT) were greater in treated compared to control pigs, SGOT (AST) significantly so. No differences in plasma calcium, phosphate, magnesium or alkaline phosphatase (ALP) were seen except for serum calcium being lower at day 21 in treated versus control pigs. No differences in urine calcium, phosphate, and hydroxyproline were seen. No differences in bone calcium, phosphate, magnesium or ash content was seen. However, metatarsal bone iron content was significantly higher in treated versus control animals. A significant correlation was seen between liver and bone iron. Osteoid surface was lower in treated compared to control pigs. Also, appositional rate and mean wall thickness were lower, and reversal surfaces greater in treated versus control pigs. The authors hypothesized that despite adverse effects on bone metabolism indicators no decrease in trabecular volume or bone ash content was seen because study length was too short (de Vernejoul et al. 1984).

In a study of 106 male and female humans with documented chronic liver disease (39 with alcoholic liver disease, 23 with hemochromatosis, 25 with chronic active hepatitis, and 19 with cholestatic liver diseases), participants were divided into cirrhotic and non-cirrhotic groups. Control data for BMD was obtained from 113 healthy

volunteers matched for age, sex, and menopausal status. Forty of the controls underwent bone biopsy. All subjects had significantly lower mean cancellous bone area compared to controls. Mean bone iron concentration was 2.5 times higher in both groups than in controls, and 22 of the participants had bone iron concentrations greater than the reference range upper limit (>36mg/kg dry bone). Bone aluminum and copper did not differ significantly in participant groups versus control group. Forty of the participants were osteoporotic as measured by bone biopsy or non-invasive criteria, and 23 of these 40 exhibited low bone formation rates. Additionally, 34 participants had diminished bone formation although not frank osteoporosis. A weak negative correlation was found between bone iron and bone formation. Participant bone area was significantly lower and mineralization lag time significantly greater compared to controls. Forearm mineral content as measured by single photon absorptiometry and vertebral BMD as measured by single energy computed tomography was significantly lower in the cirrhotic versus non-cirrhotic group. In an *in vitro* segment of the study, the rat osteosarcoma cell line UMR 106-01was cultured. Only very high (400 μmol/liter) iron concentration affected *in vitro* osteoblast function and proliferation, but testing with prolonged exposure to lower iron concentrations was not performed (Diamond et al. 1991).

In a study of 22 human males aged 35 to 62 years diagnosed with idiopathic hemachromatosis (IH) by liver biopsy, five were hypogonadal (H), nine eugonadal non-venesected (EN), and eight eugonadal venesected (EV). Healthy ambulatory men (n=20) undergoing elective orthopedic procedures acted as controls for serum biochemistry, spinal radiology, spinal quantitative computed tomography, single photon absorptiometry

of the forearm, and bone histomorphometry. Serum ferritin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) was significantly higher in H than EN, EV, and control; and significantly higher in EN than EV and control. Serum calcium was significantly higher in all participants compared to controls. Serum inorganic phosphate was higher in H compared to EN. Urine hydroxyproline excretion adjusted for creatinine was significantly higher in H versus all other groups. Radial bone density as measured by iodine-125 photon absorptiometry and trabecular bone volume as measured by double tetracycline labeled iliac crest bone biopsy were significantly decreased in H compared to all other groups, and vertebral bone density as measured by single and dual energy computed tomography (CT) was significantly decreased in H versus controls. By iliac crest bone biopsy, percent osteoclast surface and number were significantly greater in H compared to all other groups. Mineral apposition rate as measured by bone biopsy was significantly lower in all participants compared to controls, and significantly lower in EN versus EV. Percent mineralized surface and bone formation rate as measured by bone biopsy was significantly lower in EN versus EV and control groups. Mineralization lag time as measured by bone biopsy was significantly greater in both eugonadal groups versus control group. Ten of the 22 participants exhibited osteoporosis as measured by CT. The authors concluded that bone density was significantly lower in patients with IH, especially in conjunction with hypogonadism (Diamond et al. 1989).

Conte et al. (1989) compared six human males (mean age 48.8 ± 5.5 years) with primary hemachromatosis (PH) to eight human males (mean age 49.5 ± 7.9 years) with alcoholic cirrhosis (AC). A group of 30 healthy male subjects served as controls (mean

age 48.5 ± 8.3 years). Plasma ALP was significantly elevated in AC versus controls. No differences in plasma or urine calcium and phosphate were seen between PH and AC groups and controls. PH and AC groups had significantly elevated creatinine-corrected urinary hydroxyproline excretion versus controls. PH had significantly decreased forearm bone mineral density and cortical osteopenia as measured by iodine-125 photon absorptiometry versus AC and controls. Transiliac crest bone specimens indicate trabecular osteoporosis in the PH group only.

In a case study of a 48-year-old French Caucasian male diagnosed with primary hemachromatosis by liver biopsy, the patient presented initially for treatment of multiple spontaneous vertebral fractures. The patient was found to have elevated alkaline phosphatase activity but alcohol intake of less than 40 g/day. Testosterone level was significantly lower than normal. Urinary hydroxyproline excretion corrected for creatinine was elevated. Thyroid status was normal. Osteoblast and osteoclast activity were both lower than normal. Iliac crest trabecular bone volume as measured by biopsy was significantly lower and was associated with lower cortical bone thickness. Bone mineral content of the lumbar spine and femoral neck as measured by DXA were low. The authors concluded that the osteoporosis in IH is likely due to excessive bone resorption and inadequate bone formation with exact mechanisms unclear and multifactorial, e.g., hyperparathyroidism, hypogonadism, diabetes mellitus, vitamin D deficiency, vitamin C deficiency, and iron excess (Duquenne et al. 1996).

INDICATORS OF BONE METABOLISM

Insulin-like growth factor I. Insulin-like growth factor I (IGF-I) is an anabolic

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hormone which promotes cell proliferation and differentiation in many different cells. Bone is one target organ of IGF-I, and IGF-I is believed to participate in skeletal growth, regulation and bone metabolism.

In a study of 95-day-old female Sprague-Dawley rats, animals were fed a standard laboratory diet containing 0.4% calcium, 0.3% phosphorous and 3.0 units vitamin D/g diet. Animals were divided into four groups: sham plus solvent vehicle daily (sham), ovx plus solvent vehicle daily (ovx), ovx plus 200 ng 17β-estradiol/kg body weight per day (ovx LD) and ovx plus 5000ng 17β-estradiol/kg body weight daily for the first 12 days then alternating with solvent vehicle every other day thereafter (ovx HD). Animals were killed 35 days post-surgery. Final body weight was significantly higher in ovx compared to sham and ovx HD groups. Percent trabecular bone was significantly higher in sham and ovx HD versus ovx and ovx LD animals, and in ovx HD versus sham animals. Number of trabecular osteoblasts/mm and number of trabecular osteoclasts/mm were significantly higher in ovx versus sham rats and significantly lower in ovx LD and ovx HD versus ovx rats. Serum IGF-I was significantly higher in ovx and significantly lower in ovx HD compared to sham animals. The authors concluded that the increased trabecular osteoblasts and osteoclasts induced by ovariectomy and prevented by estrogen replacement was paralleled by similar increases in IGF-I also prevented by estrogen replacement. They further concluded that this implied that IGF-I may be involved in the pathogenesis of ovarian hormone deficiency-induced increased bone turnover (Kalu et al. 1994).

In a study of 300-350g female Sprague-Dawley rats, animals were ad libitum fed a

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standard diet containing 1.2% calcium and 0.9% phosphorous. Animals were randomized into five groups: control, ovx plus solvent vehicle (ovx), ovx plus 0.05 mg/kg IGF-I, ovx plus 0.2 mg/kg IGF-I, and ovx plus 0.8 mg/kg IGF-I. Surgery was performed at 6 months of age, and IGF-I treatment began 8 weeks post-surgery and ended 11 weeks post-surgery. Bones were labeled on the day prior to initiation of treatment (-1) with calcein and on days 10 and 17 of treatment with demeclocycline by intraperitoneal injection. From days 10 to 17, the 0.2 dose group exhibited significantly greater tibial longitudinal growth than ovx animals. From days -1 to 10, 10 to 17, and -1 to 17, the 0.8 dose group exhibited significantly greater longitudinal tibial growth compared to ovx animals. In addition, the 0.8 dose group exhibited significantly greater longitudinal tibial growth from days 10 to 17 and -1 to 17 than control rats. The 0.2 dose group had significantly greater femoral trabecular calcium, hydroxyproline, and trabecular bone volume compared to ovx animals. The 0.8 dose group had significantly greater trabecular bone volume compared to ovx animals. All doses of IGF-I increased the mineral apposition rate. Osteoclast number and surface was increased in the 0.8 dose group. The authors concluded that IGF-I treatment had beneficial dose-dependent effects on trabecular bone mass (Mueller et al. 1994).

In a study of 245 healthy community-dwelling Belgian women 70 years of age and older, researchers assessed the relationship between serum IGF-I as determined by radioimmunoassay and BMD of the hip as measured by DXA scan. Mean calcium intake as measured by food frequency questionnaire was 813.8 mg/day and was not related to femoral bone mass. Mean urinary pyridinoline and deoxypyridinoline excretion did not

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correlate with IGF-I. BMD of the femoral neck, Ward's triangle, and the trochanter major was positively correlated to serum IGF-I. With multivariate analysis, IGF-I was also an independent predictor of BMD at femoral neck, Ward's triangle and the trochanter major. The authors concluded that the findings suggested long-term presence of IGF-I was important in femoral integrity and that changes in the IGF regulatory system over time may be involved in age-related osteoporosis (Boonen et al. 1996).

Alkaline phosphatase and bone alkaline phosphatase. Alkaline phosphatase (ALP) is found in almost every tissue of the body. Major sources of total serum ALP are liver and bone. Total serum ALP may be elevated during periods of active bone growth, in liver disease, and in bone disease. Total serum ALP is considered an indicator of bone formation. The major source of bone ALP is osteoblasts. Bone ALP is an isoenzyme present on osteoblastic membranes, and synthesis increases during osteoblastic differentiation. Serum bone ALP is considered to be a specific indicator of bone formation, and has been shown to correlate with histomorphometric and ^{47}Ca bone formation measurements (Seibel et al. 1992, Bettica and Moro 1995). Elevated total ALP has been demonstrated in rats (Dick et al. 1996, Sims et al. 1996) and humans (Diamond et al. 1989, Duquenne et al. 1996) exhibiting greater than normal bone metabolism.

Urinary crosslinks of collagen. As previously discussed, collagen is the connective tissue foundation of bone and is composed of chains of amino acids cross-linked together. Degradation products, hydroxyproline (OH-Pr) and deoxypyridinoline (DPD) crosslinks, are produced with collagen lysis and are excreted into the urine. Urinary OH-Pr is released during both bone formation and bone resorption and is

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considered to be a marker of connective tissue degradation and of bone resorption. DPD is present mainly in bone. DPD crosslinks are released only during bone resorption and so are considered to be a specific marker for bone collagen degradation and to more specifically reflect bone resorption (Seibel et al. 1992, Bettica and Moro 1995). Elevated urinary OH-Pr and DPD crosslinks have been demonstrated in rats (Sims et al. 1996, Goulding et al. 1996) and humans (Diamond et al. 1989, Conte et al. 1989) exhibiting evidence of greater than normal bone metabolism.

Urinary mineral content. Calcium, magnesium, and phosphorous are macronutrient minerals required in the human diet. These minerals are located primarily in and are the major minerals of bone. Serum calcium content is tightly regulated as only a very narrow range of values is tolerable to the human body. Calcium released from bone and not used in one of its other biological capacities is secreted via the urine. Because calcium, phosphorous, and magnesium are involved in bone metabolism in concert with each other, bone release of calcium is accompanied by bone release of phosphorous and magnesium (Anderson 1991, Wester 1987). Elevated urinary excretion of calcium, phosphorous and magnesium are considered markers of bone demineralization. Increased urinary excretion of calcium has been demonstrated in rats (Goulding et al. 1996, Dick et al. 1996, Campos et al. 1998) experiencing greater than normal bone metabolism.

