THE EFFECTS OF DIETARY COPPER DEPLETION ON BONE METABOLISM IN TWO ANIMAL MODELS OF OSTEOPENIA

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CHAPTER I

RESEARCH PROBLEM

Introduction to the Problem

According to the World Health Organization, osteoporosis is a disease characterized by reduced skeletal mass (i.e. bone quantity) and deterioration of the microarchitecture of bone tissue (i.e. bone quality) that leads to bone fragility and increased risk of fracture (WHO Study Group 1994). Loss of bone mass, or osteopenia, and structural alterations result in diminished mechanical bone strength (Raisz and Smith 1987). Numerous factors have been reported to increase the likelihood of developing osteopenia, including factors that affect either the peak bone mass achieved or subsequent loss of bone. These factors include, but are not limited to, ovarian hormone deficiency, the absence of weight-bearing exercise and nutritional inadequacies (Albright et al. 1940; Albright et al. 1941; Lanyon 1987).

Estrogen provides some degree of protection against bone resorption. Diminished estrogen, associated with natural or surgical menopause, leads to an imbalance of bone remodeling in favor of bone resorption and therefore accelerates bone loss. Likewise, weight-bearing activity provides a stimulus for bone formation. In space, where there is absence of such biomechanical stimuli, rapid osteopenia results. Osteopenia occurring during either the postmenopausal period or spaceflight may be further exacerbated by

nutritional deficiencies. Most research examining the effect of nutritional status on bone health at menopause or during spaceflight has focused on calcium and vitamin D and their role in bone mineralization. Few studies have examined the role of trace minerals in bone health in either preventing or treating osteopenia.

Trace minerals such as copper play an important role in bone metabolism through the formation of the organic matrix (Rucker et al. 1969). The typical U.S. diet provides marginal copper intake and makes moderate deficiency possible in most age-sex groups (Pennington and Schoen 1996). The significance of this moderate deficiency may not be readily apparent in organ systems until such deficient diets are consumed over a long period of time (Levenson 1998). Therefore, the effect of copper deficiency on the osteopenia associated with postmenopausal osteoporosis and weightlessness warrants investigation.

Significance of Problems/Description of Experiments

Although major minerals such as calcium, phosphorus and magnesium make up the majority of the bone mass, the organic matrix provides a network for the precipitation of calcium salts and eventual formation of hydroxyapatite crystals. The trace mineral copper functions as a cofactor for the metalloenzyme, lysyl oxidase. Lysyl oxidase catalyzes the formation of crosslinks as collagen fibers mature and thus provides connective tissue with tensile strength (Prockop et al. 1979). Diminished mechanical strength assessed through torsion testing occurred in the bones of rats on copper restricted diets (Jonas et al. 1993). The bones from these copper-deficient rats were characterized as having abnormal levels of immature collagen fibrils and yet no significant alterations in bone calcium content. The impact of copper deficiency on bone strength in humans is

not fully understood, however, over a two year period copper supplementation in conjunction with calcium, zinc and manganese supplementation reportedly reduced bone loss in postmenopausal women (Strause et al. 1994).

Postmenopausal bone loss is by far the most common cause of osteopenia (Albright and Reifenstein 1948). In humans, the rate of bone loss accelerates during the first ten years following the cessation of menses (Stephen et al. 1987). During this initial period, the rate of total bone loss ranges from 1-5% per year or even more if only cancellous bone loss is considered (Christiansen 1993). Few studies have been performed to examine the effect of copper deficiency on bone parameters in ovarian hormone deficient animals. Experiment 1 was designed to investigate the effect of copper depletion on bone in rats with diminished estrogen. The ovariectomized rat is a suitable model for osteopenia associated with menopause due to similar patho-physiological changes in bone compared to postmenopausal women (Kalu 1991).

During spaceflight, the basic physiological alterations in humans are cephalic fluid shifts, loss of fluid and electrolytes, loss of muscle mass, motion sickness, anemia, reduced immune response, increased urinary calcium excretion and loss of mineralized bone (Hughes-Fulford 1993). Spaceflight-induced osteopenia poses a serious health risk for astronauts, especially as long duration space missions to the International Space Station and interplanetary travel become more common. Due to the prohibitive cost of space flight and the limited space aboard such missions, the tail suspended animal model has been useful model for ground-based research to study the effects of near weightlessness on bone and other physiological parameters (Morey-Holton and Globus 1998). Studies investigating the impact of dietary intake on bone in near weightlessness have focused on calcium, vitamin D and sodium (Globus et al. 1986; Halloran et al. 1986;

Navidi et al. 1995). Experiment 2 was designed to examine the effects of copper depletion on parameters of bone metabolism in mature tail suspended rats.

Objectives

The following research objectives were developed to examine the effects of copper depletion in two models of osteopenia (i.e. ovariectomized rat and tail-suspended rat).

Experiment 1: Copper and ovariectomized rats

1. To investigate the effect of copper depletion and ovariectomy on bone quality and biochemical markers of bone metabolism.
2. To explore the impact of copper depletion and diminished estrogen on the concentration of various minerals in vertebral bone.

Experiment 2: Copper deficiency and tail suspended rats

1. To determine if copper depletion and tail suspension affect bone quality (i.e. bone mineral density, mechanical strength, bone mineral content, etc.) and biochemical markers of bone metabolism in mature rats.
2. To examine the alterations in bone remodeling induced by skeletal unloading and copper depletion in mature rats.
3. To investigate the changes in concentration of selected macro and trace elements in the bone of skeletal unloaded and copper depleted rats.

Hypotheses

The following hypotheses were designed to test the effects of copper depletion and either ovariectomy or tail suspension on parameters of bone metabolism.

Part I: Ovariectomy and deficiency of copper study

1. Diminished dietary copper will not significantly alter bone quality (i.e. bone mineral density, mechanical strength, and bone mineral content) or biochemical markers of bone metabolism (e.g. urinary hydroxyproline and tartrate-resistant acid phosphatase) in ovariectomized rats.
2. There will be no statistically significant differences in vertebral bone mineral concentrations of calcium, magnesium, iron, zinc and copper as a result of ovariectomy, copper depletion or their interaction.

Part II: Tail suspension and copper deficiency study

1. Tail suspension, copper depletion or their interaction will not significantly alter the mechanical strength, bone mineral density or bone mineral content in the mature rat model.
2. There will be no statistically significant effects of tail suspension, copper depletion or their interaction on biochemical markers of bone remodeling (i.e. urinary pyridinoline crosslink excretion, serum alkaline phosphatase, and bone-specific alkaline phosphatase) in mature rats.
3. There will be no statistically significant difference in mature rats in the concentrations of bone calcium, magnesium, zinc, iron and copper due to tail suspension, copper depletion or the interaction of the two treatments.

Limitations

In both experiments included in this dissertation, the age of the animal is an important factor. For example, the ovariectomized rat has been characterized as an appropriate model for the study of postmenopausal osteoporosis, but the fact remains that most studies have continued to use young growing animals who are at different stages of skeletal maturation than females experiencing natural menopause. Likewise, much of the work using the tail-suspended rat to simulate weightlessness was performed on young growing rats. While data on young growing animals provide valuable information on the skeletal response to various stimuli, application of such findings to the mature human skeleton should be performed with caution.

The tail suspension model is a representation of only some of the physiological changes known to occur in space. Compared to space flight where the musculoskeletal system as a whole is unloaded, tail suspension prevents weight-bearing activity on the hind limbs, but the forelimbs remain loaded. The result is partial simulation of weightlessness on muscle and bone. An additional discrepancy in the model and the physiological alterations normally associated with space flight include neurological responses to weightlessness.

As with any animal study, the information should not be directly extrapolated to humans. Data from animal studies can provide the opportunity to understand not only the effects of ovarian hormone deficiency or skeletal unloading on bone, but perhaps more importantly the mechanisms involved. Understanding the underlying mechanisms of such conditions as osteopenia and osteoporosis can provide direction for future human research.

Format of the Dissertation

The two experiments included in the dissertation are organized as individual manuscripts and written using the Guide for Authors from the Journal of Nutrition. Additional data from each experiment that will be reported in future manuscripts is included in an appendix. Experiment 1 was a collaborative study that consisted of 9 dietary treatment groups and provided additional data that will be reported elsewhere.

CHAPTER II

REVIEW OF LITERATURE

Role of Copper in Bone Metabolism

Copper functions as a cofactor for specific enzymes and electron transport proteins in energy metabolism and antioxidant systems. Examples of these proteins that play either direct or indirect roles in bone health are the intracellular protein, superoxide dismutase, and the extracellular proteins, ceruloplasmin and lysyl oxidase. Superoxide dismutase functions to protect cells against oxidative damage. Ceruloplasmin carries approximately 60% of plasma copper and is involved in the metabolism of iron. And lysyl oxidase, which has the most direct effect on bone, is involved in the formation of collagen crosslinks.

Lysyl oxidase is essential for the initiation and regulatory steps for crosslink formation in the connective tissues collagen and elastin (Siegel 1978). By catalyzing the oxidative deamination of lysine side chains, lysyl oxidase initiates a series of spontaneous reactions that link collagen and elastin strands together (Linder and Hazegh-Azem 1996). Inadequate dietary copper intake leads to an increase in collagen solubility resulting from a decrease in cross-linked α-chains (Chou et al. 1969).

Increased bone fragility with copper depletion has been demonstrated in several animal species. Bennetts (1932) was the first to associate a skeletal disorder in lambs

with enzootic ataxia (i.e. swayback) to ewes grazing on certain pastures in Western Australia. The bones of these lambs were poorly developed, light and brittle, but administration of copper (7.5 mg/d) resulted in good skeletal growth and development. Baxter (1951) noted that copper-deficient dogs have bone characterized by thin cortices, wide marrow cavities, diminished trabecular bone and frequent occurrence of fracture. Rucker et al. (1969) observed that bones from copper deficient chicks became brittle and fragile. In a subsequent study, torsion testing was used to demonstrate that the femurs from chicks on copper deficient diets had less plastic deformation and could withstand less maximum torque than the birds on control diets (Rucker et al. 1975). Analysis of the collagen from copper-deficient chicks revealed fewer collagen crosslinks than normal bone. Similar increases in bone rigidity were reported by Riggins and colleagues (1979) in the tibia of chicks. Further investigation of bones from chicks led Opsahl and coworkers (1982) to conclude that a copper intake of 2 ppm is needed for crosslink formation and hence maintenance of mechanical strength, while 6-8 ppm copper is required for optimal growth. Copper deficiency in young growing pigs led to deformed and unstable legs that eventually could not support the animal's body (Teague and Carpenter 1951). While studying the effect of copper-deficiency on the bones of rats, Farquharson et al. (1989) reported collagen crosslinks were diminished in the femoral diaphysis, but remained relatively unchanged in the tibial diaphysis. Jonas and colleagues (1993) found the femur of pair-fed copper-deficient rats could sustain less maximal torque and displayed a significantly decreased ultimate angle of deformation, even though the ash weight and calcium content between the copper-deficient and control rats did not differ. Their findings substantiate the view that not only does copper

deficiency result in weakened bones, but also that the diminished strength is not necessarily a result of changes in mineral content.

Effects of Copper and Other Trace Minerals on Bone

The essential role of the trace minerals copper, manganese and zinc in the formation of bone matrix has been recognized for more than thirty years (Teague and Carpenter 1951; Leach and Muenster 1962; O'Dell et al. 1958). Strause and coworkers (1986) were one of the first groups to study the role of long-term manganese and copper deficiencies in osteopenia. Rats consuming the low copper and manganese diets for approximately six months experienced elevated serum calcium and reduced calcium concentration of the femur. Decreased mineralization and increased bone resorption were implicated in this response to the trace mineral deficiencies. However, further attempts by Strause and associates (1987) to identify the mechanism involved in the bone loss showed long-term manganese and copper restriction inhibits osteogenesis and osteoclast activity.

Zinc is another trace mineral studied in conjunction with copper, manganese and bone. In 1980, Gold (1980) described the slow healing observed in a prominent basketball player, which was associated with low serum copper, manganese and zinc. Strause et al (1994) studied the effect of trace minerals in conjunction with calcium supplements on bone loss in postmenopausal women. This study included women that either received a placebo, a trace mineral supplement, a calcium supplement, or a combination of calcium and trace mineral supplements. Spinal bone loss in older postmenopausal women on calcium supplements was further arrested by concomitant increases in trace mineral intake. These findings are limited due to the lack of statistical

power to demonstrate a significant difference between supplementation with calcium alone and supplementation with calcium and trace minerals. However, the practice of combining key trace minerals that are involved in bone metabolism with calcium supplements appears to have potential benefits beyond calcium supplementation alone.

Copper Intake, Bioavailability and Status

In Western countries the average adult consumes 0.6 to 1.6 mg Cu/d, while the estimated safe and adequate daily dietary intake (ESADDI) ranges from 1.5-3.0 mg Cu/d (Johnson et al. 1992) (National Research Council 1989). Dietary copper intake in the U.S. is described as marginal, when considering the typical diet. The best sources of copper are shellfish, organ meats, nuts, seeds, legumes and whole grains (Linder and Hazegh-Azem 1996). Pennington and Schoen (1996) indicated in their report of the 1982-1991 Total Diet Studies that the estimated intake of copper was below the National Academy of Sciences (NAS) recommendations (1989) for all eight of the age-sex groups studied. Even more of a concern are the findings that more than 60% of menus analyzed in Washington State boarding homes for the elderly were deficient (i.e. contained less than 67%) compared to the ESADDI for copper (Goren et al. 1993). Another study examining the nutrient intake of eating-dependent nursing home residents revealed that 88% of these residents had dietary intakes of less than 50% for three or more essential nutrients. The most frequent and severely deficient were zinc, vitamin B_6 and copper (Rudman et al. 1995).

However, regardless of the intake, copper's bioavailability is influenced by the gastrointestinal content of nutrients such as iron, zinc and ascorbic acid. Divalent cations such as ferrous iron compete with copper for intestinal absorption (Yu et al. 1994;

Wapnir et al. 1993). The antagonistic effect of zinc on copper absorption has been known for decades (Van Campen and Scaife 1967). Serum and liver copper decrease linearly as the logarithm of zinc concentration in the diet increases (Fischer et al. 1981). By increasing dietary zinc intake from 5 to 20 mg/d (76 to 306 μmol/d), the copper requirement needs to be 60% higher to maintain copper balance (Sandstead 1982). Only 3 mg/d (46 μmol/d) more zinc was sufficient to increase copper losses and reduce copper retention (Greger and Snedeker 1980). In general, the interaction of copper with other minerals results in copper being displaced, its intestinal absorption easily inhibited and its bioavailability decreased.

Other dietary factors may also influence the bioavailability of copper. Crude soy proteins can decrease copper bioavailability and induce copper deficiency in chicks (Davis et al. 1962). Deterioration of copper status in humans has been demonstrated when the predominant dietary carbohydrate is fructose (Reiser et al. 1985).

During periods of copper restriction, copper is conserved in a highly organ-specific manner (Levenson 1998). Organs such as the liver appear to engage in copper conservation only after a significant loss of organ copper has occurred. The effects of mild to moderate copper restriction may be significant in some organs, particularly when marginally deficient diets are consumed over long periods of time.

Copper deficiency was considered to be relatively rare, but recent findings reveal that the copper-dependent enzymes commonly used as indicators of copper status (i.e. lysyl oxidase and ceruloplasmin) may not be sensitive enough to detect mild deficiencies. In fact, copper levels had to be reduced by at least 50% of the recommended intake for aneurysm-prone mice to produce a detectable decrease in lysyl oxidase activity (Rowe et al. 1977). Decreased collagen crosslinks may occur with a less severe copper deficiency

and go undetected by usual indicators. Diamine oxidase, erythrocyte superoxide dismutase and platelet cytochrome-c oxidase have been proposed as more sensitive indicators of copper status (Wolvekamp and deBruin 1994; Milne 1998; Johnson et al. 1993). Until the use of these more sensitive indicators becomes common practice, the potential for undetected copper-related health problems is a concern.

Copper and Local Regulators of Bone Metabolism

Cytokines are non-antibody proteins that act as intercellular mediators. Three cytokines, interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) have been implicated in the pathophysiology of osteoporosis (Gowen et al. 1983; Ishimi et al. 1990; Martin and Ng 1994). IL-1 in particular has received attention due to enhanced production associated with estrogen-deficiency in oophorectomized women (Pacifici et al. 1991). Estrogen replacement therapy reportedly suppressed this increase in IL-1 production. IL-1 also enhances the production of ceruloplasmin following various stressors (Barber and Cousins 1988). Ceruloplasmin is an acute phase protein that increases in response to inflammation, infection, estrogen and dietary copper (Cousins 1985). The significance of increased plasma ceruloplasmin in response to IL-1 is yet to be determined.

Copper and Estrogen

There is considerable evidence that the actions of copper and estrogen are interrelated. Studies of women during pregnancy suggest that as estrogen increases during the second and third trimesters of pregnancy, serum copper and ceruloplasmin also increase (Scheinberg et al. 1954). Postmenopausal women on estrogen replacement

therapy have higher plasma Cu concentrations than males (Fisher et al. 1990). In rats, the administration of estrogen and the natural increases of estrogen during the estrous cycle lead to increases in plasma copper concentrations (Russ and Raymont 1956; Johnson et al. 1959; Sato and Henkin 1973). Long-term estrogen therapy has also been shown to increase lysyl oxidase activity (Sanada et al. 1978). Such changes in enzymatic activity could result in increased collagen cross-linking. Studies of the effects of estrogen as one of the active agents in oral contraceptives revealed that increased circulating copper occurred regardless of the dose, chemical form or route of administration (Briggs et al. 1970). Elevated serum copper was recorded within 10 days of starting the estrogen-containing contraceptive, but no change in serum copper was detected with progesterone. Copper also induces greater binding of estradiol to receptors in the uterus of ovariectomized rats (Fishman and Fishman 1987). Unfortunately less attention has been given to the effect of estrogen on copper status in relation to menopause compared to the research concerning the use of oral contraceptives. The only study found in the literature examining the relationship between copper deficiency and postmenopausal osteoporosis was performed by Yee and coworkers (1995). These researchers studied the effects of copper deficient and enriched diets in rats before and after ovariectomy. Ovariectomy-induced osteopenia was found to be more severe with copper deficiency, but was not alleviated by the return of normal dietary copper status. The relationship between reduced estrogen levels and tissue and/or serum copper levels needs to be further studied.

Appropriateness of the Ovariectomized Rat Model

The ovariectomized rat was first characterized as a model for postmenopausal osteopenia by Saville (1969). Because the rat does not normally experience osteopenia

until late in life, the bone loss associated with ovarian hormone deficiency is not complicated by other forms of age-related bone loss (Weiss et al. 1969; Kalu et al. 1984). Estrogen deficiency induced by bilateral ovariectomy stimulates an accelerated rate of osteopenia, similar to the bone loss observed in postmenopausal women (Kalu 1991). Thompson and colleagues (1995) found that the ovariectomized rat mimics postmenopausal osteopenia in studies of up to 12 months in duration. The appropriateness and advantages of longer-term (i.e greater than 12 months) studies of ovariectomized rats (OVX) have not been established.

Kalu et al. (1991) characterized the similarities between bone loss in ovariectomized rats and postmenopausal women as: 1) increased rate of bone turnover; 2) biphasic rates of bone loss; 3) greater loss of trabecular than cortical bone; 4) protection against bone loss by obesity; 5) skeletal response to therapies such as estrogen, tamoxifen, bisphosphonates, parathyroid hormone (PTH), calcitonin and exercise. Postmenopausal women and ovariectomized rats both experience increased bone turnover as a result of enhanced bone formation, and an even greater increase in bone resorption. The biphasic pattern of bone loss in the proximal tibia following ovariectomy was characterized by Wronski and colleagues (1989). An initial rapid phase of trabecular bone loss occurred from the time of surgery to 100 days post ovariectomy and resulted in a decrease in the percent of bone volume made up of trabecular bone from a normal value of ~30% to 5-7%. This rapid phase of osteopenia was followed by an intermediate period of cancellous bone stabilization, and then a slow phase of trabecular bone loss until trabecular bone volume was ~1% of the total bone volume. Omi and Ezawa (1995) found the bone mineral density of ovariectomized young growing and adult rats was significantly reduced at trabecular sites (i.e. lumbar spine and proximal

metaphysis of the tibia) at 1 month post surgery, but cortical sites (i.e. tibial diaphysis) were unchanged. Numerous studies have established protective effects of estrogen replacement therapy on bone in postmenopausal women and ovariectomized rats. PTH is a powerful stimulator of bone formation and completely restores lost cancellous bone in osteopenic OVX rats (Wronski et al. 1993). The bone anabolic effects of PTH are much more pronounced than the effects of estrogen or bisphosphonates. Ovariectomy also results in increased trabecular osteoblasts and osteoclasts, and increased serum alkaline phophatase levels (Kalu et al. 1993). These increases were suppressed with 17β-estradiol therapy. And even though mechanical loading of bone does not prevent bone loss associated with ovarian hormone deficiency, the loading does have a positive effect on the cortical bone of rats (Hagino et al. 1993). Although there is some question whether the same response of cortical bone will be observed in humans, weight-bearing exercise undoubtedly has a positive effect on the bone of postmenopausal women (Chow et al. 1987; Simkin et al. 1987; Dalsky et al. 1988; Smith et al. 1989). Based on these similarities in bone response at menopause and with ovariectomy we can conclude, along with Kalu and others, that the ovariectomized rat is an appropriate model to study the mechanisms of ovarian-deficient osteoporosis.

