EFFECTS OF DIETARY IRON ON BONE IN YOUNG MATURE FEMALE RATS

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Bachelor of Science

Brigham Young University

Provo, Utah

1998

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2000

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YOUNG MATURE FEMALE RATS

Thesis Approved:

Thesis Adviser

Dean of the Graduate College

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to my major advisor, Dr. Andrea Arquitt for her excellent guidance, encouragement and patience during my study. My sincere appreciation extends to my other committee members Dr. Bahram Arjmandi and Dr. Elizabeth Droke for their advice and suggestions. I would also like to extend my gratitude to Dr. Edralin Lucas, Brenda Smith and to my co-researcher Dawn Kukuk for their assistance, encouragement and friendship.

I especially wish to thank my family. They have always stood beside me providing love, support and encouragement.

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CHAPTER I

RESEARCH PROBLEM

Introduction

Two health problems facing the world today are iron intakes leading to iron deficiency or iron excess and osteoporosis (West, 1996). Iron deficiency is a world wide health problem, and iron excess is increasingly possible with the availability of nutrient supplements and with national food fortification policies. There are many factors involved in the pathophysiology of osteoporosis Therefore, prevention must take a comprehensive approach to include all factors. Studies have suggested that bone mass is affected by dietary factors and that minerals in addition to calcium may be factors (Angus et al 1988, Medeiros et al 1997). Previously, there has been little research investigating the association between bone metabolism and dietary iron. This study may propose dietary iron as one of the factors that can assist in preventive measures.

Bone Development

Bones are in a state of continual renewal consisting of resorption and formation Osteoclasts resorb old, damaged and underused bone. Osteoblasts replace the damaged bone and form new bone (Heaney, 1996). From infancy up to the age of 30, bone formation dominates, resulting in accumulation of bone mass (Matkovic, 1996). Ninety percent of peak bone mass is achieved by the age of 20, and the remaining 10% is achieved between the age 20 and 30 Beginning at about age 40, the rate of bone resorption surpasses the rate of bone formation resulting in a slow bone loss (Gallo,

1996). Comparing two populations with a difference in starting bone mass showed that with age, bone resorption occurred at the same rate. However, those with a higher initial bone mass suffered fewer hip fractures. This is attributed to a larger bone reserve provided by a high peak bone mass. Currently it is believed that the degree of attainment in peak bone mass during adolescence is a determinant for osteoporotic fracture later in life (Matkovic, 1996).

Osteoporosis

Osteoporosis is a chronic debilitating disease defined as skeletal fragility signified by a loss in bone mass and a deterioration in the microarchitectural tissue of the bone (Heaney, 1996). The effects of the disease are manifested in the elderly but the problems begin in adolescence.

Osteoporosis is very common and affects millions worldwide. Women are affected twice as frequently as men (Gallo, 1996, Barrett-Connor, 1995). This is due to the fact that men have 30% greater peak bone mass than women. Another factor is women experience bone loss at a faster rate during the first five to ten years after menopause because of estrogen deficiency. By the age of 70, men will lose 25% of cancellous and cortical bone, whereas women lose approximately 50% of cancellous bone and 35% of cortical bone (Fleming, 1992). Fractures are also more common among women because they live longer than men and they may reach the age of vulnerability more frequently (Barrett-Connor, 1995).

The most common fracture sites are the femoral neck, vertebrae, and wrist (Nichols et al, 2000). When calculating the economic costs of osteoporosis, fractures of

the hip are the main focus (Barrett-Connor, 1995). Hip fractures are easier to count because almost all cases are reported, hospitalized, and surgery is often required (Barrett-Connor, 1995, Lindsay, 1995). It was estimated that over 28 million Americans were affected equaling 1.5 million fractures and totaling $13.8 billion in 1995 (National Osteoporosis Foundation, 2000). With an increase in the elderly population it is predicted that this cost will also increase to $240 billion by the year 2040 (Cummings et al, 1990). This estimation is grossly underestimated. This total cost is not inclusive of fractures that do not require hospitalization or fractures other than the hip (Lindsay, 1995). Acute care costs were used to determine total cost, but acute care is only a fraction of the cost for overall recovery, which further underestimates economical costs associated with osteoporosis.

Osteoporotic hip fractures are associated with an increase in mortality rate (Barrett-Connor, 1995). Approximately 20% die within the first year of a hip fracture, and 13% die the next year (White et al, 1987). Each year 50,000 deaths are attributed to osteoporosis (Nichols et al, 2000). Those that live beyond 18 months following a hip fracture have a similar mortality rate as an individual equal in age that has never suffered a fracture (White et al, 1987).

In addition to economical costs, there are human costs that are often over looked. These two costs can overlap. This overlap occurs when hospital costs places financial burdens on the individual and then causes emotional stress (Barrett-Connor, 1995). Quality of life may greatly decrease. Activities of daily living that were once accomplished with little effort may become difficult to perform after fractures. This is partly due to the pain that one experiences and partly due to the fear of falling again.

Patients who undergo vertebral fractures have reported difficulty in carrying, lifting, shopping, and doing housework. Their physical appearances change and reports of low-self esteem have been high. All of these problems lead to a decreased social life (Barrett-Connor, 1995).

One of the solutions to reduce the risk of osteoporosis may be development of high peak bone mass in adolescence. Achievement of peak bone mass is a result of normal growth, and is influenced by many factors that are often interrelated These factors include genetics, age, body mass, gender, pubertal stage, physical activity, race, and nutrition (Matkovic, 1996). Many studies have shown the importance that calcium and vitamin D have on bone growth, but other minerals such as iron, copper and magnesium in addition to calcium and vitamin D have been demonstrated to influence bone growth (Angus et al 1998, Jones et al 1993, Mederios et al 1997).

Iron

Iron is an important component in the hydroxylation of collagen matrix, the connective tissue foundation of bone (O'Dell, 1981). It is this very matrix upon which insoluble mineral salts of hydroxyapatite are deposited (Koletzko et al, 1998). This allows the structure of bone to provide support to the body.

Iron Deficiency

Dietary iron intakes, both inadequate and excessive, have increasingly become a world wide problem. The most common micro-nutrient deficiency is iron (West, 1996). Looker et al (1997) estimated from data collected in the third National Health and

Nutrition Examination Survey (NHANES III) that about 700,000 children aged 1 to 2 years and 7.8 million adolescents and women in their childbearing years suffer from iron deficiency in the United States.

Those at risk for developing iron deficiency anemia are seen in particular subgroups in periods in life in which iron needs increase. Generally, this is also the period in which iron intakes are reported to be inadequate. Infancy and childhood are periods of inadequate iron intakes (Marx, 1997). Iron deficiency at such an early age can delay growth, mental and psychomotor development (Filer, 1990).

Adolescence is a period in which erythrocyte volume and muscle mass increase. For females, menstrual blood loss further increases iron needs of 0.5 to 1 mg per day of absorbed iron (Marx, 1997). Adolescent females often limit their food intake to maintain weight. Results from the Total Diet Study indicate that adolescents met less than 80% of the recommended dietary allowance (RDA) for iron (Pennington et al, 1989). Iron requirements for pregnancy increase due to the expansion of blood volume for the growth of the fetus, placenta and other tissues during the second and third trimesters (Anonymous, 1998). Iron supplements must be taken during pregnancy to meet this increased demand.

Iron Excess

Currently, health awareness has popularized the use of nutritional supplements (Greger, 1987). National surveys indicate that populations with higher incomes typically use supplements to achieve perceived ultimate health and fitness (Stewart et al, 1985). It has been estimated from the 1987 and 1992 National Health Interview survey that

approximately 24% of the population use supplements (Slesinski et al 1995). Nationwide food fortification policies are also seen as an answer to nutritional deficiencies. Toxic factors need to be considered when consuming supplements and fortified foods. Research has revealed complications of over consumption of vitamins such as vitamin A, B-6, C and D, and nicontinic acid (Greger, 1987). The toxic symptoms occurred from supplemental use rather than from food. Iron can become toxic to the body due to the fact that once absorbed, excretion is very slowly (Finch and Monsen, 1972). To maintain balance, iron absorption must keep pace with iron loss (Conrad et al, 1994). Any alteration to this balance leads to iron overload as well as iron deficiency. As seen in the patient with hemochromatosis, iron overload can cause damage to the liver, heart, pancreas and may even lead to death (Finch and Monsen, 1972). The problems associated with iron deficiency are recognized, but problems of iron excess have been underestimated (Conrad et al, 1994)

Osteoporosis is a chronic disease associated with many economical and human costs. To be able to prevent this disease, it is necessary to include all factors into a comprehensive approach. In an attempt to correct the problems of both osteoporosis and undesirable iron intakes, a common link may be found. This research has the potential to propose iron deficiency and iron excess as one of the many contributing factors leading to osteoporosis.

Purpose and Objectives

The purpose of this study was to determine the effects of inadequate, recommended, and excessive amounts of iron on bone density, bone strength, and

biochemical indicators of bone metabolism in young mature rats. The following objectives were developed for this study:

1. To determine the effect of inadequate, recommended, and excessive amounts of iron on bone density in young mature rats.
2. To determine the effect of inadequate, recommended, and excessive amounts of iron on bone strength in young mature rats.
3. To determine the effect of inadequate, recommended, and excessive amounts of iron on biochemical indicators of bone metabolism in young mature rats

Hypotheses

The following hypotheses were developed for this study:

1. There will be no statistically significant effect of dietary iron on bone density.
2. There will be no statistically significant effect of dietary iron on bone strength.
3. There will be no statistically significant effect of dietary iron on alkaline phosphatase extracted from bone.
4. There will be no statistically significant effect of dietary iron on insulin-like growth factor-I (IGF-I).
5. There will be no statistically significant effect of dietary iron on urinary excretion of deoxypyridinoline crosslinks.
6. There will be no statistically significant effect dietary iron on urinary hydroxyproline

Assumption

It is assumed the diets contained the calculated amount of iron (6 ppm, 12 ppm, 35 ppm and 150 ppm) after adjusting for iron content in cellulose.

It is assumed that the effects seen in bone densities, bone strength and biochemical indicators on bone metabolism were due to dietary iron.

Limitation

Rats lack Haversian systems; therefore, rats do not have the same pattern of bone remodeling as humans.

Abbreviations

ALP- Alkaline phosphatase

BMC- Bone mineral content

BMI- Body mass index

BMD- Bone mineral density

BD- Body weight

DXA- Dual energy x-ray absorptiometry

DPA- Dual-photon absorptiometry

Dpd- Deoxypyridinoline crosslinks

DZ- Dizygotic twins

IGF-I- Insulin-like growth factor

MZ- Monozygotic twins

NHANES III- the third National Health and Nutrition Examination Survey (1988-1994)

PBM- Peak bone mass

Pyd- Pyridinoline crosslinks

RDA- Recommended Dietary Allowance

SPA- Single-photon absorptiometry

Definitions

Bone mineral content- the amount of bone mineral per unit of area (grams).

Bone mineral density- grams of BMC per square centimeter of bone (g/cm^2).

Collagen- the protein substance of the white fibers of skin, tendon, bone, carilage, and all other connective tissue.

Endosteum- the tissue lining the medullary cavity of a bone.

Osteoblast- a cell that originates in the embryonic mesenchyme and during the early development of the skeleton and differentiates from a fibroblast to function in the formation of bone tissue. Osteoblasts synthesize the collagen and glycoproteins to form the matrix and with growth, develop into osteocytes

Osteoclast- also called osteophage. A large type of multinucleated bone cell that functions in the development and period of growth or repair, such as the breakdown and resorption of osseous tissue.

Periosteal bone- bone that forms in the perichondrium of the cartilaginous template

Proteoglycans- any of a group of polysaccharide-protein conjugates occurring primarily in the matrix of connective tissue and cartilage, composed mainly of polysaccharide chains, particularly glycosaminoglycans, as well as minor protein components.

CHAPTER II

REVIEW OF LITERATURE

There are many factors that influence the development of osteoporosis such as genetics, diet, mechanical loading, and environment. Therefore, prevention must take a comprehensive approach to include all factors. Useful indicators of bone growth and biomechanical measurements help to identify those at risk for developing osteoporosis and to monitor the effectiveness of treatment. Extensive research has been conducted on many of these risk factors; however, some areas are still lacking. Previously, there has been little research investigating the association between bone metabolism and dietary iron. This study uses a young rat model to determine the effects dietary iron has on bone density, bone strength, and biochemical indicators of bone metabolism.

Bone Development

Bone growth and the deposit of minerals begin at fetal development and continue throughout life (Koletzko et al, 1998). The structure of the bone is made up of collagen, proteoglycans and other non-collagenous proteins. This structure, bone matrix, is mineralized with insoluble mineral salts of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and small amounts of magnesium, sodium carbonate and citrate.

The matrix is controlled by two bone cells, osteoblasts and osteoclasts (Koletzko et al, 1998). Osteoblasts form bone and organize mineralization. Osteoclasts are responsible for bone resorption during which old damaged or dead bone is broken down and replaced with new bone (Heaney, 1996, Koletzko et al 1998). This process adjusts bone shape and density. The cycle of formation and resorption maintains bone

(Koletzko et al, 1998). The period from birth until longitudinal bone growth is achieved is characterized by continual bone modeling (Matkovic, 1996). This changes the structure and shape of the bone. Following this period, little change in bone volume occurs. The existing bone structure goes through the remodeling process of being formed and resorbed.

Bone mineral density (BMD) usually has reached its peak by age 18 in humans (Pocock et al 1987). However, bone mineral content (BMC) continues to increase between the ages of 20 and 40 years. Bone mineral density and BMC are influenced by many factors such as genetics, mechanical loading, diet, and environment (Chesnut,1991).

Genetics

Genetic factors accounts for 80% of the variance in BMD. The remaining 20% is related to environmental factors (Pocock et al 1987, Matkovic, 1996). Genetics can theoretically affect the development of osteoporosis in two ways (Smith et al, 1973). Genetics can affect the amount of bone mass achieved at skeletal maturity with those with a low bone mass being more susceptible to osteoporosis. Genetics can also affect the rate of bone loss during aging.

Lonzer et al (1996) studied 28 children between the ages of 5 and 20 years, and 24 parents from 16 families. Interviews were conducted to determine medical histories and family history of osteoporosis. Body mass index (BMI) was determined from height and weight measurements. Three-day food records estimated calcium intake. Dual Energy X-Ray Absorptiometry (DXA) scans determined BMC and BMD. Factors such

as BMI, height, weight and age of the children were significantly correlated with the children's BMD. There was a significant correlation between mean parental BMD and the mean of their children's BMD. When two postmenopausal mothers were excluded, there was a significant correlation between BMD of mothers and their children. Studies measuring the degree of family resemblance do not demonstrate the strength that genetics play in this resemblance (Chesnut 1991).

Another study supported genetics as a strong factor by looking at 31 adolescent females aged 14 years along with their biological parents (Matkovic et al 1990). Thirty premenopausal mothers aged 35-56 years and 24 fathers aged 38-53 years were measured for anthropometrics and bone mass at baseline. The adolescents were measured at baseline and again at 10, 18, and 24 months later. X-rays of second, third and fourth metacarpal bone were obtained on both hands to determine the external and internal metacarpal diameter as well as length. From these values total area, medullary area, and cortical area were calculated. Bone mass of the spine was determined by dual-photon absorptiometry (DPA). There was a high correlation for height and metacarpal length between mean values for parents and the daughters indicating a high genetic influence from the fathers as well as the mothers. This correlation of size and mass variables were higher than the correlation of density variables.