MEASUREMENTS OF BONE PARAMETERS

Bone mineral density and bone mineral content. Archimedes' Principle. The Archimedean Principle states a solid body immersed in liquid apparently loses as much of its own weight as the weight of the liquid it has displaced. Thus, it is possible to calculate

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a true unknown density based on liquid displacement. The density of a solid body such as a rat bone can be determined by immersion in a liquid of known density at a known temperature. Air weight of the bone is determined first followed by underwater weight. True or displacement density is calculated from the two weights and the density of the test liquid. In a study of five- and six-month-old retired female breeder rats whole and hollowed femora, density was determined by both Archimedes' principle and Hologic QDR-2000 DXA. Animals from a low-vitamin D Holtzman and a supplemental-vitamin D SD colony were used to ensure detectable treatment differences. Whole femur densities were significantly higher in the SD rats compared to the Holtzman rats as measured by both Archimedes' principle and by DXA. Whole femur density values were greater as measured by Archimedes' principle than by DXA. However, whole femur densities determined by the two methods were highly correlated ($r = 0.82$). Hollow femur densities were greater than whole femur densities as measured by Archimedes' principle but lower as measured by DXA. Femur ash weights and calcium content were highly correlated with whole bone densities as measured by both Archimedes' principle and DXA. The authors concluded that density determination is more relevant in whole than hollow bones, and both Archimedes' principle and DXA were accurate in detecting differences between the Holtzman and SD rats whole femora (Keenan et al. 1997).

Ashing. Bone mineral content may be determined by a combination of wet and dry ashing with reading of samples by atomic absorption spectrometry. Hill et al. (1986) describe a wet and dry ashing procedure in which dry ashing in a muffle furnace at 375° C for 24 to 48 hours is used. One gram samples of homogenized, dried samples of liver,

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heart, muscle, kidney, and serum which had been endogenously labeled with ^{65}Zn, ^{54}Mn, ^{59}Fe, and ^{51}Cr were placed in borosilicate tubes. Also, one gram samples of homogenized, freeze-dried wheat flour and rice flour containing known added levels of Cu, Mn, Fe, Zn, and Ca as well as bovine liver and citrus leaves containing U.S. National Bureau of Standards (NBS) certified levels of Cu, Mn, Fe, Zn, Mg, Ca, and Cr were placed in borosilicate tubes. Tubes were placed in the furnace, and the majority of sample organic material including the lipid component was burned off. Sample ash was then suspended in deionized water and nitric acid. Tubes were then placed in a heating block with addition of hydrogen peroxide until digestion was complete. Samples were analyzed by flame atomic absorption spectrometry. Percent recovery of ^{65}Zn, ^{54}Mn, ^{59}Fe, and ^{51}Cr from liver, heart, muscle, kidney, and serum was not below 94 percent as the mean of triplicate analysis. Percent recovery of added Cu, Mn, Fe, Zn, and Ca from wheat and rice flour was not below 98 percent. Analysis was within the certified range of both bovine liver and citrus leaves. The authors concluded acceptable reproducibility was achieved for all analyses and a modification of this procedure may be applicable to other elements.

Dual energy x-ray absorptiometry (DXA). Bone mineral density and content may also be determined by dual energy x-ray absorptiometry (DXA). DXA scanners directly measure body components by passing two beam energies through the measured body. Readings are generated based on the mass and type of tissue measured. In a study of seven Ellegaard research pigs weighing 35 to 95 kg and representing a range of human body weights and fatness, animals were fasted for 24 hours prior to scan. Pigs were anesthetized with pentobarbital 15 to 25 mL/kg body weight and scanned twice with a

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total-body DXA scanner without repositioning. Animals were killed by intravenous overdose of pentobarbital after scanning and carcasses frozen at -20° C for two days. Carcasses were then ground and random samples of approximately 500 g were taken. Percent body fat was determined by ether/petroleum ether extraction from one sample. Ash weight was determined from a separate sample. In a human segment of the study, six Danish women (age 24 to 49 years and body mass index 19.3 to 23.5) were scanned with the same DXA before and after having 8.8 kg of porcine lard placed on their abdomens. The scans were performed consecutively on the same day. Fat content of the porcine lard was determined by ether/petroleum ether extraction. In addition, two other Danish women and four Danish men (age 23 to 65 years and body mass index 19.2 to 29.7) were scanned twice with repositioning to evaluate within day precision. In the pigs, percent fat, fat mass of soft tissue, lean mass of soft tissue, total-body bone mineral, and total-body bone mineral density as measured by DXA did not differ significantly from values obtained from chemical fat extraction and ash weights. Measurements in the humans of simulated changes in fat mass of soft tissue and lean mass of soft tissue did not differ significantly from expected values. DXA within-day precision was acceptable. However, total-body bone mineral measurement did increase with placement of porcine lard. The authors concluded that DXA is both accurate and precise in measuring soft tissue body composition in longitudinal and cross sectional studies. They also concluded longitudinal total-body bone mineral measurements may be difficult to interpret if large changes in soft-tissue occurred (Svendsen et al. 1993).

In 18 lean and 18 obese female Zucker rats weighing 200 to 600 g, animals were

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fed 100g guar gum diet supplement/kg diet meeting AIN-93 standards for eight weeks. Rats were then anesthetized with 0.01 g xylazine /L and 0.95 g ketamine hydrochloride/L at a dose of 0.1 mL/100g body weight for lean rats and 0.12 to 0.15 mL/100g body weight for obese rats. Animals were scanned with a Hologic DXA scanner, killed by CO_2 overdose, degutted, and frozen at -20°C. In addition, one rat was scanned four times in one day, and five rats were scanned on three consecutive days. Carcasses were autoclaved, individually blended, and samples collected in triplicate for ash determination and chloroform-methanol fat extraction. Coefficient of variation for percent bone mineral content of the single rat repeatedly scanned was 11.0. Coefficient of variation for percent bone mineral content of the five rats scanned on three consecutive days ranged from 1.2 to 11.5. DXA percent fat, percent protein, and percent bone mineral content were highly correlated with carcass chemical analysis (r = 0.99, 0.96, and 0.81, respectively). The authors concluded that DXA can be used to predict chemical analysis values. They also concluded that because DXA detects body composition changes due to dietary treatment it may be useful in assessing rats in intervention studies (Rose et al. 1998).

In a study of male and female Wistar and GK (a genetic model of noninsulin-dependent diabetes mellitus) rats age two to 24 months, animals were ad libitum fed pelleted food. Twenty-one rats weighing 130 to 468 g were scanned in triplicate with a Hologic QDR 4500 DXA scanner with repositioning on seven occasions over several months to evaluate reproducibility and short-term variability. Long-term reproducibility was also assessed. Twenty-six rats weighing130 to 492 g were scanned in triplicate in one day within five days of each other. These rats were killed immediately after scanning with

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intraperitoneal injection of sodium pentobarbital, and frozen at -30°C. One rat weighing 426g was intraperitoneally injected with a 9% saline solution in amounts from five to 40 mL with a scan after each injection. Carcasses were homogenized and total lipid content determined by chloroform/methanol extraction. Short-term precision coefficient of variation for bone mass content was 1.7%. Long-term precision coefficient of variation for bone mass content was 0.7 ± 0.3%. Chemical analyses did not correlate well with DXA measurements. DXA measurements of bone mass content, fat mass, lean mass, and body weight were not significantly affected by saline injection. In rats weighing more than 200 g, it was demonstrated that single DXA scanning of body composition agreed with triplicate scanning. The authors concluded that DXA scans demonstrated reliable, accurate, and noninvasive measurements of rat body composition (Bertin et al. 1998).

Bone strength. Bone strength has been determined using a torsional device (Burstein and Frankel 1971), four-point bending or three-point bending method (Turner and Burr 1993). The three-point bending method is preferred for small bones such as rat femora. In this method, the femur is placed on two fulcra and force is delivered to the midshaft by a crosshead moving at a constant speed. Initially, the bone yields to force without sustaining damage. After the yield point, microfractures occur with continued force. Maximum strength of the bone is represented by the maximal amount of force applied to the bone at fracture (Turner and Burr 1993, Kiebzak et al. 1988).

In the Kiebzak et al. (1988) study, six-, 12- and 24-month-old male Wistar rats were fed ad libitum National Institutes of Health open formula rat chow containing 1.2% Ca and 0.9% P since weaning. Femoral breaking was performed as described above with

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a computerized Instron Universal Testing Machine on right femora. Femurs were positioned with one fulcrum just distal to the lesser trochanter and the other proximal to the medial and lateral condyles. Distance between the fulcra was 2.4 cm. Breaking force was applied at a crosshead speed of 5.0 mm/minute at the bone midshaft perpendicularly to the long axis of the bone. Bones were from anterior to posterior plane. Yield force was significantly lower in 24-month-old compared to six- and 12-month-old rats. Yield stress, yield deformation, and yield strain was significantly lower in 24-month-old compared to six- and 12-month-old rats and in six- compared to 12-month-old rats. Ultimate deformation was significantly lower compared to six-month-old rats and higher compared to 24-month-old rats in 12-month-old rats. The authors concluded that significant age-dependent changes in bone strength were seen as measured by the three-point bending method.

APPROPRIATENESS OF THE RAT MODEL

In general, the rat animal model has several practical advantages. Rats are less costly to house and care for than larger laboratory animals. The short rat life span aids in studying effects of aging. Mineral metabolism has been extensively studied in the rat and much information is available. Nutritional requirements of the rat have been defined and standard diet components are available. While the rat model is useful, its limitations must be considered. Rats lack Haversian systems and continue to gain bone mass throughout the life span. Body weight may not be an indicator of age. Longitudinal studies may not be possible if repeated bone sampling and large blood samples are required (Rodgers et al. 1993).

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The rat model's usefulness specifically for bone research should also be considered. Dick et al. (1996) determined that rats ovariectomized at six months of age and fed a 0.1% calcium diet displayed many similarities to postmenopausal women in calcium and phosphorous homeostasis. Bagi et al. (1996) determined responses in proximal femora of ovariectomized rats were similar to responses noted in proximal femora of postmenopausal women. In 1997, Bagi et al. concluded that the femoral neck was the optimal site for mechanical testing or bone histomorphometry when using the ovariectomized rat model in osteoporosis research because of the clinical significance of the proximal femur in osteoporosis in humans. The authors concluded that the rat proximal femur parallels the human proximal femur in BMD, mass, structure and strength.

In a study of 40 female Fischer-344 rats, animals were ad libitum fed a commercial rat diet containing 0.9% calcium, 0.75% phosphorous, and 600 IU/kg vitamin D_3 and given free access to tap water. Rats were killed at three, six, nine, and 12 months of age. Each rat was injected with oxytetracycline 24 days prior to kill, xylenol orange 19 days prior to kill, demeclocycline 14 days prior to kill, alizarin complexone nine days prior to kill, and calcein four days prior to kill. Longitudinal bone growth in the proximal tibia, caudal vertebra and cranial vertebra as measured by demeclocycline and calcein markers declined dramatically from three to six, nine, and 12 months. In the first lumbar vertebral body, cancellous bone remodeling increased from approximately 70% of all formation sites in three-month-old rats to approximately 85% in six-month-old rats and 91% in 12-month-old rats. In the proximal tibial metaphysis, modeling sites decreased from approximately 61% of all formation sites in three-month-old rats to approximately 40% in six-month-old

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rats and 16% in 12-month-old-rats. Proximal tibial metaphysis remodeling sites increased from approximately 21% of all formation sites in three-month-old rats to approximately 30% in six-month-old rats and 66% in 12-month-old rats. The author concluded that these findings suggested that remodeling was the prevailing activity in vertebral and tibial cancellous bone in aged rats. He also concluded that this activity was similar to higher mammals (Erben 1996). The rat model of osteoporosis has been determined to be useful so long as its limitations are considered.