Ovarian Hormone Deficiency and Bone Loss

In 1941, the term postmenopausal osteoporosis was first coined by Albright and his associates based on the diminished bone mass observed in women following menopause (Albright et al. 1941). Since that time, there has been an on-going effort to define the underlying cause of osteopenia associated with ovarian hormone deficiency. Originally, Albright proposed a theory of osteoblastic hypoactivity as the cause of this

postmenopausal bone phenomenon. In 1958, Heaney and Whedon argued that rather than reduced osteoblastic activity, enhanced bone breakdown was the cause of postmenopausal osteopenia (Heaney and Whedon 1958). This concept of elevated bone resorption continued to gain support. Jasani and colleagues (1965) further explained that ovarian hormones are involved in the regulation of bone and blood calcium. A deficiency of these hormones leads to slightly elevated plasma calcium, hypercalciuria and an overall negative calcium balance. In an attempt to unify the various concepts related to postmenopausal osteoporosis, the focus shifted in the mid-sixties to the hormones involved in calcium regulation. Estrogen deficiency was associated with enhanced parathyroid hormone sensitivity (Heaney 1965), calcitonin (Milhaud et al. 1978) and 1,25$(OH)_2$vitamin D (Riggs and Melton 1983). Although the calcium-regulating hormones received a great deal of attention, the focus in the 1970's and 1980's shifted away from systemic factors and toward local regulators of bone metabolism such as growth factors (Finkelman et al. 1992), cytokines (Pacifici et al. 1987) and prostaglandins (Feyen and Raiza 1987).

In order to study the effects of estrogen deficiency as it relates to hip fracture, Bagi and coworkers (1997) examined cancellous and cortical bone structure and strength of the femoral neck in rats. Despite pair feeding, ovariectomized rats increased body weights and fat body mass, whereas the percent lean body mass steadily declined throughout the experiment. Bone mineral density was significantly higher in the sham relative to the estrogen-deficient animals. Ovariectomy reduced trabecular number and thickness and increased trabecular separation and bone marrow space at the femoral midneck location. Cortical thickness in ovariectomized rats was reduced because of the

high endocortical resorption. Femoral necks obtained from rats had reduced strength and were less stiff relative to controls (Bagi et al. 1997).

The mechanism associated with postmenopausal osteopenia is accelerated bone turnover in which bone resorption exceeds the rate of bone formation. Recent studies have examined the involvement of cytokines such as IL-1, IL-6 and TNF-α. IL-1 production is enhanced in women who have been oophorectomized, while estrogen replacement therapy suppressed IL-1 production (Pacifici et al. 1991). Administration of IL-1 receptor antagonist to ovariectomized rats reduced bone resorption and bone loss (Kimble et al. 1994). TNF-α is also involved in the increased bone resorption observed with estrogen deficiency. Kitazawa and others (1994) observed that TNF-α binding protein, which acts as an antagonist, inhibited bone loss in ovariectomized mice. Likewise, IL-6 is another cytokine thought to stimulate bone resorption in the absence of normal estrogen (Manolagas and Jilka 1995). No change in bone mass was observed in IL-6 knockout mice when ovariectomized (Jilka et al. 1992).

Space flight and Bone

As extended stays in space become more common on the International Space Station or during planetary trips, osteopenia is expected to be a serious health problem facing crewmembers. Among the reported changes in the bone are alterations in mineral content, volume, mass, size, mechanical strength, and materials. Comparing studies of spaceflight and bone loss is complicated by the fact that experimental protocols have varied in duration of flight, age of animal, and the bone studied.

In 1985, NASA initiated a series of spaceflight experiments to study the effect of 1-week missions on the growing rat. The purpose of these experiments was to study the

acute effects of weightlessness on bone in an animal model experiencing accelerated bone remodeling. Many studies have been designed to focus on rapidly growing animals exposed to short-term spaceflight. Few studies, however, have been conducted on mature animals exposed to spaceflight due to concern over the time of exposure required to yield detectable alterations.

Spaceflight, Bone and Animal Studies

Exposure to the microgravity environment of space disrupts the bone remodeling process. Most studies have characterized this disruption as reduced bone formation rather than elevated bone resorption. Morey and Baylink (1978) first described suppressed bone formation in the tibial diameter of long bones resulting from 19.5 days of microgravity aboard the Soviet Cosmos 782 biosatellite. Histomorphometry data revealed no change in bone resorption. Rats flown on the 7-day BIOCOSMOS 1667 flight experienced significantly reduced bone formation in the femur at the trabecular and endosteal levels (Vico et al. 1991a). Bone alterations also have been reported at the humeral and tibial metaphysis in rats exposed to 18.5 days of weightlessness on COSMOS 1129 (Jee et al. 1983). Similar changes in bone formation were reported in a study with two male rhesus monkeys following 11.5 days of space flight (Zérath et al. 1996). Significant reductions in iliac trabecular bone volume, bone mineralization rate, and trabecular bone mineralization surface were observed.

Other studies, however, have found spaceflight-associated bone loss to be a result of increased bone resorption as well as decreased formation. After a 1-week flight (COSMOS 1667), Kaplanskiy et al (1987) reported increased osteoclast number in the tibial primary spongiosa. A study of eight-week-old rats flown on a 14-day mission

revealed increased osteoclastic parameters and reduced osteoblastic surfaces in regions of the femur and lumbar vertebral bodies (Vico et al. 1993b). These observed changes in the osteoclastic parameters indicate bone resorption was also being affected by microgravity. Enhanced production of two cytokines, IL-1β and interferon gamma, which are thought to promote osteoclastic activity could be responsible for increased bone resorption during space flight (Zhang and Turner 1998).

The response of bones that are normally weight-bearing versus non-weight-bearing to the microgravity environment of space differs. Wronski and Morey (1983) reported that only the weight-bearing bones (i.e. tibia) exhibited reduced rate of bone formation as a result of spaceflight. Bone response to a 14-day spaceflight is different in the less weight-bearing bone of the caudal vertebrae than the humerus and thoracic vertebrae (Zérath et al. 1996). Simmons et al. (1983) found that periosteal bone formation was not altered in the non-weight-bearing ribs and regions of the mandible covered by masticatory muscles. However, bone formation and mineralization were impaired on the molar regions of the jaw where no adjoining muscle was present. Short spaceflights with durations between 2 and 6 weeks affect primarily weight-bearing bone (Jee et al. 1983).

Martinez et al. (1988) studied the acute adaptations of the cortical bone matrix in young male rats exposed to 7 days of weightlessness aboard NASA Bioscience Shuttle Mission (SL-3). Delayed maturation of collagen crosslinks and altered microstructure of the cortical bone were found to be responsible for reduced biomechanical properties of bone. The biomechanical strength of the vertebral bodies assessed by compression testing was significantly less than synchronous and vivarium controls (Zernicke et al. 1990). The observed reduction in mechanical strength was attributed to a smaller

proportion of mature crosslinks and therefore slowed maturation of the trabecular bone. Three-point bending tests indicated that 1-week spaceflight inhibited the maturation of bone strength and stiffness. These effects were more notable in the tibia than in the humerus (Shaw et al. 1988). A defective area of collagen fiber organization called an "arrest line" was observed in growing rats flown on 19 day spaceflights on COSMOS 782 and 936 biosatellites (Turner et al. 1985). This arrest line may be partially responsible for the decreased torsional strength associated with weightlessness. Several other studies have also shown alterations in collagen maturation and decreased crosslink formation with spaceflight (Simmons et al. 1983b; Turner et al. 1985; Zérath et al. 1991; Martinez et al. 1988).

Vico and others (1988; 1993) studied the effects of 1, 2 and 3 weeks of microgravity exposure on trabecular bone. During the first two weeks, a reduction in the primary spongiosa, trabecular thinning and disappearance of the secondary spongiosa occurred. Decreased bone formation and an abrupt and transient increase in bone resorption resulted in substantial bone loss. By the end of the third week, bone loss continued but at that time appeared to be the result of decreased formation and relatively little or no change in bone resorption. Jee et al. (1983) examined the effect of 18.5 days of spaceflight on trabecular bone and determined that bone formation was inhibited as indicated by decreased osteoblast number, while bone resorption remained constant.

Alterations in the mineral concentrations have also been reported in the bone of animals exposed to weightlessness. Animals on Spacelab-3 showed significantly less calcium and phosphorus in the cortical bone of the femur than the ground-based controls (Russell and Simmons 1985). Total calcium and phosphorus in the jaws, incisors and ribs were normal after spaceflight. Bone mineral concentration of rats exposed to 14

days of weightlessness aboard the space shuttle Columbia (Space Transport System-58) was analyzed. Cervical and thoracic vertebrae were collected as weight-bearing bones, while ribs were collected as non-weight-bearing bones. Calcium and phosphorus concentrations were significantly increased and sulfur significantly decreased in the weight bearing bones of the flight animals compared to ground-based controls. Iron, zinc and magnesium were significantly reduced in both the weight-bearing and non-weight-bearing bones (Yamada et al. 1997).

Spaceflight, Bone and Human Studies

Microgravity affects the human skeletal system resulting in hypercalciuria and local osteopenia. Since histological bone data are not available on astronauts, measures of bone mineral density and biochemical markers of bone metabolism must be used as means of assessment. Alterations in biochemical markers and bone mineral density associated with bone loss occur as early as one week into space flight and are still occurring after 312 days (Smith et al. 1998; Schneider et al. 1992).

Grigoriev et al. (1998) reported bone mineral density and bone mineral content from data on 18 cosmonauts who flew missions ranging from 4.5-14.5 months in duration. The greatest bone mineral density losses were observed in bones of the lower body (i.e. pelvis, lumbar vertebra and femur), while the bone mineral density of bones of the upper body (i.e. skull, cervical vertebra, arms, ribs and sternum) was either not decreased significantly or revealed a trend toward increasing. Similar bone mineral density results were reported from two subjects who flew on separate Space Shuttle missions of either 13.7 days or 8.9 days (Miyamoto et al. 1998b). Less than 2-week missions resulted in significant reductions of bone mineral density in weight-bearing

bones (i.e. 2^{nd}-4^{th} lumbar vertebra), and significant increases in the bone mineral density of the skull.

Miyamoto et al. (1998) also reported alterations in biochemical markers of bone metabolism. These data revealed stimulation of bone resorption as shown by increased urinary pyridinoline crosslink excretion and plasma tartrate-resistant acid phosphatase activity, and increased bone specific alkaline phosphatase, an indicator of bone formation. Fractional excretion of calcium increased in both subjects during the first week of space flight. These findings coincide with earlier studies reporting hypercalciuria associated with exposure to microgravity (Whedon et al. 1976).

Bone biochemical markers in cosmonauts before, during and after a 180-day space flight (Euromir 95) also revealed decreased bone formation during flight and a return to preflight concentrations at seven days after landing (Caillot-Augusseau et al. 1998). Indicators of bone formation, intact osteocalcin, bone alkaline phosphatase, and the carboyxl-terminal propeptide of human type 1 procollagen decreased during flight and increased upon return to the 1-g environment of earth. Bone resorption was assessed by measuring the urinary excretion of free and total pyridinoline crosslinks and C-telopeptide. During spaceflight free deoxypryridinoline crosslinks and the C-telopeptides increased by 54% and 78% respectively, and slowly (i.e. ~ 30 days) returned to preflight values after landing.

Two cosmonauts who spent 1 and 6 months in the Russian MIR space station experienced decreased trabecular bone mass in the tibia. No alterations were observed in the radius or the cortical bone of either after 1 month of weightlessness (Collet et al. 1997). A trend toward a decrease in all markers of bone formation was reported along with increased pyridinoline excretion; however, none of these variables were statistically

significant. After 6 months of spaceflight, a more dramatic decrease in the tibial trabecular and cortical bone was determined by quantitative computed tomography (QCT). These changes continued to be significant even after 6 months of recovery.

Data collected on the Skylab missions revealed a significant correlation between bone loss and space flight duration (Leach et al. 1997). Bone loss in Skylab mission astronauts resulted from an increased rate of bone resorption in addition to diminished rate of formation. Examination of deoxypyridinoline crosslink data from Skylab 2, Skylab 3 and Skylab 4 revealed an increase in the rate of resorption within the first week of space flight (Smith et al. 1998).

Copper and Skeletal Unloading

Limited research has examined the relationship between skeletal unloading and copper. Prokhonchukov et al (1978) described an increase in the copper content in the femur of cosmonauts and a decrease in the copper content of the vertebral body and sternum. Krebs and colleagues (1988) found that 5 weeks of bed rest had no effect on copper balance, but decreased the zinc balance significantly. Groups that received fluoride supplementation experienced improved zinc balance compared to untreated controls. A study of longer term bed rest (i.e. 17 weeks) followed by re-ambulation (i.e. 7 weeks) observed that during bed rest individuals lose more total body zinc, and retain more zinc and copper when they return to being ambulatory. And finally, *in vitro* studies of bone cells from tail-suspended rats found no anabolic effects with copper supplementation (Yamaguchi and Ehara 1996).

Appropriateness of Tail-suspension Rat Model

Due to the prohibitive expense and space limitations on mission flights, alternative methods of investigating microgravity-induced physiological changes are needed. Ground-based research models that simulate changes in the musculoskeletal and cardiovascular systems include bed rest or immobilization in humans and tail-suspension with rats and mice. The reduction or absence of physical activity by immobilization or bed rest has continuously resulted in increased bone resorption (Aloia et al. 1978; Krolner et al. 1983; Smith et al. 1984). The tail suspended rat has been described as an appropriate model for evaluating the physiological and cellular mechanisms of bone change in weightlessness (Morey-Holton and Globus 1998). This animal model also allows for the evaluation of the effectiveness of potential countermeasures to bone loss during weightlessness.

The tail suspension model was developed in the mid-1970's to simulate the effects of weightlessness experiences during spaceflight (Morey 1979). Young growing rats were used in tail suspension studies because of their rapid rate of bone turnover and the similarities in the alteration of bone metabolism with the changes resulting from short-term spaceflight. The head-down tilt of the body at a 30° angle is accomplished using the specially designed metabolic cages (Harper et al. 1994). This design results in a cephalic fluid shift comparable to the change that occurs in space, and unrestricted movement of the forelimbs and hindlimbs. Tail suspended rats resume normal feeding, drinking and grooming in a matter of days following the initiation of suspension. Unlike space flight where unloading of the entire skeleton occurs, the hindlimbs of the tail suspended rat are subjected to weightlessness, while the forelimbs are normally loaded and support 50% of the animal's body weight (Hargens et al. 1984). The humerus and

radius provide internal controls allowing for distinction between systemic and local factors affecting the unloaded hindlimbs.

In both spaceflight and tail suspension less bone is formed. Perhaps more important is the fact that the bone that is formed is considered less mature and less mineralized (Abram et al. 1988; Bikle et al. 1987; Globus et al. 1984; Globus et al. 1986a; Kidder et al. 1990; Leblanc et al. 1985). The bone also loses mechanical strength as a result of unloading (Abram et al. 1988; Shaw et al. 1987c). Stehle (1998) reviewed the research issues related to animal models of space flight-induced bone loss and concluded that tail suspension of young animals mimics some aspects of the microgravity effect on bone. Research with mature rats is needed, however, to clarify the role of stress in the tail suspension model on markers used in bone research.

Bone Metabolism and Tail Suspension

Tail suspension disrupts bone remodeling, inhibits bone maturation and mineralization, causes transient changes in calcium regulation, and diminishes mechanical bone strength. Many studies have reported reduced bone formation as the primary cause of osteopenia associated with skeletal unloading. Bone formation was significantly inhibited by day-5 of tail suspension in young growing rats (Globus et al. 1986a). Similar changes in the rate of bone formation have been reported elsewhere in the literature (Leblanc et al. 1985; Bikle, Halloran et al. 1987; Simske et al. 1994). However, in many cases the reported decrease in bone formation is a transient response to skeletal unloading, and formation is restored to normal within 10-14 days (Globus et al. 1986a; Vico et al. 1991b). LeBlanc et al. (1985) observed "phasic alterations" in bone turnover following 14, 30, 60 and 90 days of tail suspension in mature rats. By day-14 of

suspension, the rate of bone metabolism had significantly decreased, while at day-30 the rate had returned to near control values. Depression of bone turnover during the first 14 days of unloading appeared to result from systemic alterations since both the weight-bearing (i.e. humerus) and non-weight-bearing (femur and tibia) bones were affected. Only after 60 days of tail suspension was a significant decrease in the total calcium content of the femurs reported and osteoclastic activity increased. And it was not until 90 days of suspension that the cortical bone of the femoral diaphysis appeared to have characteristics of osteoporosis.

Vico et al. (1991) examined the bone cell adaptations of mature male Wistar rats to one-, two- and six-weeks of tail suspension using histomorphometry. The acute response observed at the end of the first week was depressed formation and a dramatic increase in bone resorption. After two weeks of tail suspension, a recovery phase occurred with bone cell activities returning to equilibrium, but bone mass reaching subnormal values. By six weeks of skeletal unloading, bone cellular activity was slightly uncoupled, although not significantly, and bone loss associated with trabecular thinning and reduced trabecular number was observed. These observations indicate a rapid phase of bone loss, similar to the response in women during the early postmenopausal period, is followed by a more gradual rate of bone loss for an extended period of time. However, in contrast with these findings, Martin et al. (1990) reported no alteration in the mean mineral apposition rate at the femur midshaft by tail suspension.

Bateman and colleagues (1997) reported notable differences in responses of male and female growing mice to skeletal unloading. Males experienced decreased formation rate confined to the endocortical perimeter, while females had diminished formation in the periosteal perimeter. Since the periosteal apposition in the male mice is maintained,

normal development of structural properties of the bone are preserved. The response of the female mice, however, will prevent the increase in structural properties associated with growing animals. The authors concluded that sex hormones likely play a role in the different responses of males and females to skeletal unloading. Furthermore, Kawata and colleagues (1998) studied suspended ovariectomized mice to elucidate the histological changes associated with unloading the hindlimbs and diminished estrogen. Trabecular bone was extensively replaced by bone marrow in the suspended ovariectomized group, and an increase in osteoclasts was observed.

Etiology of Bone Loss Associated with Tail Suspension

Although the etiology of osteopenia associated with skeletal unloading is not fully understood, both systemic and local factors are believed to play key roles. The initial phase of reduced bone formation is preceded by a small transient increase in serum calcium and a decrease in 1,25 dihydroxyvitamin D (Halloran et al. 1986). Serum levels return to normal within 5-15 days. Halloran and associates (1997) examined the effects of parathyroid hormone (PTH) administration to growing rats subjected to 8 days of hindlimb suspension. Increased mRNA for osteocalcin was found, as well as increased cancellous bone volume in the proximal tibia and bone formation in the tibiofibular junction. Although PTH preserved the trabecular bone volume in unloaded animals, it did not restore periosteal formation to normal nor prevent the deficit in overall tibial mass induced by unloading. Ostoecalcin, an indicator of bone mineralization, was also depressed in response to tail suspension, but returned to normal along with calcium in 5-15 days (Patterson-Buckendal et al. 1989).

Other systemic factors implicated in bone loss in both the tail suspended model and spaceflight are the stress hormones. Plasma glucocorticoids in tail suspended rats were similar to controls (Halloran et al. 1988). Spaceflight studies, however, have reported adrenal hypertrophy in flight animals, suggestive of excess corticosteroids in response to the stress of space travel (Portugalov et al. 1976; Grindeland et al. 1990). Corticosterone does not appear to induce trabecular bone loss in rapidly growing rats, but it has been shown to inhibit cortical and trabecular bone formation (Jowell et al. 1987; Li et al. 1996). And recently a rather intriguing report by Wronski and colleagues (1998) revealed that with group housed animals aboard the space shuttle Columbia, no change in bone mass and bone formation was noted even though corticosteroid excess occurred.