At age 14, the daughters' proportion of mothers' measurements were as follows: 99% of their mothers' height and bone length, 90% of mothers' cross-sectional area of the metacarpal, 85% of mothers' cortical area of metacarpal, 87% of mothers' bone mineral content of the spine, 80% of mothers' bone density at distal radius, and 95% of mothers' metacarpal and lumbar spine bone densities. At age 14 longitudinal bone

growth diminishes, however consolidation continues to increase. This observation supports early attainment of peak bone mass. At age 16 years these percentages increased to 93% of mother's cross sectional area of the metacarpal, 90% to 97% of mother's bone mass, 91% of bone density in distal radius, 98% of bone density at the metacarpal and 101% bone density in the spine. This study suggests that longitudinal bone growth diminishes at the age of 14 years while consolidation of bone continues. From these results it appears to be beneficial to look at pediatric patient's family histories of osteoporotic fractures to ensure adequate calcium intake and counseling on avoidance of risk factors related to osteoporosis.

Twin studies provide an effective way to investigate the influence that genetics have on BMD (Pocock et al, 1987 and Smith et al, 1973). Intrapair differences of monozygotic (MZ) twins and of dizygotic (DZ) twins were compared. In analyzing the data it was assumed that any intrapair variation among MZ twins is considered to be due to environmental factors and measurement error Intrapair variation among DZ twins is not only due to environmental factors and measurement error but also to genetic factors. The juvenile group contained 28 female and 20 male MZ twin pairs and 11 female and 12 male DZ twin pairs (Smith et al, 1973). The adult group were all men consisting of 38 MZ twin pairs and 42 DZ twin pairs. Using single photon absorptiometry (SPA), measurements of bone mass and width on the right midshaft radius was obtained. For MZ and DZ groups in juvenile and adult twins, the variation of intrapair differences in height, weight, bone mass and width were computed and compared by analysis of variance. In the juvenile group, bone mass and width increased with age. The regression coefficients were significantly higher for DZ twins than for MZ twins. There were no

significant differences in mean bone mass or width in the MZ and DZ twins. For mass and width, the variance within DZ juvenile twins was four times greater than within the MZ juvenile twins. Similar results were found among the adult twins for variance within the DZ and within the MZ twins; however, the intrapair variance was greater in the adult twins than in the juvenile twins. This provides evidence that genetics influence both bone mass and bone width.

Sixty-five twin pairs, monozygotic (MZ) and dizygotic (DZ), were studied by Pocock et al (1987). Approximately half of the female twins were postmenopausal Bone mineral density and BMC were measured in various sites using DP3 dual photon absorptiometry and single photon densitometry. The sites measured were lumbar spine (L2-L4), right femur, distal radius and ulna. Anthropometrics such as weight, height and BMI were measured and calculated. Physical fitness was measured as subjects exercised on a bicycle ergometer at a known work load. The work load was used in conjunction with a pulse rate that was steady for 2 minutes to assess the VO_2max. Results showed no difference in BMI and physical fitness between MZ twins and DZ twins. Bone mineral density correlation was greater at all sites between MZ twins than DZ twins. In premenopausal women all sites had a greater correlation between MZ twins than between DZ twins. In determining heritability at different skeletal sites in premenopausal women there were significant correlations for the spine, forearm, and two sites in the proximal femur; however, at the femoral neck there was not a significant correlation. This indicates that genetics influence BMD in the spine and that environmental factors have a greater influence on variation of bone mass in the femur (Pocock 1987).

Mechanical Loading

Mechanical stimulation is shown to be a key factor in promoting bone health and is dependent upon the mode, duration and intensity of the exercise (Wheeler et al 1995). Those that have engaged in weight-bearing exercises have been shown to increase their BMC and BMD between 5% to 20%. This positive effect on BMD is directly by affecting the skeleton and indirectly by increasing muscle mass (Keen 1999). Muscle bulk and activity applies more loading to the bone. A cellular appartus (within the osteocytes) senses the degree of bending placed by mechanical loading or exercise (Heaney, 1996). This degree of bending leads to a deformation of 0.1% to 0.15% in any given dimension. Signals are sent to adjust the cycle of bone formation and resorption to either increase or decrease the bone mass. As bone renews itself it not only replaces old tissue, but allows for optimal density.

Yeh et al (1993) studied the effects of exercise and immobilization on bone formation and resorption in young growing rats. After a week of adaptation to diet and environment the rats were injected with 15 μCi/d ^{45}Ca for 2 days. After the last injection six rats were killed to determine baseline values. The remaining animals were randomized into control, immobilization, and exercise groups. Part one of the experiment ended after 21 days and part two of the experiment ended after 42 days. Exercise rats started at moderate exercise and gradually increased in intensity and duration to a final speed of 20 m/min for 60 minutes on a flatbed treadmill. Bilateral sciatic denervation at day 0 was performed for the immobilization group. Consecutive urine and fecal collections from day 1 to day 36 were obtained. The feces were ashed and then diluted to determine the excretion of ^{45}Ca. The results represented urine and

fecal radioactivity for excretion of three days. Atomic absorption spectrophotometry measured urine calcium and represented the average daily urine excretion. Both femurs were removed and ashed to determine calcium, phosphorus, and ^{45}Ca radioactivity. By using these values calculations were made to determine percent of basal bone resorption, the amount of calcium released, and the amount of calcium deposited. The left tibia was removed for histomorphometry. Cancellous bone area was measured in the mid-metaphysis below the growth plate. By dividing the mean distance from the two tetracycline bands by the days in the labeling period, longitudinal bone growth (μm/day) was calculated. Cortical bone parameters obtained were medullary area, cortical bone area, periosteal suface measuring osteoblast number, endosteal resorbing surface and tetracycline-labeled areas at the endosteal and periosteal surfaces. Periosteal bone formation rate (amount of mineralized bone formed/day at periosteum), endosteal bone formation rate (amount of mineralized bone formed/day at the endosteum), and periosteal bone apposition rate (reflecting osteoblast activity and measures the width of mineralized bone added/day) were all determined from tetracycline-labeled area.

The immobilized rats were lower than control and exercise rats in body weight, femoral dry weight, ashed weight, calcium and phosphorus content. The ^{45}Ca retention was lower at 6 weeks for the immobilized rats than control rats. Estimated bone resorption was significantly higher in the immobilized rats than the control group throughout the entire study. Estimated bone formation in exercise rats was not different from control group after 3 weeks; however, after six weeks bone formation was significantly higher by 23% and bone resorption decreased to only 60% of the control group.

Excreted levels of ^{45}Ca were consistent with formation and resorption results. Urinary and endogenous fecal paths were combined to represent the ^{45}Ca from the entire skeleton. Levels of ^{45}Ca in whole body were significantly higher in the immobilized group than the control group throughout the experiment. During the first 2 weeks, excretion was higher in exercise rats than control rats, and then continued to significantly decrease below those of the control group after 5 weeks.

Exercise rats had an increase in cortical bone area, and the length of the tibia was greater. At the periosteal surface the bone formation rate of exercise rats was not significantly different from the control group until during days 32-4. There was a decreased rate of periosteal bone formation in immobilized rats than in control rats during the first 31 days, but there were no differences from 32-41 days between the groups Studies demonstrate that bone formation decreases with age; however, these results suggest that resorption is accelerated during periods of inactivity with exercise delaying this aging induced bone change. The response of bone to immobilization occurred immediately whereas effects of exercise were not seen until one month after exercise. The reason for this delay is unknown; however, it may be due to the amount of mechanical loading Because exercise increased in intensity and duration throughout this study, the initial amount might not have exceed the minimum stimulus level that altered bone formation

The exact amount of intensity, duration, frequency and mode of exercise needed to increase BMD is not known (Wheeler et al 1995). Therefore, investigation of the effects of various intensities and duration of exercise was conducted. Eight-four female Sprague-Dawley rats at 120 days old, weighing ≈233g were randomized into nine

exercise training groups. Three intensities of low (≈ 55%VO_{2max}), medium (≈ 65% VO_{2max}), and high (≈75% VO_{2max}) were subdivided into duration of 30, 60, and 90 min/d to total nine exercise groups for a duration of ten weeks. Twelve rats refusing to run were used as the control group and activity was limited to walls of their cages.

Both femurs and right tibia were collected. The biomechanical parameters (torque at failure, twist angel at failure, energy absorbed at failure, stiffness and stress at failure) were either measured using a rapid loading torsional testing machine or were calculated. Morphometric measurements (total bone tissue area, marrow area, cortical bone area, mean cortical thickness, and polar moment of inertia) were calculated from outer and inner perimeters of cross-section from the left tibia. Dual energy x-ray absorptiometry measured BMD of vertebral segments and the left tibial diaphysis.

There was no significant difference in weight gain among rats; therefore, the differences noted in bone changes were results of exercise and not of weight gain or loss. When exercise rats were analyzed collectively, total bone tissue area, cortical bone area, and mean cortical thickness had an overall trend of significantly greater values than the control groups. Rats exercising at a high intensity had significantly smaller angles of twist before failure and less energy absorbed at failure and than control rats. The torque at failure and stress at failure were significantly lower for the high intensity rats than the low and medium intensity rats. The rats exercising for 90 minute duration exhibited statistically greater stiffness and statistically lower angle of twist at failure and energy absorbed at failure. The BMD for all exercise rats when analyzed collectively was significantly higher than the control rats for both the tibia and vertebral segment.

Adaptive changes in bone mineral density, mechanical response and morphology

was seen in the exercise groups; however, it appears than repetitive high intensity exercise may have a negative effect due to a small recovery time of bone. The increase in cortical bone mass and cortical thickness showed that the exercise rats were able to adapt to their loading environment. The measurements of bone mineral density showed an increase in bone mass.

Diet

Malnutrition is associated with delays in skeletal growth (Caulfield et al 1995). The Institute of Nutrition of Central America and Panama (INCAP) conducted a longitudinal study from 1969 to 1977 to study the effects of nutritional supplements on child growth and development in four rural communities in Gautemala. Children from the age of 0-7 years were divided into two supplemental groups Two of the villages received Atole which was a high caloric, high protein drink while the other two villages received Fresco which was a low caloric, no protein drink. Only Atole contained phosphorus and calcium but both supplements contained the same amount of iron, flouride and some vitamins. Children could consume as much supplement as they wanted, and the amount was measured daily. In 1988 a follow up study was conducted (Caulfield et al 1995) The subjects were then 11 to 27 years. Bone mineral content, radial bone width and bone density were measured using SPA. Height and weight were measured. Mean measurements appeared to be greater among those that consumed Atole than Fresco; however, the only significant differences were for mean energy intake for both boys and girls and for stature and radial bone width for girls. In comparison to NCHS reference data of children not in the study both supplements significantly

increased all measurements. When controlling for age, gender, and supplement type these measurements are still significantly greater for those consuming more calories; however, they were diminished by this adjustment. This is important to note because 33% – 65% of the variation in bone mineralization was accounted for these three factors. The amount of calories consumed, irrespective of supplement type, showed statistically significant effects on bone mineralization. The effects of supplementation on bone mineralization were no longer statistically significant once weight and height were added into the regression models. There was a positive association with weight and stature and the amount of supplemental energy when controlling for age and gender but not supplemental type. This suggests that the increases in weight and stature due to supplementation had the effect on increase bone mineralization and not the type of supplement.

Calcium

Calcium is the most studied nutrient in respect to bone studies (Angus et al 1988). Ninety-nine percent of total body calcium exists in the skeleton (Sandler et al 1985). It has been suggested that calcium intake throughout life affects peak bone mass (Angus et al 1988). If available calcium is inadequate for physiological needs, the body draws calcium from the skeleton to support serum calcium homeostatsis (Sandler et al 1985).

The Total Diet Study conducted in 1982 through 1989 estimated the average intake of adolescent female ages 14 to 16 years in the United States was 733 mg. This intake is a concern since it was 61.1% of the RDA (Pennington et al, 1989)

A two year longitudinal study was performed to determine the effect of calcium

on bone status (Matkovic et al, 1990). Using a 3 day food record the girls were stratified into two groups; the control group was placed on a diet containing <850 mg Ca/d and the calcium supplemented group was placed on a diet containing >850 mg Ca/d. Dietary interviews were conducted at six months, and anthropometerics and bone mass were measured at baseline, 10, 18 and 24 months. Over the two-year longitudinal study both groups had significant gains in bone mass and bone density; however, there was a trend for the variables to increase as calcium increased. A significant difference between the two groups was not seen possibly due to the small sample size.

A retrospective study assessed the association between adolescent consumption of milk and postmenopausal bone density (Sandler et al, 1985). Inclusion criteria was at least one year postmenopause without estrogen replacement therapy. Two hundred fifty-five white, middle to upper class women aged 49 to 66 years participated. A three-day diet record estimated current calcium intake. A food frequency questionnaire estimated calcium intake through various stages of life: childhood, adolescence, early adulthood (20-35 years), late adulthood (36-50 years), and periods of pregnancy or lactation. For each stage milk consumption was classified into the following categories: consumed with every meal, sometimes with every meal or never or rarely with meals. Current mean calcium intake was 720 mg Ca/d, but 70% were below the RDA for postmenopausal women. There was no significant correlation between baseline bone density and current calcium intake. Half of the population reported milk consumption with every meal; however, with each successive stage, milk consumption decreased to only 6% in mid-adulthood drinking milk with every meal. Those that drank milk with every meal during childhood and adolescence had significantly higher bone densities than those that

sometimes drank milk with meals. There was a positive linear relationship between bone density and the frequency of milk consumption. This study suggests that calcium intake in childhood and adolescence has protective qualities against adult bone loss.

One-hundred sixty Caucasian women aged 23-75 including pre and post menopausal women were studied to examine the influence of dietary factors on bone mass in sites more prone to fractures due to osteoporosis (Angus et al 1988). Bone mineral density was measured by DPA in the second to fourth lumbar vertebrae and three sites in the right proximal femur: the femoral neck, the Ward's triangle within the femoral neck, and the greater trochanter. Using SPA, BMC was measured in the distal radius of the forearm. Four consecutive food and fluid records were obtained along with a semiquantitative food frequency questionnaire to provide usual food habits and to cross-check the four-day food record. Food records were analyzed for nutrient content The questionnaire provided an estimate of calcium intake during childhood, adolescence and early adulthood. Body mass index was calculated from height and weight measurements.

Food records indicated that the mean calcium intake for the pre and postmenopausal women was 759 and 717 mg/day, respectively. Sixty-five percent premenopausal women and 83% postmenopausal women failed to meet the Australian Recommended Dietary Allowances (RDA) of 800 and 1000 mg of calcium/day. The majority of women failed to meet the RDA for zinc, magnesium and iron. The questionnaire and measurements of forearm BMC revealed postmenopausal women who drank more than 600 ml of milk/day before the age of 20 had a significantly higher BMC than those that drank less than 300 ml of milk/day. In premenopausal women,

magnesium and iron had a positive correlation with forearm BMC. This study suggests that bone mass is affected by dietary factors and that minerals in addition to calcium may be factors.

Iron and Bone Development

About 90% of collagen is Type 1 collagen and is found in bones and tendons to give tensile strength and rigidity (O'Dell 1981). The collagen molecule is made up of a coil of three polypetide units known as □-chains. Procollagen is first produced during the biosynthesis of collagen. From the □-chains, peptide extensions from the amino and carboxy ends are removed by a process known as hydroxylation. Within Type 1 collagen the amino acid content is about 14% hydroxyproline with lesser amounts of hydroxylysine. Hydroxyproline and hydroxylysine must be incorporated into the peptides. This is done by post-translational hydroxylation. There are three enzymes used to catalyse the hydroxylation of specific amino acid residues in the -chains which are proloyl-4-hydroxylase, lysyl hydroxylase and prolyl-3-hydroxylase Ferrous iron is required as a cofactor by all these enzymes. Hydroxylation must occur before helix formation can take place. Proline hydroxylation is necessary for triple helix formation, which is necessary for cellular secretion of collagen.