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CHAPTER III

MATERIALS AND METHODS

Experimental Design. Eighty-four weanling (21 days of age) female Sprague-Dawley rats were obtained from SASCO (Kingston, NJ). This experiment was the second part of a larger two-phase study. Animals were maintained on rat chow and deionized water for three days after arrival. After acclimation, animals were randomized into two ovarian hormone status groups (OHS) (sham-operated or ovariectomized) and four iron modified dietary regimens (calculated to be 6 ppm, 12 ppm, 35 ppm or 150 ppm as iron citrate). Animals were maintained on diets for 15 weeks, and 12 weeks following surgical treatment. The life span period of interest was from 17 weeks of age (skeletal maturity) to 29 weeks of age for both intact and induced ovarian hormone deficient animals, simulating life span iron effects on adult and postmenopausal bone metabolism.

Housing. Animals were maintained under a 12 hour light/dark cycle and in temperature-controlled conditions at the Oklahoma State University Laboratory Animal Resources (LAR) facility under the supervision of a veterinarian. The rats were housed in individual plastic cages with raised plastic floor grids. Small amounts of ground corn husk bedding was below the grids for waste absorption. Cages, feed dishes and water bottles were changed weekly.

Diet. Diet components were purchased from Teklad (Madison, WI). Diet was prepared by researchers according to AIN-93 G and AIN-93 M standards (Reeves et al. 1993) with the exception of iron concentration (Tables 1-3). Four dietary iron

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concentrations were prepared: two insufficient (6 and 12 ppm), one recommended control (35 ppm) and one excess (150 ppm). Mineral mixes and diets were prepared lowest to highest iron concentration and growth before maintenance to avoid carryover contamination. Mineral mixes were mixed in a washed and deionized water rinsed ceramic jar containing washed and deionized water rinsed ceramic balls. Diet components were mixed with a Hobart stand mixer in an acid washed plastic mixer bowl (Hobart Manufacturing Company, Troy, OH). All diets were labeled appropriately with formulation and dietary iron concentration. All diets were stored refrigerated.

Feeding. Each rat was allocated two plastic cups labeled with rat number, diet, and treatment. Feed was weighed into the cups twice a week, and cups taken to the LAR and stored refrigerated until feeding time. Biweekly feed amounts were based on animal weight and remaining feed weight prior to surgery and additionally on surgical treatment after surgery. Animals were fed daily in the late afternoon in glass crucible dishes with stainless steel grate inserts and lids.

Water. Rats were provided ad libitum access to deionized water in glass bottles with straight stainless steel sipper tubes. Fresh water was given three times per week.

Surgery. Rats were sham operated or ovariectomized at 17 weeks of age (skeletal maturity) based upon OHS group randomization. Food was withheld for 12 hours and water withheld for 6 hours prior to surgery. The procedures were performed at the Oklahoma State University LAR facility by a veterinary surgeon. Animals were anesthetized by halothane inhalation. Dorsal hair was shaved and underlying skin was scrubbed with antiseptic veterinary soap. A single dorsal incision was performed giving

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access to ovaries. Ovaries were removed by ligation in ovariectomized animals. Ovaries were lifted from and then replaced in the body in sham operated animals. In all animals, incisions were closed with liquid veterinary adhesive. Post procedure, animals were observed until fully awake and alert. Water was given to each animal after alertness returned. Feed was given to all animals at usual feeding time.

Necropsy. Necropsy was performed on 10 to 12 animals per day four days per week for two consecutive weeks. Animals were moved from the LAR facility to the Oklahoma State University Nutritional Sciences Laboratory (NSL) one day prior to scheduled necropsy. Twelve hours prior to necropsy, the rats were transferred to metabolic cages for urine and fecal collection. Feed was withheld but deionized water given. Anesthesia was achieved by intraperitoneal injection with ketamine and xylazine with a dosage of 50 and 10 mg/kg body weight, respectively. Animals were then weighed and scanned by dual x-ray absorptiometer (DXA). Blood was collected from the abdominal aorta, processed and stored appropriately. Bones (femurs, tibiae, spines, and calveri) and organs (heart, kidneys, liver, spleen, uterus, and ovaries) were collected, cleaned, weighed, and stored appropriately. Remainder of rat carcass was disposed of properly.

Tissue collection and storage. Urine was collected prior to surgery and to necropsy, centrifuged, alliquotted, and stored frozen at -20° C. Feces was collected after two feedings labeled with Blue Dye #1 aluminum (Research Diets, Incorporated, New Brunswick, NJ) prior to necropsy and stored frozen at -20° C. Whole blood collected at necropsy was analyzed immediately for hematology values. Serum for clinical chemistry

tests was analyzed immediately. Other serum collected and plasma were processed and stored frozen at -20° C. Femora and tibiae were trimmed of muscle and cartilage, taking care not to damage the periosteum. Right femur was processed first and stored frozen at -80° C. Right tibia and left femur were stored frozen at -20° C. Left tibia was stored at room temperature in 70% ethanol. Whole spines were stored frozen at -20° C, thawed individually, cleaned and separated to obtain lumbar vertebrae 3, 4 and 5. L3, 4 and 5 were stored frozen at -20° C. Mineral free gloves were used at all times during bone cleaning and bones were cleaned lowest to highest dietary iron concentration. Liver was the first organ processed and two sections were stored frozen at -80° C. One section of liver and the heart, kidneys and spleen were stored frozen at -20° C.

Analyses. Nutrition indicators. Body composition was measured immediately prior to necropsy by whole body scan with a Hologic QDR 4500A Fan Bean X-ray Bone Densitometer with appropriate software for small animals (Hologic, Inc., Waltham, MA). Hematology testing was done on whole blood immediately following necropsy with a Pentra 120 Retic (software version 3.13) and chemical solutions as appropriate for each analysis (ABX Hematology, Inc., Irvine, CA).

Clinical chemistry testing was done on serum immediately following necropsy with a COBAS Fara II (Roche Diagnostic Systems, Indianapolis, IN). Glucose testing was performed using an enzymatic approach utilizing hexokinase coupled with glucose-6-phosphate dehydrogenase as described by Neeley (1972) and appropriate Roche (Roche Diagnostic Systems, Inc., Somerville, NJ) reagent. Serum urea nitrogen (SUN) was performed with the appropriate Roche reagent which is based on a modification of the first

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totally enzymatic SUN measuring procedure. Aspartate aminotransferase (AST) was performed with the appropriate Roche reagent based on a modification of the procedure of Karmen (1955). Alanine aminotransferase (ALT) was performed with the appropriate Roche reagent based on a modification of the procedure of Wroblewski and LaDue (1956). Alkaline phosphatase (ALT) was performed with the appropriate Roche reagent based on a modification of the reference method of the American Association for Clinical Chemistry (Tietz 1983). Albumin was performed with the appropriate Roche reagent based on a modification of the bromocresol green binding assay (Doumas et al. 1971).

Bone status indicators. IGF-I testing was performed on serum stored frozen at -20° C and thawed immediately prior to assay. A Nichols Institute Diagnostics (San Juan Capistrano, CA) IGF-I by extraction kit was used with modified formic acid rat extraction method (Kalu 1994). Tube counts were performed using a Cobra II Auto-Gamma (Packard Instrument Co., Downers Grove, IL).

Alkaline phosphatase extracted from bone was performed with fractured third lumbar vertebrae (L3) utilizing a modification of the method of Farley et al. (1992). Each bone was placed in a glass test tube containing 3.0 mL of phosphate buffered saline with 0.02% sodium azide and incubated overnight (approximately 12 hours) at 4° C. After density by displacement, the bone was crushed and was then transferred to another tube containing 3.0 mL of 0.01% Triton-X100 with 0.02% sodium azide for 72 hour extraction at 4°C. Bones were centrifuged for 15 minutes at 1500 xg. The extract was decanted and analyzed by Cobas FARA II clinical analyzer with a Roche ALP reagent.

Urinary creatinine level and urinary excretion of Ca, Mg, and P were performed

with urine stored frozen at -20 ° C and thawed immediately prior to testing. A COBAS Fara II with appropriate Roche reagent for each analysis was used. The Roche reagent for urinary creatinine content is based on a kinetic modification of the Jaffe reaction (Jaffe 1886, Larson 1972). The Roche reagent for urinary calcium content is based on Arsenazo III, a very sensitive and specific reagent which requires slightly acidic conditions (Michaylova and Illkova1971). The Roche reagent for urinary magnesium content utilizes chlorophosphonazo III (Ferguson et al. 1964), a sensitive dye for magnesium and calcium determination at neutral pH, modified to prevent calcium interference. The Roche reagent for urinary inorganic phosphorous content is based on a modification of Daly and Ertingshausen's (1972) procedure.

Urinary DPD was performed on urine stored frozen at -20 ° C and thawed immediately prior to testing. A Pyrilinks-D kit (Metra Biosystems, Inc., Mountain View, CA) was used. The Pyrilinks-D reagent is based on a monoclonal antibody which is specific for DPD (Robins et al. 1994). Due to hyperconcentration, samples were diluted once based on urinary creatinine and a second time by combining 50 μL of the previously described dilution with 500 μL of assay buffer (personal communication from Brenda Smith, Ph.D., November 1999).

Table 4 Urinary sample dilution for DPD analysis in female SD rats

First Dilution	Urinary Creatinine (mg/dL)	Sample	Deionized Water
A	< 30	0.05 ml	0.2 ml
B	30-75	0.05 ml	1 ml
C	75-150	0.05 ml	2.5 ml
D	>150	0.05 ml	4.5 ml

Plate washing was performed by El$_x$ 405 Auto Plate Washer (Bio-Tek Instruments, Inc., Winooski, VT) and plate counting by El$_x$ 808I Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT).

Urinary hydroxyproline was determined using samples collected prior to surgery and necropsy and stored frozen at -20° C until analysis. The method of Bergman and Loxley (1970) was utilized. A Tecator 1024 Shaking Water Bath (Sweden) and Beckman DU® 640 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) set at 562 nm were used.

Fracture force were measured on femurs stored frozen at -20° C until testing. Bones were thawed overnight and soaked in 0.9% saline for 24 hours. Bone length was measured to the nearest millimeter, and bones were fractured with an Instron Universal Testing Machine Model TM-S (Instron Corporation, Canton, MA) with a crosshead speed of 1.0 mm/minute according to the method of Kiebzak et al. (1988). Marrow was removed , and bones were soaked in type I water in the dessicator with vacuum for 1.5

hours.

Bone mineral density and bone mineral content were measured in the intact rat immediately prior to necropsy by whole body scan with a Hologic QDR 4500A Fan Bean X-ray Bone Densitometer (Svendsen et al. 1993, Bertin et al. 1998, Rose et al. 1998) with appropriate software for small animals (Hologic, Inc., Waltham, MA). Rats were scanned positioned straight and flat on ventral surface, limbs spread, tail lying to the side. Scan was initiated in the center position and lasted approximately two minutes.

Bone mineral density of the left femur was also determined by Archimedes' principle (Keenan et al. 1997). Underwater and air weighing were performed with a top loading Mettler H18 balance and Density Determination Kit ME-33340 (Mettler Instrument Corporation, Princeton, NJ). Bone density was then calculated by using the formula $\rho_2 = A/P \times \rho_0$ where ρ_2 = density of the femur, A = weight of the femur in the air, P = buoyancy of solid body in type I water, and ρ_0 = density of type I water at a given temperature.

Bone mineral content was also determined by wet and dry ashed using a modification of the method of Hill et al. (1986) using mineral free gloves. Wet ashing was performed under an acid hood. Acid washed tubes were double labeled with a high temperature pencil when hot. After being allowed to cool, tubes were weighed empty, and re-weighed after sample placement. Bones were dried at 100°C for 24 hours. Weighed tubes were placed in an Isotemp Dry Bath 145 heating block (Fisher Scientific, Pittsburgh, PA). Fifty μL each of type I deionized water, Ultrex hydrogen peroxide (H_2O_2) (Baker), and double distilled nitric acid (GFS Chemicals) were pipetted into each

sample tube using a clean and separate mineral free pipette tip for each solution and a pipette not having a metal mechanism. Heating block was turned on and set at 80° C. After 15 minutes, temperature was raised to 90° C. After 15 minutes, 100 μL of H_2O_2 was added to each tube, and heating block temperature was raised to 100° C. After 30 minutes, 100 μL of H_2O_2 was added to each tube, and heating block temperature was raised to 105° C. Hydrogen peroxide (100 μL) was added three more times after 15 or 30 minute waiting periods before being allowed to evaporate to dryness.