Local regulators of bone metabolism are also affected by skeletal unloading. Molecular studies indicate that both tail suspension and spaceflight transiently increase mRNA for insulin-like growth factor 1 (IGF-1), insulin-like growth factor-1 receptors (IGF-1R), and alkaline phosphatase, but decrease mRNA for osteocalcin (Bikle et al. 1994). The shifts in osteocalcin and alkaline phosphatase are consistent with decreased bone maturation whereas the increase of IGF-1 and IGF-1R indicate a compensatory response to decreased bone formation. Machwate et al. (1994) infused suspended animals with IGF-1 and found enhanced osteoblast recruitment, increased trabecular bone formation, and a reduced decrement in trabecular bone loss. The authors concluded that IFG-1 may be one of the mediators of the effects of mechanical loading on bone. Other local factors such as nitric oxide and prostaglandins have also been implicated in the mechanisms of bone alterations associated with skeletal loading (Chow et al. 1998).

Tail suspension also affects cell proliferation and differentiation. Following 5 days of tail suspension, tibial bone marrow stromal cells exhibit 50% less c-fos, an index

of cell proliferation, and 61% more alkaline phosphatase, an indicator of early osteoblast differentiation (Kostenuik et al. 1997). Osteocalcin mRNA is reduced by 35%. Barou and colleagues (1998) examined the cellular response to 6 days of tail suspension in 5-week-old rats and observed decreased proliferation of periosteal and trabecular preosteoblasts in primary and secondary spongiosa as well as bone marrow cells. Clearly, the memory of loading *in vivo* in cultured osteoprogenitor cells is retained so that osteoprogenitor cells *in vitro* have inhibited proliferation and differentiation.

There are conflicting reports concerning the effect of tail suspension on intestinal calcium absorption. Over a 14-day calcium balance study, Navidi and associates (1995) observed a significant decrease in net calcium absorption. Other experiments using the duodenal loop method and an *in vitro* everted gut technique revealed no change in duodenal calcium transport (Globus et al. 1986; Globus et al. 1984).

Along with affecting bone turnover, unloading of the hind limbs also retards bone mineralization and depresses skeletal maturation (Bikle et al. 1987). Immature bone can result from impaired osteoblast differentiation, matrix maturation and/or mineralization. Vitamin D deficiency is known to decrease bone formation and maturation in part due to immature cross-link formation of the collagen matrix (Mechanic 1976). Van Loon, J., et al. (1995) reported decreased mineralization and increased calcium release in isolated fetal mouse long bones under near weightlessness.

One aspect of tail suspension and spaceflight that may play an important role in osteopenia is the cephalic fluid shift. Leach (1979) indicates that the space flight-induced disturbances in the cardiovascular, renal, hormonal, autonomic and biochemical systems can be attributed to two major effects of weightlessness: 1) the absence of hydrostatic forces leading to fluid-electrolyte shifts within the body, and 2) the absence of

mechanical forces resulting in the degradation of normally weight-bearing tissues. When Vico and associates (1995) studied the effects of 14 days of tail suspension in 6-month-old rats, they found lower calcium content, ash weight, bone mineral content (BMC) and bone mineral density (BMD) in the tibia and femur. No significant changes were detected in the humerus or skull. However, one group of animals was re-ambulated at the end of the study and the skull showed a decrease in BMC, ash and dry weights. The researchers concluded the changes observed in the skull emphasize the importance of the fluid shifts on bone metabolism during tail suspension. Roer and Dillaman (1990) have found bone growth to be influenced by the cardiovascular fluid changes. Since mean arterial blood flow began to decrease immediately at suspension and continued until reaching a plateau at ~60% of the controls by day 5, the authors suggest a possible role of diminished blood flow in the bone loss and muscle atrophy associated with tail suspended rats and weightlessness.

Ultimately the major risk associated with osteopenia is reduced bone strength, and increased incidence of fracture. More than seventy-five years ago, Allison and Brooks (1921) described significant geometric property changes in bone of immobilized or denervated limbs. Osteopenia and reduced structural properties (i.e. bone rigidity and ultimate torque) associated with skeletal unloading are a result of both geometrical and material changes (Abram et al. 1988). These material changes include ash percent of bone and calcium content. Histomorphometric analysis of the femur revealed diminished formation resulting in a breakdown of the maturation process of the bone. Growing rats, undergoing tail suspension, also experienced differences in femoral strength. Shaw and associates (1987) examined the influence of a 4-week weight-bearing program on mechanical strength of young female rats classified as either hindlimb suspended-

sedentary, hindlimb suspended-high intensity exercise (i.e. highly intensive treadmill running) or controls. Region-specific cortical thinning and endosteal resorption in the tibial and femoral diaphysis occurred in conjunction with decreased mechanical properties of the bone. Even though the intensive running group experienced a positive effect of the exercise in terms of muscle atrophy, the strenuous exercise did not counteract the effects of suspension on mechanical properties. Simske et al. (1990) demonstrated that age is an important determinant in the duration of tail suspension necessary to alter bone mechanical characteristics. Mice ranging in age from 1.3-6 months that were suspended for 2 weeks experienced significant decreases in mechanical properties, but mice older than six months experienced no change due to suspension.

Tail Suspension and Other Physiological Parameters

Muscle atrophy also occurs during spaceflight and tail suspension. Decreased muscle mass has been observed in the soleus, gastrocnemius and extensor digitorum longus (Morey-Holton and Wronski 1981; Musacchia et al. 1980; Oganov et al. 1980). LeBlanc and colleagues (1985) observed a greater atrophic response in the soleus compared to the gastrocnemius muscles. Slow-twitch muscle fibers appear to respond to skeletal unloading with a greater degree of atrophy than fast-twitch fibers. This response results in an increase in the percentage of fast-twitch fibers. Muscle mitochondrial density also decreases from skeletal unloading.

Shaw et al. (1987) studied the effects of skeletal unloading on muscle and the use of intense treadmill running as a countermeasure. Female rats were separated into one of three groups; control, sedentary suspended, or exercise suspended. Suspension produced generalized atrophy of skeletal muscle especially the slow twitch muscle fibers.

Although the treadmill running counteracted the muscular atrophy during the suspension, the exercise did not mitigate the changes in bone mechanical properties and cross-sectional morphologies and in some cases exacerbated the changes.

The responses of bone and muscle tissue to tail suspension have been widely reported in the literature, but alterations in various blood parameters have not been thoroughly studied. Nakaya and associates (1990) compared the changes in male and female rats to tail suspension. In male rats, serum albumin as well as creatinine, uric acid, glucose, triglycerides, hemoglobin and serum iron were significantly reduced by tail suspension. Tail suspension in female rats resulted in a significant decrease in serum albumin and iron, while statistically insignificant trends toward decreased glucose, triglycerides, hemoglobin and uric acid were observed. No change in creatinine was observed in the female rats due to suspension. Apparently alterations in common clinical blood parameters were more marked in male verses female rat. Understanding the effect of tail suspension on clinical blood chemistry parameters as well as muscle provides valuable insight into the changes in bone occurring as a result of weightlessness.

CHAPTER III

THE EFFECT OF COPPER DEFICIENCY AND ESTROGEN DEPLETION ON PARAMETERS OF BONE METABOLISM IN RATS

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Abstract

Copper, as a cofactor for lysyl oxidase, is essential for collagen crosslink formation. The relationship between copper and estrogen makes the effects of copper depletion on the bone health of postmenopausal women a particular health concern. The purpose of this study was to examine the effects of copper depletion and ovariectomy (OVX) on bone. Weanling female Sprague Dawley rats (n=32) were fed the AIN-93G diet for 5 weeks before OVX or sham-OVX (Sham). Five to six days after surgery, rats were randomly assigned to dietary treatments (control—AIN-93M diet or $^{-}$Cu with 0.6 mg added copper as cupric carbonate per kg of diet). Ten weeks after OVX rats were food deprived for 12 h, anesthetized and exsanguinated by cardiac puncture. Increased

weight gain ($p<0.01$) became significant due to OVX by week 4 after surgery. Thymus weight ($p<0.001$) as percent body weight was increased while femur ($p<0.01$), tibia ($p<0.01$) and 4th lumbar vertebrae (L4) ($p<0.01$) were decreased by OVX. The dry weight of the tibia ($p<0.01$) was increased by OVX, but L4 dry weight and ash weight were not affected. OVX reduced 5th lumbar vertebra bone mineral density (BMD) ($P<0.001$), but no change was observed in the femur. The mechanical strength of the femur was increased ($p<0.05$) by OVX, but not by diet. L4 magnesium and zinc concentrations were reduced ($p<0.05$) by OVX. Copper depletion had no effect on the calcium, magnesium, zinc, iron or copper concentrations of L4. Serum phosphorus ($p<0.05$) and liver copper concentrations ($p<0.05$) were reduced in the copper depleted animals. Urinary excretion of calcium, magnesium and phosphorus were not altered by OVX. Urinary hydroxyproline was increased ($p<0.01$) in response to OVX, while other markers of bone metabolism were unchanged. These findings suggest that weight gain provided skeletal loading that countered the bone loss associated with OVX. Copper depletion had no effect on the mechanical strength, bone mineral density or bone concentrations of calcium, magnesium, zinc, iron and copper. However, further studies examining the role of copper depletion on the osteopenia associated with ovarian hormone deficiency are needed.

Key Words: osteopenia, copper, postmenopausal, bone

Introduction

Loss of bone mass observed in women during the postmenopausal period is the most common cause of osteopenia (Albright and Reifenstein 1948). Estrogen provides

protection to the bone by acting directly on osteoblast cells through high affinity estrogen receptors (Eriksen et al. 1988). Diminished estrogen results in an increased rate of bone turnover, with a greater increase in bone resorption than bone formation (Heaney et al. 1978). Nordin et al. (1990) reported that the hormonal changes at menopause lead to the loss of approximately 11% of bone mass during the first 5 years following menopause and an additional 5% over the next 20 years. This rapid rate of bone loss results in nearly half of all women over the age of 50 suffering from a fracture related to osteoporosis (McBean et al. 1994).

In addition to estrogen, nutritional factors are also involved in the pathogenesis of postmenopausal osteoporosis. Heaney (1993) discussed most of the nutrients related to adult bone health and categorized them into essential (i.e. calcium, phosphorus and vitamins C and D), interactive (i.e. protein and sodium) and uncertain (i.e. vitamin K, manganese, zinc and copper) nutrient groups. Calcium, phosphorus and vitamin D have undoubtedly been the most extensively studied. Protein and sodium increase calcium urinary excretion and excessive intake of either of these "interactive" nutrients can lead to significant urinary calcium loss. (Heaney 1993b; Barrett-Connor et al. 1994). Of the group characterized as having an "uncertain" effect on adult bone health, copper is of special interest due to its relationship with estrogen and known role in bone health.

Considerable evidence exists that copper and estrogen are interrelated. In rats, ceruloplasmin and serum copper concentrations increased with the administration of exogenous estrogen and with the natural increases in estrogen during the estrous cycle (Russ and Raymont 1956; Johnson et al. 1959; Sato and Henkin 1973). Copper also increased the affinity of estradiol for receptors in the uterus of ovariecomtized rats (Fishman and Fishman 1987). Women typically have a higher plasma copper

concentration than men (Fischer et al. 1990). Studies of women taking oral contraceptives with estrogen as one of the active ingredients revealed increased serum copper and ceruloplasmin (Briggs et al. 1970). During the second and third trimesters of pregnancy when estrogen is increased, circulating copper also increases (Scheinberg et al. 1954). And estrogen replacement therapy reportedly elevated plasma copper concentrations in postmenopausal women (Fisher, L'Abbe´, and Giroux 1990). Estrogen therapy has also been shown to enhance lysyl oxidase activity in the bone (Sanada et al. 1978).

The role of copper in bone health has been related to the activity of lysyl oxidase. Copper functions as a cofactor for lysyl oxidase. Lysyl oxidase is essential for the crosslink formation of collagen and elastin (Siegel 1978). Deficiencies of copper have been shown to produce osteoporotic-like symptoms and increase bone fragility in several animal species and in humans (Jonas et al. 1993; Opsahl et al. 1982; Bennetts 1932; Baxter 1951; Dollwet and Sorenson 1988).

Dietary copper intake by adults in Western countries averages between 0.6 and 1.6 mg Cu/d (Johnson et al. 1992). Compared to the estimated safe and adequate daily dietary intake (ESADDI) recommendation of 1.5-3.0 mg Cu/d, dietary intake in the U.S. is marginal. Futhermore, copper absorption can be diminished by high fructose consumption, food processing and certain divalent cations (i.e. zinc, iron and stannous tin) (Wapnir 1998). Postmenopausal women who are not on estrogen replacement therapy may have reduced copper plasma concentrations that are further compromised by moderately deficient copper diets and reduced bioavailability.

Yee et al. (1995) investigated the effects of moderately copper-deficient (1.79 mg Cu/kg diet) or copper enriched diets (22.7 mg Cu/kg diet), and estrogen depletion on rats. Diets were altered independently prior to and following ovariectomy. The osteopenia

observed in the ovariectomized rats was slightly more pronounced with copper deficiency. And copper enrichment following ovariectomy did not alleviate bone loss due to prior copper deficiency and diminished estrogen.

The purpose of this study was to further examine the effects of a diet lower in copper on bone quantity and bone quality in ovariectomized rats.

Methods

Thirty-two weanling female Sprague Dawley rats (Sasco) were individually housed and fed the AIN-G diet with no added chromium for five weeks prior to surgery (Reeves et al. 1993). Room conditions were maintained at 20-24°C with 12 h light-dark cycles. Animals were assigned to weight matched ovariectomized (OVX) and sham-operated (Sham) groups, anesthetized with halothane and underwent the surgical procedure.

Following a 5-6 day post-operative recovery period, the weight matched groups were randomly assigned to either the control diet (control=AIN-93M) or copper depletion diet ($^{-}$Cu = 0.6 mg Cu added per kg of diet). The copper concentration of the control and $^{-}$Cu diet was determined by ashing diet samples and analyzing by atomic absorbance spectrophotometry (Perkin Elmer, Norwalk, CT). Atomic absorbance indicated that the copper concentration of the copper deficient diet was 1.3 mg/kg diet and the control diet was 5.8 mg/kg diet. Animals were weighed on a weekly basis with the exception of week-2.

Ten weeks after surgery, rats were placed in metabolic cages and food deprived. After a 12 h urine collection, rats were anesthetized using ketamine (100 mg/kg body

weight) and xylazine (5 mg/kg body weight), placed in the EM-Scan (Springfield, IL) for body composition analysis and then exsanguinated by cardiac puncture.

Sample Collection

Liver, kidney and spleen were excised, cleaned of all adhering connective tissue, and stored at -20°C for future analysis. Both femurs and tibias, and the 4th and 5th lumbar vertebrae (L4-L5) were removed and carefully cleaned in an attempt to prevent damage to the pereosteum, and stored at -20°C.

Serum Analysis

Blood samples were allowed to sit on ice for approximately 30 minutes and then centrifuged at 1500 X g for 20 minutes. Aliquots of serum were taken to assess calcium, magnesium, phosphorus, iron, creatine and alkaline phosphatase using the clinical analyzer (Cobas Fara II). Creatinine and all minerals with the exception of iron were assayed using Roche Reagents (Nutley, NJ). Raichem (San Diego, CA) reagents were used to analyze serum iron. Serum creatinine was analyzed using a modification of the method by Larson (1972) and absorbance read at a wavelength of 520 nm. Magnesium was measured based on the method of Ferguson and colleagues (1964) and the change in absorbance was determined at 550 nm. Calcium was analyzed on the serum using a modified version of the Michaylova and Illkova method (1971)and read at 600 nm. Serum inorganic phosphorus was determined based on a modification of the method of Daly and Ertingshausen (1972). Serum alkaline phosphatase was also assayed as an indicator of bone formation, using a modification of the method of Tietz (1983)

Urine Analysis

The twelve-hour urine collections were centrifuged at 2100 rpm for 30 minutes and then stored at -20°C. Urinary creatinine, magnesium, calcium, and phosphorus excretion were analyzed on the clinical analyzer using the same reagents and similar methodology as previously described on the serum. As an indicator of collagen degradation, urinary hydroxyproline was assessed based on the method of Bergman and Loxley (1970).

Tissue Mineral Analysis

Liver samples were weighed (wet weight of ~200 mg) into acid-washed borosilicate glass tubes and dried for 24 h at 100° C. Tissue dry weights were recorded. Samples were then exposed to a series of wet and dry ashing until all organic material was removed using a modification of the method of Hill and colleagues (1986). Wet ashing was accomplished by adding 50-100 µl of double distilled concentrated nitric acid (GFS Chemicals, Columbus, OH) and hydrogen peroxide (J.T. Baker, Phillipsburg, NJ)). Dry ashing was carried out using a ramping protocol with temperature settings of 275°C for 600 minutes and 375°C for 1440 minutes in a muffle furnace (Lindberg, Watertown, WI). The removal of all organic material was considered complete when samples were either white in color or lucent. Ash weights were recorded and then samples were diluted with 0.5% double distilled nitric acid (GFS Chemicals, Columbus, OH). Samples were analyzed for magnesium, zinc, iron, and copper using a Perkin-Elmer Zeeman 5100 flame atomic absorption spectrophotometer (Perkin Elmer, Norwalk, CT). L4 vertebral samples were analyzed following the same basic procedure as described for the liver with the exception of only one dry ashing temperature set at 375°C for 1440 minutes.

Samples were then analyzed for calcium, magnesium, iron, zinc, and copper by flame atomic absorption.

Bone Densitometry Analysis

Dual-energy-x-ray densitometry (DEXA) scans were performed on the femur and L5 of each animal using the small animal high-resolution scan module (Hologic QDR-2000). During the scan, soft tissue was simulated by placing each bone in deionized water at a depth of approximately 2 cm. Bone mineral area (BMA in cm^2), bone mineral content (BMC in g) and bone mineral density (BMD in g/cm^2) were recorded from the DEXA.

Mechanical Strength and Bone Morphology

Three-point bending tests were performed on femurs (Instron Model 1122). Samples were allowed to reach room temperature prior to testing. Femur length was measured with a vernier caliber (accuracy: 0.1 mm) from the head of the femur to the distal condyles. The external diameter of the femur was measured at the midshaft by taking two measurements 90° apart. The femurs were then placed in the three-point bending fixture and positioned so that the posterior bone surface rested on the lower supports and the upper supports contacted the anterior surface of the bone. During the test, the anterior surface was compressed in the midshaft region at a displacement rate of 3 mm/min. The load displacement curve was recorded simultaneously throughout the test so that break load and modulus could be determined from the curve. Immediately following the tests, cortical thickness was assessed by taking four measurements, 90°

apart, at the point of fracture. Cortical thickness was determined as the average of these four measurements.

Biochemical Analyses of Bone Metabolism

Bone-specific indicators of bone formation (i.e. alkaline phosphatase) and bone resorption (i.e. tartrate-resistant acid phosphatase (TRAP) and bone hydroxyproline) were assessed to determine alterations bone metabolism. The left tibia from each animal was de-fatted and protein extracted using a modification the method of Martin et al (1997). Extracts were then analyzed on the clinical analyzer (Cobas Fara II) for alkaline phosphatase, TRAP and hydroxyproline. Bone-specific alkaline phosphatase, TRAP and hydroxyproline were then calculated per gram of dry bone.

Results

Weight gain was significantly greater ($p<0.05$) in the OVX group by the fourth week after surgery (Table 1). By the final week of treatment, the OVX group's body weight was approximately 27% greater than the weight of the sham group. Copper depletion produced a transient increase ($p<0.05$) in body weight at weeks five and six, but failed to reach the level of significance at any other time during the treatment period. Body composition analysis revealed no significant alteration in percent fat despite the considerable weight gain (Table 2). Tissue weights expressed as a percentage of body weight resulted in the thymus ($p<0.001$) being significantly greater in the OVX group, compared to the sham-operated controls (Table 3). In contrast, the weights of the kidney ($p<0.001$), femur ($p<0.001$), tibia ($p<0.001$) and L4 ($p<0.001$) as percent body weight were significantly decreased by OVX. Copper depletion significantly reduced kidney

weight ($p<0.05$) expressed as a percentage of body weight. Tibia dry weight was significantly increased by OVX, but L4 dry weight and ash weight were not altered by diminished estrogen or dietary treatments (Table 4).

Assessment of BMC, BMA and BMD by DEXA revealed a significant decrease in the BMC ($p<0.05$) and BMD ($p<0.001$) of L5 of the OVX group, but femur BMC and BMD were not altered (Table 5). Copper depletion failed to produce alterations in either vertebral or femoral BMD. And BMA was not significantly affected by OVX or dietary treatment.

Bone strength was tested at the midshaft of the femur by three-point bending and provided some unexpected results. The femurs of OVX animals withstood greater break load ($p<0.05$) than the femurs from the sham group (Table 6). The modulus tended to be increased in the OVX group, although not to a level of statistical significance. Mechanical strength of the bones was not significantly influenced by copper depletion. Femur length was significantly greater in the OVX compared to the Sham group, but cortical thickness and midshaft diameter were not altered significantly by OVX or copper depletion.