Effects of Iron Deficiency on Bone.

There is little research to support the hypothesis that iron deficiency affects collagen metabolism (O'Dell 1981). This may be due to the fact that other pathologic signs, such as anemia, are manifested before changes in collagen hydroxylation

takes place.

Rothman et al (1971) induced anemia in rats to investigate the effect iron may play in fracture healing. Rats were randomly assigned into two groups. The experimental group received a low iron diet and had four milliliters of blood withdrawn weekly for four weeks to induce anemia. The control group had the same diet with adequate iron. Hematocrit and serum iron levels verified iron deficiency anemia in the experimental rats. The right fibula was surgically fractured in the mid-shaft for the experimental and control rats. The three time periods studied after the fracture were 3, 6, and 8 weeks. At each time period forty rats were sacrificed. Fracture healing was evaluated by tensile strength and by microscopic examination of histologic serial sections of the site of fracture.

Tensile strength at three weeks showed the fractured fibula of the control group to be 89% of the geometric mean compared to the normal intact fibula. The fractured fibula of the anemic group did not have enough strength to bear the minimum load of the instrument. At 6 weeks the control group was 98% of the geometric mean and the anemic group was 36% of the geometric mean compared to the normal intact fibula for tensile strength. Eight weeks showed the control group to be 96% of the geometric mean and the anemic group was 66% of the geometric mean compared to the normal intact fibula for tensile strength. These differences were significant.

The stages of healing begin as the initial site of injury shows the presence of the hematoma, which notes the damage to the bone and soft tissue (Rothman et al, 1971). Then during the second stage beginning in only a few days, the hematoma organizes, and after a week a fibrocartilaginous callus forms. Slowly this fibrocarilaginous callus is

converted to bony callus. The histologic examination of the control group at three weeks showed the cartilaginous callus had begun transformation. At the fracture site the cartilage had formed a plate where active osteogenesis had started with the formation of trabeculae of fibrous bone. At six weeks the transformation to bony callus was complete, and the process toward normal configuration by remodeling and reorganization was occurring. Remodeling continued at 8 weeks with wideness of the fracture site fading. The anemic group did not follow similar stages of healing. The fracture site closed off from the medullary canal and showed non-union. After 3 weeks osteogenesis occurred but to a lesser extent. At six and eight weeks, retardation of fracture healing remained.

Similar results were found on fractures of rabbits when induced with normovolemic or with hypovolemic anemia (Heppenstall and Brighton 1977). Thirty rabbits were divided into three groups after fracture of the fibulae occurred. Group A had 20 ml of blood removed and then reinfused into the same animal. Group B had the same amount of blood drawn; however, only the plasma was re-injected into the animal. In group C the blood was removed and discarded. After 21 days the animals were sacrificed and a three point bending test was perfomed on the fibulae. Reoentgenograms and histology were performed. The control group (A) results were compared to groups B and C. Groups B and C had a mean hemacocrit statistically lower than group A indicating anemia. Evidence from the roentgenograms revealed healing among groups A and B though C showed a delay. The strength of the fibula in group C was significantly lower than that of groups A and B which were similar. It appears from this study that if iron deficiency anemia exists and the blood volume is maintained, then oxygen delivery does not change. Group C was unable to repair the fracture due to decreased oxygen delivery

secondary to blood loss as well as to loss of plasma constituents.

Effects of Iron Excess on Bone. Many diseases that exhibit iron overload have demonstrated signs of osteoporosis (de Vernejoul et al 1984). A study involving ten female pigs in which five pigs were treated daily with 300 mg of iron dextran was conducted to determine whether iron overload triggers bone changes. After 36 days of treatment the pigs in the treatment and control groups were killed. Blood samples, urine, and two epiphyses of main metatarsal bones for bone mineral evaluation were collected. The pigs had been double tetracyline labeled, and a fragment of trabecular bone from the iliac crest was taken for measurement of trabecular bone volume, osteoblast surfaces, osteoclast resoption surfaces, depth of the lacunae, mean osteoid thickness and appositional rate. Using these measurements, total labeled surfaces, formation at the tissue level, and duration of the formation period were calculated.

Iron treatment had no effect on 24-hour urine calcium, phosphate and hydroxyproline, or on serum 25-OHD and serum 1,25-$(OH)_2D$ levels. Bone minerals and ash content were unchanged except an increase in bone iron content in treated pigs. Histomorphometry showed that there was no difference in trabecular bone volume and osteoid thickness between the groups. Osteoblast, appositional rate and formation rate at tissue level decreased in treated animals. The duration of the formation period was not significantly different. The osteoclast surface and the depth of lacunae resulting from resorption showed no difference in treated pigs.

From these results there appeared to be an imbalance between bone formation and bone resorption. However, this imbalance did not decrease bone mass as indicated by trabecular bone volume and bone ash content. The length of the experiment may explain

why a decrease in bone mass was not observed. The experiment may have been too short to give an opportunity to see changes in these values.

Dietary Iron

Iron Deficiency. Dietary iron intakes, both deficiencies and excesses, have increasingly become a world wide problem. The most common nutrient deficiency is iron. It was estimated from data collected in NHANES III that about 700,000 children aged 1 to 2 years and 7.8 million adolescents and women in their childbearing years suffer from iron deficiency in the United States (Looker et al, 1997).

Those at risk for developing iron deficiency anemia are seen in particular subgroups of the population. There are periods in life in which iron needs increase. Generally, these are also the periods in which iron intakes are reported to be inadequate. Infancy and childhood are one of these periods of inadequate iron intakes. A full term infant has iron stores that will meet the body's requirements until four to six months of age. During this stage of life growth is rapid and iron intake is inadequate, therefore, iron deficiency anemia is usually seen around age nine months. For a preterm or low-birth-weight infant the risk of iron deficiency anemia is even greater due to lower iron stores. Iron deficiency at such an early age can delay growth, mental and psychomotor development (Marx, 1997)

Adolescence is another period of rapid growth in which erythrocyte volume and muscle mass increase (Marx, 1997). For females, menstrual blood loss further increases iron needs of 0.5 to 1 mg per day of absorbed iron. Adolescent females often limit their food intake to maintain weight (Anonymous, 1998). The National Health and Nutrition

Examination Survey (NHANES III) data indicate that only 25% of adolescents meet the RDA for iron. Eleven percent of non-pregnant women from ages 16 to 49 are iron deficient (Anonymous, 1998). Iron requirements for pregnancy increase due to the expansion of blood volume for the growth of the fetus, placenta and other tissues during the second and third trimesters. Iron supplements must be taken during pregnancy to meet this increased demand.

Iron Excess. Currently, health awareness has popularized the use of nutritional supplements (Greger, 1987). National surveys indicate that populations with higher incomes typically use supplements to achieve perceived ultimate health and fitness. It has been estimated from the 1987 and 1992 National Health Interview survey that approximately 24% of the population use supplements (Slesinski et al 1995). Nationwide food fortification policies are also seen as an answer to nutritional deficiencies (Greger, 1987). Toxic factors need to be considered when consuming supplements and fortified foods. Research has revealed complications of over consumption of vitamins such as vitamin A, B-6, C and D, and nicontinic acid. The toxic symptoms occurred from supplemental use rather than from food. Iron can become toxic to the body due to the fact that once absorbed, excretion is very slow To maintain balance iron absorption must keep pace with iron loss. Any alteration to this balance leads to iron deficiency as well as iron overload (Conrad et al, 1994) As seen in the patient with hemochromatosis, iron overload can cause damage to the liver, heart, pancreas and may even lead to death (Finch and Monsen 1972). The problems associated with iron deficiency are recognized, but problems of iron excess have been underestimated (Conrad et al, 1994).

Adolescents

The highest level of bone mass is achieved during adolescence and is the result of normal growth (Matkovic, 1996). It is important to understand the age of peak bone mass (PBM) attainment to prevent osteoporosis.

The Saskatchewan Pediatric Bone Mineral Accrual Study began in 1991 (Bailey 1997). The purpose of this study was to examine bone mineral accretion in growing children. Two hundred twenty-eight students from age of eight to fourteen years participated. The objective was to develop bone mineral density and bone mineral content standards for normal developing children based on information on bone mineral accretion. Each year BMC of the whole body, lumbar spine (L1-L4) and proximal femur were measured by DXA. Twenty-four hour recalls and physical activity questionnaires were administered three times a year for the first three years, then twice a year for the remainder of the study. Macro- and micronutrient intakes including calcium were determined from analyzing the 24-hour recalls. Distance and velocity growth curves for height and BMC for the lumbar spine, the femoral neck and the total body were developed by using the data collected over a 5 year period. The results (when pooling by 1 year age groups) showed that the boys reached peak linear growth at age 13.5 years and girls reached it at age 11.6 years. At these ages both boys and girls attained 90% of adult status in height, 70% in BMC at the femoral neck, and 60% for the total body and lumbar spine. One year after peak linear growth the rate of bone mineral uptake peaked for both males and females showing a dissociation between linear growth and bone mineral accrual. Two years before and after peak linear growth is the period known as the growth spurt and is a critical time for bone mineral accretion. During this four year period, over

35% of total body and spine bone mineral and over 27% of the bone mineral at the femoral neck were deposited. The bone mineral content accumulation during this time is more than the amount most people will lose during adult life. Peak skeletal mass attained during the growth years accounts for 50% of the variability in bone mass in the elderly. Therefore, the growing years determine much of the fracture risk in the elderly

Matkovic et al (1994) found similar results to the Saskatchewan Pediatric Bone Mineral Accrual Study when determining the timing of PBM. Other multiple skeletal sites such as spine, proximal femur, neck, Ward's triangle, trochanter, and forearm were measured in the same subject. All measurements of bone mass were measured by DXA with exception of the forearm which was measured on a SPA. Two hundred sixty-five premenopausal Caucasian females from eight to 50 years were studied. Subjects with a BMI above 29.9 were excluded from the study. At several sites, accumulation of bone minerals continued to increase one to seven years after skeletal height had been reached at the age of 16 years. The attainment of peak height by 16 years of age indicates decrease in longitudinal bone growth; however, accumulation of bone mass continues at various sites. Between the ages of 11 and 15 years 37% of total skeletal mass had been accumulated as compared to 35% found in Bailey's study (1997) Between the ages of eight and 16 years the average gain in height was 2.4% of the peak adult height for women per year, and the accumulation of total bone mineral between these ages was 6% per year Between the ages of 18 and 50 years the BMC and BMD changed only slightly and was not a statistically significant change. Peak bone mass of the proximal end of the femur appeared to be ≈17 years of age, and older premenopausal women had significantly lower BMD than the younger women. This suggests that once PBM of the

proximal end of the femur was achieved there was an immediate decline in BMD.

At the age of 14, females had similar values to their mothers' for bone size, mass and density. Longitudinal growth ceased while consolidation continued to increase. Most epiphyses closed at the age of 16. For both male and female, 95-99% of PBM was achieved by the age of 18 (Koletzko et al., 1998).

The risk of developing osteoporosis was lower for those who had higher bone mass as young adults. When the two communities with different calcium intakes were studied they found that the rate at which bone loss occurred was the same; however, the risk of hip fracture for those that started with a greater PBM was lower (Matkovic, 1996)

Osteoporosis

Osteoporosis is defined as skeletal fragility characterized by low bone mass and by a degeneration of structural bone tissue (Heaney, 1996). As defined by The World Health Organization, a bone mass value more than 2.5 standard deviations below a normal young adult mean is considered to be at risk of fracture (Nichols et al, 2000, Heaney, 1996). The assessment of bone health is accomplished through measurements of BMC. Bone mineral content is expressed as the amount of bone mineral per unit of area (grams). Bone mineral density is expressed as grams of BMC per square centimeter of bone (g/cm^2). The spine, hip and wrist are sites commonly measured because these are the sites most common for fracture risk in osteoporosis.

The pathophysiology is multifactorial (Nichols et al, 2000). Factors that increase the risk of osteoporosis are diseases (anorexia nervosa, HIV, hyperparathyroidism, insulin-dependent diabetes, renal failure, hyperthyroidism, inflammatory bowel disease,

and multiple sclerosis), inherited factors (race, gender, and familial history of osteoporotic fracture), and environmental factors (low body weight, cessation of menstrual period, low calcium intake, inactivity, corticosteroid use, smoking, alcohol, and caffeine). Treatment of osteoporosis depends on the cause.

Osteoporosis is a major public health concern (Nichols et al, 2000). It is related to significant increases in morbidity, mortality and economic burden. Despite the advances made in the last few year in understanding of prevention strategies, screening, detection and treatment, osteoporosis continues to escalate. One out of two women is affected by osteoporosis at some time in her life. It affects 25% of white, postmenopausal women in Western countries (Angus et al 1988). Each year medical costs associated with osteoporosis are close to $13.8 billion (National Osteoporosis Foundation, 2000). Each year 50,000 deaths are attributed to osteoporosis (Nichols et al, 2000).

Indicators of Bone Growth

A focus on decreasing the cost of osteoporosis has interested many in developing and improving indicators of bone remodeling (Calvo et al 1996). By using hisomorphometry of a biopsy specimen, bone status can accurately be assessed (Calvo et al 1996 and Russell 1997). This technique is invasive, however Ideal measurements should be easily and frequently obtained without risk or discomfort; biochemical markers of serum and urine could function in this manner. These indicators should identify those at risk, diagnose early, and determine effective therapy for those with osteoporosis. Indicators must be unique to bone, reflect total skeletal metabolism and be validated by

comparing with traditional measures of bone remodeling.

Bone Specific Alkaline Phosphatase

Bone specific alkaline phosphatase is a biochemical marker of bone formation. It is an enzyme found in the membrane of the osteoblasts and is an indirect measure of osteoblast activity (Delmas 1993, Bikle 1997, Russell, 1997). Alkaline phosphatase expression starts once cessation of cell proliferation occurs and it reaches a maximum during matrix maturation and decreases as matrix mineralization declines (Risteli and Risteli, 1993). Several roles have been proposed for alkaline phosphatase in bone formation: increased local concentrations of inorganic phosphate destroy inhibitors of mineral crystal growth, transport phosphate, or act as a calcium binding protein (Calvo et al 1996).

Demineralized bone matrix powder was implanted subcutaneously into the shaved upper chest of rats of various ages (Nishimoto et al 1985). Alkaline phosphatase activity, total calcium and histological examinations were measured at the site of implants in 1-, 3-, 10-, and 16-month-old rats to assess bone formation. Results showed that alkaline phosphatase activity peaked two weeks after injection in 1- and 3- month old rats and was significantly different from 10- and 16-month-old rats In the 16-month-old rats alkaline phosphatase levels were nearly half the alkaline phosphatase levels in 1- and 3-month-old rats. There was a significant reduction of bone formation between 1- and 3-month-old rats as measured by total calcium accumulated in demineralized bone matrix powder implants. Bone histology at 2 weeks postimplantation showed new bone in 1-month old rats, while evidence of new bone was lacking in 16-month old rats. These results

demonstrate that indicators of osteogenic activity decrease with age. The most surprising result was the significant decrease in bone-forming capacity between 1- and 3-month-old rats.

