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The Relationship of Nutritional Copper to the Development of Postmenopausal Osteoporosis in Rats

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ABSTRACT

Factors that influence tissue copper concentration include age, diet, hormones, and pregnancy. In this study we altered diet independently, hormone (estrogen) independently, and various combinations of diet and hormone in animals of the same age to study the effects of ovariectomy complicated with dietary copper deficiency; a deficiency that has been demonstrated to cause bone defects. Sprague-Dawley rats were placed on various combinations of copper deficient or enriched diets before and/or after ovariectomy to determine if copper deficiency aggravated osteoporosis and if return to a copper-adequate diet alleviated it. In this study, ovariectomy did induce an osteopenia that was characterized by decreased trabecular bone. This osteopenia was slightly more severe with copper deficiency, but was not necessarily alleviated by the return of normal copper levels to the diet.

Index Entries: Osteopenia; copper; bone development; osteoporosis.

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INTRODUCTION

Bone is a tissue that is critically affected by nutritional factors such as the vitamins, A, C, D, K, calcium, phosphorus, magnesium, and several trace elements, such as fluoride, silicon, and zinc. The possibility exists that diet is important in its pathogenesis. Dietary copper is important for bone matrix formation, and it is marginally deficient in many Western diets *(1–4)*. The safe and adequate range of dietary copper intake for adults is 1.5–3 mg/d, but the above studies document median daily copper intakes of less than 2 mg copper/d and many as low as 0.78 and 0.76 mg/d. Potentially compounding the dietary problem are factors that decrease copper absorption from the intestine, such as high levels of dietary protein and the formation of insoluble copper complexes with phytate, dietary fiber *(4)*, and phosphate *(1)*. In addition, certain health behaviors, such as overzealous ascorbic acid supplementation *(4,5)* and even excessive calcium supplementation *(6,7)* have a tendency to decrease intestinal copper absorption.

Alcohol consumption or any simple sugar that is capable of producing an aberration of carbohydrate metabolism also aggravates a copper deficiency *(8)*.

The known effects of copper deficiency differ between species, resulting in various conditions. It is known to produce osteoporotic bone lesions and to reduce bone strength in many animals and in humans *(9–14)*. In humans, confirmed copper disorders include Menkes' steely hair disease and Wilson's disease. Some of the bone defects observed in these diseases are similar to those seen in osteoporosis *(10)*. There are also reports of infants developing osteoporosis-like symptoms because of a nutritional copper deficiency *(15,16)*. Premature neonates often will have inadequate copper stores. Infants who were placed on a relatively long-term milk diet became copper deficient *(16,17)*, because milk is such a poor source of copper, and calcium chelates the copper *(7)*. Prior to the discovery for the need to supplement copper, infants who were on total parenteral nutrition for extended periods of time developed osteoporotic lesions *(16)*. Low serum concentrations of copper are associated with slow bone fracture healing *(16)*. A study of 46 elderly patients with femoral neck fractures demonstrated serum copper levels to be significantly lower than those of a group of age-matched controls *(18)*.

The objective of the present study was to determine if a mildly deficient copper diet has a modulating effect of either increasing or alleviating osteopenia in ovariectomized rats.

MATERIALS AND METHODS

Animals

Female (8-wk-old, 140 ± 1.8 g), Sprague-Dawley rats were randomly divided into the five groups listed in Table 1. They were housed two per

Table 1
Experimental Paradigm

Group (*n*)	Diet, 8–11 wk	Treatment, 11 wk	Diet, 11–22 wk
NØN (9)	Normal	No treatment	Normal
NON (8)	Normal	OVX	Normal
NOL (8)	Normal	OVX	Low copper
LON (8)	Low copper	OVX	Normal
LOL (9)	Low copper	OVX	Low copper

cage with raised stainless steel grid floors to prevent coprophagy and ingestion of adventitious trace elements. Environmental conditions were maintained at 20–24°C with a 12 h/12 h light–dark cycle. Food was provided to each animal in an amount equal to that previously consumed by the rats fed the control diet. This was initially 12 g/(rat/d) but was increased to 15 g/ (rat/d) at 17 wk of age. The rats were given free access to deionized water (dH_2O) for the entire experimental period. The animals were fed every other day whereas dH_2O, in dH_2O washed and rinsed bottle, were changed once a week. The protocol for this project was reviewed and approved by the Texas A&M University Laboratory Animal Care committee.