Tubes were then placed in an acid washed beaker with open ends of the tubes facing the bottom of the beaker and an acid washed petri dish placed on the beaker to close off air. The beaker of tubes was placed inside a Lindberg 847 ashing oven (General Signal, Watertown, WI). Samples were processed for 2880 minutes at 375° C and then held for 25 minutes at 100° C.

Wet and dry ashing procedures were alternately repeated until all samples turned white and bone was not recognizable. Ashed samples will be analyzed for minerals at a later date with a Perkin Elmer 5100 PC flame atomic absorption spectrometer (Perkin Elmer Corporation, Norwalk, CT).

Animals were injected intraperitoneally at 8 days and 24 hours prior to necropsy with calcein at a dosage of 0.75mg/100 grams body weight. Bone appositional growth rate and histology will be determined at a later date.

Statistics. Data were analyzed by PC SAS version 7.0 (SAS Institute, Cary, NC) and significance was set at $P < 0.05$. Means of variables of necropsy animals in experiment one fed same diets as those in the second experiment for 15 weeks were subtracted by diet

group as measures of change due to treatment.

Table 1 American Institute of Nutrition 1993 Purified Diet Components for Laboratory Rodents							
Ingredient		**Growth**	**Maintenance**				
Cornstarch		397.49 g	465.69 g				
Casein		200.0 g	140.0 g				
Dextrose		132.0 g	155.0 g				
Sucrose		see Table 2	see Table 3				
Soybean oil		70.0 g	40.0 g				
Celufil fiber		50.0 g	50.0 g				
Mineral mix		35.0 g	35.0 g				
Vitamin mix		10.0 g	10.0 g				
L-Cystine		3.0 g	1.8 g				
Choline		2.5 g	2.5 g				

Table 2 American Institute of Nutrition 1993 Growth Mineral Mix					
Ingredient		**6 ppm**	**12 ppm**	**35 ppm**	**150 ppm**
Calcium carbonate	40.04% Ca	357	357	357	357
Potassium phosphate monobasic	22.76 P, 28. 73% K	196	196	196	196
Potassium citrate, Tri-K monohydrate	36.16% K	70.78	70.78	70.78	70.78
Sodium chloride	39.34% Na, 60.66% Cl	73.275	73.275	73.275	73.275
Potassium sulfate	44.87% K, 18.39% S	46.6	46.6	46.6	46.6
Magnesium oxide	60.32% Mg	24	24	24	24
Ferric citrate	16.5% Fe (35 ppm)	0.88	1.86555	5.8883	25.8126
Zinc carbonate	52.14% Zn	1.65	1.65	1.65	1.65
Manganous carbonate	47.79% Mn	0.63	0.63	0.63	0.63
Cupric carbonate	57.47% Cu	0.3	0.3	0.3	0.3
Potassium iodate	59.3% I	0.01	0.01	0.01	0.01
Sodium senenate, anhydrous	41.79% Se	0.01025	0.01025	0.01025	0.01025
Ammonium paramolybdate, 4 hydrate	54.34% Mo	0.00795	0.00795	0.00795	0.00795
Sodium meta-silicate, 9 hydrate	9.88% Si	1.45	1.45	1.45	1.45
Chromium potassium sulfate, 12 hydrate	10.42% Cr	0.275	0.275	0.275	0.275
Lithium chloride	16.38% Li	0.0175	0.0175	0.0175	0.0175
Boric acid	17.5% B	0.0815	0.0815	0.0815	0.0815
Sodium fluoride	45.24% F	0.0635	0.0635	0.0635	0.0635
Nickel carbonate	45% Ni	0.0318	0.0318	0.0318	0.0318
Ammonium vanadate	43.55% V	0.0066	0.0066	0.0066	0.0066
Total mineral elements		773.069	774.0546	778.0773	798.4516
Sucrose		217.365	216.3795	212.3567	191.9824

Table 3 American Institute of Nutrition 1993 Maintenance Mineral Mix					
Ingredient		**6 ppm**	**12 ppm**	**35 ppm**	**150 ppm**
Calcium carbonate	40.04% Ca	357	357	357	357
Potassium phosphate monobasic	22.76 P, 28. 73% K	250	250	250	250
Potassium citrate, Tri-K monohydrate	36.16% K	28	28	28	28
Sodium chloride	39.34% Na, 60.66% Cl	73.275	73.275	73.275	73.275
Potassium sulfate	44.87% K, 18.39% S	46.6	46.6	46.6	46.6
Magnesium oxide	60.32% Mg	24	24	24	24
Ferric citrate	16.5% Fe (35 ppm)	0.88	1.8655	5.8883	25.8126
Zinc carbonate	52.14% Zn	1.65	1.65	1.65	1.65
Manganous carbonate	47.79% Mn	0.63	0.63	0.63	0.63
Cupric carbonate	57.47% Cu	0.3	0.3	0.3	0.3
Potassium iodate	59.3% I	0.01	0.01	0.01	0.01
Sodium senenate, anhydrous	41.79% Se	0.01025	0.01025	0.01025	0.01025
Ammonium paramolybdate, 4 hydrate	54.34% Mo	0.00795	0.00795	0.00795	0.00795
Sodium meta-silicate, 9 hydrate	9.88% Si	1.45	1.45	1.45	1.45
Chromium potassium sulfate, 12 hydrate	10.42% Cr	0.275	0.275	0.275	0.275
Lithium chloride	16.38% Li	0.0174	0.0174	0.0174	0.0174
Boric acid	17.5% B	0.0815	0.0815	0.0815	0.0815
Sodium fluoride	45.24% F	0.0635	0.0635	0.0635	0.0635
Nickel carbonate	45% Ni	0.0318	0.0318	0.0318	0.0318
Ammonium vanadate	43.55% V	0.0066	0.0066	0.0066	0.0066
Total mineral elements		784.289	785.2745	789.2973	809.2216
Sucrose		206.145	205.1595	201.1367	181.2124

CHAPTER IV

RESULTS AND CONCLUSIONS

EFFECTS OF DIETARY IRON AND OVARIECTOMY ON BONE IN SKELETALLY MATURE OPERATED RATS [1]

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ABSTRACT

Eighty-four weanling Sprague-Dawley rats were randomized to dietary Fe (6, 12, 35 and 150 ppm) and to ovarian hormone status (ovariectomized or sham-operated) groups three days after receipt. Four animals were dropped from all analyses due to illness during the experiment. Animals were fed Fe modified AIN-93 G for seven weeks and Fe modified AIN-93 M thereafter; both formulations were adjusted for the four Fe levels. Pre-surgery, animals were pair fed to the diet group weighing the least. Following surgery, animals were pair fed to the 150 ppm sham group but where necessary were fed less to match for weight. Animals were fed for 15 weeks then ovariectomized or sham-operated under halothane anesthesia. Twelve weeks post-surgery, animals were fasted for 12 hours in plastic metabolic cages for urine and fecal collections. Animals were then anesthetized with ketamine/xylazine, DXA scanned and exsanguinated by abdominal aorta puncture. Tissues were perfused with cold saline (9 g/L) prior to collection. No diet or ovarian hormone status (OHS) effects were observed for bone strength as measured by three-point bending. Diet x OHS interactions occurred for femur area and length and for change in femur area and length. Ovarian hormone status effect was seen for femoral bone mineral density (BMD) and displacement density of L3 vertebra. Diet effects were noted for change in femoral BMD, cortical area, bone mineral content (BMC), and alkaline phosphatase (ALP) extracted from L3. Diet and OHS effects were seen in L3 true density change, hemoglobin (Hb) concentration and insulin-like growth factor-I (IGF-I). Final body weight was significantly greater in the ovx versus sham rats regardless of iron status. Diet effect occurred for urinary calcium, magnesium, hydroxyproline and

deoxypyridinoline crosslinks excretion pre-surgery. Diet x OHS interaction was seen for final urinary calcium excretion. Ovarian hormone status effect was observed for final urinary deoxypyridinoline (DPD) crosslinks, hydroxyproline excretion, change from surgery to final urinary hydroxyproline and DPD crosslinks excretion. As expected, OHS affected bone. Dietary iron intake also affected bone. Dietary iron played a role in bone metabolism, and adequate iron intake may be a preventative factor in the chronic debilitating disease, osteoporosis.

KEY WORDS: iron, bone, density, rats, ovariectomy, osteoporosis

INTRODUCTION AND BACKGROUND

In the United States alone, a conservative estimate is that nearly half of all women over the age of 50 will suffer a fracture related to osteoporosis, resulting in more than 1.5 million fractures per year including painful and often debilitating hip fractures (32). Other common osteoporotic fracture sites include the vertebrae and distal forearm (10).

Osteoporosis affects twice as many women as men, and is especially prevalent in post-menopausal women. Both natural and surgical menopause are associated with an initial phase of rapid bone loss which may last seven to ten years (8).

It has been estimated that only 50% of individuals regain mobility within one year of hip fracture (20) and reported that within one year of hip fracture 21.5% of men and 35.4% of women followed were deceased (30). Medical costs associated with osteoporotic fractures in1995 have been estimated at $13.8 billion (23).

Although there are numerous alternatives available in the fight against osteoporosis, none can completely prevent bone loss. Hence, it is important to investigate other factors which may prevent bone loss.

Iron is integral in the hydroxylation of collagen matrix, the connective tissue foundation of bone (24). As iron is key in this reaction, both inadequate and excessive iron intake may affect connective tissue metabolism. Yet, the association between dietary iron intake and bone metabolism is unclear. While inadequate iron intake is found across racial, gender, and socioeconomic boundaries worldwide (11), excess iron intake is increasing due to the availability of iron supplements and food fortification policies (25). Because type I or postmenopausal osteoporosis is the most common form of osteoporosis,

the purpose of this study was to investigate the effects of dietary iron in ovarian hormone sufficient and deficient skeletally mature rats and to determine if there were differences between iron and/or ovarian hormone status (OHS) groups in bone density and mineral content as well as in blood and urine indicators of bone metabolism.

MATERIALS AND METHODS

Experimental design. This study was a completely randomized 2 x 4 factorial block design. Independent variables were dietary iron levels and ovarian hormone status. This experiment was the second part of a larger two-phase study.

Animals. Eighty-four weanling female Sprague-Dawley (SD) rats (SASCO, Kingston, NJ) were acclimated on standard rat chow and deionized water for 3 days after arrival prior to randomization. Animals were maintained on dietary treatment for 15 weeks before surgery.

Diets. Diets were calculated to contain 6, 12, 35 or 150 ppm iron as iron citrate. All other minerals met the AIN-93 recommendations (28). Animals were fed AIN-93 G for seven weeks and then switched to the respective mineral concentration AIN-93 M diets for the remainder of the experiment. Animals were pair fed to the 150 ppm sham group but where necessary were fed less to match for weight.

Treatment. After 15 weeks of initial dietary treatment, animals were anesthetized by halothane induction and operated. Ovaries were located through a single dorsal incision and ligated in ovx animals or replaced in sham animals. Animals were monitored post-procedure until fully alert.

Necropsy. Twelve weeks post-surgery, animals were food deprived but provided

with DI water for twelve hours in plastic metabolic cages for urine and fecal collections. At the time of sacrifice, animals were anesthetized with ketamine/xylazine and dual energy x-ray absorptiometry (DXA) scans were performed. Animals were exsanguinated by abdominal aorta puncture, and tissues were perfused with cold saline (9g/L) prior to collection.

Analyses. Biochemical tests including albumin, glucose, serum urea nitrogen, serum creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were performed with a COBAS Fara II (Roche Diagnostic Systems, Indianapolis, IN) and appropriate reagents. Hematological analyses were performed with a Pentra 120 Retic (ABX Hematology, Inc., Irvine, CA). Body composition was determined with a Hologic QDR 4500A DXA scanner.