Insulin-Like Growth Factor

Bone growth and development are directly affected by insulin-like growth factor (IGF-I). IGF-I is known to stimulate chondrocytes in the growth plate and to increase the synthesis of DNA, proteoglycan, protein and collagen leading to an increase in long bone growth (Thompson et al, 1996 and Burtis and Ashwood, 1994). The circumference of long bones increased as IGF-I stimulated osteoblastic periosteal bone formation. An increase in skeletal and serum IGF-I levels has been shown to be parallel to increases in bone mass growth during childhood (Mora et al 1999). Other studies have demonstrated that the increase in bone mass is mainly due to the increase in bone size. Even though high levels of IGF-I results in an increase in size the bone mineral density tends to be unaffected.

Skeletal parameters were compared to IGF-I levels to determine an association between the two (Mora et al 1999). One hundred ninety-seven healthy white children and adolescents participated in this study. Blood was drawn after an overnight fast to determine serum IGF-I levels. Cortical bone density, cross-sectional area, and cortical bone area were measured at the midshaft of the femur by computed tomography (CT) The results showed that a direct relationship between serum IGF-I levels and age, weight, and other anthropometric measurements, as well as femoral cortical bone and femoral cross-sectional area. Even after accounting for the confounding variables of femoral

length and body weight there was still a positive correlation between IGF-I and femoral cross-sectional and cortical bone areas. There was no correlation between IGF-I levels and cortical bone density. These results are in conformity with other studies that suggested that IGF-I is a determinant of bone geometry, but not of bone density.

Growth hormone (GH) stimulates the largest of the IGF binding proteins (IGFBP-3). Thompson et al (1996) studied the effects of IGF-I on bone growth by injecting neonatal rats with monosodium glutamate (MSG) to deplete hypothalamic growth hormone-releasing hormone (GHRH) producing neurons and chronically decrease serum GH levels. Of interests were the changes in size, mineral and connective tissue density and content in rat femurs. Results demonstrated that naso-anal length, femur length and humerus length of MSG injected rats were significantly lower than controls. This suggests that the decrease in GH levels due to a deficiency in hypothalamic GHRH, leads to diminished production and activity of circulating growth factors such as IGF-I which is GH dependent.

Urinary Deoxypyridinoline Crosslinks

Organic matrix of bone is 90% type 1 collagen which is crosslinked by specific molecules to give rigidity and strength. These crosslinking amino acids are the pyridinium crosslinks, pyridinoline (Pyd) and deoxypyridinoline (Dpd) (Branca et al, 1992, Riis 1993, Delmas 1993). The highest concentration of Pyd is found in type II collagen of cartilage and lower concentrations are found in type I collagen of bone; however, Dpd is found only in type I collagen of bone (Riis, 1993, Seibel et al 1992). Dpd is formed during the maturation of collagen fibrils in cartilage and bone. During the

bone resorption process Dpd is released into the cirulation and excreted unmetabolized in urine. Because urinary excretion is unaffected by diet, Dpd is a suitable marker for assesing resorption and providing analytical data on the rate of bone metabolism. In patients with vertebral osteoporosis, Dpd levels were correlated with bone turnover measured by bone histomorphometry (Delmas 1993).

Thirty-six postmenopausal women with vertebral fractures were studied to compare values obtained from pyridinoline assay to histomorphometry (Delmas et al 1991). None of the patients were being treated for osteoporosis at the time. The proximal and distal forearm bone mass measured by SPA was observed to be significantly lower than in sex- and age-match controls of postmenopausal women without vertebral fractures. Pyridinoline and Dpd were measured on fasting urine samples. An iliac crest biopsy after labeling with demethylchlortetracycline and oxytetracycline was performed. The following parameters were measured: the eroded surface and the osteoclast surface, bone formation ratio (BFR), BFRs (surface based), BFRv (volume based), and BFRt (tissue based).

When compared with normal healthy premenopausal controls, Pyd and Dpd were higher in 50% of the osteoporotic patients. There was a positive correlation between urinary Pyd and Dpd and both osteoclast surface and bone formation. There were significant correlations between the osteoporotic patients and osteoclast surface and bone formation rate. Possibly a decrease in the maintenance of some coupling between resorption and formation may explain these results. It is believed that there are limitations of histologic parameters of resorption that decrease the validity of the assessment of the rate of resorption. Histologic parameters are static, and they are limited

to the cancelous envelope and not cortical bone, which is 80% of the skeleton. Despite these limitations, urinary Pyd and Dpd, especially Dpd, were significantly correlated with the osteoclast surface suggesting that it is a useful bone resorption marker

Hydroxyproline

Hydroxyproline is found in collagen and is an indirect meausre of bone breakdown (Rao and Rao 1980). Urinary excretion is used to measure the changes in collagen metabolism. Since the breakdown products of collagen are not reused for collagen biosynthesis it has been shown to be a reliable index of collagen catabolism. Any changes in urinary hydroxyproline were due to changes in collagen synthesis rate, the conversion rate of one form of collagen to another, or the rate at which either form is degraded (Rao and Rao 1980).

Urinary hydroxyproline is expressed as a ratio to urinary creatinine to provide estimates of excretion rates in relation to lean body mass (Horowitz et al 1984). This ratio also corrects for bladder emptying error detected in short, timed urine collections

Horowitz et al (1984) used urinary hydroxyproline to measure the effects of calcium supplementation on bone resorption. Fourteen postmenopausal women were admitted after a 12 hour overnight fast, had blood and urine samples collected on admittance, and then were fed a diet containing 800 mg of calcium plus a 1000 mg calcium supplement for eight days. Blood and urine samples were collected eight days later after an overnight fast Plasma calcium, phosphate, creatinine, alkaline phosphatase and urinary phosphate, creatinine, and hydroxyproline were measured. Results demonstrated that daily supplemental calcium had a protective effect on bone resorption

The hydroxyproline/creatinine ratio significantly decreased from 0.022 to 0.017 in a matter of eight days.

Male and female rats varying in age 4, 8, 12, 16 and 20 months were studied to establish the influence of age on urinary hydroxyproline (Blanusa et al 1978). Hydroxyproline was measured from 24-hour urine samples. Results from analysis of variance showed that urinary hydroxyproline significantly decreased with age in both sexes. This may reflect a decrease in metabolic activity of collagen.

Measurements of Bone

Dual Energy X-Ray Absorptiometry (DXA)

In the past dual-energy projection methods (DPA) were used to estimate body composition (Going et al 1993). Dual photon absorptiometry was originally developed to estimate bone mineral content but was also able to estimate total and regional-body soft tissue fat and fat free mass. The accuracy was good but there were precision errors. Dual photon absorptiometry was replaced with dual energy x-ray absorptiometry (DXA) to provide better precision while accurately estimating lean mass of soft tissue, fat free mass of soft tissue and total-body bone mineral (Going et al 1993, Svendsen et al, 1993)

Seven pigs were studied to compare DXA with chemical analysis (Svendsen et al, 1993). After DXA measurements the pigs were killed and homogenized with a total body grinder and a total-body pelletizer Random samples of approximately 500 g were taken divided to analyze for percent fat determined by chemical fat extraction and the ash weight Calculations made from percent fat determined fat mass of soft tissue and total-body bone mineral. Results showed total-body bone mineral measured by DXA was

highly correlated with ash weight, and percent fat, fat mass of soft tissue, and lean body mass was highly correlated with chemical analysis. The fatty and lean elements of the body were directly measured by DXA. Dual energy x-ray absorptiometry measurements had high accuracy with chemical analysis.

Densitometry estimates of fat have shown significant errors due to fluctuations in body water and in bone mineral. Going et al (1993) investigated whether DXA was able to detect small changes in soft-tissue composition induced by changing the hydration status of 17 males and females. Over three consecutive days, patients were placed in dehydration-rehydration periods to induce changes of approximately two percent of body weight. Body composition measurements of standing height (SHt), body weight (BW), body density (BD), total-body water, total body bone mineral content, and soft-tissue composition, were taken at baseline after a 12 hour fast with out exercise and on the following two days during the dehydration period. For eight hours they ate and drank ad libitum. A second 12-hour period without food and exercise prepared them for the following day's measurements. After day two, subjects were maintained without liquid and exercise for 24 hours to induce weight loss by dehydration. The last 12 hours food was also withheld, and measurements were taken at the end of the 24-hour fast. The rehydration period began on day three; a volume of water equal by weight to 75% of the weight lost during the dehydration period was replaced. After a complete void 1 ½ hours later, BW was measured again. Water was ingested equal to 120% of the weight difference from baseline plus the amount equal to the volume lost during urination. All measurements were repeated after a final void one hour later. Urine specific gravity was measured to confirm changes in hydration status. Dual energy x-ray absorptiometry

measurements were compared with measurements from hydrodensitometry. A high correlation was found between baseline measurements of BW and total mass from DXA. After the dehydration and rehydration periods the same relationship was found between BW and total mass. At baseline there was also a high correlation between percent fat and fat free mass (FFM) from the densitometry and DXA (percent fat in males, $r = 0.92$ and FFM, $r = 0.95$; percent fat in females $r = 0.98$ and FFM, $r = 0.99$). The same correlation was seen after the dehydration and rehydration periods. During the dehydration and rehydration periods total-body bone mineral remained approximately unchanged Because water is found primarily in lean tissue, the change found in BW should be equal to the changes seen in lean tissue mass, and bone mass would remain unchanged. These findings supported DXA as an accurate measure for estimating small changes in individual components of body composition.

Biomechanical Measurements

Biomechanical testing provides information on mechanical integrity of bone (Turner and Burr 1993). A useful way to measure the mechanical properties of small animal bones is by conducting bending tests. Both tensile and compression stresses are a result of bending. Tensile stress occurs when the material is stretched Compression stress occurs when the material is compacted The fracture usually occurs on the tensile side, because bone is weaker in tension.

Either a three-point or a four-point bending test can be performed (Turner and Burr 1993). In a three-point bending test the force is applied perpendicular to the long axis of the bone at the mid-shaft by a crosshead moving at a constant speed, while fulcra

support the two ends of the bone (Kiebzak et al 1988). Three-point bending is the preferred method when using rodent bones (Turner and Burr 1993) due to it's simplicity. A four-point bending test requires that the force at all four loading points be equal, and this is difficult to ensure when using irregularly shaped bones from rodents.

Second moment of area is a mechanical parameter used to describe the geometry of the cross-sectional area of the bone (Kiebzak et al 1988). The equation is as follows:

$$\text{Second Moment of Area (for an ellipse) } (cm^4) = \frac{\Pi}{64}[(BD^3) - (bd^3)]$$

B = outside diameter perpendicular to the point of applied force.

D = outside diameter parallel to the point of applied force.

b = inside diameter perpendicular to the point of applied force.

d = inside diameter parallel to the point of applied force.

This equation is necessary when calculating bending stress.

A second mechanical parameter is stress, which is expressed as force per unit area of bone. The equation is as follows:

$$\text{Stress } (kgf/cm^2) = \frac{\text{force x length x C}}{\text{4 x second moment of area}}$$

force = the force used by the mechanical testing instrument at the yield point

C = ½ of the bone diameter (D) that is parallel to the direction of applied force

Stress takes into account geometry of the cross-sectional area of the bone and the distance between the two supports. It is dependent on the tissue composition of the bone rather than it's structure. Therefore stress measurements allow comparisons of tissue strength of different bone sizes and shapes.

The mechanical properties of bone vary according to the method of preparation

and hydration. Freezing the bone at -20°C in saline-soaked guaze has been shown to be an effective method of long term preservation (Turner and Burr 1993). A human femur frozen at -20°C for 20 days rather than -70°C exhibited no change in bending properties of the bone. Peng et al (1994) tested the effects of freezing on femur and tibia in 20 of the 30 rats. The remaining ten rats were selected to measure the strength of fresh bone. The contralateral leg was stored at -20°C in plastic test tubes with some soft tissue remaining for one week. The leg was then thawed at room temperature and cleaned of adhering tissue. In both frozen and fresh bones, all femur fractures occurred at the basal part of the femoral neck. Measurements included inner and outer diameters of cross section, transectional areas of the marrow cavity and cortical bone, and failure loads of the femoral neck, and three-point bending of the tibia. There were no significant differences between fresh and frozen bones for all measurements.

A dry bone will increase in strength but decreases in toughness making it brittle (Turner and Burr 1993). For test results to be accurate, hydration is necessary. This can be achieved by soaking the bone in physiological saline.

Copper is a mineral that is also involved in collagen formation (Jonas et al, 1993) Copper is the cofactor for lysyl oxidase, an enzyme needed for intra- and inter-molecular cross-links in collagen. A deficiency of copper has been shown to be associated with bone fragility in man and in animals. To determine whether a decrease in bone strength is due to changes in the inorganic material or due to a collagen defect, copper deficiency was induced in seven pairs of weanling Wistar rats, and mechanical strength was tested. One of the rats in each pair was fed a copper deficient diet while the other rat was fed a copper sufficient diet. To control for significant differences in weight, the animals were

pair fed. Copper deficiency may decrease appetite and may result in lower growth rate than the control rats. The rats were killed after 56 days, and both femora were cleaned of soft tissue and tested on a torsional loading instrument. The liver was also collected to analyze copper content. The torsional loading method was used because it produced the same type of loading found in humans during trauma (Burstein and Frankel, 1971). Measurements (length of femur, outside diameters at the midshaft, and cortical thickness measured at midshaft once bone was fractured) were obtained to estimate biological variability. From the load-deformation graphs, the maximal torque, the torsional stiffness, the ultimate angular deformation, and the absorbed energy was determined. The bones were ashed to measure ash weight and calculate percentage of ash in fat-free dry bone. The calcium content of bone was also determined. Rats were similar in weight, femur length, measures of midshaft external diameter, and cortical thickness at the midshaft. Severe copper deficiency was confirmed by liver copper assays. The maximal torque, ultimate angular deformation and energy absorption during bone loading was significantly lower in the femora of the copper-deficient rats as compared to the control rats. There were no significant differences in percentage bone ash or calcium content, showing that the decrease in bone strength was not due to changes in inorganic material. The decrease in bone strength was possibly due to changes in the collagen matrix of the bone.

The effects of copper and iron deficiency on mechanical strength and mineral composition of bone were studied (Medeiros et al, 1997). Three week old, male rats were randomly assigned to either copper deficient diet, iron deficient diet or the control diet Rats remained on diet for six week and then were killed for analysis. Femurs were dried

to constant weight in an oven at 37°C before testing for breaking strength using an Instron. The force was applied at a rate of 200 mm/min. Radiograms of the left femur were used to calculate total area, cortical area and medullary area. Single photon absorptiometry was used to measure BMC and BMD of femurs. Cortical area was significantly lower in the copper and iron deficient rats than controls. Medullary area was significantly greater in the copper and iron deficient rats. There were no significant differences in the copper and iron deficient diet groups compared to controls in total area, BMC and BMD. Fracture force was significantly lower in both the copper and iron deficient rats than controls. Fracture force in copper deficient rats was similar to the results found by Jonas et al (1993). It appeared that the decrease in bone strength was possibly due to inability to form collagen crosslinks rather than to a decrease of inorganic material.