Diets

Animals were fed a Purina test diet* (Purina Mills, St. Louis, MO) The control basal diet contained 22.7 mg/kg of copper, which exceeds the 6.0 mg/kg minimum recommended by the American Institute of Nutrition *(19)*. The copper concentration of the diet fed to low copper groups was 1.79 mg/kg. These copper concentrations were confirmed by atomic absorption analysis by CenTech Laboratories (The Analytical Laboratories Group of Ralston Purina Co., Checkerboard Square, St. Louis, MO). This copper concentration was intentionally not sufficient to induce copper deficiency, but does represent a marginal copper intake.

Experimental Procedure

Animals were anesthetized with an ip injection of ketamine (60 mg/kg) and xylazine (4 mg/kg) just prior to being ovariectomized (OVX) at 11 wk of age. With the back shaved, a dorsal midline skin incision and

*Source of minerals in mineral mix: $CaCO_3$, $CaHPO_4$, $MgSO_4$ anhydrous, ferric ammonium citrate, $MnSO_4$, $CuSO_4$ anhydrous, K_2HPO_4, NaCl, NaH_2PO_4, $(NH_4)_6Mo_7O_{24}$, KIO_3, $Na_2SeO_3{\cdot}5H_2O$, NaF, $ZnCl_2$, $CrCl_2$, $CoCl_2$, and solka floc as the carrier. Composition of mineral mix (mg/kg diet): Ca, 0.6 (%); P, 0.4(%); K, 0.4(%), Mg, 0.065(%); Na, 0.2(%); F, 5; Fe, 60; Zn, 20; Mn, 65; Cu, 15; Co, 3.2; I, 0.6; Cr, 3; Mo, 0.8; and Se, 0.2.

two lateral flank incisions were made to remove the ovaries. Each flank incision was closed with one suture, and the skin was stapled and washed with alcohol. At the time of ovariectomy groups 3, 4, and 5 (Table 1) reversed diets. At 22 wk of age, each animal was anesthetized by metofane inhalation, weighed, and decapitated. Blood was collected in heparinized test tubes, centrifuged, and the plasma was collected and stored at –80°C for later analysis. The right femur and tibiae were removed and dissected free of soft tissue. The femur was placed in a small plastic vial and stored at –80°C for later analysis, whereas the left tibia was kept in a vial of Carson's fixation *(20)*, remaining at room temperature.

Plasma Analysis

Plasma calcium, diluted 50-fold in a 20 mmol/L lanthanum chloride solution prepared with dH_2O, and plasma copper were determined by atomic absorption spectrophotometry in a nitrous oxide-acetylene flame at the wavelengths 422.7 and 324.7 nm, respectively. Inorganic phosphorus was measured by colorimetry with a Sigma (St. Louis, MO) diagnostics kit using the method of Fiske and SubbaRow *(21)*. Plasma was assayed for estradiol levels with a radioimmunoassay kit (Coat-A-Count, DPC, Los Angeles, CA) to confirm ovariectomy. Readings were performed on a Beckman [Fullerton, CA] Gamma 5500 counter.