Insulin-like growth factor I (IGF-I) was analyzed using a Nichols Institute Diagnostics (San Juan Capistrano, CA) kit and modified formic acid rat extraction method (16). Alkaline phosphatase in bone was measured using a modification of the method of Farley et al. (13) by placing third lumbar vertebra (L3) into a glass test tube containing 3.0 mL of phosphate buffered saline with 0.02% sodium azide. Vertebrae were incubated approximately 12 hours at 4° C to remove contaminants. After density by displacement determination, vertebrae were crushed and transferred into another tube containing 3.0 mL of 0.01% Triton-X100 in phosphate buffered solution with 0.02% sodium azide for 72 hour extraction at 4° C. Crushed vertebrae were centrifuged for 15 minutes at 1500 x/g to remove insoluble materials. The extract was then decanted and processed with a Cobas FARA II and Roche alkaline phosphatase reagent. Urinary hydroxyproline was

determined using the method of Bergman and Loxley (3). Urinary deoxypyridinoline (DPD) crosslinks were determined using a Pyrilinks-D kit (Metra Biosystems, Inc., Mountain View, CA). Double dilution was performed based on urinary creatinine content due to hyperconcentration (personal communication, Brenda Smith, Ph.D.). Urinary creatinine level and urinary excretion of calcium (Ca), magnesium (Mg), and phosphorous (P) were analyzed with a COBAS Fara II and appropriate Roche reagents for each analysis.

Bone mineral content (BMC) and bone mineral density (BMD) were analyzed for whole body and right femur by DXA (Hologic QDR 4500A Fan Beam X-ray Bone Densitometer, Hologic, Inc., Waltham, MA) with appropriate software for small animals. BMD of the right femur and L3 was also determined by Archimedes' principle (17), weighing the sample using a Density Determination Kit ME-33340 (Mettler Instrument Corporation, Princeton, NJ).

Femur fracture force was measured at the rate of 1mm/min according to the method of Kiebzak et al. (18) using an Instron Universal Testing Machine Model TM-S (Instron Corporation, Canton, MA).

Data analysis. Means of variables of necropsy animals fed the same diets as those in the second experiment were subtracted by diet group as measures of change due to treatment. Data were analyzed by PC SAS version 7.0 (SAS Institute, Cary, NC), and significance was set at $P < 0.05$.

RESULTS

Despite being heavier, the ovx rats had lower femoral DXA BMD and L3 true

density than the sham rats (Tables 5 and 6). Rats given the 6 ppm diet had greater change from surgery to necropsy in femur BMD, BMC and cortical area than rats fed other diets (Table 5). Animals fed the 6 ppm diet and sham animals regardless of iron status had the greatest change from surgery to necropsy in L3 true density (Table 6). No diet or OHS effects were seen for bone strength as measured by three-point bending (data not shown).

Diet x OHS interactions were seen in femur length and area and change in femur length and area from surgery to necropsy with 6 ppm ovx animals having greater length and change in length than all other groups, significantly more than 6 and 150 ppm sham groups (Table 5).

Although mean weights of rats did not differ initially between diet and OHS groups and despite pair feeding to the 150 ppm sham group following surgery, regardless of iron status the ovx rats weighed significantly more at necropsy than the sham rats (Figure 1). Femur area was not correlated with body weight.

All of the ovariectomized rats regardless of iron status and animals fed the 6 ppm diet had significantly higher concentrations of IGF-I than did all other rats (Figures 2 and 3). Animals given the 6 ppm diet exhibited a significantly greater change from surgery to necropsy in alkaline phosphatase extracted from the third lumbar vertebra (L3) than did animals fed the 12 and 150 ppm diets (Table 6).

At pre-surgical urine collection, animals given the 6 ppm diet excreted more urinary DPD crosslinks than animals given all other diets, significantly more than the animals given the 35 and 150 ppm diets. The 6 ppm diet animals also excreted more urinary hydroxyproline than all other animals, significantly more than rats fed the 12 ppm

and 35 ppm diets (Table 7). The rats fed the 6 ppm diet excreted significantly more urinary Ca and Mg than all other rats (Table 8).

Ovariectomized rats regardless of iron status excreted significantly more urinary DPD crosslinks and hydroxyproline and had a significantly greater change in excretion from pre-surgery to necropsy than the sham-operated rats (Table 7). A significant diet x OHS interaction was seen for urinary Ca excretion with the 6 ppm sham and 6 ppm ovx groups tending to excrete more urinary calcium than all other groups, significantly more than the 12 ppm sham and 150 ppm sham groups (Table 8).

An OHS effect occurred for albumin and ALT; however, mean values remained within normal limits (data not shown). A diet x OHS interaction occurred in serum urea nitrogen with the 150 ppm sham animals having significantly higher values than all other animals except 12 ppm sham rats. A diet x OHS interaction was seen in serum glucose with 6, 12, 35 and 150 ppm ovx rats having significantly higher values than their respective sham groups (data not shown). Animals fed the 6 ppm diet exhibited iron deficiency as evidenced by increased red blood cell (RBC) numbers, mean cell volume (MCV) and reticulocyte (RETIC) numbers and decreased hemoglobin (Hb) concentrations. Ovariectomized rats regardless of iron status exhibited greater Hb concentrations than sham animals although values remained within normal limits (Table 9).

DISCUSSION

The increased and greater change in femur area and length in the 6 ppm ovx animals together with the lower femoral and L3 BMD in ovx animals suggests possible increased longitudinal growth without accompanying increase in density. This is also

supported by the animals fed the 6 ppm diet having greater change in femur BMD, BMC and cortical area than animals fed other diets as well as by the rats given the 6 ppm diet and sham rats regardless of iron status having the greatest change in L3 true density. Significantly increased femur and tibial length in ovx compared to sham animals has been previously reported (26, 27, 35). Increased bone formation and resorption with overall net loss in ovx animals compared to sham animals has been previously reported in the rat femur (1, 2, 15, 19, 26, 27, 29, 31, 33) and tibia (21, 34, 35). Increased femoral cortical area has been demonstrated in ovx rats (15). Related to the development of osteoporosis, this suggests that low lifetime dietary iron intake may exacerbate hormone-induced bone loss.

Despite pair feeding, ovx weighed significantly more at necropsy than sham animals. This is similar to other research in which animals were pair fed or ad libitum fed (1, 2, 7, 15, 19). Similar to other research, it appears no partial protection from osteopenia was provided by greater weight (29, 35).

The significantly higher IGF-I in rats fed the 6 ppm diet and in the ovx rats regardless of iron status suggests greater bone remodeling in these groups compared to the other groups. This increased IGF-I in ovariectomized versus sham animals corresponds with Kalu et al's (16) findings although their IGF-I values were higher than ours. The increased level of IGF-I is similar to other findings of increased bone regulatory hormones of serum cortisol and parathyroid hormone in iron deficient rats (5). Increased IGF-I in the ovx group corresponds with increased bone metabolism seen in ovx rats supplemented with medium doses of IGF-I exhibiting increased bone formation and in ovx

rats supplemented with high doses of IGF-I exhibiting enhanced bone resorption (22). It has also been found that serum IGF-I is an independent predictor of human female femoral BMD and suggested that IGF-I plays a direct role in the endocrine regulation of bone remodeling (4).

Increased bone remodeling is also suggested in rats given the 6 ppm diet by the greater change in alkaline phosphatase extracted from L3. This is similar to findings of elevated ALP in ovariectomized versus sham animals at 9 days and 1, 3, and 6 weeks post-surgery; 12 months post-surgery; and in cultured tibial surface cells in ovariectomized versus sham animals at 1 and 6 months post-surgery (31, 12, 6, 21). However, ovariectomized rats were found to show no difference in ALP compared to sham animals at 3 months post-surgery (21).

Further evidence of increased bone metabolism was seen in animals fed the 6 ppm diet with the greater pre-surgical excretion of urinary hydroxyproline and DPD crosslinks. Increased bone metabolism was also evidenced by the ovx animals' greater excretion and greater change in excretion of urinary hydroxyproline and DPD crosslinks. Studies have shown increased urinary excretion one or both of these bone resorption markers in ovx versus sham animals (31, 14, 6), and prevention of this increase in ovx animals with estrogen replacement (14). Iron overload but not deficiency been associated with elevated bone resorption markers in a study of men with PH who exhibited elevated urinary hydroxyproline compared to control men (9). A paucity of information exists on bone metabolism and iron in human females.

Considering that the majority of body calcium, magnesium and phosphorous is

contained in bone and the narrow tolerable serum range of calcium, the increased urinary calcium and magnesium excretion in rats fed the 6 ppm diet at surgery suggests greater bone resorption than bone formation in this group. Animals fed the 12 ppm diet also showed greater resorption than formation indicating increased resorption in both low iron groups. It has been demonstrated elsewhere that iron deficient rats excreted significantly more urinary calcium than control rats (5).

The increased urinary calcium excretion of the 6 ppm sham and 6 ppm ovx groups at final urine collection would also suggest greater bone resorption than bone formation. Others have reported greater Ca excretion following ovariectomy in rats (6, 12, 14, 26).

The diet x OHS interaction for serum urea nitrogen with the greatest value in the 150 ppm sham group indicates generalized tissue degradation in this group. This is supported by their significantly lower final body weight. This group also had lower food intake than all other groups (data not shown).

Our findings indicate significantly different blood and urinary indicators of bone metabolism based on both surgical and dietary treatment. Diet effects were seen in Hb; IGF-I; pre-surgical urinary DPD, hydroxyproline, calcium, and magnesium excretion; and change in alkaline phosphatase extracted from L3; change in femur BMD, BMC, and cortical area. Ovarian hormone status effects were seen in final body weight; Hb; IGF-I; urinary DPD and hydroxyproline excretion; change in urinary DPD crosslinks and hydroxyproline excretion; and femoral BMD as measured by DXA and L3 true density. Diet x OHS interactions were seen in urinary calcium excretion; femoral area; and femoral length; and in change in femoral area and length. Based on our findings, both estrogen

and iron deficiency seem to exert detrimental effects on bone metabolism.

Despite dietary effects prior to surgical treatment, in change from surgery to necropsy and diet x OHS interaction at necropsy on the above listed bone metabolism parameters, no dietary effects were noted for bone strength at necropsy. It has been hypothesized that bone strength may not be affected by ovariectomy as the greater appositional bone growth associated with ovariectomy partially compensates for cancellous bone loss (26). Also, it is possible that the dietary effects may have become overshadowed by the strong and well-known effects of hormone deficiency on bone. Another explanation may be that the necropsy timing was too long after surgery as rats continue to gain bone mass throughout the life span whereas humans do not; partial recovery from hormone-induced bone loss may have occurred in our rats. More elevated indices of bone resorption at fewer weeks post-ovariectomy have been previously described (6, 34).

In this study, dietary iron concentration impacted bone metabolism. Adequate iron intake may contribute, in part, to the prevention of osteoporosis, a chronic debilitating disease which is high in human and economic cost.