Hogan et al (2000) evaluated different methods for testing mechanical properties of cancellous bone in the proximal tibia. With most methods of testing bone strength, cortical bone is tested along with cancellous bone; however, with estrogen deficiency osteoporosis the greatest loss occurs in the cancellous bone. The proximal tibia metaphysis was tested using two different methods: whole slice compression that compresses the entire specimen (cortical and cancellous bone) and reduced-platen compression that compresses only the cancellous bone in the center of the sample. A three-point bending test was performed on the femur, and all three methods were compared. Twenty six rats aged 15 weeks were divided into two groups; half were ovariectomized (OVX) and half were sham operated for controls. The rats were killed at 19 weeks, and femurs and tibias were collected, cleaned, weighed, and measured. Both

extrinsic and intrinsic measurements were made. Extrinsic measurements refer to the whole bone considering the tissue material properties in addition to bone size and shape. Extrinsic measurements include maximum force, stiffness, displacement or deformation at maximum force and energy to maximum force. Intrinsic measurements refer only to tissue material properties. Intrinsic measurements include ultimate stress, elastic modulus, and strain at ultimate stress. The mean weight of the OVX group was significantly greater than the sham group. There were significant increases in length of the femur and tibia in the OVX group, and cross-sectional moment of inertia of the femur was significantly higher indicating continued modeling of cortical bone and longitudinal bone growth. All extrinsic and intrinsic measurements, except for displacement at maximum force, were significantly lower in the OVX group compared to the sham group in the reduced-platen compression test. In the whole-slice compression test all measurements (except displacement at maximum force, specimen thickness, and total cross-sectional area) were significantly lower in OVX than sham; however, the values were half of the values from the reduced-platen compression test. From the three-point bending test, only maximum force was significantly higher in OVX than sham, this result is expected because three point bending also tested cortical bone. The effects of ovariectomy were minimal on cortical bone. After comparisons of testing methods it was clear that cortical bone decreased the sensitivity of the test's ability to detect changes in the cancellous bone. Therefore, a test in which only the strength of cancellous bone is determined, such as reduced-platen compression test, is more sensitive to changes in bone than the three point bending test.

Rat Model

Animal models should have anatomic and physiologic similarities to humans (Aufdemorte et al, 1993). Animal models should parallel closely to humans in disease pathogenesis and progression, have similar exposure to risk factors, and be able to be manipulated to induce disease or control for disease. Convenience, relevance and appropriateness are characteristics an animal model should possess (Aufdemorte et al, 1993 and Kalu, 1991).

Convenience

Convenience refers to the ease of using the animal model (Kalu, 1991). The rat model is convenient to maintain and work with. In comparison to other models such as dogs and non-human primates rats are relatively inexpensive and are associated with fewer ethical constraints

Relevance

Relevance refers to comparisons made from animals to humans concerning a phenomenon being studied (Kalu, 1991). There are many skeletal similarities between rats and humans. In aged ovariectomized rat models, bone loss was greater in the vertebra than in the femur (cancellous loss was greater than cortical loss), this loss was similar to that in postmenopausal women, (Kalu, 1991 and Hogan et al, 2000) In both aged ovarectomized rats and postmenopausal women high levels of biochemical markers of bone turnover with bone resorption exceeding formation occur immediately after menopause, and increase medullary area have been shown (Kalu, 1991). Other

characteristics shared by the ovarectomized rat model and postmenopausal women are decreased intestinal calcium absorption, rapid loss of cancellous bone followed by a slower steady decrease, and response to treatment. The effects of treatment (estrogen, bisphosphonates, calcitonin, vitamin D and its analogs, tamoxifen, parathyroid hormone, and exercise) had similar preventive effects on bone loss in ovariectomized rats and postmenopausal women. These similarities among others provide evidence that the rat model is suitable for studying osteoporosis.

The human skeleton consists of 80% cortical bone and 20% trabecular bone (O'Flaherty 1991). Following the blood flow through rat bones demonstrates these percentages also apply to rats. Microshpere technique was used to measure the blood flow in bone of a rat (Buckberg et al, 1971). The blood flow rate was highest in trabecular and hematopoietic marrow and lowest to cortical bone showing a pattern of flow to immature, growing bone greater than to mature bone. It has been estimated that 3% of cardiac output flows through the skeleton. It was further estimated that the blood flow rate is 35 ml/min/100g to trabecular bone and 5 ml/min/100g to cortical bone. Using these estimates the skeleton was further compartmentalized to show that 80% by weight was cortical bone and 20% was trabecular bone.

Appropriateness

Appropriateness refers to other factors that make a model acceptable for studying a particular phenomenon (Kalu, 1991). Rat models follow the pattern of growth similar to humans. From 1-3 months of age rapid increases in length, weight, density and calcium content of femurs were found that were similar to childhood and adolescence in

humans. During this period there was enormous modeling, remodeling and growth occurring. From six months of age on, the increase was more gradual. There were minimal changes in the femur calcium content or density from six months to 12 months similar to adulthood. All bone parameters reached a plateau at 12 months of age and no further change up to 24 months of age occurred.

Acheson et al (1959) studied similarities of skeletal development between human development and rat models. Ten male and 13 female Sprague-Dawley rats were weaned on the 20th day, separated by sex, and feed ad libitium. Measurements of total-length, tail-length and weight were obtained between the 16th and 110th day of life. Animals were anesthetized during radiography in which standard Kodak 'no-screen' film was used. For assessing skeletal maturation the principles of the Oxford method was used. The Oxford method was based on the fact that there are irreversible processes during maturation, and certain changes occur in the shape of the epiphyses of long bone, or in round bones. These changes were seen radiographically, and they occurred in a defined order. These changes were referred to as maturity indicators. Each indicator was given point values to total 100 at the age of 110 days. Serial radiograms were taken at 10 day intervals and given a maturity score. Similarities in skeletal development were seen among the rat model and humans. The female rat matured more quickly than the male rat. Between the 70th and 80th day the rate body length increase of the female rat decreased while the male rat continued to grow for a longer period of time. The male rats were heavier and longer than the female rats during all stages of growth. Besides these similarities it is important to note that the rat and man are both skeletally immature at birth. At birth the skeleton is incompletely mineralized (O'Flaherty 1991). The skeleton

consists mainly of cartilage which is considerably less dense than fully mineralized bone. Mineralization continues after birth when body calcium rises rapidly and steadily during growth and then stabilizes during sexual maturity. Since much of the transformation of cartilage into bone happens outside of the uterus this makes the rat an appropriate model for studying the effects that environmental stress plays on skeletal development.

Associated Problems with the Rat Model

Many feel that because osteoporosis is not a rare disease there are many people available for studying osteoporosis instead of using a rat model (Kalu, 1991). However, there are ethical constraints that limit the ability to test new hypothesis or potential therapies.

Another problem is that the skeletal mass of a rat is stable for a prolonged period during life (Kalu, 1991). The estrus cycles continue to about 19 months of age spanning most of the animal's life. Bone mass is still maintained during this period due to the amount of sex hormones that are still being secreted by the ovaries. However, rats can be ovarectomized to induce an ovarian hormone deficiency.

It has been stated that rats are continuously growing (Kalu, 1991). When rats were fed ad libitum they continued to grow and increase in body weight, but this increase in weight was a result of more deposition of fat than increase in lean body mass. Once rats began to age they lost both adiposity and lean body mass. Several studies have shown that there is a decrease in osteogenesis in the epiphyseal growth plate of a rat at age six to 18 months. Rats suffer from senile bone loss if they live long enough.

Rats lack a Haversian system and do not have the same pattern of bone

remodeling as humans (Kalu, 1991). Remodeling activities of activation, resorption, and formation found in several sites were found to be similar to the remodeling activity of cancellous in humans (Baron et al, 1984). This suggests that rats have the same mechanisms of bone turnover that exists in humans. The rat model has been shown to possess the characteristics of convenience, relevance and appropriateness to validate it's use in studying bone.

Rat Model and Iron Absorption

Iron absorption is higher in rats than in humans limiting the ability to predict the absorptive response of iron in humans (Reddy and Cook, 1994). Human studies have demonstrated that ascorbic acid enhances iron absorption. The differences in iron absorption among rats and humans can possibly be due to the fact that rats can synthesize ascorbic acid; therefore, provide an effective substrate for increasing iron absorption in this species.

CHAPTER III

MATERIALS AND METHODS

Experimental Design

Forty weanling female Sprague-Dawley rats (Sasco, Kingston, NY) were received at 21 days of age. A completely random design with four dietary regimens was used in this experiment. Animals were fed an AIN-93-G diet for seven weeks post weaning and then diets were changed to an AIN-93-M formulation for the remainder of the experiment (eight weeks). At 18 weeks of age at which point the rats had reached skeletal maturity, the rats were killed for analysis of bones and tissues. The project was approved by the Institutional Animal Care and Use Committee protocol #709 (Appendix A)

Treatment Protocol

Housing

The animals were housed in the Oklahoma State University Laboratory Animal Resource Center (LAR) where proper care for the animals was monitored. The rats were caged in individual plastic cages with raised plastic floor grids that were changed weekly The light, temperature and humidity were controlled by the animal facility.

Diets

Each group was fed a purified diet based on AIN-93 (Reeves et al., 1993) recommendations except for iron (Tables 1 and 2) Two groups had iron inadequate diets

calculated at 6 ppm, and 12 ppm, one control group calculated at 35ppm and one excess level of iron calculated at150ppm. Previously the cellulose was lot tested for contamination of trace minerals. The same lot numbers for all ingredients were used for all diets. Mineral mixes were prepared by weighing macro and micro-nutrients, then combining and mixing in a burundum-fortified porcelain jar on a roller type mill for 6 hours. Mineral mixes were prepared one kilogram at a time to equal 2.5 kg sufficient for the entire experiment.

Feeding Schedule

Animals were fed late in the afternoon to minimize spilling of the diet Remaining diet and animals were weighed twice a week. In order to match animal weights, diet fed was based on feed consumption of animals that gained the least weight. Deionized water ad libitum was provided to ensure adequate hydration.

Necropsy

Animals were fasted for 12 hours in individual plastic metabolic cages with access to deionized water. Urine was collected for the 12 hours, volume determined, centrifuged, and frozen for future analysis. Animals were anesthetized with 50 mg/kg of body weight ketamine and 2.5 mg/kg of body weight xylazine based on last LAR weight and were weighed immediately prior to body composition determinants. Body composition, whole body BMD and BMC were determined by Dual Energy X-ray Absorptiometry (DXA) (Hologic QDR 4500A, Waltham, MA) using the small animal analysis mode.

Table 1

Mineral Mixes

Component	AIN93-G g/kg	AIN93-M g/kg
Calcium Carbonate	357	357
Potassium Phosphate	196	250
Potassium Citrate	70.78	28
NaCl	73.275	73.275
Potassium Sulfate	46.6	46.6
Magnesium Oxide	24	24
Zinc Carbonate	1.65	1.65
Manganous Carbonate	0.63	0.63
Cupric Carbonate	0.3	0.3
Postassium iodate	0.01	0.01
Sodium selenate	0.01025	0.01025
Amonium paramolybdate	0.00795	0.00795
Sodium meta-silicate	1.45	1.45
Chromium potassium sulfate	0.275	0.275
Lithium chloride	0.0174	0.0174
Boric acid	0.0815	0.0815
Sodium Flouride	0.0635	0.0635
Nickel carbonate	0.0318	0.0318
Ammonium vanadate	0.0066	0.0066

Levels of dietary iron for AIN93-G and AIN93-M
(corrected for amount of iron in cellulose)

	6 ppm	12 ppm	35 ppm	150 ppm
Ferric Citrate g/kg	0.88	1.86555	5.8883	25.8126

Powdered sucrose corrected for amount in titrated minerals for AIN93-G/AIN93-M

Sucrose g/kg	217.37/ 206.15	216.38/ 205.16	212.36/ 201.14	191.98/ 181.21

Table 2

Composition of the Diet

Component	AIN-93G g/kg	AIN-93M g/kg
Corn starch[1]	397.5	582.1
Casein[2]	200.0	140.0
Dextrinized corn starch[3]	132.0	155.0
Sucrose[4]	100.0	100.0
Soybean oil[5]	70.0	40.0
Cellulose[6]	50.0	50.0
Mineral mix (AIN-93G/93M-MX)[7]	35.0	35.0
Vitamin mix (AIN-93G/93M-VX)[8]	10.0	10.0
L-Cystine[9]	3.0	1.8
Choline[10]	2.5	2.5

[1] Harlan Teklad, Madison, WI. Lot # 98302
[2] Harlan Teklad, Madison, WI. Lot # 98308
[3] Clo-Dex 10 – Maltodextrin Lot # 98227
[4] Great Value Extra Fine Granulated Sugar
[5] Crisco All Natural Pure Vegetable Oil
[6] Harlan Teklad, Madison, WI. Lot # 98197
[7] Mineral mix was prepared in the lab
[8] Harlan Teklad, Madison, WI. RX 892395
[9] Harlan Teklad, Madison, WI. Lot # 98295
[10] Harlan Teklad, Madison, WI. Lot # 98101

Blood was collected by abdominal aorta puncture. EDTA treated syringes were used for hematological analyses, and syringes without anticoagulant for serum. Once the blood was collected for serum it was clotted on ice, centrifuged and distributed to tubes for either freezing or immediate analysis. Hematology values were obtained using the ABX Pentra 120 Retic instrument (Irvine, CA.).

Tissues were perfused with saline using a peristatic pump through the aorta puncture site. Those tissues that were collected were the heart, kidneys, liver and spleen. They were trimmed, weighed and then frozen for future mineral analyses. The heart, kidneys, and spleen were stored in plastic tissue bags and placed in a -20°C freezer. The liver was divided into three portions: the 1st portion was frozen in a plastic tissue bag at -70°C for enzyme assays, the 2nd portion was also frozen in a plastic tissue bag at -70°C, and the 3rd was frozen in a plastic tissue bag at -20°C for mineral analysis.

Bones collected were femurs, tibias and lumbar vertebrae. At necropsy all bones, except vertebrae and sterum were cleaned of adhering flesh carefully as not to remove the periostium and to avoid mineral contamination. Clean bones were stored appropriate for the specific analysis. The left tibia was stored in a scintillation vial with 70% ethanol and left at room temperature. The right tibia and left femur were frozen in plastic bags at -20°C. The spine was frozen whole in a 50mL centrifuge tube at -20°C. Later the spine was cleaned to separate lumbar 3, 4, and 5 vertebrae and placed into individual plastic tissue bags and refrozen at -20°C. The right femur was frozen in liquid nitrogen and stored in a plastic tissue bag at -70°C.

Analyses

Growth, nutritional status, bone mineral content and bone metabolism were evaluated. These measures will help to explain the effects that inadequate, adequate and excessive dietary iron have on skeletal growth in young mature rats.

Nutritional Status

Albumin, glucose, serum urea nitrogen, calcium, magnesium, and phosporus were measured in serum for overall nutritional status. The presence or absence of hepatic damage was measured by the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST). All analyses were performed on the COBAS Fara II clinical analyzer using appropriate reagents from Roche Diagnostics (Roche Diagnostic Systems, Indianapolis, IN).

The analysis of albumin uses a modification of Doumas' bromocresol green binding assay (Doumas et al 1971). Once bromocresol green binds to albumin there is a spectral shift that occurs in the absorbance at 630 nm. The change in absorbance is directly proportional to the concentration of albumin in the sample.

The analysis of glucose is determined as glucose is phosphorylated by hexokinase to produce glucose-6-phosphate and adenosine diphosphate (Neeley, 1972). Glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase to 6-phosphonogluconate and nicotinamide adenine dinucleotide (NAD) is reduced to nicotinamide adenine dinucleotide (NADH). One micromole of NADH is produced for each micromole of glucose The NADH absorbs light at 340 nm and is directly proportional to the concentration of glucose in the sample.