Bone Analysis

The right femurs were allowed to reach room temperature, dissected free of soft tissue, and weighed. Bones were handled only with forceps and gloves. Femur length was measured with a metric rule from the proximal end of the head to the distal end of the distal epiphysis. Diameters of the femur midshaft and the proximal and distal metaphyseal–diaphyseal junctions were measured with a caliper. Total femur volume was determined by displacement of dH_2O in a graduated cylinder. Dry weight, after drying overnight at 60°C, and fat-free dry wt, was also determined following ethanol and ether extraction and drying overnight at 60°C. The bone was then sawed in half at the midshaft for measurement of the inner diameter with a calibrated eyepiece. Cortical thickness index (CTI) was calculated as a ratio of the difference between the outer diameter and inner diameter to the outer diameter. Each femur was transferred to a small, tared acid-washed beaker. Ash weight was determined after heating the femurs to 600°C in a muffle furnace for 20 h. Ash was completely dissolved in approx 2 mL of nitric acid and was slowly dried at 100°C. The remaining residue of each sample was brought up to a total volume of 2 mL with dH_2O. Aliquots were diluted with dH_2O and 20 mmol/L chloride for sampling of bone calcium by atomic absorption spectrophotometry at the wavelength of 422.7 nm. Additional aliquots were diluted with dH_2O for bone copper determination by atomic absorption spectrophotometry at 324.7 nm and for inor-

ganic phosphorus content by the colorimetric method of Fiske and SubbaRow *(21)* (Sigma).

The tibia, fixed in Carson's fixative, was embedded in paraffin. Longitudinal sections of the tibia were cut at 7 μm, dried on glass slides, and prepared for light microscopy by staining with hematoxylin and eosin *(20)*. These stained longitudinal sections of the tibia, specifically the metaphyseal area, were photographed through a light microscope at low magnification (objective = 2.5×) and printed to magnification of 50×. Trabecular bone, trabecular bone area, and cortical bone area were determined by morphometric point counting. Each photograph was counted three times. The first count was performed after a random dropping of a grid on the photograph while the other counts were a 120° rotation from the previous grid placement.

Bone Density

Bone density, as specific gravity, was calculated as a ratio of femur dry weight to actual bone volume. Ash weight per unit volume was a ratio of the total femur ash weight to actual bone volume.

Statistical Analysis

Results are reported as mean ± SE and are considered statistically significant at the level $p < 0.05$. NON was compared to NøN by Student's *t*-test in order to determine the effect of ovariectomy. The four ovariectomized groups were then analyzed by two-way ANOVAs and Tukey's test to determine the effect of diet.

RESULTS

Rats weighing 140.75 ± 1.81 g were randomly divided into the six experimental groups, with no initial significant difference in body weight between the control and experimental groups. Estradiol levels of the ovariectomized groups were confirmed to be significantly lower (6.64 ± 1.40 pg/mL) than those of the nonovariectomized groups. (18.92 ± 8.41 pg/mL). Ovariectomy also caused a significant percent (108.09 ± 26) increase in body weight, but there were no differences in body weight among the ovariectomized animals owing to diet.

Plasma Mineral Concentrations

Plasma calcium and phosphorus concentrations were not altered solely by the ovariectomized state, but in general, ovariectomized animals tended to have higher calcium levels than nonovariectomized, and low copper diets tended to further increase these levels, but not to a statistically significant amount.

Compared to controls, plasma copper was significantly lower in groups that had some combination of a low copper diet and ovariectomy. Animals that had a more recent copper deficiency reflected a significantly lower plasma copper (0.40 ± 0.2 μg/100 mL) than the animals that had a copper deficiency that occurred prior to ovariectomy (1.12 ± 0.2 mg/100 mL). Separately, ovariectomy or a copper deficient diet each resulted in a significantly lower plasma copper level than the control.

Bone Morphology

Femur volume of the copper deficient rats was slightly greater than the controls (Table 2), but ovariectomy or diet did not significantly increase or decrease bone volume. Other femur morphometric characteristics including length (36.32 ± 0.27 mm), diameter (midshaft, 3.74 ± 0.06 mm; proximal, 6.29 ± 0.19 mm and distal metaphyseal–diaphyseal junction, 6.52 ± 0.06 mm), and cortical thickness index (0.356 ± 0.01) were not significantly affected by either a low-copper diet or ovariectomy.

Bone Density Measurements

Neither ovariectomy nor any of the variations of copper deficient diet, resulted in a significantly lower bone density than the control when measured as either specific gravity or ash weight per unit volume (Table 2).