Evaluation of the effect of OVX and copper depletion on the distribution of selected minerals was accomplished by measuring serum levels, and urinary output, as well as liver and L4 concentrations. Assays of serum calcium, magnesium, phosphorus and iron revealed that serum calcium and magnesium were not influenced by OVX or copper depletion (Table 7). Serum phosphorus was depressed ($p<0.05$) by copper depletion, but unaffected by OVX. Neither OVX nor the dietary intervention alone affected serum iron, but the interaction of OVX and the low copper diet resulted in a significant ($p<0.05$) reduction in serum iron. Urinary excretion of magnesium was

significantly reduced ($p<0.01$) in the OVX group, but no alterations in calcium and phosphorus excretion were observed (Table 8). Copper depletion did not result in a change in the urinary excretion of calcium, phosphorus or magnesium. The liver concentrations of magnesium, zinc, iron and copper were analyzed and the results presented in μg / g of dry weight (Table 9). No statistically significant change was found in the liver mineral concentrations due to OVX, although a tendency toward decreased liver copper ($p<0.06$) was observed. As expected, the animals on the copper depleted diet had reduced liver copper concentrations ($p<0.01$), but no other alterations in liver minerals were noted. The bone concentrations of calcium, magnesium, zinc, iron and copper were analyzed and reported in Table 10. L4 magnesium ($p<0.05$) and zinc ($p<0.01$) were significantly reduced due to OVX. The calcium concentration of L4 was not statistically significant ($p<0.06$), but a tendency toward calcium loss with OVX was observed. Copper depletion had no significant effect on the bone mineral concentrations assessed, including no change in the copper content.

Biochemical markers of bone metabolism were also assayed to determine whether alterations in bone resorption or bone formation had caused the observed changes in bone quantity and bone quality (Table 11). Bone formation was not altered by either the OVX or dietary treatment in this study based on the results of serum alkaline phosphatase and bone-specific alkaline phosphatase analyses. Bone TRAP, bone hydroxyproline and urinary hydroxyproline were assessed to evaluate changes in bone resorption. Urinary hydroxyproline was the only indicator of bone resorption that was significantly affected by OVX. Excretion of hydroxyproline was elevated ($p<0.01$) in response to OVX. No effect of copper depletion was observed on any biochemical indicators of bone formation or bone resorption.

Discussion

In the present study the gain in body weight experienced by the OVX animals is in agreement with previous findings from studies where ovariectomized rats were not pair-fed (Kalu et al. 1983; Kalu et al. 1989). After only a period of four weeks, the weight gain observed in this study became significant. Similar increases in body weight have been reported in perimenopausal and postmenopausal women (Lovejoy 1998; Kirchengast et al. 1998). The *ob* protein, leptin, has been proposed as the cause of weight gain associated with diminished estrogen. Chu et al. (1999) recently reported that the ovariectomy-induced weight gain was accompanied by an early drop in leptin and subsequently followed by a rise in leptin production. The rise in leptin, which began around the 8^{th} week post ovariectomy, was believed to be in response to increased body weight and reduced leptin sensitivity. Estrogen replacement eliminated these alterations in serum leptin caused by ovariectomy.

No significant alterations in body composition were observed despite the dramatic increase in body weight. Percent fat was 29.81 ± 3.38 and 32.57 ± 3.38 in the sham-operated and OVX groups respectively. Clark and Tarttelin (1982) reported that in young growing Sprague-Dawley rats, ovariectomy increased overall body weight without increasing fat deposition. However, a redistribution of body fat was noted as adipose tissue appeared to be shifted away from the carcass and more toward subcutaneous sites. Yu et al. (1979) observed that the increase in body weight in F344 rats after adulthood was due more to increased deposition of fat than to greater lean mass. Although Yu and colleagues did not examine changes in body composition of ovariectomized rats, their study raises the issue that the maturity of the animal can influence changes in body composition.

Tissue weights expressed as a percentage of body weight were altered. The 2-fold increase in thymus observed in this study has also been noted elsewhere (Kalu et al. 1993). Kalu and colleagues (1993) conducted histological examination of the thymus and found that the increase in thymus weight associated with ovariectomy was a result of an increase in parenchymal tissue and not fat deposition. The effects of these changes in the thymus are not yet fully understood.

The decrease in bone weight expressed as a percentage of body weight in the OVX group is perhaps a reflection of the increase in body weight rather than an indication of osteopenia. Tibia dry weight actually increased in the OVX group, whereas L4 dry weights and ash weights were not affected. This increase in the dry weight of the tibia is also likely related to the weight gain experienced by the OVX animals. The increase in body weight serves as a potential load to the limbs and results in an improvement in bone mineral density. Similar improvements in vertebral bone mineral density have been correlated with weight gain in postmenopausal women (Travas et al. 1999).

DEXA scans of L4 revealed a significant loss of bone as a result of the OVX treatment, while the femoral BMD was not altered. Copper depletion had no effect on the BMD in this study. A more rapid response to OVX would be expected in the BMD of the vertebra compared to the femur due to a greater proportion of trabecular bone in the vertebra. The higher rate of bone turnover in trabecular bone results in greater change in vertebral bone mass (Gallagher 1990; Kalu et al. 1989; Wronski et al. 1989). Little or no change in the femur BMD would be expected if skeletal loading countered the effects of diminished estrogen.

As a result of natural or surgical menopause, bone loss occurs and bones become more fragile. In this study, however, improved mechanical strength (i.e. break load) occurred in the femur of the OVX group. The ability of the femur to withstand a greater force at the midshaft would again support the concept of greater body weight loading the limbs. Although studies of the effects of various exercise regimens on bone health in postmenopausal women have provided confounding results, physical activity is generally accepted as having a positive effect on bone mass and bone strength. Recently, Peng and colleagues (1997) reported that treadmill running inhibited the increase in osteoclast number typically observed in ovariectomized rats. Slow treadmill running strengthened the bones of the OVX animals. Apparently the combination of weight gain and slow running provided optimal loading to the skeleton. Turner (1999) reported increased mechanical loading reduced bone loss in the metaphysis, the major site of trabecular bone loss in the ovariectomized rat. Perhaps some of the variability in the results from exercise studies on postmenopausal women could be explained by the type of exercise performed and the site-specific responses of the bone to weight-bearing exercise.

While weight gain may have influenced the results of three-point bending tests in the OVX animals, it does not explain the basis for no reduction in mechanical strength due to copper depletion. One possible explanation for no increase in bone fragility is that the animals were not depleted enough to decrease collagen crosslink formation and reduce the bone mechanical strength. Numerous studies have reported diminished bone strength as a result of copper-deficient diets, however, in most cases torsion testing was used as opposed to three-point bending. The type of force exerted during mechanical testing may offer another possible explanation the lack of change in mechanical strength due to copper depletion. Assessment of bone mechanical strength by applying a blunt

force to the femur diaphysis may not be a sensitive means of quantifying bone strength. This is especially true since the proportion of cortical bone is greater at the femur midshaft compared to the metaphysis where fractures tend to occur. Assessment of femural strength by torsion testing and vertebral strength by compression testing would perhaps have provided more accurate and sensitive assessments of bone mechanical strength.

No differences were observed in femur midshaft diameter and cortical thickness due to OVX or copper depletion. Femur length was, however, greater in the OVX group. Accelerated linear bone growth has been reported in ovariectomized rats, although this phenomenon is usually transient (Wronski et al. 1988). Increased metaphyseal bone volume, due to bone growth, may be partly responsible for the lack of change observed in bone mineral density of the femur.

Bone loss in the OVX animals was not associated with deviations in serum calcium and magnesium. Similar results were reported in a previous study by Turner and associates (1987). Copper depletion decreased serum phosphorus. The decrease in serum iron observed in the OVX animals on low copper diets may result from the effects of reduced estrogen combined with low copper intake. OVX also resulted in reduced urinary magnesium loss, but no change in calcium and phosphorus excretion.

Liver concentrations of magnesium, iron, and zinc are apparently not affected by ovariectomy. The tendency toward decreased liver copper may be a partial reflection of the relationship between copper and estrogen. Reduced plasma concentrations of copper have been reported in ovariectomized rats and postmenopausal women who were not on estrogen (Russ and Raymont 1956; Johnson et al. 1959). Hormone replacement therapy has been shown to elevate plasma copper in postmenopausal women (Fisher et al. 1990).

Although liver zinc, iron and magnesium concentrations were not influenced by the dietary treatment, the reduction in liver copper confirmed that some degree of copper depletion was achieved.

OVX significantly reduced L4 magnesium and zinc. The calcium concentration of L4 did not reach the level of statistical significance; however, there was a tendency toward decreased calcium concentration as a result of OVX. The level of copper depletion used in this study was not sufficient to produce a change in L4 copper concentrations. This finding corresponds with the fact that no alterations in BMD and mechanical strength of the femur were observed. Previous findings by Jonas and colleagues (1993) found that in weanling rats, 8 weeks of copper depletion (0.4 mg Cu/kg diet) was sufficient to produce reduced torsional strength in the femur. Copper depletion in this study apparently results in diminished copper status of the soft tissues of the liver prior to changes occurring within the bone.

Based on the biochemical markers of bone metabolism, one would conclude that the osteopenia observed in this study resulted from enhanced bone resorption and not diminished bone formation. However, it is important to note that hydroxyproline was the only marker altered, which leads to that conclusion. Hydroxyproline is not specific to bone degradation due to the presence of hydroxyproline in many types of collagen found throughout the body. The urinary excretion of a deoxypyridinoline crosslinks, a more specific indicator of bone resorption, or histomorphometric analysis is needed to confirm these findings. Copper depletion had no effect on parameters of bone formation or bone resorption. This finding is in agreement with the BMD data, mechanical strength assessment and L4 copper concentrations, concerning the effect of copper depletion on bone. One might question whether or not the level of dietary copper used and/or the

duration of depletion were adequate to alter parameters of bone quantity and bone quality.

In summary, the weight gain that occurred in the OVX animals likely played a role in the response of the hind limb bones observed in this study. Pair feeding would have minimized weight gain, but may have altered the natural weight changes that mimic those often seen in postmenopausal women. Copper depletion did not affect bone strength, which may have been a result of insufficient copper depletion or the fact that the method of testing employed in this study was not sensitive to changes associated with copper deficiency. Future studies of copper depletion, diminished estrogen and osteopenia should consider varying levels of copper deficiency, torsion and compression testing, and histomorphometric analyses of bone.

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Table 1. Body weights (g) in sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper (1.3 mg/kg of diet) diets.

	Weight 1 (g)	Weight 2 (g)	Weight 3 (g)	Weight 4 (g)	Weight 5 (g)	Weight 6 (g)	Weight 7 (g)	Weight 8 (g)	Weight 9 (g)
Low Copper x Sham	168.9 ± 4.8	196.6 ± 6.9	195.5 ± 8.8	222.9 ± 10.1	212.0 ± 7.5	234.1 ± 10.2	224.6 ± 8.2	233.1 ± 8.7	232.4 ± 9.6
Low Copper x OVX	170.1 ± 4.8	204.1 ± 9.3	248.2 ± 8.8	240.0 ± 10.1	261.3 ± 7.5	256.7 ± 10.2	281.2 ± 8.2	290.9 ± 8.7	270.8 ± 9.6
Control x Sham	156.8 ± 4.8	186.7 ± 8.3	187.8 ± 7.7	199.4 ± 9.0	198.6 ± 7.5	209.8 ± 9.1	211.0 ± 8.2	217.5 ± 8.5	216.7 ± 9.6
Control x OVX	158.2 ± 4.8	192.4 ± 8.3	224.9 ± 7.7	223.0 ± 9.0	240.6 ± 7.5	243.9 ± 9.1	263.4 ± 8.2	270.4 ± 8.7	278.0 ± 9.6
Sham	162.8 ± 3.8	191.7 ± 6.9	191.6 ± 5.8	211.2 ± 7.6	205.3 ± 5.3	221.9 ± 7.7	217.8 ± 5.8	225.3 ± 6.1	224.6 ± 6.7
OVX	164.1 ± 3.4	198.2 ± 6.9	236.5 ± 5.8	231.5 ± 7.6	250.9 ± 5.3	250.3 ± 7.7	272.3 ± 5.8	280.7 ± 6.1	284.4 ± 6.7
Low Copper	169.5 ± 3.4	200.4 ± 7.9	221.8 ± 6.2	231.5 ± 8.6	236.7 ± 5.4	245.4 ± 8.7	252.9 ± 5.9	262.0 ± 6.2	264.6 ± 6.8
Control	157.5 ± 3.4	189.6 ± 6.2	206.3 ± 5.4	211.2 ± 8.6	219.6 ± 5.4	226.8 ± 6.9	237.2 ± 5.9	243.9 ± 6.2	247.3 ± 6.8
Source of Variation					*p*-Values				
OVX	0.79	0.38	<0.01	<0.05	<0.01	<0.05	<0.001	<0.001	<0.001
DIET	.022	0.19	0.09	<0.05	<0.05	0.05	0.07	0.05	0.20
OVX x DIET	0.99	0.90	0.37	0.69	0.63	0.49	0.80	0.78	0.88

[1]Values represent least squares means ± SEM (OVX, n=16, Low copper, n=16).

Table 2. Body composition of sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Percent Fat (%)	Percent Lean (%)
Low Copper x Sham	34.36 ± 4.66	65.64 ± 4.66
Low Copper x OVX	27.88 ± 4.66	72.12 ± 4.66
Control x Sham	30.79 ± 4.95	69.21 ± 4.95
Control x OVX	31.75 ± 4.95	68.25 ± 4.95
Sham	32.57 ± 3.38	67.43 ± 3.38
OVX	29.81 ± 3.38	70.19 ± 3.38
Low Copper	31.12 ± 3.32	68.88 ± 3.32
Control	31.27 ± 3.51	68.73 ± 3.51
	p-Values	
OVX	0.57	0.57
DIET	0.98	0.98
OVX x DIET	0.44	0.44

[1]Values represent least squares means ± SEM (OVX, n=15, Low copper, n=16).

Table 3. Tissue weights expressed as percentage of body weight from sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Thymus (%)	Spleen (%)	Kidney (%)	Femur (%)	Tibia (%)	L4 (%)
Low Copper x Sham	0.10 ± 0.01	0.20 ± 0.02	0.67 ± 0.02	0.30 ± 0.01	0.22 ± 0.01	0.11 ± 0.01
Low Copper x OVX	0.21 ± 0.01	0.22 ± 0.02	0.53 ± 0.02	0.25 ± 0.01	0.19 ± 0.01	0.09 ± 0.01
Control x Sham	0.10 ± 0.01	0.23 ± 0.02	0.67 ± 0.02	0.31 ± 0.02	0.25 ± 0.01	0.11 ± 0.01
Control x OVX	0.18 ± 0.01	0.23 ± 0.02	0.61 ± 0.02	0.28 ± 0.01	0.20 ± 0.01	0.08 ± 0.01
Sham	0.10 ± 0.01	0.21 ± 0.01	0.67 ± 0.01	0.30 ± 0.01	0.24 ± 0.01	0.11 ± 0.01
OVX	0.20 ± 0.01	0.23 ± 0.01	0.57 ± 0.01	0.26 ± 0.01	0.20 ± 0.01	0.08 ± 0.01
Low Copper	0.15 ± 0.01	0.21 ± 0.01	0.60 ± 0.01	0.27 ± 0.01	0.21 ± 0.01	0.10 ± 0.01
Control	0.14 ± 0.01	0.23 ± 0.01	0.64 ± 0.01	0.29 ± 0.01	0.23 ± 0.01	0.10 ± 0.01
			p-Values			
OVX	<0.001	0.27	<0.001	<0.01	<0.01	<0.01
DIET	0.39	0.16	<0.05	0.13	0.15	0.71
OVX x DIET	0.16	0.48	<0.05	0.61	0.54	0.79

[1]Values represent least squares means ± SEM (OVX, n=15-16, Low copper, n=16).

Table 4. Lumbar vertebra (L4) and tibia wet weight, dry weight and percent moisture, and L4 ash weight in sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	L4 Wet Weight (g)[1]	L4 Dry Weight (g)	L4 Ash Weight (g)	Moisture (%)	Tibia Wet Weight (g)	Tibia Dry (g)	Moisture (g)
Low Copper x Sham	0.25 ± 0.02	0.17 ± 0.01	0.09 ± 0.01	30.44 ± 4.00	0.49 ± 0.03	0.42 ± 0.02	13.43 ± 1.31
Low Copper x OVX	0.26 ± 0.02	0.18 ± 0.01	0.10 ± 0.01	29.23 ± 4.00	0.56 ± 0.03	0.48 ± 0.02	13.20 ± 1.31
Control x Sham	0.23 ± 0.02	0.16 ± 0.01	0.09 ± 0.01	27.04 ± 4.25	0.49 ± 0.03	0.41 ± 0.02	15.27 ± 0.92
Control x OVX	0.24 ± 0.02	0.17 ± 0.01	0.10 ± 0.01	28.86 ± 4.25	0.51 ± 0.03	0.44 ± 0.02	13.14 ± 1.31
Sham	0.24 ± 0.02	0.17 ± 0.01	0.09 ± 0.01	28.74 ± 2.90	0.49 ± 0.02	0.41 ± 0.01	14.35 ± 0.92
OVX	0.25 ± 0.02	0.18 ± 0.01	0.10 ± 0.01	28.55 ± 2.90	0.53 ± 0.02	0.46 ± 0.01	13.17 ± 0.92
Low Copper	0.26 ± 0.02	0.18 ± 0.01	0.10 ± 0.01	29.83 ± 2.85	0.52 ± 0.02	0.45 ± 0.01	13.31 ± 0.93
Control	0.24 ± 0.02	0.17 ± 0.01	0.10 ± 0.01	27.45 ± 3.01	0.50 ± 0.02	0.42 ± 0.01	14.21 ± 0.93
				p-Values			
OVX	0.73	0.34	0.47	0.96	0.11	<0.01	0.37
DIET	0.31	0.36	0.76	0.57	0.37	0.13	0.51
OVX x DIET	0.99	0.76	0.85	0.81	0.42	0.49	0.47

[1]Values represent least squares means ± SEM (OVX, n=15-16, Low copper, n=16).

Table 5. Femur and lumbar (L5) BMC, BMA and BMD of sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Femur BMC (g)	Femur BMA (cm^2)	Femur BMD (g/cm^2)	L5 BMC (g)	L5 BMA (cm^2)	L5 BMD (g/cm^2)
Low Copper x Sham	0.590 ± 0.068	5.610 ± 0.542	0.101 ± 0.004	0.075 ± 0.005	0.395 ± 0.016	0.184 ± 0.005
Low Copper x OVX	0.576 ± 0.063	5.941 ± 0.542	0.097 ± 0.004	0.062 ± 0.004	0.376 ± 0.014	0.166 ± 0.005
Control x Sham	0.550 ± 0.072	5.700 ± 0.542	0.092 ± 0.004	0.073 ± 0.005	0.385 ± 0.016	0.190 ± 0.005
Control x OVX	0.689 ± 0.067	6.741 ± 0.542	0.100 ± 0.004	0.061 ± 0.005	0.363 ± 0.016	0.168 ± 0.005
Sham	0.569 ± 0.050	5.654 ± 0.380	0.097 ± 0.003	0.074 ± 0.003	0.390 ± 0.012	0.187 ± 0.003
OVX	0.633 ± 0.046	6.341 ± 0.380	0.098 ± 0.003	0.061 ± 0.003	0.370 ± 0.011	0.167 ± 0.003
Low Copper	0.582 ± 0.047	5.775 ± 0.387	0.099 ± 0.003	0.069 ± 0.003	0.386 ± 0.011	0.175 ± 0.003
Control	0.620 ± 0.050	6.220 ± 0.387	0.096 ± 0.003	0.067 ± 0.003	0.374 ± 0.012	0.180 ± 0.003
			p-Values			
OVX	0.35	0.21	0.68	<0.05	0.21	<0.001
DIET	0.59	0.43	0.49	0.72	0.47	0.47
OVX x DIET	0.27	0.51	0.20	0.92	0.90	0.71

[1]Values represent least squares means ± SEM (OVX, n=12-14, Low copper, n=12-16).