The analysis of serum urea nitrogen depends upon hydrolyzing urea by urease to ammonia and carbon dioxide (Tiffany et al, 1972). Ammonia and α-ketoglutarate are catalyzed by glutamate dehydrogenase to glutamate and water with concurrent oxidation of NADH to NAD. For each mole of urea present two moles of NADH are oxidized The decrease in absorbance at 340 nm is proportional to the urea concentration in the sample.

The analysis of calcium is determined by a highly specific and sensitive reagent. Arsenazo III binding to calcium forming a colored complex with absorbance maxima at 600 nm and 650 nm (Michaylova and Illkova, 1971). The absorbance levels are proportional to calcium concentration in the sample. Addition of the EDTA reagent corrects for nonspecific absorbance interference by removing calcium from the calcium-Arsenazo III complex and allows for an accurate sample blank measurement. The difference in absorbance between the calcium-Arsenazo III complex and the EDTA complex is the absorbance caused by calcium alone

The analysis of magnesium depends upon a sensitive dye, chlorophosphonazo III (CPZ III) which binds to magnesium causing an absorbance decrease at 550 nm and an absorbance increase at 675 nm (Ferguson et al, 1964). Calcium is inhibited from binding to CPZ III by EDTA used in reagent 1 Nonspecific absorbance interferences are decreased by the EDTA reagent which removes magnesium from the magnesium-CPZ III complex allowing for an accurate sample blank measurement. The differenence in absorbance between the magnesium-CPZ III complex and the EDTA complex is the absorbance caused by magnesium alone.

The analysis of phosphorus is determined by using the reagent based on

modification of the procedure developed by Daly and Ertingshausen (1972). Phosphorus reacts with ammonium molybdate in dilute sulfuric acid and forms the unreduced phosphomolybdate complex and is measured at 340 nm.

The analysis of ALT depends upon the transfer of an amino group between L-alanine and α-ketoglutarate to form pyruvate and L-glutamate (Bergmeyer and Horder, 1980). Pyruvate reacts with NADH in the presence of lactate dehydrogenase to form NAD. NAD reacts with glucose-6-phosphate in the presence of glucose-6-phosphate dehydrogenase and NADH is generated. The rate of oxidation of NADH is measured at 340 nm to determine the ALT activity.

The analysis of AST depends upon the transfer of an amino group between L-aspartate and α-ketoglutarate to form oxaloacetate and L-glutamate (Bergmeyer and Horder, 1986). In the presence of malate dehydrogenase, oxaloacetate reacts with NADH to form NAD. When NAD reacts with glucose-6-phosphate in the presence of glucose-6-phosphate dehydrogenase NADH is generated. The rate of oxidation of NADH is measured at 340 nm to determine the AST activity

Hematology included total leukocyte counts and differentials, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocytes, and platelet counts. All variables were measured on an automated hematology instrument (ABX Pentra 120 retic, Irvine, CA).

Bone

In preparation for femur analyses, the left femur was soaked in 9% saline in a scintillation vial at room temperature over night. Each bone was placed in a weigh boat

with saline and then individually scanned by DXA to obtain bone mineral density and bone mineral content. The DXA instrument was standardized to a known phantom before measuring the bones.

Bone length and diameters were measured with calipers. The midpoint was marked for fracture testing. Fracture force was determined by an Instron Universal Testing Machine (Model TM-S, Instron Corporation, Canton, MA.) at a loading rate of 1 mm/min with a three-point compression bend test. Force value is the minimum amount of force required to fracture the femur applied parallel to the mid-shaft diameter (Thompson et al, 1996). Once the femur was fractured, the marrow was cleaned from the bone by rinsing with a syringe and Type I water. The inside and outside diameters were measured to use in the calculations of second moment of area, stress, cortical area and medullary area (Kiebzak et al,1988).

The bones were soaked in Type I water over night and then placed in the dessicator with vacuum for 1 hour. Wet weight and air weight were measured using a Density Determination Kit Mettler ME-33340 (Mettler Instruments, Grelfensee, Switzerland), and density by displacement was calculated using Archimedes' principle. The fractured, cleaned femurs were then transferred to an acid washed, labeled and pre-weighed tube and placed in the drying oven for 48 hours at 100° C. After the bones cooled in the dessicator the dry weight was then recorded, and bones were ashed by wet and dry ashing using a modification of the method of Hill et al (1986). The tubes containing the bone samples were placed in a heating block and wet ashed at 105° C with 100µl each of double distilled concentrated nitric acid (GFS Chemicals, Columbus, OH), and 30% Ultrex hydrogen peroxide (J.T. Baker, Phillipsburg, NJ) and Type I water. An

additional 100µl of hydrogen peroxide was repeatedly added until bubbling ceased. The tubes were dried in the heating block until liquid evaporated, and then placed in a muffle furnace at ashing temperature of 375° C for 48 hours. The cycle of wet and dry ashing was repeated until bones were completely ashed. Once ashed, the bones were analyzed for calcium, phosphorus, magnesium, iron, copper, zinc and manganese by flame or graphite furnace atomic absorption spectroscopy as appropriate using a Perkin Elmer 5100PC AAS with deterium and Zeman background correction.

Bone Metabolism

Bone formation was assessed by alkaline phosphatase extracted from L3 vertebra and serum insulin-like growth factor I (IGF-I). The third lumbar was measured for density by displacement using a Density Determination Kit Mettler ME-33340 (Mettler Instruments, Grelfensee, Switzerland) and calculated using Archimedes' principle. A detailed method for extraction and analysis of alkaline phosphatase extracted from L3 vertebra followed from Farley et al (1992) is listed in Appendix B.

Insulin-like growth factor-I was measured using a RIA method The IGF-1 kit (Nichols Institute Diagnostics, San Juan Capistrano, CA.) was used and extraction followed formic acid-acetone extraction procedure from Kalu et al (1994).

Urinary excretion of deoxypyridinoline (Dpd) crosslinks and hydroxyproline (Bergman et al, 1970) were measured as indicators of bone resorption. The final overnight fasting urine was used to assess Dpd using a competitve ELISA method (Metra Biosystems, Montain View, CA). The results were corrected for urinary concentration by urinary creatinine A detailed method for extraction and analysis of urinary

hydroxyproline is listed in Appendix C.

Urinary excretion of calcium, magnesium, phosphorus and creatinine were assessed spectrophotometrically using the COBAS Fara II clinical analyzer. Urinary minerals were corrected for concentration by urinary creatinine.

Statistical Analyses

Data were analyzed using SAS (version 7.0, SAS Institute, Cary, NC). The completely random model was analyzed using the generalized linear model (GLM) procedure in SAS to analyze for effects of dietary iron on bone and growth. Significance level was set at 0.05. Difference of means was tested by LS means testing

CHAPTER IV

EFFECTS OF DIETARY IRON ON BONE IN YOUNG MATURE RATS

L. McDonald, D. Kukuk, A.B. Arquitt, M.J. Fisher

ABSTRACT

The effects of inadequate, recommended and excessive amounts of iron on bone density and strength and on biochemical indicators of bone metabolism were investigated in young mature female rats. Forty weanling female Sprague Dawley rats were randomized into four levels of dietary iron as iron citrate; control (AIN-93; 35 ppm), two iron deficient levels (calculated to be 6 ppm and 12 ppm), and iron excess (calculated to be 150 ppm). After 15 weeks of treatment the rats were fasted for 12 hours, anesthetized, scanned via Dual Energy X-Ray Absorptiometry (DXA), exsanguinated, and blood and tissues of interest were collected for various analyses. There were no significant differences in body weight among the treatment groups initially or at the end. Red blood cell numbers ($p< 0.001$) and hemoglobin concentration ($p< 0.0001$) were significantly lower in the 6 ppm than 12, 35, and 150 ppm groups. Reticulocyte numbers were significantly ($p< 0.01$) higher in 6 ppm compared to 12, 35 and 150 ppm diets. Insulin-like growth factor was significantly ($p< 0.05$) higher in 6 ppm than all other diets.

Whole body bone mineral density as shown by DXA was significantly ($p< 0.05$) lower in 6 ppm than 12 ppm and 150 ppm diets. Femur bone mineral content was significantly ($p< 0.05$) lower in the 6 ppm than all other diet groups, and the 12 ppm diet

was significantly ($p< 0.05$) lower than the 150 ppm diet group. Femur bone mineral density was significantly ($p< 0.01$) lower in the 6 ppm diet group than in the 35 and 150 ppm diet groups, and the 12 ppm diet group was significantly ($p< 0.05$) lower than 150 ppm diet group. Density as measured by Archimedes' principle in L3 vertebrae was significantly ($p< 0.01$) lower in 6 ppm than 35 and 150 ppm, and significantly ($p< 0.05$) lower in 12 ppm than 150 ppm. Iron deficiency appeared to delay bone growth. Adequate iron intake may be one factor in achieving peak bone mass

INTRODUCTION

Bones are in a state of continual renewal consisting of resorption and formation (8). Osteoclasts resorb old, damaged and under used bone, and osteoblasts replace the damaged bone and form new bone. From infancy up to the age of 30, bone formation dominates, resulting in accumulation of bone mass (14). It is believed that the degree of attainment of peak bone mass during adolescence is the determinant of osteoporotic fracture later in life, and may delay the onset of osteoporosis (14). Osteoporosis is a chronic debilitating disease defined as skeletal fragility signified by a loss in bone mass and a deterioration in the microarchitectural tissue of the bone (8). It was estimated that over 28 million Americans were affected by osteoporosis equaling 1.5 million fractures and totaling $13.8 billion in 1995 (18). With an increase in the elderly population it is predicted that this cost will also increase to $240 billion by the year 2040 (4). Achievement of peak bone mass is a result of normal growth but is influenced by many factors that are often interrelated. Many studies have demonstrated the importance that calcium and vitamin D have on bone growth, but other minerals such as iron, copper

and magnesium in addition to calcium and vitamin D have been demonstrated to influence bone growth. (1, 10, 16).

Iron is an important component in the hydroxylation of collagen matrix, the connective tissue foundation of bone (19) upon which insoluble mineral salts of hydroxyapatite are deposited (13) allowing the structure of bone to provide support to the body. Dietary iron intakes, both deficiencies and excesses, have increasingly become a world wide problem. Results from the Total Diet Study indicate that adolescents met less than 80% of the recommended dietary allowance (RDA) for iron (20). Currently, health awareness has popularized the use of nutritional supplements (6). It was estimated from the 1987 and 1992 National Health Interview survey that approximately 24% of the population used supplements (24). Nationwide food fortification policies are also seen as an answer to nutritional deficiencies and contribute to excess intakes (7). Toxic factors need to be considered when consuming supplements and fortified foods.

Osteoporosis is a chronic disease associated with many economical and human costs. To be able to prevent this disease, it is necessary to include all factors into a comprehensive approach. Diet has been demonstrated as one of the many factors influencing peak bone mass. Iron is necessary for the hydroxylation of collagen matrix (19) and is also the most common micronutrient deficiency (26). In an attempt to correct the problems of both osteoporosis and undesirable iron intakes, a common link may be found. The purpose of this project was to investigate the effect of iron in bone metabolism, attainment of bone density and on strength during growth.

MATERIALS AND METHODS

Forty weanling female Sprague Dawley rats at 21 days of age (Sasco, Kingston, NY.) were randomized into four levels of dietary iron as iron citrate; calculated iron concentrations were control (35 ppm), two iron deficient levels (6 ppm and 12 ppm), and iron excess (150 ppm). All other minerals were at AIN-93 (21) recommendations including the potentially beneficial mineral elements. The rats were fed the iron modified AIN-93 G for 7 weeks and then switched to the respective mineral concentration AIN-93M diets for the remainder of the experiment (eight weeks). Animals and remaining diet were weighed twice a week. In order to match animal weights, diet fed was based on feed consumption of animals that gained the least weight Deionized water ad libitum was provided to ensure adequate hydration.

Necropsy

At 15 weeks of treatment the rats were fasted with access to deionized water in plastic metabolic cages for 12 hours for urine and fecal collections. Animals were anesthetized with ketamine/xylazine (50 and 2.5 mg/kg of body weights, respectively). Bone mineral density, bone mineral content, percent lean tissue, and percent body fat were determined by DXA (Hologic QDR 4500 A, Waltham, MA). Animals were exsanguinated via abdominal aorta puncture. The tissues were then perfused with cold saline (9 g/L) before collection. Bones were cleaned of adhering flesh and stored appropriately for the specific analysis. The left femur was frozen in plastic tissue bags at -20°C. The spine was frozen whole in a 50 mL centrifuge tube at -20°C. Later the spine

was cleaned to separate lumbar 3, 4, and 5 vertebrae and placed into individual plastic tissue bags and refrozen at -20°C.

Analyses

Nutritional Indicators: Albumin, glucose, serum urea nitrogen, alkaline phosphatase, calcium, magnesium, and phosphorus were measured in serum for overall nutritional indicators. The presence or absence of hepatic damage was measured by the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST). All analyses were performed on the COBAS Fara II clinical analyzer using appropriate reagents from Roche Diagnostics (Roche Diagnostic Systems, Indianapolis, IN)

Hematological parameters included leukocyte counts, red blood cells, reticulocytes, and hemoglobin to assess iron status. All variables were measured on an automated hematology instrument (ABX Pentra 120 retic, Irvine, CA).

Bone and bone metabolism analyses: Bone density and total area were analyzed for whole body, left femur and L3 vertebrae by DXA and/or Archimedes' principle. Fracture force was determined by an Instron Universal Testing Machine (Model TM-S, Instron Corporation, Canton, MA.) using the left femur. Second moment of area, cortical area, medullary area and fracture stress were calculated. Alkaline phosphatase (ALP) extracted from the L3 vertebrae and serum insulin-like growth factor (IGF-I) were analyzed for bone metabolism. IGF-I was measured using a RIA kit (Nichols Institute Diagnostics, San Juan Capistrano, CA) following extraction using the formic acid-acetone extraction procedure from Kalu et al (11). Alkaline phosphatase was extracted from L3 vertebrae following a modified method from Farley et al (6) The L3 vertebrae

were incubated with 3 ml of phosphate buffered saline with 0.02% sodium azide for 12 hours at 4° C to remove contaminants. The L3 vertebrae were then crushed and transferred into another tube containing 3 ml of 0.01% triton-X 100 with 0.02% sodium azide in Type I water and incubated for 72 hours at 4° C for extraction of ALP. The supernatant was tested for ALP using the COBAS Fara II clinical analyzer and reagents from Roche Diagnostic.

Urinary deoxypyridinoline crosslinks (Dpd) and hydroxyproline, bone excretion products, were also analyzed along with urinary minerals. Dpd was measured using a competitve ELISA method (Metra Biosystems, Montain View, CA). Hydroxyproline was determined following the method by Bergman et al (2). Urinary minerals were analyzed on the FARA II clinical analyzer using Roche Diagnostics reagents and controls from Sigma Chemical Co and BioRad.

Data were analyzed using PC SAS (version 7.0, SAS Institute, Cary, NC). The data from this completely random design were analyzed using the generalized linear model (GLM) procedure in SAS to analyze for effects of dietary iron on bone and growth. Difference of means was tested by LS means testing.

RESULTS

Nutritional Indicators. Weight gain was similar ($p > 0.05$) in all diet groups (Figure 1). The 6 ppm diet group was significantly ($p < 0.001$) lower than all other diet groups in hemoglobin and in red blood cell count (Table 3). The 6 ppm diet group had significantly ($p < 0.01$) higher reticulocyte counts than all other diets. All red cell indicators confirm iron inadequacy. ALT was significantly ($p < 0.05$) higher in 6 ppm

than 12 ppm and 150 ppm diet groups (Table 3). AST was significantly ($p < 0.05$) higher in 6 ppm than 35 ppm and 150 ppm diet groups. Alkaline phosphatase activity, an enzyme involved in bone metabolism, was significantly ($p < 0.05$) greater in the 6 ppm diet compared to the 35 ppm control diet. There were no significant ($p > 0.05$) differences observed either in the remaining serum nutritional indicators or in urinary mineral excretion.