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Table 5 Effect of Diet and Ovarian Hormone Status (OHS) on Femur Variables of Female SD Rats[1,2]

	Area	Area Change	Length	Length Change	BMD	BMD Change	BMC
	mm^2	mm^2	cm	cm	g/cm^2	g/cm^2	grams
Diet and OHS							
6 ppm Sham	1.75 ± 0.03^{a}	0.18 ± 0.03^{a}	3.70 ± 0.03^{a}	0.19 ± 0.03^{a}	0.163 ± 0.002	$0.025 \pm 0.004^{a,*}$	9.24 ± 0.21
6 ppm Ovx	1.85 ± 0.03^{b}	0.28 ± 0.03^{b}	3.80 ± 0.03^{b}	0.29 ± 0.03^{b}	0.160 ± 0.002	$0.008 \pm 0.004^{a,\#}$	9.27 ± 0.20
12 ppm Sham	$1.78 \pm 0.03^{a,b}$	0.13 ± 0.03^{a}	3.70 ± 0.03^{a}	0.18 ± 0.03^{a}	0.158 ± 0.002	$0.013 \pm 0.004^{b,*}$	8.96 ± 0.21
12 ppm Ovx	$1.81 \pm 0.03^{a,b}$	0.17 ± 0.03^{a}	3.72 ± 0.03^{a}	0.20 ± 0.03^{a}	0.157 ± 0.002	$0.00041 \pm 0.004^{b,\#}$	9.13 ± 0.21
35 ppm Sham	$1.79 \pm 0.03^{a,b}$	0.16 ± 0.03^{a}	3.68 ± 0.03^{a}	0.17 ± 0.03^{a}	0.163 ± 0.002	$0.012 \pm 0.004^{b,*}$	9.47 ± 0.22
35 ppm Ovx	$1.79 \pm 0.03^{a,b}$	0.15 ± 0.03^{a}	3.71 ± 0.03^{a}	0.20 ± 0.03^{a}	0.159 ± 0.002	$(-0.0009) \pm 0.004^{b,\#}$	9.23 ± 0.21
150 ppm Sham	1.66 ± 0.03^{c}	$(-0.03) \pm 0.03^{c}$	3.58 ± 0.03^{c}	0.01 ± 0.03^{c}	0.157 ± 0.002	$(-0.0064) \pm 0.004^{c,*}$	8.59 ± 0.20
150 ppm Ovx	$1.81 \pm 0.03^{a,b}$	0.12 ± 0.03^{a}	$3.74 \pm 0.03^{a,b}$	0.17 ± 0.03^{a}	0.159 ± 0.002	$(-0.0072) \pm 0.004^{c,\#}$	9.29 ± 0.22
P Value							
Diet	NS	<0.0001	0.034	<0.0001	NS	<0.0001	NS
OHS	0.0024	0.0024	0.0003	0.0003	NS	0.0006	NS
Diet x OHS	0.0822	0.0822	0.0633	0.0633	NS	NS	NS

1 Diet groups not sharing the same letter are significantly different. 2 OHS groups not sharing the same symbol are significantly different.

Table 5 Effect of Diet and Ovarian Hormone Status (OHS) on Femur Variables of Female SD Rats[1,2]

BMC Change	Cortical Area	Cortical Area Change
grams	mm^2	mm^2
0.08 ± 0.01^a	6.37 ± 0.22	0.87 ± 0.22^a
0.07 ± 0.01^a	6.79 ± 0.21	1.28 ± 0.21^a
0.05 ± 0.01^b	6.39 ± 0.22	0.20 ± 0.22^b
0.04 ± 0.01^b	6.36 ± 0.22	0.16 ± 0.22^b
0.06 ± 0.01^b	6.68 ± 0.23	0.57 ± 0.23^b
0.03 ± 0.01^b	6.66 ± 0.22	0.56 ± 0.22^b
$(-0.02) \pm 0.01^c$	6.12 ± 0.21	$(-0.12) \pm 0.21^b$
0.02 ± 0.01^c	6.78 ± 0.23	0.54 ± 0.23^b
<0.0001	NS	0.0003
NS	0.1095	0.1095
NS	NS	NS

1 Diet groups not sharing the same letter are significantly different. 2 OHS groups not sharing the same symbol are significantly different.

Table 6 Effect of Diet and Ovarian Hormone Status (OHS) on L3 Variables in Female SD Rats[1,2]

	True Density	True Density Change	ALP	ALP Change
	g/cm^3	g/cm^3	uKat/g bone	uKat/g bone
Diet and OHS				
6 ppm Sham	$1.32 \pm 0.01^{*}$	$0.064 \pm 0.009^{a,*}$	25.21 ± 3.48^{a}	7.44 ± 3.48^{a}
6 ppm Ovx	$1.27 \pm 0.01^{\#}$	$0.020 \pm 0.008^{a,\#}$	23.20 ± 3.32^{a}	5.43 ± 3.32^{a}
12 ppm Sham	$1.32 \pm 0.01^{*}$	$0.048 \pm 0.009^{a,b,*}$	$17.15 \pm 3.48^{a,c}$	$(-5.57) \pm 3.48^{b}$
12 ppm Ovx	$1.28 \pm 0.01^{\#}$	$0.006 \pm 0.009^{a,b,\#}$	$20.80 \pm 3.48^{a,c}$	$(-1.92) \pm 3.48^{b}$
35 ppm Sham	$1.33 \pm 0.01^{*}$	$0.041 \pm 0.010^{b,c,*}$	$19.65 \pm 3.67^{a,c}$	5.86 ± 3.67^{a}
35 ppm Ovx	$1.28 \pm 0.01^{\#}$	$(-0.016) \pm 0.009^{b,c,\#}$	$19.16 \pm 3.48^{a,c}$	5.36 ± 3.48^{a}
150 ppm Sham	$1.33 \pm 0.01^{*}$	$0.021 \pm 0.009^{c,*}$	$16.80 \pm 3.32^{b,c}$	0.15 ± 3.32^{b}
150 ppm Ovx	$1.30 \pm 0.01^{\#}$	$(-0.010) \pm 0.010^{c,\#}$	$13.68 \pm 3.67^{b,c}$	$(-2.98) \pm 3.67^{b}$
P Value				
Diet	NS	0.0009	0.0873	0.0082
OHS	<0.0001	<0.0001	NS	NS
Diet x OHS	NS	NS	NS	NS

1 Diet groups not sharing the same letter are significantly different. 2 OHS groups not sharing the same symbol are significantly different.

Table 7 Effect of Diet and Ovarian Hormone Status (OHS) on Urinary Hydroxyproline (OH-Pr) and Deoxypyridinoline (DPD) Excretion

	OH-Pr Surgery	OH-Pr Necropsy	OH-Pr Change	DPD Surgery	DPD Necropsy	DPD Change
Diet and OHS	umol/umol cr	umol/umol cr	umol/umol cr	umol/umol cr	umol/umol cr	umol/umol cr
6 ppm sham	0.74 ± 0.081^{a}	$0.58 \pm 0.094^{*}$	$(-0.16) \pm 0.10^{*}$	8.2 ± 0.79^{a}	$3.8 \pm 0.84^{*}$	$(-4.4) \pm 0.80^{*}$
6 ppm ovx	0.68 ± 0.077^{a}	$0.82 \pm 0.090^{\#}$	$0.14 \pm 0.09^{\#}$	9.2 ± 0.75^{a}	$10.4 \pm 0.80^{\#}$	$1.2 \pm 0.80^{\#}$
12 ppm sham	0.51 ± 0.081^{b}	$0.39 \pm 0.094^{*}$	$(-0.13) \pm 0.10^{*}$	$7.0 \pm 0.79^{a,b}$	$3.2 \pm 0.84^{*}$	$(-3.8) \pm 0.80^{*}$
12 ppm ovx	0.57 ± 0.081^{b}	$0.73 \pm 0.094^{\#}$	$0.16 \pm 0.10^{\#}$	$8.7 \pm 0.79^{a,b}$	$8.6 \pm 0.84^{\#}$	$(-0.10) \pm 0.80^{\#}$
35 ppm sham	0.47 ± 0.091^{b}	$0.43 \pm 0.099^{*}$	$(-0.041) \pm 1.10^{*}$	6.6 ± 0.83^{b}	$3.5 \pm 0.89^{*}$	$(-3.1) \pm 0.85^{*}$
35 ppm ovx	0.54 ± 0.081^{b}	$0.55 \pm 0.094^{\#}$	$0.013 \pm 0.10^{\#}$	6.9 ± 0.79^{b}	$8.8 \pm 0.84^{\#}$	$1.9 \pm 0.80^{\#}$
150 ppm sham	$0.68 \pm 0.077^{a,b}$	$0.46 \pm 0.090^{*}$	$(-0.22) \pm 0.10^{*}$	5.5 ± 0.75^{b}	$2.4 \pm 0.80^{*}$	$(-3.1) \pm 0.77^{*}$
150 ppm ovx	$0.49 \pm 0.085^{a,b}$	$0.64 \pm 0.099^{\#}$	$0.16 \pm 1.00^{\#}$	7.9 ± 0.83^{b}	$8.0 \pm 0.89^{\#}$	$(-0.048) \pm 0.86^{\#}$
P value						
Diet	**0.0737**	NS	NS	**0.0363**	NS	NS
OHS	NA	**0.0013**	**0.0006**	**NA**	**0.0001**	**<0.0001**
Diet x OHS	NA	NS	NS	NA	NS	NS

1 Diet groups not sharing the same letter are significantly different. 2 OHS groups not sharing the same symbol are significantly different.

Table 8 Effect of Diet and Ovarian Hormone Status (OHS) on Urinary Mineral Excretion in Female SD Rats[1]

	Mg Surgery	Mg Necropsy	P Surgery	P Necropsy	Ca Surgery	Ca Necropsy
Diet and OHS	umol/umol/cr	umol/umol/cr	umol/umol/cr	umol/umol/cr	umol/umol/cr	umol/umol/cr
6 ppm sham	9.37 ± 0.81^{a}	8.11 ± 1.01	3.89 ± 0.40	4.00 ± 0.40^{a}	5.42 ± 0.52^{a}	7.38 ± 0.85^{a}
6 ppm ovx	7.70 ± 0.78^{a}	8.86 ± 0.96	3.84 ± 0.38	3.00 ± 0.38^{b}	3.74 ± 0.49^{a}	6.57 ± 0.85^{a}
12 ppm sham	6.14 ± 0.81^{b}	6.56 ± 1.01	3.91 ± 0.40	3.49 ± 0.40^{a}	2.18 ± 0.52^{b}	$3.83 \pm 0.85^{b,c}$
12 ppm ovx	7.62 ± 0.81^{b}	6.13 ± 1.01	4.57 ± 0.40	3.19 ± 0.40^{b}	3.77 ± 0.52^{b}	$5.89 \pm 0.85^{a,c}$
35 ppm sham	7.34 ± 0.86^{b}	6.82 ± 1.07	3.49 ± 0.42	3.69 ± 0.42^{a}	2.75 ± 0.58^{b}	$5.34 \pm 0.89^{a,c}$
35 ppm ovx	5.98 ± 0.81^{b}	6.00 ± 1.01	4.10 ± 0.40	2.85 ± 0.40^{b}	2.18 ± 0.52^{b}	$5.12 \pm 0.85^{a,c}$
150 ppm sham	7.32 ± 0.78^{b}	7.21 ± 0.96	3.90 ± 0.38	4.26 ± 0.38^{a}	2.49 ± 0.49^{b}	2.57 ± 0.81^{b}
150 ppm ovx	5.91 ± 0.86^{b}	7.60 ± 1.07	3.30 ± 0.42	2.99 ± 0.42^{b}	2.01 ± 0.54^{b}	6.23 ± 0.89^{a}
P value						
Diet	**0.0339**	NS	NS	NS	**0.0002**	**0.0197**
OHS	NA	NS	NA	**0.0033**	NA	**0.057**
Diet x OHS	NA	NS	NA	NS	NA	**0.0401**

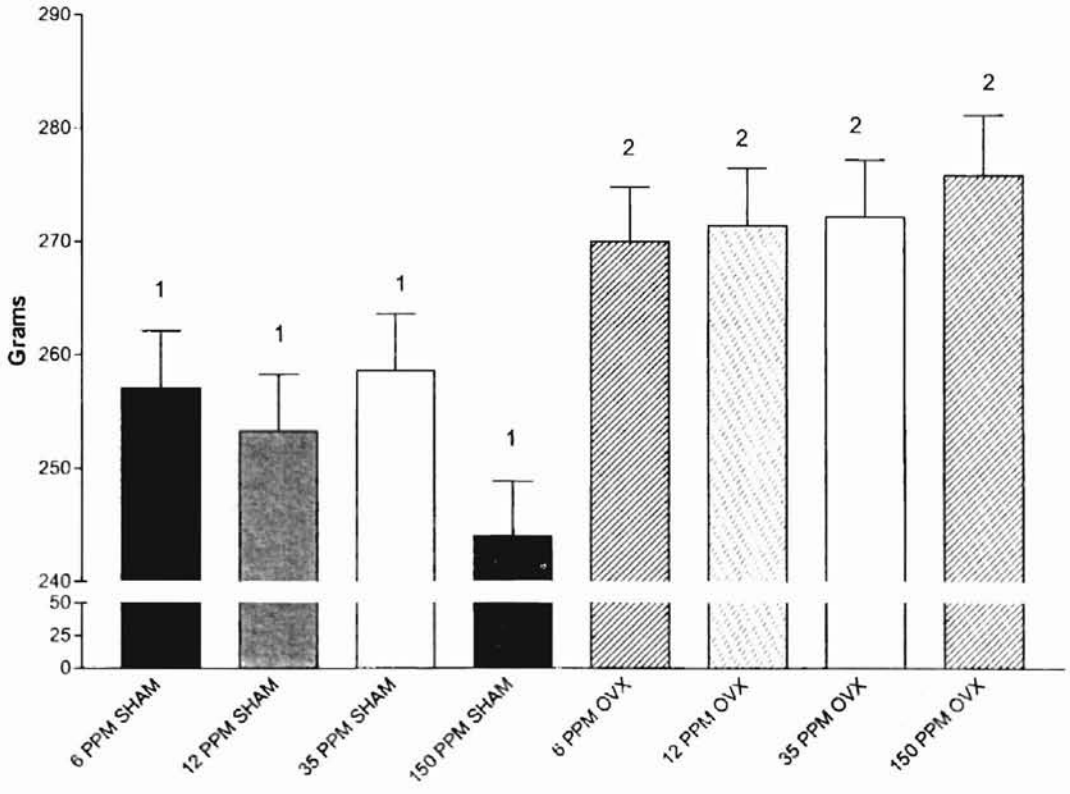
1 Diet groups not sharing the same letter are significantly different. 2 OHS groups not sharing the same symbol are significantly different.