Table 6. Femur mechanical strength and femur morphology in sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Modulus	Break Load (N)	Length (mm)	Cortical Thickness (mm)	Midshaft Diameter (mm)
Low Copper x Sham	2401.50 ± 122.84	11.18 ± 0.56	34.98 ± 0.37	0.61 ± 0.02	3.16 ± 0.06
Low Copper x OVX	2538.77 ± 122.84	11.86 ± 0.56	35.87 ± 0.34	0.62 ± 0.02	3.28 ± 0.06
Control x Sham	2097.92 ± 122.84	9.79 ± 0.56	34.05 ± 0.39	0.59 ± 0.02	3.14 ± 0.06
Control x OVX	2449.54 ± 122.84	12.04 ± 0.56	35.44 ± 0.36	0.64 ± 0.02	3.19 ± 0.06
Sham	2249.71 ± 86.14	10.49 ± 0.39	34.51 ± 0.27	0.60 ± 0.01	3.15 ± 0.04
OVX	2494.15 ± 86.14	11.95 ± 0.39	35.66 ± 0.25	0.63 ± 0.01	3.24 ± 0.04
Low Copper	2470.13 ± 87.57	11.52 ± 0.40	35.43 ± 0.25	0.62 ± 0.01	3.22 ± 0.04
Control	2273.73 ± 87.57	10.92 ± 0.40	34.74 ± 0.26	0.61 ± 0.01	3.16 ± 0.04
			p-Values		
OVX	0.05	<0.05	<0.01	0.10	0.16
DIET	0.13	0.30	0.08	0.75	0.34
OVX x DIET	0.39	0.17	0.50	0.24	0.54

[1]Values represent least squares means ± SEM (OVX, n=16, Low copper, n=16).

Table 7. Serum mineral concentrations in sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Serum Calcium (mg/dl)	Serum Magnesium (mg/dl)	Serum Phosphorous (mg/dl)	Serum Iron (mg/dl)
Low Copper x Sham	10.53 ± 0.31	2.05 ± 0.08	6.87 ± 0.44	318.34 ± 29.5
Low Copper x OVX	10.56 ± 0.31	2.11 ± 0.08	6.9 ± 0.44	210.48 ± 31.7
Control x Sham	10.11 ± 0.31	2.00 ± 0.08	8.02 ± 0.51	266.05 ± 30.2
Control x OVX	10.09 ± 0.31	2.04 ± 0.08	7.84 ± 0.44	292.76 ± 32.17
Sham	10.32 ± 0.22	2.03 ± 0.05	7.45 ± 0.33	292.20 ± 20.90
OVX	10.33 ± 0.27	2.08 ± 0.06	7.37 ± 0.31	251.62 ± 22.60
Low Copper	10.54 ± 0.22	2.08 ± 0.06	6.88 ± 0.31	264.41 ± 21.65
Control	10.10 ± 0.22	2.02 ± 0.06	7.93 ± 0.34	279.40 ± 22.46
		p-Values		
OVX	0.98	0.54	0.86	0.20
DIET	0.17	0.51	<0.05	0.64
OVX x DIET	0.92	0.87	0.82	<0.05

[1]Values represent least squares means ± SEM (OVX, n=12-16, Low copper, n=13-16).

Table 8. Urinary calcium, magnesium and phosphorus excretion per unit creatinine (mg/cre) in sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Urinary Calcium (mg Ca/mg cre)	Urinary Magnesium (mg Mg/mg cre)	Urinary Phosphorus (mg P/mg cre)	Urinary Creatinine (mg/dl)
Low Copper x Sham	0.15 ± 0.03	0.13 ± 0.02	1.81 ± 0.20	1.26 ± 0.13
Low Copper x OVX	0.15 ± 0.03	0.08 ± 0.02	1.26 ± 0.20	1.45 ± 0.13
Control x Sham	0.20 ± 0.03	0.13 ± 0.02	1.63 ± 0.20	1.78 ± 0.13
Control x OVX	0.11 ± 0.03	0.07 ± 0.02	1.43 ± 0.20	1.70 ± 0.13
Sham	0.18 ± 0.02	0.13 ± 0.01	1.72 ± 0.14	1.52 ± 0.09
OVX	0.13 ± 0.02	0.08 ± 0.01	1.35 ± 0.14	1.58 ± 0.09
Low Copper	0.15 ± 0.02	0.11 ± 0.01	1.53 ± 0.14	1.36 ± 0.09
Control	0.15 ± 0.02	0.10 ± 0.01	1.53 ± 0.14	1.74 ± 0.09
		p-Values		
OVX	0.17	<0.01	0.08	0.68
Diet	0.98	0.62	0.98	<0.01
OVX x Diet	0.20	0.96	0.39	0.29

[1]Values represent least squares means ± SEM (OVX, n=16, Low copper, n=16).

Table 9. Liver magnesium, zinc, iron and copper concentrations (μg/g dry weight) in sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Liver Magnesium (μg/g)	Liver Zinc (μg/g)	Liver Iron (μg/g)	Liver Copper (μg/g)
Low Copper x Sham	547.36 ± 153.60	88.52 ± 7.95	1121.99 ± 202.56	13.66 ± 2.08
Low Copper x OVX	492.28 ± 164.02	76.20 ± 9.21	946.64 ± 221.11	11.20 ± 2.25
Control x Sham	506.58 ± 141.60	101.86 ± 8.44	965.54 ± 202.55	22.13 ± 2.08
Control x OVX	640.07 ± 141.60	84.90 ± 9.99	971.91 ± 221.11	16.47 ± 1.90
Sham	526.97 ± 102.89	95.20 ± 5.76	1043.76 ± 142.81	17.89 ± 1.45
OVX	566.18 ± 107.17	80.55 ± 6.75	959.28 ± 154.25	13.84 ± 1.47
Low Copper	519.82 ± 114.26	82.36 ± 6.15	1034.32 ± 150.86	12.43 ± 1.55
Control	573.32 ± 101.07	93.39 ± 6.56	968.73 ± 150.86	19.30 ± 1.42
			p-Values	
OVX	0.79	0.11	0.69	0.06
DIET	0.74	0.24	0.76	<0.05
OVX x DIET	0.53	0.80	0.67	0.45

[1]Values represent least squares means ± SEM (OVX, n=11-14, Low copper, n=11-14).

Table 10. Lumbar (L4) calcium, magnesium, zinc, iron and copper concentrations (mg/g dry weight or μg/g dry weight) in sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Lumbar Calcium (mg/g)	Lumbar Magnesium (mg/g)	Lumbar Zinc (μg/g)	Lumbar Iron (μg/g)	Lumbar Copper (μg/g)
Low Copper x Sham	180.50 ± 10.99	2.89 ± 0.21	398.61 ± 20.35	196.16 ± 23.00	5.00 ± 0.40
Low Copper x OVX	173.25 ± 10.99	2.68 ± 0.21	332.61 ± 20.35	183.91 ± 23.00	5.25 ± 0.40
Control x Sham	203.47 ± 11.67	3.56 ± 0.22	395.22 ± 21.62	264.05 ± 24.44	5.45 ± 0.42
Control x OVX	167.04 ± 11.67	2.81 ± 0.22	341.51 ± 21.62	198.52 ± 24.44	5.32 ± 0.42
Sham	191.98 ± 7.98	3.22 ± 0.15	396.92 ± 14.78	230.11 ± 16.70	5.23 ± .27
OVX	170.14 ± 7.98	2.75 ± 0.15	337.06 ± 14.78	191.21 ± 16.70	5.29 ± .29
Low Copper	176.87 ± 7.83	2.79 ± 0.15	365.61 ± 14.51	190.03 ± 16.40	5.13 ± .28
Control	185.25 ± 8.27	3.19 ± 1.58	368.36 ± 15.33	231.29 ± 17.32	5.38 ± .30
			p-Values		
OVX	0.06	<0.05	<0.05	0.11	0.88
DIET	0.47	0.08	0.90	0.10	0.55
OVX x DIET	0.21	0.22	0.77	0.27	0.65

[1]Values represent least squares means ± SEM (OVX, n=15, Low copper, n=16).

Table 11. Biochemical markers of bone metabolism in sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Serum Alkaline Phosphatase (mg/dl)	Bone Alkaline Phosphatase (mg/g bone)	Bone Trap (mg/g bone)	Bone Hydroxyproline (mg/g bone)	Urinary Hydroxyproline (per creatinine)
Low Copper x Sham	49.29 ± 12.19	2.37 ± 0.35	2.58 ± 0.80	20.99 ± 2.48	0.12 ± 0.02
Low Copper x OVX	77.29 ± 12.19	2.76 ± 0.35	2.28 ± 0.80	18.24 ± 2.32	0.18 ± 0.02
Control x Sham	78.63 ± 11.24	1.96 ± 0.316	2.63 ± 0.80	14.75 ± 2.14	0.14 ± 0.02
Control x OVX	78.75 ± 11.24	2.22 ± 0.35	1.45 ± 0.80	18.70 ± 2.14	0.21 ± 0.02
Sham	63.96 ± 8.17	2.17 ± 0.24	2.61 ± 0.56	17.87 ± 1.62	0.13 + 0.01
OVX	78.02 ± 8.17	2.49 ± 0.24	1.87 ± 0.56	18.47 ± 1.55	0.19 ± 0.01
Low Copper	63.29 ± 8.82	2.56 ± 0.25	2.43 ± 0.57	19.62 ± 1.72	0.15 ± 0.01
Control	78.69 ± 8.02	2.09 ± 0.25	2.04 ± 0.57	16.73 ± 1.53	0.17 ± 0.01
			p-Values		
OVX	0.23	0.36	0.36	0.79	<0.01
DIET	0.22	0.20	0.64	0.23	0.26
OVX x DIET	0.24	0.86	0.59	0.15	0.75

[1]Values represent least squares means ± SEM (OVX, n=15-16, Low copper, n=14-16).

[2]Markers of bone formation (i.e. serum alkaline phosphatase and bone-specific alkaline phosphatase) and markers of bone resorption (i.e.bone TRAP (tartrate-resistant acid phosphatase), bone hydroxyproline, and urinary hydroxyproline are reported.

CHAPTER IV

THE EFFECTS OF DIETARY COPPER DEPLETION ON BONE METABOLISM IN MATURE TAIL-SUSPENDED RATS

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Abstract

Information concerning the role of micronutrient depletion on bone loss associated with weightlessness is limited. The trace mineral, copper, is essential in collagen crosslink formation and the extent to which low dietary copper intake impacts bone metabolism in near weightlessness has not been investigated. Using a 2 X 2 factorial design, thirty-six 5½ -month old male Sprague Dawley rats were either tail-suspended (TS) or kept ambulatory (AMB). Rats in each group, were randomly assigned to one of two dietary treatments (control, AIN-93M or $^{-}$Cu treatment, AIN-93M no added Cu) and had free access to deionized water. The $^{-}$Cu treatment group also received a five day treatment of the copper chelator triethylenetetramine tetrahydrochloride (TETA at 0.83% of diet), two weeks prior to the 28-d suspension period. TETA reduced ($p<0.001$) plasma ceruloplasmin. Bone, liver, and kidney copper concentration was reduced in the

$^{-}$Cu animals. TS animals experienced significant ($p<0.01$) losses in bone mineral density (BMD) and bone mineral content (BMC) of both the 5th lumbar vertebra (L5) and femur, while $^{-}$Cu animals only lost BMD in L5. Neither copper status nor tail suspension had effects on the BMD or BMC of the humerus. No significant changes were observed in serum or bone specific alkaline phosphatase activities, while urinary excretion of deoxypyridinoline crosslinks was significantly increased at week 2 of suspension ($p<0.0001$) and remained elevated at week 4 ($p<0.05$). The $^{-}$Cu animals had significantly ($p<0.05$) greater zinc and reduced ($p<0.01$) iron in L5 and significantly ($p<0.05$) lower iron and magnesium concentrations in humerus. These findings suggest that bone loss associated with skeletal unloading occurs as a result of increased bone resorption and not altered formation. Further studies are needed to investigate the potential protective role of copper in weightlessness conditions.

Key Words: copper, bone, tail-suspension, osteopenia, TETA

Introduction

In the absence of weight-bearing activity (e.g. during space flight and prolonged bed rest) osteopenia occurs (Morey and Baylink 1978;Donaldson et al. 1970). The tail-suspended rat provides a valuable model for studying alterations in bone metabolism associated with skeletal unloading without the prohibitive cost and limitations of space flight and human bed rest studies. Most tail-suspension studies have been conducted on young growing rats and in these animals osteopenia resulted from a decreased rate of periosteal bone formation (Wronski and Morey 1983). Often, however, this decrease in bone formation appeared to be transient and the rate of formation returned to normal within 10-14 days (Globus et al. 1986a;Vico et al. 1991). A few studies of mature

animals have been reported in the literature, and they revealed lower calcium content, ash weight, bone mineral density (BMD) and bone mineral content (BMC) in unloaded limbs (Vico et al. 1995). A phasic response to tail suspension was also observed in mature rats. Formation was decreased during the first two weeks and then bone remodeling (i.e. formation and resorption) was increased for a period of up to 90 days (Leblanc et al. 1985).

The tail-suspended rat model simulates not only the skeletal unloading of the hind limbs as seen in the microgravity environment, but also the cephalic fluid shift known to occur (Roer and Dillaman 1994;Charles and Bungo 1991). Similar shifts occur in downward-head-tilt bed rest studies. The electrolyte balance, cardiovascular function and renal filtration are among the physiological systems affected by these fluid shifts (Leach 1979;Cintron et al. 1990;Leach et al. 1991).

Osteopenia associated with skeletal unloading may be further exacerbated by altered nutritional status. Globus and co-workers (1986) found that although calcium supplementation did not reverse the effects of tail-suspension in young growing rats, it did increase the calcium content of weighted and non-weighted bones by reducing the rate of bone resorption. Navidi et al. (1995) reported high salt intake in tail-suspended animals enhanced urinary calcium excretion, but did not worsen the bone loss. The response of the calcium endocrine system and calcium absorption to tne high salt diet was depressed in the tail-suspended animals. Krebs and colleagues (1993) observed decreased zinc balance as a result of 17 weeks of bed rest, but no effect on copper balance. Fluoride supplementation increased zinc and nitrogen balances during bed rest compared to control subjects (Krebs et al. 1988). No studies to date have examined the effects of dietary copper intake on bone parameters of animals experiencing skeletal unloading.

The trace mineral copper plays an important role in collagen crosslink formation as a metalloenzyme for lysyl oxidase (Siegel 1978). Collagen crosslinks provide bone with tensile strength and decrease bone rigidity (Riggins et al. 1979). Studies of copper deficiency have been performed on lambs, dogs, chicks, pigs and rats and in all species skeletal abnormalities have resulted (Bennetts 1932; Baxter 1951; Rucker et al. 1969; Teague and Carpenter 1951; Jonas et al. 1993). According to Pennington and Schoen (1996) the average copper intake in the U.S. is below the National Academy of Sciences standards for all age groups. Combine a marginal copper intake with skeletal unloading and the potential for compromised skeletal health is substantive.

Studies examining the physiological response of mature bone to skeletal unloading are needed. The tail-suspended rat can be used to study the response of bone tissue to unloading and the mechanisms involved, particularly in mature animals. Additionally, the influence of nutrients such as copper on the bone loss that occurs during skeletal unloading has not been investigated. Therefore, this study was designed to examine the effect of dietary copper status on parameters of bone quantity and quality in mature tail-suspended rats.

Methods

Animals and Diets

Thirty-six, male Sprague Dawley rats (Charles River Laboratories) were individually housed in a temperature- and light-controlled room. Rats were maintained on commercial laboratory diets until the initiation of dietary treatments and had *ad libitum* access to distilled water throughout the study. The experiment was designed so that rats in each of the three replications (n=12) were approximately 4 ½ months of age at the beginning of the suspension treatment. Rats were assigned by weight to one of the

two dietary treatments (control, AIN-93M or $^{-}$Cu treatment, AIN-93M no added Cu) (Reeves et al. 1993). To further promote copper depletion in these mature animals, the $^{-}$Cu treatment group started receiving the copper chelator triethylenetetramine tetrahydrochloride (TETA at 0.83% of diet) for five days (Cohen et al. 1983a;Cohen et al. 1983b) starting twelve days prior to tail suspension. Pre-TETA and post-TETA tail blood was drawn and ceruloplasmin measured to verify copper depletion. During the twenty-one days prior to the start of the suspension treatment, the two dietary treatment groups were pair-fed to control for variations in weight gain/loss. No difference in body weight due to diet was observed, so rats were assigned to either the suspension or the ambulatory group in a 2x2 factorial design. Tail-suspension was accomplished via the method of Wronski and Morey-Holton (1979), using orthopedic tape, a suspension mechanism and a metabolic cage especially designed for suspension studies. Ambulatory rats served as controls and were pair-fed to the tail-suspended group on their respective diets throughout the 28-day suspension period. Twelve-hour fasting urine and fecal samples were collected at three points throughout the treatment (i.e. prior to suspension, 14-day and 28-day) and stored at $^{-}$20°C. Body weights were determined weekly prior to suspension and every third day throughout the remainder of the experiment. A device was used to obtain body weight while the animals were suspended so that weight-bearing activity on the hind limbs was avoided. At the end of the twenty-eight days of tail-suspension, rats were food-deprived for 12 h, anesthetized with an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (5 mg /kg body weight), and exsanguinated via cardiac puncture.

Sample Collection

Blood samples were collected via cardiac puncture at the time of necropsy. Serum was separated by centrifugation at 1500 x g for 20 minutes at 4°C and a sample of whole blood was removed using a heparinized syringe for ionized calcium analysis. Aliquots of serum were then frozen at ˜20°C for later analyses. Liver, kidney, heart, testes and spleen samples were freed of excess tissue, weighed and stored at -20°C. Both femurs and humeri, and 3rd-5th lumbar vertebrae (L3-L5) were removed, cleaned and stored at -20°C. The right femur was stored in phosphate buffered saline (PBS) for mechanical strength testing. The left femur, L5, and right humerus were stored until densitometric measurements and bone mineral analysis could be completed.

Densitometric Measurements

The left femur, L5 and right humerus of each animal were scanned using a dual-energy x-ray densitometry (DEXA) small animal high-resolution scan module (Hologic QDR-2000). To simulate soft tissues, each bone was placed in deionized water approximately 2 cm deep during analysis. Bone mineral area (BMA), bone mineral content (BMC) and bone mineral density (BMD) were recorded from the DEXA.

Mechanical Strength Testing

Femur samples were transported to NASA-Ames Research Center (Moffet Field, CA) for torsion testing. Prior to testing, femur geometry was measured using a vernier calliper (accuracy: 0.1mm). The length of the femur was assessed from the greater trochanter to the distal condyles. The external diameter of the bone was measured at the midshaft by taking two measurements 90° apart. Specimens were then individually

prepared for mechanical testing by potting both the proximal and distal ends of the femur in a low melting point metal, while being held with precision fixtures. The fixtures were then aligned in the torsion testing system and rotated at a speed of 1 deg/sec. Femoral rotation was in the internal direction with torque and angular displacement data recorded until the point of fracture. Each bone was then visually inspected to insure the characteristic spiral pattern of fracture. Maximum torque (Newton*meter^2) and maximum angular displacement (degrees) were determined and analyzed.

Tissue Mineral Analysis

Bone samples (i.e. L5 and humerus) were analyzed for copper, iron, zinc, calcium and magnesium content, while only copper, iron, zinc and magnesium were analyzed on soft tissues (i.e. liver and kidney). The samples were weighed (wet weight of ~200 mg) into acid-washed borosilicate glass tubes and dried for 24 h at 100° C. Tissue dry weights were then recorded. Next, samples were exposed to a series of wet and dry ashing steps until all organic material was removed using a modification of the method of Hill and colleagues (Hill et al. 1986). After ashing, the samples were diluted with 0.5% double distilled nitric acid (GFS Chemicals, Columbus, OH) and analyzed using a Perkin-Elmer Zeeman 5100 atomic absorption spectrophotometer (Perkin Elmer, Norwalk, CT).

Biochemical Analyses

Plasma ceruloplasmin was measured to assess the effects of the copper depletion diet and of TETA before and immediately after feeding TETA (0.83% of diet) for five days. Tail blood was collected in microcentrifuge tubes. Plasma was separated and a modification of the method of Sunderman and Nomoto (1970) was used to measure

ceruloplasmin on the clinical analyzer (Cobas Fara II). Ceruloplasmin was expressed as mg of ceruloplasmin per dl of plasma.

Serum alkaline phosphatase and bone-specific alkaline phosphatase were assayed as indicators of bone formation. Serum samples were thawed and analyzed on the clinical analyzer using alkaline phosphatase calibrators and standards (Roche Reagents, Nutley, NJ). Bone-specific alkaline phosphatase was analyzed on both L3 and the left humerus to identify any alterations in bone formation occurring in either weight-bearing or non-weight-bearing bones. The bones were de-fatted and protein extracted using the method of Farley and colleagues (1992). Extracts were then analyzed on the clinical analyzer for alkaline phosphatase as previously described for serum samples. Total bone protein was also analyzed and expressed as mg protein per dl of extract. Bone-specific alkaline phosphatase was then calculated per unit of protein.

Urinary excretion of deoxypyridinoline crosslinks was assayed using an ELISA, Pyrilinks-D (Metra Biosystems, Inc.) on samples collected at baseline (i.e. prior to suspension), day-14 and day-28. DPD crosslink excretion provided a measure of type-I collagen degradation and therefore served as an indicator of bone resorption (Seibel et al. 1992). The DPD data was presented as total nM of DPD excreted per twelve hours. These values were not expressed as DPD per unit of creatinine as often reported in the literature, because tail suspension is known to enhance protein catabolism (Leblanc et al. 1985).