Bone and Bone Metabolism. Whole body BMC was significantly ($p < 0.05$) lower in 6 ppm than 150 ppm diet groups (Table 4). Whole body BMD was significantly ($p < 0.05$) lower in 6 ppm than 12 ppm and 150 ppm. Whole body BMC and BMD in the 35 ppm diet was not significantly different from the 6 ppm and 150 ppm diet groups. There were no significant differences in other body composition measures (percent lean tissue, and percent body fat) measured by DXA among any of the groups (data not shown). BMC of femur was significantly ($p < 0.05$) lower in the 6 ppm than in all other diet groups, and the 12 ppm diet was significantly ($p < 0.05$) lower than 150 ppm diet. BMD of femur was significantly ($p < 0.01$) lower in the 6 ppm than in the 35 ppm and 150 ppm diet groups, and the 12 ppm diet group was significantly ($p < 0.05$) lower than the 150 ppm diet group. The significant differences seen in BMD and BMC of femur measured by DXA were not verified by density by displacement (Table 4). There was no significant difference found in density when femur was measured by Archimedes' principle. Using Archimedes' principle, the density of L3 vertebrae was significantly ($p < 0.01$) lower in 6 ppm than 35 ppm and 150 ppm, and significantly ($p < 0.05$) lower in 12 ppm than 150 ppm. These indicators suggest that iron deficiency during growth affects the attainment of maximal density.

Bone growth and fracture strength differed among diet groups. While there were no significant differences in femur length or medullary area, femur area was significantly ($p<0.01$) lower in 6 ppm than 150 ppm. Cortical area was significantly ($p< 0.05$) lower in 6 ppm than all other diet groups. Second moment of area was significantly ($p< 0.05$) lower in 6 ppm than in 35 ppm and 150 ppm. The 6 ppm diet was lower but not significant than all other diet groups in fracture force. Fracture stress was higher in 6 ppm diet than all other diet groups; however, this difference was not significant (Table 4)

Bone metabolism indicators are included in Table 5. IGF-1 was significantly ($p< 0.05$) higher in 6 ppm than all other diets. ALP extracted from L3 vertebrae was higher but not significantly in 6 ppm and 12 ppm than 35 ppm and 150 ppm. There were no significant differences between diets for urinary crosslinks excretion or hydroxyproline.

DISCUSSION

We were successful in inducing iron deficiency in the 6 ppm diet as indicated by hemoglobin, red blood cell count and reticulocyte counts. The higher concentrations in ALT and AST in 6 ppm diet were also similarly seen in a study by Rodriguez-Matas et al (23). Significantly higher levels of AST, but not ALT, were seen in their iron deficient rats from ten to 30 days after an iron deficient diet. The reason for the increase in AST in iron deficient rats is unknown. The higher concentrations of AST and ALT in our study indicates that iron deficiency influences liver function.

Whole body and femur BMD and BMC as shown by DXA scans in 6 ppm diets were significantly lower than in 150 ppm diets, which was not different than the control

diet. This agrees with the finding from Angus et al (1) who demonstrated iron intakes were positively correlated with forearm BMC in postmeopausal women. The lower BMD and BMC did not result in weaker bones as shown by fracture force, but the three point bending test is not a sensitive test because it assesses cortical bone as well as trabecular bone (9, 10). In our study, there was no significant difference in fracture force between the diet groups. Medeiros et al (16) found a decrease in femur strength in iron deficient rats. Our results in mechanical strength were not in agreement with this study. Several reasons may be attributed to our findings. Currey (5) suggested that using a higher speed to account for the limiting factor of bone deformation was more sensitive. Medeiros et al (16) used a rate at 200 mm/min in comparison to the rate of 1 mm/min used in this study. However, Medeiros et al (16) prepared bones for fracture by first drying at 37°C to constant weight before breaking strength was determined. Drying has been known to decrease toughness and increase brittleness (25). Thus, the bones of iron deficient rats in Medeiros et al (16) study may have been more brittle than control rats.

In our rats total area, second moment of area and cortical area were significantly lower in 6 ppm diet than in the 35 and 150 ppm diet groups, showing a similar pattern as DXA measurements. When fracture force is adjusted to account for bone gemetry it is referred to as fracture stress (12). Others (12, 9) have reported that geometric distribution of bone influences fracture force; however, when adjustments were made for geometry of the bone significant differences was seen in fracture stress. When we adjusted for differences in the geometric distribution of bone in our results, fracture stress was not significant, in fact fracture stress was higher in 6 ppm than all other diet groups. Kiebzak et al (12) saw no significant difference in fracture force in rats between the ages of six

and 24 months; however, when adjusting for geometry of bone, fracture stress was significantly lower in the 24 month old rats than in the six month old rats. Hogan et al (9) found fracture force was significantly higher in ovariectomized rats than in sham operated rats, but when adjusting for cross-sectional area the ovariectomized rats were lower but not significant than sham operated rats. Even though the 150 ppm diet is significantly higher than 6 ppm diet in total area, second moment of area and cortical area, the integrity and quality of the bone may be diminished. Matkovic et al (15) reported that bones compensate for a decrease in trabecular volume by increasing bone mass from periosteol expansion and maintenance. Kiebzak et al (12) stated that adjustment in architecture of bone was an attempt to maintain strength. Therefore, a bone that has lost it's integrity may appear strong due to adjustment in increasing cross-section area, total area, cortical area and medullary area.

There were no differences seen in the bone resorption indicators urinary Dpd crosslinks and hydroxyproline. In growing animals these metabolites should be low due to bone formation, which dominates over bone resorption during this age.

Mora et al (17) studied children and found that high levels of IGF-I did not result in an increase in bone mineral density; however, the length, cross-sectional area and cortical area of the femur was greater. Our results were inconsistent with Mora et al (17) findings. The 6 ppm diet group had IGF-1 levels significantly higher than all other diet groups; however, cross-sectional area and cortical area were significantly higher in 35 and 150 ppm diet group and all diet groups, respectively than in the 6 ppm group. IGF-I enhances the synthesis of collagen and proteoglycans, which also has positive effects on the homeostasis of calcium, magnesium and potassium (3). The increased levels of IGF-I

in the 6 ppm diet may be in an attempt to overcome lower BMC, BMD, and smaller bone geometry.

Serum alkaline phosphatase activity was significantly higher in 6 ppm than 35 ppm suggesting increased bone formation in the 6 ppm group. Risteli and Risteli (22) stated that alkaline phosphatase expression started once cessation of cell proliferation occurred, reached a maximum during matrix maturation, and decreased as the rate of matrix mineralization declined. Higher serum ALP and IGF-I in the 6 ppm group indicates that the bone matrix may still be maturing in an attempt to increase bone while the 35 ppm diet may have approached matrix maturation. Similar but not significant results were found when alkaline phosphatase was extracted from L3 vertebra. IGF-I levels agree with serum alkaline phosphatase as the iron deficient diet is higher than in the control. Perhaps the higher values in the 6 ppm diet is leading to catch up growth.

In summary, dietary iron played a role in bone metabolism during growth. The two indicators of bone formation measured, IGF-I and alkaline phosphatase, indicated that there was an increase in bone metabolism in the iron deficient rats. Perhaps this was an attempt to overcome the slower growth that was apparent by a lower BMD, BMC and bone measurements in the 6 ppm diet group. The lack of difference found in measurements of biomechanical strength indicated that iron deficiency or iron excess did not impede strength as measured by three point bending. Even though 150 ppm diet was significantly higher in BMD, BMC and bone measurements than 6 ppm diet they appeared to be weaker, but not significantly, when fracture force was adjusted for cross-sectional area and geometry of bone. The length of this study may have been too short for the impact of iron deficiency and excess to be definitive A study examining all

stages of life in the rat could be conducted to determine if the higher levels in the 6 ppm diet for IGF-I could lead to catch up growth increasing the BMC, BMD and bone measurements. Iron deficiency appeared to delay bone growth. Adequate iron intake may be one factor in achieving peak bone mass.

ACKNOWLEDGMENTS

The research was supported by OCAST Project HR 98-015 and OKLO2132.

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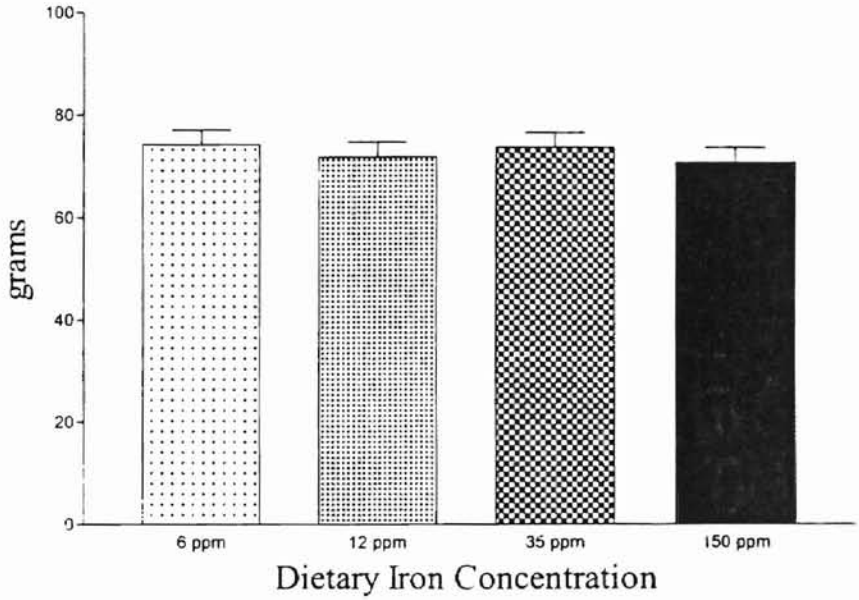
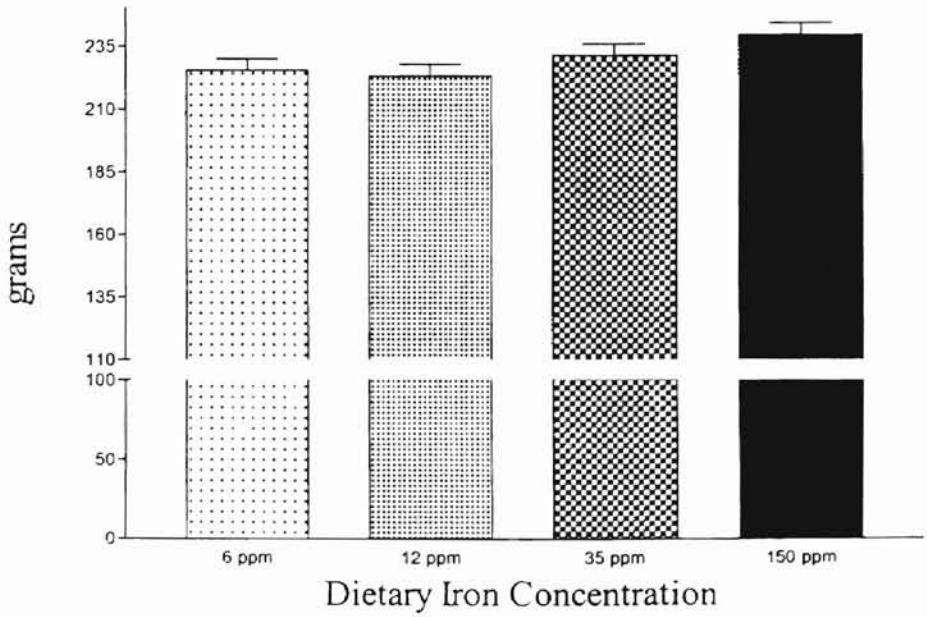
Figure 1. Initial and Final Weights of Young Mature Female Rats

1a. Initial Weights *



*No significant difference

1b. Final Weights*



*No significant difference

Table 3 Effects of Dietary Iron on Indicators of Nutritional Adequacy, Biochemical Measurements and Hemotology in Young Mature Rats[1,2,3]

Indicators of Nutritional Status	6 ppm	12 ppm	35 ppm	150 ppm
Serum Albumin (g/L)	37±1	35±1	38±1	37±1
Serum Glucose (mmol/L)	10.0±1.6	9.5±2.8	13.4±1.7	10.5±2.5
Serum Urea Nitrogen (mmol/L)	0.5^{a}±0.0	0.7^{b}±0.1	$0.6^{a,b}$±0.0	$0.6^{a,b}$±0.1
Serum Alkaline Phosphatase (uKat/L)	1.2^{1}±0.1	$1.0^{1,2}$±0.1	0.8^{2}±0.1	$0.9^{1,2}$±0.1
Serum Calcium (mmol/L)	9.25±0.76	8.98±0.80	8.10±0.820	8.90±0 74
Serum Magnesium (mmol/L)	1.00±0.24	1.02±0.22	0.89±0.22	0.92±0.24
Serum Phosphorus (mmol/L)	6.69±0.70	6.64±0.70	6.15±0.70	6.15±0.75
Alanine Aminotransferase (uKat/L)	0.41^{1}±0.02	0.33^{2}±0.02	0.37^{1}±0.02	0.30^{2}±0.02
Aspartate Aminotransferase (uKat/L)	1.55^{1}±0.11	$1.34^{1,2}$±0.11	1.14^{2}±0.11	1.07^{2}±0.11
Urinary Calcium (µmol/µmol creatinine)	0.12±0.02	0.11±0.02	0.11±0.02	0.11±0.02
Urinary Magnesium (µmol/µmol creatinine)	0.23±0.03	0.31±0.03	0.26±0.03	0.28±0.04
Urinary Phosphorus (µmol/µmol creatinine)	0.17±0.02	0.19±0.02	0.14±0.02	0.14±0.02
Leukocyte counts (10^{3}/mm^{3})	2.75±0.66	2 43±0.66	2 73±0.69	3.22±0.57
Red Blood Cells (10^{6}/mm^{3})	8.97^{1}± 0.24	7.51^{2}±0.26	$7\ 05^{2}$±0.27	7.32^{2}±0.22
Reticulocytes (10^{6}/mm^{3})	4.45^{1}±0.42	2.57^{2}±0.56	2.02^{2}±0.52	$2\ 64^{2}$±0.40
Hemoglobin g/L	11.19^{1}±0.33	13.73^{2}±0.36	14.06^{2}±0.38	$14\ 59^{2}$±0.31

[1] LS Means ± SE

[2] Variables in rows with different numerals are significantly ($P< 0.05$) different

[3] Variables in rows with different characters approach significance ($p=0.0568$)

Table 4 Effects of Dietary Iron on Properties of Whole Body, Femur and L3 Vertebrae[1,2]