Table 9 Effect of Diet and Ovarian Hormone Status on Hematological Status of Female SD Rats[1,2]

	RBC	HB	HCT	MCV	MCHC	PLT	RETIC
Diet and OHS	$10^6/mm^3$	g/dl	%	um^3	g/dl	$10^3/mm^3$	$10^6/mm^3$
6 ppm sham	7.80 ± 0.18^{a}	$13.41 \pm 0.24^{a,*}$	33.44 ± 1.43	47.30 ± 1.31^{a}	36.79 ± 1.30	597.00 ± 60.97	0.33 ± 0.025^{a}
6 ppm ovx	8.43 ± 0.19^{a}	$14.11 \pm 0.25^{a,\#}$	38.73 ± 1.51	46.89 ± 1.38^{a}	36.47 ± 1.38	531.00 ± 105.60	0.29 ± 0.028^{a}
12 ppm sham	7.46 ± 0.18^{b}	$14.27 \pm 0.24^{b,*}$	38.38 ± 1.43	51.70 ± 1.31^{b}	37.19 ± 1.30	638.33 ± 35.20	0.20 ± 0.025^{b}
12 ppm ovx	7.63 ± 0.21^{b}	$14.97 \pm 0.26^{b,\#}$	40.01 ± 1.60	52.38 ± 1.46^{b}	37.43 ± 1.46	566.57 ± 39.91	0.19 ± 0.028^{b}
35 ppm sham	7.38 ± 0.19^{b}	$14.40 \pm 0.25^{b,*}$	38.94 ± 1.51	53.13 ± 1.46^{b}	37.04 ± 1.46	541.33 ± 43.11	0.23 ± 0.030^{b}
35 ppm ovx	7.32 ± 0.21^{b}	$14.60 \pm 0.26^{b,\#}$	38.56 ± 1.60	52.75 ± 1.46^{b}	37.85 ± 1.46	587.86 ± 39.91	0.18 ± 0.028^{b}
150 ppm sham	7.50 ± 0.18^{b}	$14.17 \pm 0.24^{b,*}$	38.58 ± 1.43	51.60 ± 1.31^{b}	33.41 ± 1.30	579.38 ± 37.34	0.20 ± 0.027^{b}
150 ppm ovx	7.58 ± 0.21^{b}	$14.94 \pm 0.26^{b,\#}$	40.19 ± 1.60	53.13 ± 1.46^{b}	37.21 ± 1.46	571.75 ± 37.34	0.21 ± 0.028^{b}
P value							
Diet	**0.0012**	**0.0022**	NS	**0.002**	NS	NS	**0.0001**
OHS	NS	**0.0013**	NS	NS	NS	NS	NS
Diet x OHS	NS	NS	NS	NS	NS	NS	NS

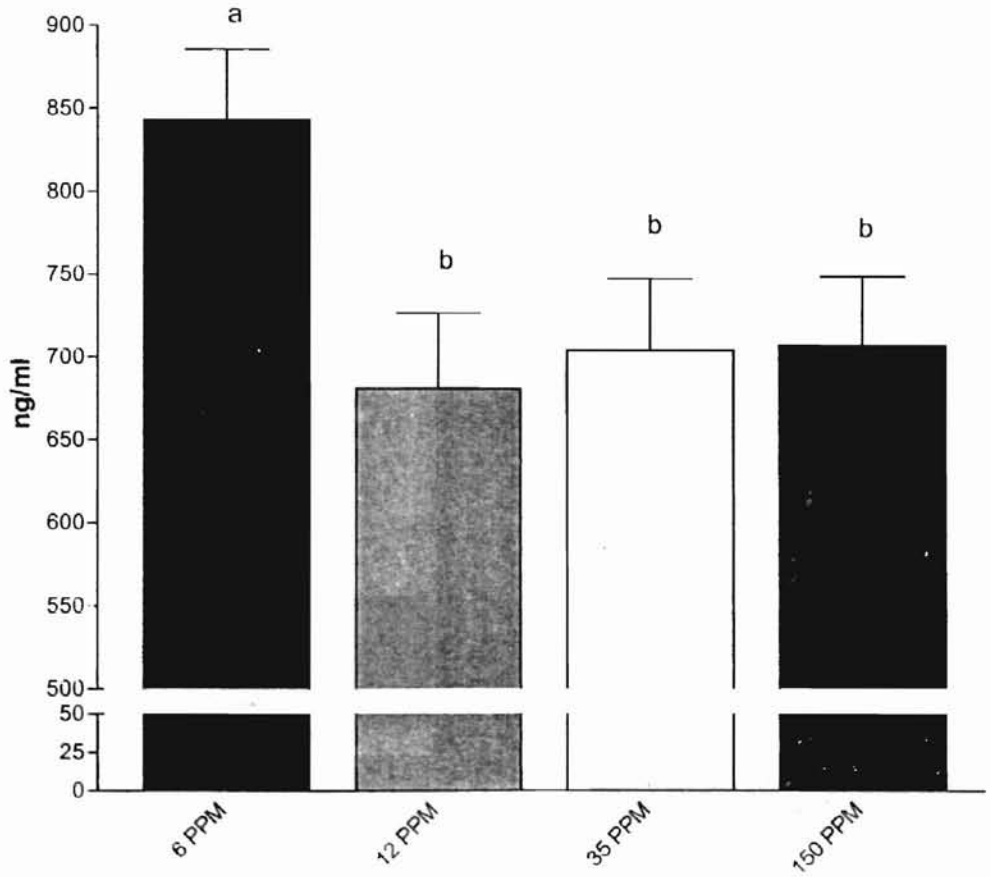
1 Diet groups not sharing the same letter are significantly different. 2 OHS groups not sharing the same symbol are significantly different.

Figure 1 Effect of dietary iron and ovarian hormone status on final body weight in female SD rats[1,2]



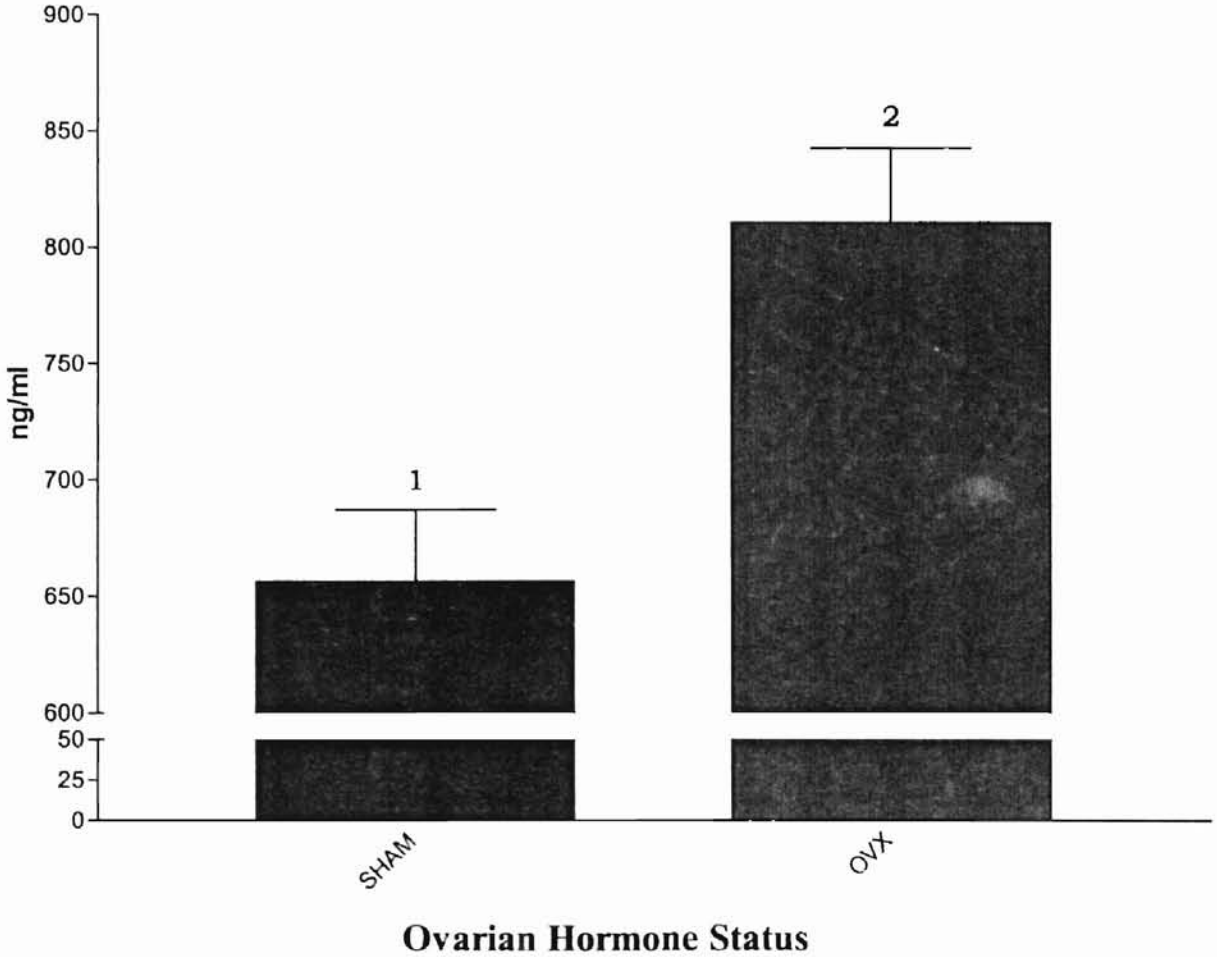
1 Treatment groups not sharing the same number are significantly (P<0.05) different.
2 No diet effect seen.

Figure 2 Effect of dietary iron on serum insulin-like growth factor I (IGF-I) in female SD rats[1]



1 Dietary groups not sharing the same letter are significantly (P <0.05) different.

Figure 3 Effect of ovarian hormone status on serum insulin-like growth factor I (IGF-I) in female SD rats[1]



1 Treatment groups not sharing the same number are significantly (P<0.05) different.

CHAPTER V

SUMMARY AND CONCLUSIONS

The purpose of this research was to further clarify the association between dietary iron consumption, hormonal status, and bone metabolism. We attempted to determine if significant differences would exist in blood, urinary, and radiographic indicators of bone metabolism and in bone parameters based on surgical and dietary treatment.

HYPOTHESIS TESTING

The results of hypothesis testing are given below.

1) There will be no significant differences by diet or OHS group and no significant diet x OHS interaction in final body weight. This was rejected for OHS as the ovx group was significantly heavier than the sham group. This failed to be rejected for diet and diet x OHS interaction as no significant differences were seen.