Statistical Analysis:

The data were analyzed as a 2x2 factorial design using the Statistical Analysis System (SAS) version 6.11. Analysis of variance and least squares means were

calculated using the general linear model (GLM) procedure and $p<0.05$ was accepted as significant. Data are reported as mean $\pm$ SEM unless otherwise indicated.

Results

Copper depletion was accomplished prior to suspension as evidenced by a significant reduction ($p< 0.001$) in plasma ceruloplasmin (Table 1). Despite pair-feeding, tail-suspension by day-18 significantly reduced ($p<0.05$) the body weight of the suspended animals compared to the ambulatory controls (Table 2). This discrepancy in body weight continued throughout the remainder of the experiment and became even more pronounced ($p<0.01$) at day-27 as ambulatory animals continued to regain body weight but tail-suspended animals did not. As a result of tail suspension, heart expressed as a percentage of body weight was significantly increased ($p<0.01$) and the weight of the testicles significantly decreased ($p<0.001$) (Table 3). No effects on tissue weights were observed due to the $^{-}$Cu dietary treatment.

Tail-suspension, but not copper depletion, resulted in a significant reduction ($p<0.05$) in torsional strength of the femur (Table 4). No statistical difference in the angle of deformation was detected with tail suspension or copper depletion, although there was a trend toward a reduced angle prior to fracture in the copper depleted rats. The DEXA data indicated a significant loss in femoral BMC ($p<0.05$) and BMD ($p<0.01$) and an even greater decrease in the BMA ($p<0.01$), BMC ($p<0.001$) and BMD ($p<0.001$) of L5 in response to 28-days of tail-suspension (Table 5). No change, however, was observed in the bone densitometry measures of the humerus due to tail-suspension.

L5 dry weight ($p<0.05$) and ash weight ($p<0.01$) were significantly reduced by tail suspension (Table 6). Similar changes were not found in the humerus, liver or

kidney. Tail suspension significantly increased L5 ($p<0.05$) copper (Table 7). Liver iron was significantly increased ($p<0.001$) in response to tail suspension, but no effect of suspension was noted on L5, humerus or kidney (Table 8). The zinc concentration of tail suspended animals was significantly increased in only the humerus ($p<0.05$) and liver ($p<0.01$) (Table 9). A trend toward increasing calcium concentration of the humerus was apparent, but not significant ($p<0.07$) as a result of tail suspension (Table 10). There was no effect on tissue concentrations of magnesium due to tail suspension (Table 11).

As expected, the ⁻Cu treatment group experienced a significant reduction in the copper content of L5 ($p<0.001$), liver ($p<0.001$), kidney ($p<0.001$) and humerus ($p<0.05$) (Table 7). The ⁻Cu treatment significantly decreased the iron concentration of L5 ($p<0.01$), humerus ($p<0.05$), and kidney ($p<0.05$), and tended to increase, although not significantly, liver iron (Table 8). The zinc concentration of L5 was significantly increased ($p<0.05$) in the ⁻Cu group (Table 9), but tended to decrease ($p<0.07$) in the kidney. The magnesium content of the humerus was significantly ($p<0.05$) decreased by the ⁻Cu diets and significantly increased ($p<0.01$) in the liver (Table 11).

Ionized calcium was not affected by tail-suspension or the ⁻Cu diet. No significant alterations in serum or bone specific alkaline phosphatase were detected from either tail suspension or ⁻Cu dietary treatment. However, urinary excretion of DPD had significantly increased ($p<0.001$) in tail suspended animals at 2- weeks of suspension and remained elevated, although not as significantly ($p<0.05$), at 4-weeks of suspension (Table 12). In suspended rats, ⁻Cu diets significantly decreased ($p<0.05$) urinary DPD excretion.

Discussion

The results of this study help to elucidate the effects of tail suspension and copper depletion on parameters of bone metabolism. Twenty-eight days of hindlimb suspension were long enough to produce significant alterations in indicators of bone quality and quantity in mature rats. Feeding TETA for five days successfully lowered copper as evidenced by ceruloplasmin values. The combination of TETA and the $^{-}$Cu diet produced diminished copper concentration of both soft (i.e. liver and kidney) and hard (L5 and humerus) tissues.

The suspended rats lost weight for much of the 28-day suspension period. Weight loss was greatest during the first nine days of suspension, but due to pair feeding was not significantly less than the ambulatory group until day-18. As the average intake of suspended animals returned to amounts that more closely approximated dietary intake prior to suspension, ambulatory rats began to regain weight, but the tail suspended animals did not. The occurrence of weight loss due to tail suspension in this study differs from the results of studies on young growing rats (i.e. body weight <200 g) (Bikle et al. 1987; Halloran et al. 1986; Sessions et al. 1989; Roer and Dillaman 1990). However, patterns of weight loss in tail suspension studies on rats that were >200 g at the beginning of suspension were similar to this study (Vico et al. 1995; Leblanc et al. 1985; Globus et al. 1986b). Reduced muscle and bone mass due to atrophic effect of tail suspension may be partly responsible for the decline in body weight (Leblanc et al. 1985). Due to the ~30° angle at which the rat's body is suspended, the cephalic fluid shift is another possible factor involved in weight loss. Fluid that tends to pool in the head and upper torso distends the surrounding vasculature (Feldman and Brunner 1994). During space flight, this distention causes a physiological response similar to fluid-volume overload

and thus a decrease in total body water results (Leach 1979). Such a decrease in total body water in addition to bone and muscle loss could result in significant weight loss during tail suspension.

While these explanations seem plausible, the potential role of stress on body weight and other physiological responses to tail suspension remains in question. Halloran and colleagues (1988) found that decreased bone formation in young tail-suspended rats was not a consequence of increased plasma glucocorticoids or sensitivity to glucocorticoids. The effects of tail suspension on older rats, however, have not been established. Until further investigated, the possibility remains that some of the alterations observed in this and other studies of older animals could reflect in part a stress response. However, since the humerus provides an internal control for distinguishing between systemic and local responses to skeletal unloading of the hind limbs, alterations in bone parameters occurring in the femur or other unloaded bone and not in the humerus would appear to be a result of locally-mediated responses.

The torsional strength of the femur was diminished by tail suspension, but unaffected by diet. Neither skeletal unloading nor copper depletion altered the maximum angle of deformation. Reduced mechanical strength has been reported in young growing rats exposed to tail-suspension (Shaw et al. 1987; Martin 1990; Abram et al. 1988). Jonas et al (1993) had previously reported a significant reduction in both the torsional strength and ultimate angular deformation in young growing rats fed a copper-deficient diet for eight weeks. A lack of tolerance of bone from copper-deficient chicks to torsional force and deformation was also reported by Opsahl et al. (1982), but the twenty-one day experiment was conducted on very young (i.e. one-day-old) chicks. No previous

studies have examined the effects of copper-deficiency on torsional strength of the femur in mature animals.

Although copper content of the femur was not analyzed in this study, the copper concentration in L5 and the humerus confirm that the $^{-}$Cu diet was sufficient to significantly reduce the copper concentration of bone. Several studies have attributed the diminished mechanical strength in copper-deficient animals to a decrease in the number of collagen crosslinks (Opsahl et al. 1982; Rucker et al. 1975; Jonas et al. 1993; Riggins et al. 1979). Due to copper's role as a cofactor for lysyl oxidase, diminished crosslink formation is considered the likely cause of the reduced mechanical strength in tail-suspended animals. In this study of mature animals, however, the reduced copper concentrations observed in L5 and the humerus are not reflected in the mechanical strength of the femur. Compression testing the vetebrae would help to further explain the relationship between diminished copper concentration of the bone and mechanical strength.

Farquharson and colleagues (1989) reported that dietary copper affected deoxypyridinium crosslink formation in the femur diaphysis in young growing rats. Urinary excretion of DPD is a useful indicator of the type I collagen degradation products, since DPD is the collagen crosslink specific to type I collagen, and is only found in appreciable amounts in bone and teeth (Eyre et al. 1984). The DPD data and results from mechanical testing in the current study indicate that the reduced mechanical strength of the femur in the mature tail-suspended animal is likely a result of increased collagen breakdown. The use of the copper-chelator, TETA, plus 7 weeks of copper depletion diet was not sufficient to produce a statistically significant reduction in mechanical strength of the femur in 5 ½ month-old rats. Urinary excretion of DPD was

altered by copper depletion only in tail suspended animals. Future studies of copper depletion and skeletal unloading examining collagen crosslinks in bone would provide insight into the relationship between copper and collagen formation in the mature animal.

Since the excretion of DPD is a biochemical indicator of bone resorption, it appears that enhanced bone resorption was occurring as a result of tail suspension and led to decreased mechanical strength of the femur. Bone resorption appeared to be more rapid during the first fourteen days of suspension. The increased rate of bone resorption due to skeletal unloading in this study supports the previous findings of Smith et al (1998) in bed rest and space flight studies. In contrast, indicators of bone formation (i.e. serum alkaline phosphatase and bone-specific alkaline phosphatase) were not significantly altered by tail suspension or copper depletion. Kostenuik et al (1997) had previously reported a decrease in alkaline phosphatase activity in young growing rats that underwent hind limb elevation for five days. Such decreases in the rate of bone formation, as indicated by reduced alkaline phosphatase, were not observed in the mature animal despite the fact that analyses were performed on both normally loaded (i.e. humerus) and unloaded (i.e. L3) bones.

DEXA analysis indicated that 28 days of tail suspension was adequate to produce significant decreases in the BMD and BMC of L5 and the femur. The greatest changes were observed in L5 as might be expected since the vertebrae have a higher proportion of trabecular bone than the femur. Despite the fact that the femur has greater cortical bone content, the period of skeletal unloading utilized in this study resulted in significant osteopenia in the femur. The humerus was not significantly altered by tail suspension. This finding supports the contention that in the ~30° head down tilt position, the humerus is normally loaded (Hargens et al. 1984a). The densitometry measurements performed in

this study are in agreement with results by Vico and colleagues (1995), who found similar changes in the BMD and BMC of 6-month-old rats.

The effects of the copper depletion on the BMD were only observed in the vertebra but not in the femur. This finding is of particular interest not only to crewmembers aboard space flights, but also to earthbound individuals exposed to normal gravity. Due to the incidence of vertebral fractures, the limited numbers of older individuals performing weight-bearing activity, as well as the poor copper intakes reported among elderly populations (Rudman et al. 1995), the potential for detrimental effects on vertebral bone are a concern. Further studies of the effects and mechanisms of low copper diets on bone undergoing skeletal unloading are warranted.

L5 copper was increased by tail suspension, but there were no significant changes in iron, zinc, magnesium or calcium. Reduced calcium concentrations in the tibia of young growing tail-suspended animals have been previously reported (Halloran et al. 1988; Sessions et al. 1989). And Vico and colleagues (1995) noted a significant reduction in the calcium content of the tibia of 6-month-old tail-suspended rats, but no signicant reduction in the femur. Novikov and Ilyin (1981) determined that the skeletal reaction to near weightlessness varies depending on bone studied (i.e. tibia vs femur) and the age of the animal.

A trend toward increased calcium concentration of the humerus (p=0.06) was observed in tail-suspended rats. This trend may be partly explained by increased fluid volume in response to the head-down tilt position. Another possible explanation is that the humerus of tail-suspended animals is actually hyper-loaded as opposed to normally loaded. Increased loading would result in increased bone mineral. However, the DEXA

results in this study and the findings of Hargens and colleagues (1984) do not support hyper-loading of the humerus.

Tail suspension significantly increased the liver copper, iron and zinc. The increase in the liver concentration of these minerals may in part be a result of fluid shifts toward the head and forelimbs that may alter hepatic circulation. Another possible explanation is that redistribution of minerals occurred in response to stress. Nonetheless, in the present study the cause of the altered mineral content of the liver cannot be fully explained. No alterations in kidney minerals were observed in response to tail suspension, even though in other models of weightlessness (i.e. chronic bed rest) glomerlular filtration rate is decreased (Leach et al. 1983).

The copper depletion ($^{-}$Cu) group significantly reduced the copper concentrations in all tissues measured. The iron concentration of L5, humerus and kidney also were reduced by the dietary treatment. Liver iron concentrations in this study were 19% higher in the controls than the $^{-}$Cu group. Enhanced liver iron sequestration during copper depletion has been reported by Linder and Hazegh-Azem (1996). Previous reports by Cohen et al (1983) examining the effects of TETA found no significant reduction in liver iron, hematocrit or hemoglobin. The increased zinc concentration of L5 and not the humerus in response to the copper depletion diet could be explained by a possible copper or iron interaction with zinc, but it is unlikely that such interactions would be localized in one bone verses the skeleton as a whole. Further investigation into the cause of elevated zinc in the L5 vertebra is needed. The magnesium concentration of humerus was significantly decreased in the copper depleted group and liver magnesium was significantly decreased. No known interaction between copper and magnesium has been reported, making the interpretation of the magnesium data difficult. The potential

exists that alterations in tissue magnesium are a result of the chelator used in the study, however further investigation is required.

In this study, copper depletion did not produce the statistically significant decrease in bone mechanical strength and increase in bone rigidity seen in studies with younger animals. However, trends toward similar changes in the bone strength and rigidity were observed. Perhaps a longer copper depletion would be required with these older animals to produce significant results. The issue of long-term copper deficiency and its effect on bone undergoing skeletal unloading should be further investigated. Additionally, the potential protective role of copper supplementation in near weightless conditions is of interest.

While feeding TETA made copper depletion of the adult rats in this study possible in a relatively short period of time, it also complicated the interpretation of mineral data. Discerning whether or not an observed change in mineral status was a direct result of copper depletion, chelation by TETA or a combination of these two factors was difficult. Future studies evaluating the effects of TETA on zinc and iron concentrations in the bone would help elucidate the results of copper depletion in this study.

Twenty-eight days of tail suspension was an adequate period of time to produce osteopenia in the unloaded bones of 5 ½-month-old rats. These data from mature animals provide valuable insight into the skeletal responses to space flight. Not only was bone loss observed, but also actual mineral composition of the bone was altered. The osteopenia reported in this study apparently resulted from increased bone resorption as opposed to diminished bone formation. Further histomorphometric analyses and

investigations into molecular mechanisms are needed to more fully clarify the results of this study.

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Table 1. Ceruloplasmin measured prior to 5-day TETA (0.83% diet) treatment (Pre-TETA) and immediately following the 5-day TETA treatment (Post-TETA).

	Pre-TETA (mg/dl)	Post-TETA (mg/dl)
AMB x $^{-}$Cu	29.1 ± 7.7	8.2 ± 5.0
AMB x Control	39.3 ± 7.7	45.7 ± 4.1
SUSP x $^{-}$Cu	34.3 ± 7.7	6.6 ± 4.1
SUSP x Control	45.4 ± 7.7	45.8 ± 4.1
AMB	34.2 ± 5.4	26.9 ± 3.2
SUSP	39.9 ± 5.4	26.2 ± 2.9
$^{-}$Cu	31.7 ± 5.4	7.4 ± 3.2
Control	42.3 ± 5.4	45.8 ± 2.9
Source of Variation	*p*-values	
SUSP	0.48	0.87
DIET	0.20	<0.001
SUSP x DIET	0.96	0.85

[1]Values represent least squares means ± SEM (SUSP, n = 6; $^{-}$Cu, n = 5-6).

Table 2. Body weight of ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and copper depleted ($^{-}$Cu) diets.

	Day 0 (g)	Day 3 (g)	Day 6 (g)	Day 9 (g)	Day 12 (g)	Day 15 (g)	Day 18 (g)	Day 21 (g)	Day 24 (g)	Day 27 (g)
AMB x $^{-}$Cu	536.1 ± 10.0	552.4 ± 10.7	537.4 ± 11.3	521.3 ± 11.1	517.3 ± 10.7	519.1 ± 10.7	522.9 ± 10.5	530.4 ± 10.7	528.6 ± 10.9	536.8 ± 10.7
AMB x Control	530.4 ± 10.0	543.1 ± 10.7	531.5 ± 11.3	515.5 ± 11.1	513.2 ± 10.7	517.6 ± 10.7	520.3 ± 10.5	524.2 ± 10.7	527.6 ± 10.9	533.9 ± 10.7
SUSP x $^{-}$Cu	532.7 ± 10.0	541.7 ± 10.7	515.4 ± 11.3	502.7 ± 11.1	501.2 ± 10.7	499.4 ± 10.7	500.7 ± 10.5	502.2 ± 10.7	502.3 ± 11.6	501.5 ± 11.4
SUSP x Control	541.6 ± 10.7	556.1 ± 11.4	529.8 ± 12.0	507.0 ± 11.8	501.0 ± 11.4	498.2 ± 11.4	494.4 ± 11.2	499.1 ± 11.4	496.9 ± 11.6	496.0 ± 11.4
AMB	533.2 ± 7.1	547.7 ± 7.6	534.5 ± 8.0	518.4 ± 7.8	515.3 ± 7.6	518.3 ± 7.6	521.6 ± 7.4	527.3 ± 7.6	528.12 ± 7.7	535.4 ± 7.5
SUSP	537.1 ± 7.3	548.9 ± 7.8	522.6 ± 8.2	504.8 ± 8.1	501.1 ± 7.8	498.8 ± 7.8	497.6 ± 7.7	500.6 ± 7.8	499.6 ± 8.2	498.8 ± 8.0
$^{-}$Cu	534.4 ± 7.1	547.0 ± 7.6	526.4 ± 8.0	512.0 ± 7.8	509.3 ± 7.6	509.3 ± 7.6	511.8 ± 7.4	516.3 ± 7.6	515.5 ± 7.9	519.2 ± 7.8
Control	536.0 ± 7.3	549.6 ± 7.8	530.6 ± 8.2	511.3 ± 8.1	507.1 ± 7.8	507.9 ± 7.8	507.3 ± 7.7	511.6 ± 7.8	512.2 ± 7.9	515.0 ± 7.8
Source of Variation						*p*-values				
SUSP	0.70	0.92	0.31	0.24	0.20	0.08	<0.05	<0.05	<0.05	<0.01
DIET	0.88	0.82	0.72	0.95	0.84	0.90	0.68	0.67	0.78	0.71
SUSP x DIET	0.48	0.28	0.38	0.66	0.86	0.99	0.87	0.89	0.85	0.91

[1] Values represent least squares means ± SEM (SUSP, n = 17; $^{-}$Cu, n = 17).

[2] Rats were weighed on the day of suspension (Day 0) and every third day throughout the 28-day treatment period.

Table 3. Tissues expressed as a percentage of body weight for ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and the copper depleted ($^{-}$Cu) diets.

	Heart (%)	Testes (%)	Liver (%)	Kidney (%)
AMB x $^{-}$Cu	0.248 ± 0.006	0.696 ± 0.019	2.649 ± 0.085	0.553 ± 0.017
AMB x Control	0.247 ± 0.006	0.683 ± 0.019	2.535 ± 0.085	0.559 ± 0.017
SUSP x $^{-}$Cu	0.270 ± 0.007	0.265 ± 0.020	2.615 ± 0.090	0.583 ± 0.018
SUSP x Control	0.270 ± 0.007	0.276 ± 0.020	2.419 ± 0.090	0.583 ± 0.018
AMB	0.247 ± 0.004	0.689 ± 0.013	2.592 ± 0.060	0.556 ± 0.012
SUSP	0.270 ± 0.005	0.270 ± 0.014	2.517 ±0.063	0.583 ± 0.013
$^{-}$Cu	0.259 ± 0.005	0.480 ± 0.014	2.632 ± 0.062	0.568 ± 0.012
Control	0.258 ± 0.004	0.479 ± 0.014	2.477 ± 0.062	0.571 ± 0.012
Source of Variation		*p*-Values		
SUSP	<0.01	<0.001	0.40	0.13
DIET	0.97	0.96	0.08	0.85
SUSP x DIET	0.98	0.55	0.64	0.86

[1]Values represent least squares means ± SEM (SUSP, n = 17; $^{-}$Cu, n = 17).

Table 4. Femur mechanical strength assessed by torsion testing on ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and copper depleted ($^{-}$Cu) diets.

	Maximum Torque ($N*m^2$)	Maximum Angle (deg)
AMB x $^{-}$Cu	569.4 ± 26.8	12.9 ± 0.9
AMB x Control	561.8 ± 26.8	13.0 ± 0.9
SUSP x $^{-}$Cu	484.1 ± 30.5	12.6 ± 0.9
SUSP x Control	507.4 ± 30.5	14.7 ± 0.9
AMB	565.6 ± 18.9	12.8 ± 0.6
SUSP	496.0 ± 21.5	13.6 ± 0.6
$^{-}$Cu	527.0 ± 20.3	12.6 ± 0.6
Control	534.6 ± 20.3	13.8 ± 0.6
Source of Variation	*p*-Values	
SUSP	<0.05	0.35
DIET	0.60	0.18
SUSP x DIET	0.79	0.37

[1]Values represent least squares means ± SEM (SUSP, n = 14-17; $^{-}$Cu, n = 17).