Property	6 ppm	12 ppm	35 ppm	150 ppm
Whole Body				
BMC (grams)	7.73[1]±0.19	7.92[1]±0.18	8.09[1,2]±0.19	8.40[2]±0.18
BMD (g/cm^2)	0.148[1]±0.002	0.154[2]±0.002	0.153[1,2]±0.002	0.158[2]±0.002
Femur				
BMC (grams)	0.33[1]±0.01	0.36[2]±0.01	0.37[2,3]±0.01	0.39[3]±0.01
BMD (g/cm^2)	0.212[1]±0.004	0.220[1,2]±0.004	0.227[2,3]±0.004	0.233[3]±0.004
Density by Displacement (g/cm^3)	1.4832±0.04166	1.4672±0.04166	1.4794±0.04166	1.4793±0.04166
Femur Length (cm)	3.51±0.02	3.52±0.02	3.51±0.02	3.58±0.02
Total Area (cm^2)	1.57[1]±0.03	1.65[1,2]±0.03	1.63[1,2]±0.03	1.69[2]±0.03
Second Moment of Area (cm^2)	3.86[1]±0.23	4.48[1,2]±0.23	4.60[2]±0.23	4.93[2]±0.23
Cortical Area (mm^2)	5.50[1]±0.19	6.20[2]±0.19	6.11[2]±0.19	6.24[2]±0.19
Medullary Area (mm^2)	2.40±0.15	2.35±0.15	2.48±0.15	2.65±0.15
Fracture Force (mm/min)	9.41±0.46	10.36±0.46	9.88±0.48	10.75±0.46
Fracture Stress (kgf/ cm^2)	3.17±0.11	3.08±0.11	2.97±0.12	3.08±0.12
L3 Vertebrae				
Density by Displacement (g/cm^3)	1.2521[1]±0.009	1.2734[1,2]±0.009	1.2925[2,3]±0.009	1.3064[3]±0.009

[1] LS Means ± SE

[2] Variables in rows with different numerals are significantly ($p<0.05$) different

Table 5 Effects of Dietary Iron on Biochemical Markers of Bone Formation and Resorption[1,2]

Biochemical Marker	6 ppm	12 ppm	35 ppm	150 ppm
Insulin-like Growth Factor (IGF-I) (ng/mL)	652.10[1]±37.58	505.40[2]±39.67	537.62[2]±37.58	538.02[2]±39.67
Alkaline Phosphatase extracted from L3 vertebrae (uKat/g bone)	17.77±3.22	22.72±3.22	13.80±3.22	16.66±3.22
Urinary Deoxypyridinoline Crosslinks (mmol/L/12 hours)	2.50±0.33	2.57±0.33	2.57±0.33	2.25±0.33
Hydroxyproline (mmol/L/12 hours)	0.20±0.03	0.19±0.03	0.16±0.03	0.17±0.03

[1] LS Means ± SE

[2] Variables in rows with different numerals are significantly ($p<0.05$) different

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary

Forty weanling female Sprague Dawley rats were randomized into four levels of dietary iron as iron citrate; control (AIN-93), two iron deficiency levels (calculated at 6 ppm and 12 ppm), and iron excess (calculated at 150 ppm). At 18 weeks of age the rats were fasted for 12 hours, anesthetized, DXA scanned, and exsanguinated via abdominal aorta puncture. Blood, urine, tissues, the femurs, tibias and the spine were collected for analyses.

Whole body BMC was significantly ($p< 0.05$) lower in 6 and 12 ppm than 150 ppm. Whole body bone mineral density was significantly ($p< 0.05$) lower in 6 ppm than 12 and 150 ppm. Femur BMC was significantly ($p< 0.05$) lower in the 6 ppm than all other diet groups, and the 12 ppm diet was significantly ($p< 0.05$) lower than 150 ppm diet. BMD of femur was significantly ($p< 0.01$) lower in the 6 ppm than in the 35 and 150 ppm diet groups, and the 12 ppm diet group was significantly ($p< 0.05$) lower than the 150 ppm diet group. There was no significant difference in fracture force in rat femur Serum alkaline phosphatase, an enzyme involved in bone metabolism, was significantly ($p< 0.05$) higher in the 6 ppm diet compared to the 35 ppm control diet. Alkaline phosphatase extracted from L3 vertebrae tended to be higher but not significant in 6 and 12 ppm than 35 and 150 ppm. Insulin-like growth factor was significantly ($p< 0.05$) higher in 6 ppm than all other diets. There was no significant differences between diets for urinary crosslinks excretion or hydroxyproline.

Results of Hypothesis Testing

The following hypotheses were developed for this study:

1. There will be no statistically significant effect of dietary iron on bone density.

Hypothesis #1 was rejected because DXA scans showed significantly lower whole body bone mineral density ($p < 0.05$) and bone mineral content ($p < 0.05$) in 6 ppm than 150 ppm diet group as well as significant differences in the low iron diets, 6 ppm and 12 ppm, compared to 35 and 150 ppm for bone mineral density ($p < 0.05$) and bone mineral content ($p < 0.05$) in rat femur. Using Archimedes' principle, the density of L3 vertebrae was significantly ($p < 0.01$) lower in 6 ppm than 35 and 150 ppm, and less in 12 ppm than 150 ppm.

2. There will be no statistically significant effect of dietary iron on bone strength.

Hypothesis #2 failed to reject because there was no significant difference in fracture force in rat femur between levels of dietary iron; however, 6ppm diet was lower than 150 ppm. Cortical area was significantly ($p < 0.05$) lower in 6 ppm than all other diet groups. Second moment of area was significantly ($p < 0.05$) lower in 6 ppm than in 150 ppm. After adjusting for cross-sectional area (second moment of area) and geometry of bone, fracture stress was higher, but still not significant in the 6ppm diet than the 150 ppm diet.

3. There will be no statistically significant effect of dietary iron on alkaline phosphatase extracted from bone.

Hypothesis #3 failed to reject because there was no significant difference in alkaline phosphatase extracted from L3 vertebrae among dietary iron groups The 6 ppm diet was significantly ($p < 0.05$) higher than 35 ppm in the serum alkaline phosphatase,

while alkaline phosphatase extracted from bone was higher in the 6 and 12 ppm groups the difference was not significant from 35 and 150 ppm.

4. There will be no statistically significant effect of dietary iron on insulin-like growth factor-I (IGF-I).

Hypothesis #4 was rejected because IGF-I was significantly ($p < 0.05$) higher in 6 ppm than all other diets indicating increased bone metabolism in the 6 ppm diet.

5. There will be no statistically significant effect of dietary iron on urinary deoxypyridinoline crosslinks.

Hypothesis #5 failed to reject because there was no significant effect of dietary iron on urinary deoxypyridinoline crosslinks. In young mature animals this metabolite should be low due to bone formation, which dominates over bone resorption during this age.

6. There will be no statistically significant effect of dietary iron on urinary hydroxyproline.

Hypothesis #6 failed to reject because there was no significant effect of dietary iron on urinary hydroxyproline. In young mature animals this metabolite should be low due to bone formation, which dominates over bone resorption during this age.

Conclusions

In this experiment dietary iron played a role in bone metabolism during the growth phase. The two indicators of bone formation measured, IGF-I and alkaline phosphatase, indicated that there was an increase in bone metabolism in the iron deficient

rats. Perhaps this was in an attempt to overcome lower BMD, BMC and bone measurements (total area, second moment of area and cortical area) which were higher in the 35 and/or 150 ppm groups. The two indicators of bone resorption measured, urinary deoxypyridinoline crosslinks and hydroxyproline were not significantly different among diet groups. This was expected because bone formation exceeds resorption during this age of the animals. Bone metabolism appears to be greater in the 6 ppm diet than in the 150 ppm diet.

Bone mineral content and BMD measured in whole body using DXA revealed the 6 ppm diet group was significantly lower than the 150 ppm diet, which was not different than the control. Bone mineral content and BMD measured in the femur using DXA revealed the 6 ppm diet group was significantly lower than 35 and 150 ppm diet. Density by displacement in the L3 vertebrae also revealed the 6 ppm diet group was significantly lower than the 35 and 150 ppm. Total area, cortical area and second moment of area all reveal the 6 ppm diet to be significantly lower than the 150 ppm diet. Bone metabolism in the 6 ppm is higher than the 150 ppm diet as indicated by IGF-I and alkaline phosphatase. The higher bone metabolism and the lower BMC, BMC and bone measurements suggest that iron deficiency delays bone growth. If IGF-I and alkaline phosphatase levels continued to be elevated perhaps catch up growth might occur.

Fracture force was not significant among diet groups but there was a tendency for fracture force to increase as dietary iron increased This supports the significant findings in higher BMC, BMD, and bone measurements in the 150 ppm diet. Once fracture force was adjusted for cross-sectional area and geometry of bone, the opposite occurred. Fracture stress was greater but not significantly in the 6 ppm diet than all other diets.

Even though BMC, BMD and bone measurements were greater in the 150 ppm the quality and integrity of the bone may be poor. Histomorphometric analysis would reveal if there is a reduction in trabecular volume. When there is a decrease in trabecular volume the bone will compensate by increasing in cross-sectional area, total area, cortical area and medullary area. These results suggest that the 150 ppm diet may have compensated by increasing the architecture of the bone in an attempt to maintain strength.

In conclusion, iron deficiency affects bone density, mineral content and geometry Adequate iron intake is one factor in achieving peak bone mass during adolescence. This study has found a common link between bone development and undesirable iron intakes

Recommendations

The following recommendations for future research were developed from this study.

1) Experiments similar to the present experiment should be conducted directly measuring the iron dependent enzymes prolyl and lysyl hydroxylase necessary for hydroxylation of the matrix.

2) Long term studies are needed to evaluate whether results seen in bone density will reflect on bone strength over a longer period of time.

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APPENDIXES

Appendix A

Institutional Animal Care and Use Committee protocol # 709

OKLAHOMA STATE UNIVERSITY

OSU

College of Veterinary Medicine
Laboratory Animal Resources Unit
Stillwater, Oklahoma 74078-2002
405-744-7631

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

OKLAHOMA STATE UNIVERSITY

OSU

College of Veterinary Medicine
Laboratory Animal Resources Unit
Stillwater, Oklahoma 74078-2002
405-744-7631

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

Appendix B

Method for Alkaline Phosphatase Extracted from Bone

Appendix B

Method for Alkaline Phosphatase Extracted from Bone

The determination of alkaline phosphatase extracted from bone is measured by a modified method from Farley et al (1992).

- The lumbar vertebra was removed from -20° C freezer and placed in tube for extraction with 3 ml of phosphate buffered saline with 0.02% sodium azide at a pH of 7.2 and incubated for 12 hours at 4° C to remove contaminants
- The bones were then placed in Type I water and a dessicator with vacuum for one hour.
- Wet weight and air weight were measured using a Density Determination Kit Mettler ME-33340 (Mettler Instruments, Grelfensee, Switzerland) and density by displacement was calculated using Archimedes' principle.
- Using a mallet the vertebrae were crushed.
- Vertebrae were weighed in preweighed acid washed tubes to express alkaline phosphatase per gram of crushed bone.
- 3 ml of 0.01% triton-X 100 with 0.02% sodium azide in Type I water was added to the tube containing the crushed vertebrae and incubated for 72 hours extraction at 4° C.
- The tubes were centrifuged for 15 minutes at 1500xg and then decanted.
- The supernate was tested for alkaline phosphatase using the COBAS Fara II clinical analyzer and reagents from Roche Diagnostic (Roche Diagnostic Systems, Indianapolis, IN).

The analysis of alkaline phosphatase is determined as orthophosphate monoesters are hydrolyzed by alkaline phosphatases into inorganic phosphate and alcohol (Tietz, 1983) Some alkaline phosphatases can form a new ester by transferring a phosphate from the substrate to an alcohol. This is accomplished at a faster rate than the rate of hydrolyzing the substrate. The Roche reagent for alklaline phosphatase uses 4-nitro-phenylphospate as the orthophospate monoester and the phosphate acceptor and buffer is 2-amino-2-methyl-1,3-propanediol. The 4-nitrophenoxide ion has a strong absorbance at 405nm and is proportional to the enzyme activity.

Appendix C

Determination of Hydroxyproline

Appendix C

Determination of Hydroxyproline

The determination of hydroxyproline in the urine is measured by a method from Bergman et al (1970).

- Mix 1.0 mL of urine and 1.0 mL of 12 N hydrochloric acid in marked polypropylene tubes.
- The tubes are placed in an oven overnight (at least 16 hours) at 100-105° C.
- Let the tubes cool down to about 40°C before adding one drop of phenolphthalein solution (1%phonolphthalein – 0.1 g in 10 ml alcohol) and solid lithium hydroxide slowly to each sample until phenolphthalein turns pink
- Vortex until solids are in solution.
- Hydrochloric acid is added until the solution is clear again.
- Distilled water is added to the solution to make 10 ml.

All samples can be prepared up to this point. The following steps were completed on 40 samples at a time due to the lengthy duration of procedures

- Centrifuge for 10 minutes at 1500xg
- Six tests tubes are marked as a, b (for duplicate samples), 1, 2, 3 (for standards), and B (for blank)
- Transfer 250µl of the neutralized hydrolysate into the six test tubes
- In the blank tube 250µl of distilled water is added
- Add 0.5 ml isopropanol to tubes labed a, b, and B

- Standard solutions are prepared by 0.100g of hydroxyproline diluted to 100 ml distilled water. Then the standard is diluted to different concentrations:

  Standard I – 0.5 µg/ml (50 µl of stock diluted to 100 ml with isopropanol)

  Standard II – 1.0 µg/ml (100 µl of stock diluted to 100 ml with isopropanol)

  Standard III – 2.5 µg/ml (250 µl of stock diluted to 100 ml with isopropanol)

- Add 0.5 ml of standard I to the tube labeled 1, 0.5 ml standard II to the tube labeled 2, and 0.5 ml standard III to the tube labeled 3
- The oxidant is made with one part of 7% aqueous chloramine-T and four parts of buffer. The 7% aqueous choramine-T is made fresh daily. It consists of 0.7 g chloramine-T added with distilled water to make a 10 ml solution. The buffer consists of 57 g of sodium acetate, 37 5 g of trisodium citrate, 5.5 g of citric acid and 385 ml of isopropanol. Distilled water is added to make 1 liter of buffer. Add 0.25 ml of oxidant solution to all tubes except the blank tube. Each tube is vortex for 3 seconds, then wait for 4 minutes before adding Ehrlich's reagent
- Ehrlich's reagent is prepared by dissolving 17 6 g of p-dimethylamino-benzaldehyde in 40.8 g of 60% perchloric acid and adding isopropanol to make up 100 ml. This is made just before use. Add 0.5 ml Ehrlich's reagent to all tubes
- Then add 0.25 ml oxidant solution to the blank and vortex all tubes for 10 seconds.
- The tubes are then placed in a 60°C water bath for 21 minutes. Incubate the tubes for one hour at room temperature and then vortex each tube for 5 seconds
- The spectrophotometer (Beckman DU 640, Fullerton, CA) is warmed up 15 minutes before the end of the hour and set at 562 nm to read the absorbance of all tubes. This

is accomplished by reading one at a time. This method relies on the formation of pyrrole upon oxidation of hydroxyproline (Prockop and Undenfriend, 1960). Pyrrole forms a chromophore with the p-dimethylaminobenzaldehyde in the Ehrlich`s reagent

VITA

Lauren McDonald

Candidate for the Degree of

Master of Science

Thesis: EFFECTS OF DIETARY IRON ON BONE IN YOUNG MATURE FEMALE RATS

Major Field: Nutritional Sciences

Biographical:

Personal Data: Born in Lancaster, California, On April 23, 1971, the daughter of Jay and Darla McDonald.

Education: Attended Antelope Valley College, Lancaster, California and Utah Valley State College, Orem, Utah; received Bachelor of Science degree in Dietetics from Brigham Young University, Provo, Utah in April 1998. Completed the requirements for the Master of Science degree with a major in Nutritional Sciences at Oklahoma State University in July, 2000.

Professional Memberships: American Dietetic Association.