2) There will be no significant differences by diet or OHS group and no significant diet x OHS interaction in bone metabolism as measured by:

a) insulin-like growth factor-I (IGF-I) at necropsy. This was rejected for diet and OHS as the animals fed the 6 ppm diet and the ovx group exhibited higher serum levels than other groups. This failed to be rejected for diet x OHS interaction as no significant differences were seen.

b) alkaline phosphatase (ALP) extracted from bone at necropsy. This was rejected for diet, OHS and diet x OHS interaction as no significant differences were seen. However, this was rejected for change for diet as the animals fed the 6 ppm diet exhibited greater change from surgery than did other groups. This failed to be rejected for change

for OHS and diet x OHS interaction as no significant differences were seen.

c) urinary excretion of calcium (Ca) pre-surgery and at necropsy. This was rejected pre-surgery as the animals fed the 6 ppm diet exhibited greater excretion than other groups. At necropsy, this was rejected as a diet x OHS interaction was seen with 6 ppm sham and 6 ppm ovx exhibiting greater excretion than other groups.

d) urinary excretion of magnesium (Mg) pre-surgery and at necropsy. This was rejected pre-surgery as the animals fed the 6 ppm diet exhibited greater excretion than other groups. At necropsy, this failed to be rejected for diet, OHS, and diet x OHS interaction as no significant differences were seen.

e) urinary excretion of phosphorous (P) pre-surgery and at necropsy. This failed to be rejected pre-surgery as no significant differences were seen. At necropsy, this was rejected for OHS as the sham group exhibited higher excretion compared to the ovx group. This failed to be rejected at necropsy for diet and diet x OHS interaction as no significant differences were seen.

f) urinary excretion of free deoxypyridinoline (DPD) pre-surgery and at necropsy. This was rejected pre-surgery as the animals fed the 6 ppm diet exhibited greater excretion than other groups. At necropsy, this was rejected for OHS as the ovx group exhibited greater excretion and greater change from surgery than the sham group. This failed to be rejected at necropsy for diet and diet x OHS interaction as no significant differences were seen.

g) urinary excretion of hydroxyproline pre-surgery and at necropsy. This was rejected pre-surgery as the animals fed the 6 ppm diet exhibited greater excretion than

other groups. At necropsy, this was rejected for OHS as the ovx group exhibited greater excretion and greater change from surgery than the sham group. This failed to be rejected at necropsy for diet and diet x OHS interaction as no significant differences were seen.

3) There will be no significant differences by diet or OHS group and no significant diet x OHS interaction in bone size at necropsy as measured by:

a) femur length. This was rejected for diet x OHS interaction as the animals fed the 6 ppm ovx group exhibited greater length and greater change from surgery compared to 6 and 150 ppm sham groups.

b) femur area. This was rejected for diet x OHS interaction as the 6 ppm ovx group exhibited greater area and greater change from surgery compared to 6 and 150 ppm sham groups.

4) There will be no significant differences by diet or OHS group and no significant diet x OHS interaction in bone density of the femur (DXA and Archimedes') or L3 (Archimedes') at necropsy as measured by:

a) dual energy x-ray absorptiometry (DXA). This was rejected for diet as the animals fed the 6 ppm diet had greater change from surgery in femur BMD and cortical area than animals fed other diets. This was rejected for OHS as the sham group exhibited greater femoral density than the ovx group. This failed to be rejected for diet x OHS interaction as no significant differences were seen.

b) Archimedes' principle. This was rejected for diet as the animals fed the 6 ppm diet had greater change from surgery in L3 true density than animals fed the other diets. This was rejected for OHS for L3 as the sham group exhibited greater density than the

ovx group. This failed to be rejected for L3 for diet x OHS interaction as no significant differences were seen. This failed to be rejected for femur for diet, OHS, and diet x OHS interaction as no significant differences were seen.

5) There will be no significant differences by diet or OHS group and no significant diet x OHS interaction in bone mineral content at necropsy as measured by:

a) dual energy x-ray absorptiometry (DXA). This was rejected for diet as the animals fed the 6 ppm diet had greater change from surgery in femoral BMD than animals fed the other diets. This failed to be rejected for OHS and diet x OHS interaction as no significant differences were seen.

6) There will be no significant differences by diet or OHS group and no significant diet x OHS interaction in bone strength at necropsy as measured by:

a) femur fracture force. This failed to be rejected for diet, OHS, and diet x OHS interaction as no significant differences were seen.

SUMMARY AND CONCLUSIONS

Our findings indicate significantly different blood, urinary and DXA indicators of bone metabolism based on both surgical and dietary treatment. Diet effects were seen in Hb; IGF-I; pre-surgical urinary DPD, hydroxyproline, calcium, and magnesium excretion; change in bone ALP; and change in femoral BMD, BMC, and cortical area. Ovarian hormone status effects were seen in final body weight; Hb; IGF-I; urinary DPD and hydroxyproline excretion; change in urinary DPD and hydroxyproline excretion from surgery; and femoral and L3 BMD. Diet and OHS interactions were seen in urinary calcium excretion; femoral area and length; and change in femoral area and length from

surgery.

The low dietary iron regimen was adequate to produce iron deficiency in the animals given the 6 ppm diet. These rats were iron deficient as demonstrated by hematological indices whereas the other rats were not.

Despite pair feeding to the 150 ppm sham group after surgery, the ovariectomized animals were significantly heavier than the sham animals at necropsy. Reports on load bearing providing partial protection against ovarian hormone deficiency induced bone loss in rats have been conflicting (Mosley et al. 1997, Peng et al. 1994, Wronski et al. 1987, Westerlind et al. 1997, Roudebush et al. 1993). In this study, no correlation between final body weight and femur area and length was found. An association between dietary iron intake and bone density has been previously established in Caucasian women (Angus et al. 1988) but not fully investigated. Although our ovariectomized animals were heavier than the sham animals, no protection from bone loss appears provided as femoral BMD was significantly lower in the ovx versus sham group.

Increased bone metabolism is apparent in the ovariectomized rats compared to sham rats. Elevated serum IGF-I in the ovx versus sham animals indicates increased bone formation and corresponds with other research in rats and humans (Kalu et al. 1994, Mueller et al. 1994, Boonen et al. 1996). Elevated total urinary DPD crosslinks and OH-Pr excretion along with greater change in excretion of both from surgery in ovariectomized versus sham rats indicate increased bone resorption (Seibel et al. 1992). These findings are similar to other research findings in rats and humans (Sims et al. 1996, Goulding et al. 1996, Diamond et al. 1989, Conte et al. 1989). Although indicators of

both formation and resorption were increased in ovariectomized animals, net bone loss is apparent in the significantly decreased femoral BMD, decreased L3 true density, and the smaller change in these animals in L3 true density from surgery compared to sham animals. A trend of lower femoral BMC and greater medullary area in the ovariectomized versus sham rats also support greater bone resorption than bone formation.

While the effects of ovariectomy on rat bone are well-documented, the effect of dietary iron intake on bone has not been extensively investigated (Angus et al. 1988, Malecki et al. 2000). Diet effect on bone metabolism was seen in the animals given the 6 ppm diet. Animals fed the 6 ppm diet exhibited increased bone formation with higher serum IGF-I and bone ALP extracted from L3 compared to animals fed the other diets. In addition, diet x OHS interaction was noted in femur area and length with 6 ppm ovx animals having greater area, length and change in area and length from surgery compared to other groups. The rats given the 6 ppm diet also had the greatest change in femur BMD, femur BMC, femur cortical area, and L3 true density although there were no significant differences in the mean values of these variables. The animals given the 6 ppm diet also demonstrated increased bone resorption with increased urinary excretion of DPD crosslinks, OH-Pr, Ca, and Mg pre-surgery compared to animals given the other diets. Diet x OHS interaction occurred for urine Ca excretion at final urine collection with the 6 ppm sham and 6 ppm ovx groups excreting more than the other groups.

Although diet effects, OHS effects, and diet x OHS interactions occurred in the above listed bone metabolism parameters, no effects were noted for bone strength at necropsy. There are several possible explanations for this finding. The midshaft of the

femur may not be the optimal site for testing femur fracture force. The midshaft contains primarily cortical bone as opposed to the femoral neck which is predominantly composed of trabecular bone. Trabecular bone is more affected by ovarian hormone deficiency than is cortical bone. Also, human hip fractures most often occur in the femoral neck. Another explanation may be that necropsy was performed too long after surgery. More elevated indices of bone resorption at fewer weeks post-ovariectomy have been previously described (Wronski et al. 1987, Chan and Swaminathan 1998). Since rats continue to gain bone mass throughout the life span, partial recovery from hormone-induced bone loss may have occurred in our rats. As to lack of diet effect in strength testing, it is possible that the strong effect of ovarian hormone deficiency overwhelmed diet effects seen earlier. It is also possible that the rats fed the 6 ppm diet were recovering bone mass as evidenced by the greater changes from surgery to necropsy in femur BMD, BMC and cortical area and L3 true density. It has also been hypothesized that bone strength may not be affected by ovariectomy as the greater appositional bone growth associated with ovariectomy partially compensates for cancellous bone loss (Peng et al. 1997).

Looking at the larger experiment including the skeletally mature and the ovarian hormone altered rat phases, it appears that adequate dietary iron intake could represent an important factor in proper bone metabolism, particularly during growth. In our study, iron deficiency adversely affected bone during rapid growth, and the iron deficient animals experienced accelerated bone growth after skeletal maturity. As such, optimal iron nutrition, especially in growth, could possibly play a role in the prevention of the chronic and debilitating osteoporosis, a disease high in human and economic cost.

SUGGESTIONS FOR FURTHER RESEARCH

In this study, dietary iron intake impacted bone metabolism; however, additional research is necessary to further investigate this effect. Possibilities for further research include a larger study with an increased number of necropsy dates, including necropsy dates in the early weeks post-ovariectomy.

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APPENDIX

APPENDIX A
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE PROTOCOL APPROVAL

OSU

College of Veterinary Medicine
Laboratory Animal Resources
[illegible]

Memorandum

DATE: February 3, 1998

TO: Dr. Andrea Arquitt
Nutritional Sciences

FROM. Dr. K. Vargas
IACUC Veterinarian

SUBJECT: Protocol Approval

Your protocol, #709, entitled "Effects of Iron on Bone in Growing and in Mature Rats", has been approved for 108 rats by the Institutional Animal Care and Use Committee. The protocol is approved through January 31, 2001.

A modification must be submitted to the committee for approval prior to any changes in the protocol.

Institutional Assurance number A3722-01

Bringing Dreams to Life

OSU

College of Veterinary Medicine
Laboratory Animal Resources
Stillwater, Oklahoma 74078-2
405-744-6745

Memorandum

DATE: August 21, 1998

TO: Dr. Andrea Arquitt
Nutritional Sciences

FROM Dr. Archie Clutter
IACUC Chairman

SUBJECT: Modification Approval

The modification to protocol, #709, entitled "Effects of Iron on Bone in Growing and in Mature Rats", for addition of 12 rats has been approved by the Institutional Animal Care and Use Committee.

dgm

Bringing Dreams to Life

VITA

Dawn C. Kukuk

Candidate for the Degree of

Master of Science

Thesis: EFFECTS OF DIETARY IRON AND OVARIECTOMY ON BONE IN SKELETALLY MATURE OPERATED RATS

Major Field: Nutritional Sciences

Biographical:

Personal Data: Born in Oklahoma City, Oklahoma, on July 27, 1969, the daughter of Larry and Charlene Clark.

Education: Graduated from Perry High School, Perry, Oklahoma in May 1987; received Bachelor of Science degree in Nutritional Sciences from Oklahoma State University, Stillwater, Oklahoma in May 1998. Completed Dietetic Internship Program at Oklahoma State University, Stillwater, Oklahoma in August 1999. Completed the requirements for the Master of Science degree with a major in Nutritional Sciences at Oklahoma State University in July 2000.

Experience: Employed as a medical records clerk at Perry Memorial Hospital from1988 to 1992; employed as a medical transcriptionist by Perry Family Practice from 1992 to 1998; employed as a research and teaching assistant by Oklahoma State University and the Department of Nutritional Sciences from 1998 to 2000; employed as a banquet chef manager by St. Mary's Mercy Hospital 1999 to present.

Professional Memberships: Oklahoma Dietetic Association and American Dietetic Association.