Table 5. Bone mineral area (BMA), bone mineral content (BMC) and bone mineral density (BMD) determined by dual-energy x-ray densitometry (DEXA) on ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and copper depleted ($^{-}$Cu) diets.

	L5BMA (cm^2)	L5BMC (g)	L5BMD (g/cm^2)	FemBMA (cm^2)	Fem BMC (g)	FemBMD (g/cm^2)	Hum BMA (cm^2)	Hum BMC (g)	Hum BMD (g/cm^2)
AMB x $^{-}$Cu	0.620± 0.017	0.152 ± 0.006	0.245 ± 0.004	2.437 ± 0.057	0.600 ± 0.020	0.246 ± 0.004	1.261 ± 0.028	0.266 ± 0.008	0.211 ± 0.002
AMB x Control	0.619 ± 0.017	0.156 ± 0.006	0.253 ± 0.004	2.414 ± 0.057	0.604 ± 0.020	0.250 + 0.004	1.290 ± 0.028	0.269 ± 0.008	0.208 ± 0.002
SUSP x $^{-}$Cu	0.570 ± 0.017	0.128 ± 0.006	0.223 ± 0.004	2.305 ± 0.057	0.532 ± 0.020	.0230 ± 0.004	1.256 ± 0.028	0.259 ± 0.008	0.206 ± 0.002
SUSP x Control	0.560 ± 0.018	0.132 ± 0.006	0.236 ± 0.004	2.416 ± 0.060	0.583 ± 0.021	0.241 ± 0.004	1.282 ± 0.030	0.271 ± 0.008	0.211 ± 0.002
AMB	0.619 ± 0.012	0.154 ± 0.004	0.249 ± 0.003	2.425 ± 0.040	0.602 ± 0.014	0.248 ± 0.003	1.276 ± 0.020	0.267 ± 0.006	0.209 ± 0.002
SUSP	0.564 ± 0.012	0.130 ± 0.004	0.230 ± 0.003	2.361 ± 0.041	0.558 ± 0.015	0.236 ± 0.003	1.269 ± 0.021	0.265 ± 0.006	0.209 ± 0.002
$^{-}$Cu	0.595 ± 0.012	0.140 ± 0.004	0.234 ± 0.003	2.371 ± 0.040	0.565 ± 0.014	0.238 ± 0.003	1.256 ± 0.020	0.262 ± 0.006	0.208 ± 0.002
Control	0.589 ± 0.012	0.145 ± 0.004	0.244 ± 0.003	2.415 ± 0.041	0.593 ± 0.015	0.245 ± 0.003	1.290 ± 0.021	0.270 ± 0.006	0.210 ± 0.002
Source of Variation					*p*-Values				
SUSP	<0.01	<0.001	<0.001	0.27	<0.05	<0.01	0.81	0.84	0.89
DIET	0.74	0.41	<0.01	0.45	0.19	0.09	0.35	0.37	0.59
SUSP x DIET	0.76	0.99	0.46	0.26	0.27	0.44	0.96	0.56	0.08

[1]Values represent least squares means ± SEM (SUSP, n = 17; $^{-}$Cu, n = 17).

Table 6. Wet weight, dry weight and ash weight of L5 and humerus in ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and copper depleted ($^{-}$Cu) diets.

	L5 Wet Weight (g)	L5 Dry Weight (g)	L5 Ash Weight (g)	Humerus Wet Weight (g)	Humerus Dry Weight (g)	Ash Weight (g)
AMB x $^{-}$Cu	0.56 ± 0.03	0.35 ± 0.01	0.22 ± 0.01	0.61 ± 0.02	0.42 ± 0.01	0.32 ± 0.01
AMB x Control	0.62 ± 0.03	0.35 ± 0.01	0.22 ± 0.01	0.61 ± 0.02	0.42 ± 0.01	0.34 ± 0.01
SUSP x $^{-}$Cu	0.55 ± 0.03	0.32 ± 0.01	0.19 ± 0.01	0.58 ± 0.02	0.40 ± 0.01	0.32 ± 0.01
SUSP x Control	0.53 ± 0.03	0.32 ± 0.01	0.20 ± 0.01	0.57 ± 0.02	0.41 ± 0.01	0.32 ± 0.01
AMB	0.59 ± 0.02	0.35 ± 0.01	0.22 ± 0.01	0.61 ± 0.01	0.42 ± 0.01	0.33 ± 0.01
SUSP	0.54 ± 0.02	0.32 ± 0.01	0.20 ± 0.01	0.57 ± 0.01	0.41 ± 0.01	0.32 ± 0.01
$^{-}$Cu	0.55 ± 0.02	0.34 ± 0.01	0.20 ± 0.01	0.59 ± 0.01	0.41 ± 0.01	0.32 ± 0.01
Control	0.57 ± 0.02	0.33 ± 0.01	0.21 ± 0.01	0.59 ± 0.01	0.41 ± 0.01	0.33 ± 0.01
Source of Variation			*p*-Values			
SUSP	0.10	<0.05	<0.01	<0.05	0.19	0.35
DIET	0.56	0.94	0.38	0.89	0.87	0.65
SUSP x DIET	0.25	0.71	0.75	0.91	0.80	0.38

[1]Values represent least squares means ± SEM (SUSP, n = 17; $^{-}$Cu, n = 17).

Table 7. Tissue copper concentration (μg/g dry weight) of ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and copper depleted ($^{-}$Cu) diets.

	L5 μg/g	Humerus μg/g	Liver μg/g	Kidney μg/g
AMB x $^{-}$Cu	3.05 ± 0.09	0.26 ± 0.06	6.67 ± 0.69	57.34 ± 5.65
AMB x Control	3.53 ± 0.09	0.38 ± 0.06	12.43 ± 0.65	114.11 ± 5.65
SUSP x $^{-}$Cu	3.17 ± 0.09	0.17 ± 0.06	7.45 ± 0.65	60.15 ± 5.65
SUSP x Control	3.81 ± 0.09	0.37 ± 0.06	13.36 ± 0.65	93.66 ± 5.65
AMB	3.29 ± 0.06	0.32 ± 0.04	9.55 ± 0.47	85.72 ± 3.99
SUSP	3.49 ± 0.06	0.27 ± 0.04	10.40 ± 0.50	76.90 ± 3.99
$^{-}$Cu	3.11 ± 0.06	0.22 ± 0.04	7.06 ± 0.47	58.74 ± 4.01
Control	3.67 ± 0.06	0.37 ± 0.05	12.90 ± 0.50	103.88 ± 3.99
Source of Variation		*p*-Values		
SUSP	<0.05	0.46	0.22	0.13
DIET	<0.001	<0.05	<0.001	<0.001
SUSP x DIET	0.35	0.50	0.92	<0.05

[1]Values represent least squares means ± SEM (SUSP, n = 16-17; $^{-}$Cu, n = 17).

Table 8. Tissue iron concentration (μg/g dry weight) of ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and copper depleted ($^{-}$Cu) diets.

	L5 (μg/g)	Humerus (μg/g)	Liver (μg/g)	Kidney (μg/g)
AMB x $^{-}$Cu	2.4 ± 0.2	46.3 ± 3.6	621.6 ± 77.2	167.9 ± 23.3
AMB x Control	3.5 ± 0.2	55.9 ± 3.6	434.4 ± 72.5	231.5 ± 23.3
SUSP x $^{-}$Cu	2.6 ± 0.2	46.1 ± 3.6	871.5 ± 72.5	187.6 ± 24.8
SUSP x Control	3.0 ± 0.2	53.5 ± 3.9	815.4 ± 83.8	241.3 ± 24.8
AMB	2.9 ± 0.2	51.1 ± 2.6	528.0 ± 53.0	199.7 ± 16.5
SUSP	2.8 ± 0.2	49.8 ± 2.7	843.4 ± 55.4	214.5 ± 17.6
$^{-}$Cu	2.5 ± 0.2	46.2 ± 2.6	746.6 ± 53.0	177.8 ± 17.0
Control	3.2 ± 0.2	54.7 ± 2.7	624.9 ± 55.4	236.4 ± 17.0
Source of Variation		*p*-Values		
SUSP	0.72	0.73	<0.001	0.55
DIET	<0.01	<0.05	0.12	<0.05
SUSP x DIET	0.14	0.78	0.40	0.84

[1]Values represent least squares means ± SEM (SUSP, n = 16-17; $^{-}$Cu, n = 17).

Table 9. Tissue zinc concentration (μg/g dry weight) of ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and copper depleted ($^{-}$Cu) diets.

	L5 (μg/g)	Humerus (μg/g)	Liver (μg/g)	Kidney (μg/g)
ABM x $^{-}$Cu	251.5 ± 10.2	238.8 ± 6.8	119.1 ± 7.0	68.8 ± 2.3
AMB x Control	223.0 ± 10.2	252.4 ± 6.8	119.4 ± 6.5	74.6 ± 2.3
SUSP x $^{-}$Cu	248.0 ± 10.2	261.8 ± 6.8	141.4 ± 6.5	70.0 ± 2.4
SUSP x Control	227.1 ± 10.9	258.6 ± 7.2	147.3 ± 7.6	72.9 ± 2.4
AMB	237.3 ± 7.2	245.6 ± 4.8	119.3 ± 4.8	71.7 ± 1.6
SUSP	237.6 ± 7.5	260.2 ± 4.9	144.3 ± 5.0	71.4 ± 1.7
$^{-}$Cu	249.8 ± 7.2	250.3 ± 4.8	130.3 ± 4.8	69.3 ± 1.7
Control	225.1 ± 7.5	255.5 ± 4.9	133.3 ± 5.0	73.8 ± 1.7
Source of Variation		*p*-Values		
SUSP	0.98	<0.05	<0.01	0.92
DIET	<0.05	0.46	0.66	0.07
SUSP x DIET	0.72	0.23	0.69	0.55

[1]Values represent least squares means ± SEM (SUSP, n = 16-17; $^{-}$Cu, n = 17).

Table 10. Bone calcium concentration (mg/g dry weight) of ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and copper depleted ($^{-}$Cu) diets.

	L5 (mg/g)	Humerus (mg/g)
AMB x $^{-}$Cu	185.2 ± 12.6	245.9 ± 7.1
AMB x Control	162.2 ± 12.6	242.0 ± 7.1
SUSP x $^{-}$Cu	164.5 ± 12.6	253.4 ± 7.1
SUSP x Control	189.5 ± 13.4	262.0 ± 7.6
AMB	173.7 ± 8.9	243.9 ± 5.0
SUSP	177.0 ± 9.2	257.7 ± 5.1
$^{-}$Cu	174.9 ± 8.9	249.6 ± 5.0
Control	175.9 ± 9.2	252.0 ± 5.2
	p-Values	
Source of Variation		
SUSP	0.80	0.07
DIET	0.94	0.75
SUSP x DIET	0.07	0.40

[1]Values represent least squares means ± SEM (SUSP, n = 17; $^{-}$Cu, n = 17).

Table 11. Tissue magnesium concentration (mg/g dry weight) of ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and copper depleted ($^{-}$Cu) diets.

	L5 (mg/g)	Humerus (mg/g)	Liver (mg/g)	Kidney (mg/g)
AMB x $^{-}$Cu	3.26 ± 0.13	3.92 ± 0.08	0.61 ± 0.02	0.41 ± 0.04
AMB x Control	3.24 ± 0.13	3.99 ± 0.08	0.51 ± 0.02	0.40 ± 0.04
SUSP x $^{-}$Cu	2.92 ± 0.13	3.88 ± 0.08	0.61 ± 0.02	0.39 ± 0.04
SUSP x Control	3.27 ± 0.14	4.15 ± 0.09	0.54 ± 0.03	0.40 ± 0.04
AMB	3.25 ± 0.09	3.96 ± 0.06	0.26 ± 0.02	0.41 ± 0.03
SUSP	3.10 ± 0.09	4.02 ± 0.06	0.57 ± 0.02	0.39 ± 0.03
$^{-}$Cu	3.09 ± 0.09	3.90 ± 0.06	0.61 ± 0.02	0.40 ± 0.03
Control	3.26 ± 0.09	4.07 ± 0.06	0.52 ± 0.02	0.40 ± 0.03
Source of Variation		*p*-Values		
SUSP	0.24	0.48	0.77	0.74
DIET	0.21	<0.05	<0.01	0.89
SUSP x DIET	0.13	0.24	0.57	0.78

[1]Values represent least squares means ± SEM (SUSP, n = 16-17; $^{-}$Cu, n = 17).

Table 12. Total 12h urinary excretion of deoxypyridinoline (DPD) crosslinks (nM) of ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and copper depleted ($^{-}$Cu) diets.

	Baseline (nM)	2-Week (nM)	4-Week (nM)
AMB x $^{-}$Cu	1289.92 ± 253.22	742.05 ± 189.44	576.80 ± 261.73
AMB x Control	1042.46 ± 253.22	599.89 ± 189.44	816.76 ± 261.73
SUSP x $^{-}$Cu Diet	1327.44 ± 253.22	1638.28 ± 189.44	1331.07 ± 261.73
SUSP x Control	1528.47 ± 269.57	2411.04 ± 201.68	1336.31 ± 278.63
AMB	1164.70 ± 179.05	670.97 ± 133.96	696.78 ± 185.07
SUSP	1427.96 ± 184.92	2024.66 ± 138.35	1333.69 ± 191.14
$^{-}$Cu	1307.18 ± 179.05	1190.17 ± 133.96	953.94 ± 185.07
Control Diet	1285.47 ± 184.92	1505.47 ± 138.35	1076.54 ± 191.14
Source of Variation		*p*-Values	
SUSP	0.31	<0.001	<0.05
DIET	0.93	0.11	0.65
SUSP x DIET	0.40	<0.05	0.66

[1]Values represent least squares means ± SEM (SUSP, n = 17; $^{-}$Cu, n = 17).

[2]Urine collection was performed on the day prior to suspension (Baseline), at the end of two weeks (2-week) and the final urine collection (4-week)

CHAPTER V

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary

The purpose of this research was to investigate the effects of copper depletion on bone quantity (i.e. bone mass) and quality (i.e. bone mineral density, mechanical strength and bone mineral content) in two animal models of osteopenia; the ovariectomized animal model and the tail-suspended rat model.

In Chapter I of this dissertation, five objectives and hypotheses are listed. The hypotheses are separated into Part I, related to the ovariectomized rat, and Part II, pertaining to the tail-suspended rat. Each hypothesis will be individually addressed in this summary, and then general conclusions and recommendations for future research will be discussed.

Part I, hypothesis one states that diminished copper will not significantly alter bone quality or biochemical markers of bone metabolism (e.g. urinary hydroxyproline, and tartrate-resistant acid phosphatase) in ovariectomized rats.

The copper depletion diet (0.6 mg Cu added / kg of diet) did not alter bone quantity or quality. Bone ash weight, dry weight, bone mineral density and mechanical strength were not affected by copper depletion. Biochemical markers indicated that no alterations in bone metabolism resulted from copper depletion. However, diminished estrogen, due to ovariectomy, produced significant increases in body weight, which

appeared to "load" the limbs. This skeletal loading served to protect the hind limb bones as indicated by no significant change in bone mineral density of the femur, increased mechanical strength of the femur and increased tibia dry weight, despite the decreases in vertebral bone mineral density. Only one biochemical marker (i.e. urinary hydroxyproline) was altered by ovariectomy. Increased urinary excretion of hydroxyproline ($p<0.01$) indicated a possible increase in bone resorption was occurring, but no changes in the rate of bone formation were detected. Based on no alterations being observed in bone quantity or quality due to copper depletion in this study, hypothesis 1 is accepted.

Part I, hypothesis two states that there will be no statistically significant differences in vertebral bone mineral concentrations of calcium, magnesium, iron, zinc and copper as a result of ovariectomy or copper depletion.

Ovariectomy resulted in significant decreases ($p<0.05$) in vertebral magnesium and zinc. A trend toward decreased calcium concentrations ($p<0.06$) of the vertebrae was evident, but no alterations in vertebral iron or copper concentrations were observed. The copper depletion diet produced no statistically significant alterations in the selected vertebral mineral concentrations analyzed in this study, including copper. A trend toward decreased magnesium and iron was noted. Copper depletion was apparently not low enough or the duration of the treatment was too short to produce symptoms of copper deficiency in the bone in animals of this age. Results from this research support the rejection of Part I, hypothesis 2.

Part II, hypothesis one states that tail suspension or copper depletion will not significantly alter the mechanical strength, bone mineral density or bone mineral content in the mature rat model.

The bone mineral density of the femur ($p<0.01$) and vertebrae ($p<0.001$), which are considered "unloaded" in this model, was significantly reduced. No change was observed in the bone mineral density of the humerus, however, which experienced "normal loading". Bone mineral concentration was also significantly reduced in the femur ($p<0.0001$) and vertebra ($p<0.05$) due to tail suspension, but not in the humerus. The mechanical strength of the femur was significantly reduced ($p<0.05$) by skeletal unloading.

Copper depletion significantly reduced the bone mineral density of vertebral bone ($p<0.01$), but had no effect on the femur or humerus. The bone mineral concentrations of the vertebra, femur and humerus were not altered due to copper depletion. Likewise, the mechanical strength of the femur was not affected.

These findings indicate that tail suspension does affect the bone mineral density, mechanical strength and bone mineral content in mature animals. And although copper depletion did not alter mechanical strength and bone mineral content, vertebral bone mineral density was reduced. Based on these results, hypothesis 1 of Part II is rejected.

Part II, hypothesis two states that there will be no statistically significant effect of tail suspension or copper depletion on biochemical markers of bone remodeling (i.e. urinary pyridinoline crosslink excretion, serum alkaline phosphatase, and bone-specific alkaline phosphatase) in mature rats.

Tail suspension caused the urinary excretion of pyridinoline crosslinks to significantly increase ($p<0.001$) by the second week and to remain elevated through the end of the study. Copper depletion failed to alter crosslink excretion. Neither tail suspension or copper depletion altered serum alkaline phosphatase or bone-specific

alkaline phosphatase. Based on the increase in crosslink excretion in relation to tail suspension, hypothesis 2 of Part II is rejected.

Part II, hypothesis three states that in mature rats there will be no statistically significant difference in the concentrations of bone calcium, magnesium, zinc, iron and copper due to either tail suspension or copper depletion.

Only the copper concentration of vertebral bone was significantly reduced ($p<0.05$) as a result of tail suspension. The zinc concentration of the humerus was increased significantly ($p<0.05$) , while the calcium concentration of the humerus tended to increase ($p<0.07$) in tail-suspended animals. Copper depletion resulted in the expected reduction in copper concentrations of the vertebra ($p<0.001$) and humerus ($p<0.05$) and a reduction in the concentration of iron in the vertebra ($p<0.01$) and humerus ($p<0.05$). Zinc concentrations were greater in the vertebra ($p<0.05$), while magnesium concentrations in the humerus were reduced ($p<0.05$) with copper depletion. These results indicate that both tail suspension and copper depletion influenced the bone concentrations of calcium, magnesium, iron, zinc and copper. Based on these findings, hypothesis 3 of Part II is rejected.

Conclusions

From experiment 1 it can be concluded that 10 weeks of copper depletion, at the dietary level used in this study (0.6 mg Cu added /kg of diet), was not a severe enough deficiency to produce alterations in bone parameters in animals of this age. Osteopenia occurred in the vertebra as a result of ovarian hormone deficiency, but not in the hind limbs. Weight gain appeared to "load" the bones of the hind limbs and to provide protection against the loss of bone mass and strength normally associated with

ovariectomy. Arguably, pair feeding would have reduced the degree of weight gain that occurred in this study, but may not have as closely resembled the natural weight gain observed in postmenopausal women.

In experiment 2, mature rats exposed to four weeks of tail suspension experienced significant alterations in unloaded bones. Normally loaded bones were not affected by tail suspension, and therefore indicate that the changes in bone quality and quantity are likely the result of local factors and not systemic factors of bone metabolism. Increased bone resorption was apparently the primary factor involved in the bone loss in mature, tail-suspended rat. The effect of tail suspension on the copper concentration of the vertebra should be further investigated

Copper depletion had the greatest effect on vertebral bone and resulted in reduced bone mineral density. No copper effect was observed on the bone mineral density of the femur or humerus. Copper depletion was evidently not severe enough to reduce bone mechanical strength. However, copper depletion did alter bone concentrations of iron, zinc, copper, magnesium and calcium. The role of TETA in the alterations of bone mineral concentrations requires further examination.

Recommendations

In older rats, more severe copper depletion diets may be necessary to produce the increase in bone fragility associated with copper deficiency in younger animals. A longer depletion period at the dietary intake used in this study also may have resulted in more the typical alterations in bone associated with copper deficiency. Future experiments should investigate lower copper intakes as well as longer duration depletion periods to create a more severe deficiency. Due to the function of copper in collagen crosslink

formation and typical Western dietary intakes, the possible role of copper deficiency in postmenopausal osteoporosis deserves further investigation.

Future studies should also include analysis of the bone mineral concentrations of the bone corresponding to bones submitted to mechanical strength testing. Such analysis would allow for better correlation between various mineral concentrations and mechanical strength. Also, the use of compression testing or torsion testing may have provided biomechanical information that more closely reflects the types of forces typically involved in bone fractures. Although these alternative methods provide a more realistic force than 3-point bending, a device is not yet available that produces fractures at one of the most common fracture sites, the head and trochanter region of the femur, as a result of a torsional type of force.

Future studies should also consider the use of alternative biochemical markers of bone metabolism. Although urinary hydroxyproline, bone hydroxyproline and alkaline phosphatase provide insight into the metabolic changes occurring within bone, better markers that are more specific to bone tissue are now available. Future studies should consider assessing alterations in bone formation and resorption using assays such as the pyridinoline crosslinks, N-telopeptides and osteocalcin.

While Experiment 2 provided insight into the role of copper and skeletal unloading on bone health, the issue of stress should be addressed. The model may produce a transient stress during the first few days of tail-suspension, but studies examining serum glucocorticoids on younger animals have not supported this theory. Research on the glucocorticoid response to tail suspension has not been conducted on mature and aged rat. Studies are needed to clarify the alterations in bone resulting from skeletal unloading.

Future studies should also consider the use of older animals and the changes associated with recovery from tail suspension. Although 5 ½-month-old rats were used in this study, rats that are at least 1 year-of-age may more closely represent the maturation of the typical crewmember in space. Also, the effects of copper depletion and the lack of weight-bearing exercise should be of particular interest with the growing elderly population in the U.S. Studies examining the effects of a return to normal weight-bearing or recovery may provide additional insight into the skeletal response to unloading.

Investigation into the chelating action of TETA and its effect on bone mineral concentration also warrants further exploration. If the role of copper deficiency in the bone health of aging animals is to be studied, a quick chelating drug such as TETA that produces a rapid depletion could prove to be very beneficial. However, based on the results of this research, it appears that TETA may be chelating or altering the metabolism of such minerals as iron and zinc. Whether or not the changes in iron and zinc are a result of copper deficiency or TETA should be determined.

In both of the studies, volumetric determination of bone mineral density, histomorphometric analyses and molecular studies would have provided valuable insights. DEXA measures of bone mineral density are relatively quick and inexpensive, but some researchers have questioned the accuracy of determining density based on a 2-dimensional measure (i.e. area) verses a 3-dimensional measure (i.e. volume). Both measures should be performed in future studies to verify results. Additionally, histo-morphometric analyses would provide insight beyond the biochemical markers into the actual changes occurring at bone remodeling sites in various bones exposed to either skeletal loading or unloading. And finally, molecular studies in conjunction with

histomorphometric analyses could provide important information concerning the mechanisms of bone loss at a cellular level. Determination of the mechanisms involved in changes in bone quality and quantity, and the influence of nutrients such as copper on these mechanisms are critical in the understanding, prevention and treatment of osteoporosis.

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APPENDIX A

SUPPLEMENTAL MATERIALS FOR CHAPTER III

Serum Analyses

Blood was drawn and samples were placed on ice for approximately 30 minutes. The samples were then centrifuged at a speed of 1500 x g for 22 minutes. Aliquots of serum were analyzed immediately on the clinical analyzer (Cobas Fara II) for glucose, fructosamine, albumin, cholesterol, triglycerides, high density lipoprotein (HDL) and creatinine.

Serum samples were thawed and insulin was determined using a radioimmuno assay (Linco, St. Charles, MO) based on the method of Morgan and Lazarow (Morgan and Lazarow 1963). The concentration of insulin is expressed as ng /ml.

Results of serum albumin, creatinine, glucose, fructosamine and insulin are presented in Appendix A, Table 1. Serum lipid data are presented in Appendix A, Table 2.

Liver Lipid Analysis

Liver samples were cleaned, weighed and stored at -20° at necropsy. Liver lipids were analyzed from frozen samples based on the method of Carr et al. (Carr et al. 1993). Lipid extraction was performed on samples of liver weighing approximately 0.2000 g. Total liver lipids, liver total cholesterol, liver triglycerides and liver phospholipids were

determined via enzymatic assay. Results of the liver lipid analyses are presented in Apprendix A, Table 3.

Liver Trace Element Analyses

Liver samples, which had been stored on the day of necropsy at -20°C, were thawed. All samples were removed using a glass knife and handled with mineral free gloves and plastic forceps to avoid mineral contamination. Samples of liver (weighing ~200 mg) were weighed into acid-washed borosilicate glass tubes and dried for 24 h at 100°C. Tissue dry weights were recorded. Next, liver samples were exposed to a series of wet and dry ashing until all organic material was removed using a modification of the method of Hill and colleagues (Hill, Patterson, Veillon, and Morris 1986). Details of the wet and dry ashing protocol have been outlined in Chapters III & IV. Percent moisture lost was calculated based on the formula below:

$$\% \text{ Moisture Lost} = \frac{\text{Wet Weight} - \text{Dry Weight}}{\text{Wet Weight}}$$

Liver wet weight, dry weight, ash weight and percent moisture lost are reported in Appendix A, Table 4.

Hematology

At the time of necropsy, a sample of whole blood was removed for hematology (Serano System 9000). Results of these analyses are presented in Appendix A, Table 5.

Appendix A Table 1. Clinical chemistry parameters in sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Serum Albumin (mg/dl)	Serum Creatinine (mg/dl)	Serum Glucose (mg/dl)	Serum Fructosamine (μmol/l)	Serum Insulin (ng/ml)
Low Copper x Sham	3.8 ± 0.1	0.62 ± 0.04	162.18 ± 10.3	130.0 ± 3.8	0.2 ± 0.1
Low Copper x OVX	3.5 ± 0.1	0.62 ± 0.04	189.53 ± 11.0	126.0 ± 4.1	0.4 ± 0.1
Control x Sham	3.6 ± 0.1	0.69 ± 0.04	129.11 ± 11.2	131.3 ± 4.2	0.3 ± 0.1
Control x OVX	3.4 ± 0.1	0.62 ± 0.04	198.32 ± 11.0	122.8 ± 4.1	0.4 ± 0.1
Sham	3.7 ± 0.1	0.66 ± 0.03	145.65 ± 7.5	130.5 ± 2.8	0.3 ± 0.1
OVX	3.4 ± 0.1	0.62 ± 0.03	193.93 ± 7.7	124.4 ± 2.9	0.4 ± 0.1
Low Copper	3.6 ± 0.1	0.62 ± 0.03	175.86 ± 7.6	127.8 ± 2.8	0.3 ± 0.1
Control	3.5 ± 0.1	0.66 ± 0.03	163.72 ± 7.9	127.0 ± 2.9	0.3 ± 0.1
Source of Variation			*p*-Values		
OVX	<0.01	0.40	<0.001	0.14	0.16
DIET	0.16	0.42	0.28	0.85	0.74
OVX x DIET	0.24	0.43	0.07	0.55	0.81

[1]Values represent least squares means ± SEM (OVX, n=16, Low copper, n=16).

Appendix A Table 2. Serum cholesterol, triglycerides and HDL cholesterol in sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Serum Cholesterol (mg/dl)	Serum Triglycerides (mg/dl)	Serum HDL (mg/dl)
Low Copper x Sham	71.91 ± 5.8	49.62 ± 5.6	57.15 ± 6.15
Low Copper x OVX	113.16 ± 5.8	62.82 ± 5.2	92.38 ± 5.7
Control x Sham	70.59 ± 5.8	38.93 ± 5.2	51.80 ± 7.1
Control x OVX	89.46 ± 5.8	49.18 ± 5.2	79.40 ± 7.1
Sham	71.25 ± 4.1	44.28 ± 3.8	54.48 ± 4.8
OVX	101.3 ± 4.1	56.0 ± 3.6	85.89 ± 4.6
Low Copper	92.5 ± 4.2	56.22 ± 3.9	74.76 ± 4.2
Control	80.0 ± 4.2	44.05 ± 3.7	65.60 ± 5.1
Source of Variation		*p*-Values	
OVX	<0.001	<0.05	<0.001
DIET	<0.05	<0.05	0.17
OVX x DIET	0.06	0.78	0.56

[1]Values represent least squares means ± SEM (OVX, n=16, Low copper, n=16).

Appendix A Table 3. Liver lipids in sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Total Liver Lipids (mg/g liver)	Liver Cholesterol (mg/g liver)	Liver Free Cholesterol (mg/g liver)	Liver Triglycerides (mg/g liver)	Liver Phospholipids (mg/g liver)
Low Copper x Sham	92.8 ± 11.8	3.0 ± 0.5	2.4 ± 0.7	10.5 ± 4.8	9.6 + 1.1
Low Copper x OVX	113.1 ± 13.6	4.9 ± 0.6	4.5 ± 0.8	30.0 ± 5.6	12.0 + 1.3
Control x Sham	75.2 ± 11.8	2.6 ± 0.5	1.8 ± 0.7	8.0 ± 4.8	7.0 + 1.1
Control x OVX	85.0 ± 11.8	3.3 ± 0.5	2.3 ± 0.7	12.2 ± 4.8	8.3 + 1.1
Sham	84.0 ± 8.2	2.8 ± 0.4	2.1 ± 0.5	9.3 ± 3.4	8.3 + 0.8
OVX	99.0 ± 8.9	4.1 ± 0.4	3.4 ± 0.5	21.1 ± 3.7	10.1 + 0.8
Low Copper	102.9 ± 9.1	3.5 ± 0.3	3.4 ± 0.5	20.2 ± 3.7	10.8 ± 0.8
Control	80.1 ± 8.4	3.3 ± 0.2	2.1 ± 0.5	10.1 ± 3.4	7.6 ± 0.8
Source of Variation			*p*-Values		
OVX	0.23	<0.05	0.09	<0.05	0.12
DIET	0.08	0.09	0.07	0.06	<0.05
OVX x DIET	0.67	0.28	0.25	0.14	0.64

[1]Values represent least squares means ± SEM (OVX, n=16, Low copper, n=16).

Appendix A Table 4. Liver dry weight, ash weight and percent moisture in sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	Wet Liver (mg)	Dry Liver (mg)	Moisture (%)	Ash Liver (mg)
Low Copper x Sham	196 ± 18	66 ± 7	65.78 ± 1.69	3 ± 1
Low Copper x OVX	162 ± 20	61 ± 8	62.17 ± 1.96	2 ± 1
Control x Sham	177 ± 18	57 ± 7	67.60 ± 1.69	3 ± 1
Control x OVX	176 ± 18	58 ± 7	67.24 ± 1.69	3 ± 4
Sham	186 ± 12	61 ± 6	66.69 ± 1.19	3 ± 0.3
OVX	169 ± 13	63 ± 7	64.71 ± 1.28	2 ± 0.3
Low Copper	179 ± 14	63 ± 5	63.98 ± 1.31	2 ± 0.3
Control	177 ± 13	58 ± 5	67.42 ± 1.21	3 ± 0.3
Source of Variation			*p*-Values	
OVX	0.34	0.83	0.27	0.32
DIET	0.92	0.44	0.07	0.16
OVX x Diet	0.38	0.68	0.36	0.58

[1]Values represent least squares means ± SEM (OVX, n=11-14, Low copper, n=11-14).

Appendix A—Table 5. Hematology data from sham-operated (Sham) and ovariectomized (OVX) rats fed the control (AIN-93M) and low copper diets.

	WBC (10^3/mm^3)	RBC (10^6/mm^3)	HGB (g/dl)	HCT (%)	MCV (μm^3)	MCH (pg)	MCHC (g/dl)
Low Copper x Sham	4.6 ± 1.0	6.8 ± 0.2	14.9 ± 0.4	34.7 ± 0.9	50.9 ± 0.7	21.8 ± 0.4	42.8 ± 0.5
Low Copper x OVX	6.2 ± 1.0	6.9 ± 0.2	14.8 ± 0.4	35.3 ± 0.9	50.9 ± 0.7	21.5 ± 0.4	42.2 ± 0.5
Control x Sham	7.0 ± 1.0	7.1 ± 0.2	15.2 ± 0.4	36.5 ± 0.9	51.5 ± 0.7	21.4 ± 0.4	41.6 ± 0.5
Control x OVX	7.4 ± 1.0	6.9 ± 0.2	15.2 ± 0.4	35.9 ± 0.9	52.1 ± 0.7	22.1 ± 0.4	42.4 ± 0.5
Sham	5.8 ± 0.7	7.0 ± 0.1	15.0 ± 0.3	35.6 ± 0.6	51.2 ± 0.5	21.6 ± 0.3	42.2 ± 0.3
OVX	6.8 ± 0.7	6.9 ± 0.1	15.0 ± 0.3	35.6 ± 0.6	51.5 ± 0.5	21.8 ± 0.3	42.3 ± 0.3
Low Copper	5.4 ± 0.7	6.9 ± 0.1	14.9 ± 0.3	35.0 ± 0.6	50.9 ± 0.5	21.6 ± 0.3	42.5 ± 0.4
Control	7.2 ± 0.8	7.0 ± 0.1	15.2 ± 0.3	36.2 ± 0.6	51.8 ± 0.5	21.7 ± 0.3	42.0 ± 0.4
Source of Variation				*p*-Values			
OVX	0.33	0.74	0.99	0.96	0.61	0.60	0.87
DIET	0.11	0.54	0.37	0.19	0.25	0.79	0.32
OVX x DIET	0.58	0.42	0.93	0.50	0.67	0.15	0.13

[1] Values represent least squares means ± SEM (OVX, n = 16; Cu, n = 16).

[2] Variables reported include white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC).

APPENDIX B

SUPPLEMENTAL MATERIALS FOR CHAPTER IV

Hematology

At the time of necropsy a sample of whole blood was removed and analyzed for hematology (ABX Vega, Montpellier, Cedex 04). Results of these analyses are presented in Appendix B—Table 1

Carbohydrate Analyses

Blood samples were placed on ice for approximately 30 minutes then centrifuged at 1500 x g for 22 minutes. Aliquots of serum were taken for the clinical analyzer (Cobas Fara II) assay of glucose and fructosamine. Glucose and fructosamine were analyzed using Roche reagents (Nutley, NJ). Results are presented in Appendix B— Table 2.

Lipid Analyses

At necropsy an aliquot of serum was stored for cholesterol and triglyceride analyses. Both lipid assays were performed using the clinical analyzer and Roche reagents (Nutley, NJ). Serum cholesterol and serum triglyceride results are presented in Appendix B—Table 2.

Liver samples were cleaned, weighed and stored at -20° necropsy. Liver lipids were analyzed based on the method of Carr et al. (Carr, Andresen, and Rudel 1993). Lipid extraction was performed on samples of liver weighing approximately 0.2000 g. Total liver lipids, liver total cholesterol, liver triglycerides and liver phospholipids were determined via enzymatic assay. Results of the liver lipid analyses are presented in Apprendix B—Table 3.

Appendix B—Table 1. Hematology data from ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and the copper depleted ($^{-}$Cu) diets.

	WBC (10^3/mm^3)	RBC (10^6/mm^3)	HGB (g/dl)	HCT (%)	MCV (μm^3)	MCH (pg)	MCHC (g/dl)
AMB x $^{-}$Cu	5.3 ± 1.1	6.7 ± 0.2	11.2 ± 0.3	32.5 ± 0.8	48.1 ± 2.1	16.7 ± 0.8	34.6 ± 0.7
AMB x Control	3.4 ± 1.1	7.0 ± 0.2	12.3 ± 0.3	36.3 ± 0.8	52.2 ± 2.1	16.6 ± 0.8	34.0 ± 0.7
SUSP x $^{-}$Cu	3.7 ± 1.1	7.1 ± 0.2	12.2 ± 0.3	34.4 ± 08	49.6 ± 2.1	16.7 ± 0.8	35.5 ± 0.7
SUSP x Control	4.1 ± 1.2	7.0 ± 0.2	12.1 ± 0.3	35.8 ± 0.8	50.1 ± 2.2	17.2 ± 0.9	33.9 ± 0.7
AMB	4.3 ± 0.7	6.9 ± 0.2	11.7 ± 0.2	34.4 ± 0.6	20.2 ± 1.5	17.2 ± 0.6	34.3 ± 0.5
SUSP	3.9 ± 0.8	7.1 ± 0.2	12.2 ± 0.2	35.1 ± 0.6	50.2 ± 1.5	17.4 ± 0.6	34.7 ± 0.5
$^{-}$Cu	4.9 ± 0.7	6.9 ± 0.2	11.7 ± 0.2	33.4 ± 0.6	48.8 ± 1.5	17.2 ± 0.6	35.0 ± 0.5
Control	3.7 ± 0.8	7.0 ± 0.2	12.2 ± 0.2	36.1 ± 0.6	51.5 ± 1.5	17.4 ± 0.6	33.9 ± 0.5
Source of Variation				p-Values			
SUSP	0.68	0.36	0.23	0.37	0.99	0.76	0.57
DIET	0.47	0.71	0.16	<0.01	0.22	0.78	0.11
SUSP x DIET	0.28	0.51	0.10	0.15	0.49	0.40	0.51

[1]Values represent least squares means ± SEM (SUSP, n = 17; $^{-}$Cu, n = 17).

2Variables reported include white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC).

Appendix B—Table 2. Serum glucose, fructosamine, cholesterol and triglycerides of ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and the copper depleted ($^{-}$Cu) diets.

	Glucose (mg/dl)	Fructosamine (μmol/l)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
AMB x $^{-}$Cu	343.4 ± 27.4	131.9 ± 3.7	72.6 ± 5.4	79.2 ± 7.6
AMB x Control	285.0 ± 27.4	137.0 ± 3.7	71.8 ± 5.4	77.8 ± 7.6
SUSP x $^{-}$Cu	247.6 ± 29.2	133.4 ± 3.7	93.2 ± 5.4	49.3 ± 7.6
SUSP x Control	202.2 ± 29.2	133.2 ± 4.0	86.1 ± 5.7	44.5 ± 8.1
AMB	314.2 ± 19.4	134.4 ± 2.6	72.2 ± 3.8	78.5 ± 5.4
SUSP	224.9 ± 20.6	133.3 ± 2.7	89.7 ± 3.9	46.9 ± 5.5
$^{-}$Cu	295.5 ± 20.0	132.7 ± 2.6	82.9 ± 3.8	64.3 ± 5..4
Control	243.6 ± 20.0	135.1 ± 2.7	78.9 ± 3.9	61.1 ± 5.5
Source of Variation		*p*-Values		
SUSP	<0.01	0.76	<0.01	<0.001
DIET	0.08	0.53	0.47	0.69
SUSP x DIET	0.82	0.48	0.57	0.83

[1]Values represent least squares means ± SEM (SUSP, n = 17; $^{-}$Cu, n = 17).

Appendix B—Table 3. Liver lipids from ambulatory (AMB) and tail-suspended (SUSP) rats fed the control and the copper depleted (¯Cu) diets.

	Total Liver Lipids (mg/g liver)	Total Liver Cholesterol (mg/g liver)	Liver Triglycerides (mg/g liver)	Liver Phospholipids (mg/g liver)
AMB x ¯Cu	77.4 ± 7.2	4.0 ± 0.5	11.3 ± 5.0	25.8 ± 1.4
AMB x Control	77.8 ± 7.2	3.2 ± 0.5	13.4 ± 5.0	23.7 ± 1.4
SUSP x ¯Cu Diet	88.5 ± 7.2	4.8 ± 0.5	17.3 ± 5.0	24.1 ± 1.4
SUSP x Control	90.0 ± 7.7	4.9 ± 0.5	19.4 ± 5.3	22.8 ± 1.5
AMB	77.6 ± 5.1	3.6 ± 0.3	12.4 ± 3.5	24.7 ± 1.0
SUSP	83.9 ± 5.3	4.8 ± 0.3	18.3 ± 3.6	23.5 ± 1.1
¯Cu	83.0 ± 5.1	4.4 ± 0.3	14.3 ± 3.5	24.98 ± 1.0
Control Diet	83.9 ± 5.3	4.0 ± 0.3	16.4 ± 3.6	23.2 ± 1.1
Source of Variation		*p*-Values		
SUSP	0.12	<0.05	0.25	0.39
DIET	0.90	0.41	0.68	0.24
SUSP x DIET	0.94	0.38	0.99	0.79

[1]Values represent least squares means ± SEM (SUSP, n = 17; ¯Cu, n = 17).

VITA

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