Although neither ovariectomy or a copper-deficient diet was shown to alter the proportion of cortical or trabecular area in the tibial metaphysis, ovariectomy did significantly decrease the percent of trabecular bone present, as well as the percent of trabecular bone of the trabecular area (Table 3). Copper deficiency was not found to significantly alter trabecular bone or percent trabecular bone of trabecular area in these animals.

Bone Composition

No difference was observed between treatments in the wet weight (0.759 g ± 0.014) of the femurs. However, the percentage of water in the bone was significantly higher in ovariectomized animals fed a normal diet (24.78% ± 0.39) compared to the control (23.94% ± 0.28). The fat component of the femur was significantly higher in ovariectomized animals (Table 4). Copper deficiency increased the bone fat content when given early or late in the experiment, but the later diet deficiencies were of greater magnitude. Differences in percent mineral followed a similar trend as in bone density, expressed as specific gravity or ash weight per unit volume. When bone mineral composition was analyzed as percent dry weight, a significantly lower percent mineral following ovariectomy was also found (Table 4).

Bone calcium, analyzed as mmole/g bone, was unaffected by ovariectomy or diet. Bone phosphorus (Table 5) was consistently higher in ovariectomized animals fed a low copper diet.

Table 2
Bone Mass

Group (*n*)	Bone volume, mL	Bone density	
		Specific gravity, dry wt/bone vol	Ash wt/unit vol
NØN (9)	0.454 ± 0.013	1.251 ± 0.030	0.8100 ± 0.0206
NON (8)	0.483 ± 0.013	1.178 ± 0.026	0.7423 ± 0.0185[a]
NOL (8)	0.517 ± 0.019	1.110 ± 0.036	0.6939 ± 0.0303[b]
LON (8)	0.519 ± 0.019	1.122 ± 0.021	0.6379 ± 0.0855[b]
LOL (9)	0.498 ± 0.013	1.130 ± 0.034	0.7192 ± 0.0253[a]

Within a column, means not sharing a common superscript are significantly different ($p < 0.05$).

Table 3
Bone Morphometrics

Group (*n*)	Cortical area, %	Trabecular area, %	Trabecular bone, %	Trabecular bone of trabecular area, %
NØN (9)	20.19 ± 1.20	80.49 ± 1.45	35.79 ± 2.89*	44.62 ± 3.32*
NON (8)	21.04 ± 1.04	78.98 ± 1.04	25.06 ± 1.87	31.46 ± 2.28
NOL (8)	20.91 ± 1.38	80.20 ± 2.13	19.79 ± 1.73	25.03 ± 2.11
LON (8)	21.72 ± 1.06	78.31 ± 1.05	23.19 ± 2.24	29.88 ± 3.19
LOL (9)	20.59 ± 1.48	79.39 ± 1.49	25.99 ± 2.07	31.06 ± 1.91

*Indicates a significant difference ($p < 0.05$) for NØN vs NON.

Table 4
Bone Composition as Percent Dry Weight

Group (*n*)	Dry wt, g	Fat-free wt, g	Fat, %	Mineral, %
NØN (9)	0.5705 ± 0.0083	0.5659 ± 0.0081	0.80 ± 0.10*	64.19 ± 0.23*
NON (8)	0.5725 ± 0.0094	0.5666 ± 0.0093	1.03 ± 0.06[a]	61.91 ± 0.57
NOL (8)	0.5794 ± 0.0154	0.5702 ± 0.0151	1.57 ± 0.07[b]	61.42 ± 0.81
LON (8)	0.5868 ± 0.0115	0.5795 ± 0.0115	1.23 ± 0.15[a]	63.17 ± 0.33
LOL (9)	0.5761 ± 0.0096	0.5671 ± 0.0096	1.56 ± 0.08[b]	61.71 ± 0.43

*Indicates a significant difference ($p < 0.05$) for NØN vs NON.
Within a column, means not sharing a common superscript are significantly different ($p < 0.05$).

Bone copper per gram bone was significantly lower when the copper deficiency was long term or following ovariectomy as opposed to copper deficiency, only before ovariectomy. Copper content per gram bone was also lower in the ovariectomized animals fed a normal diet than in the controls.

Table 5
Bone Mineral Composition as MMol/g bone

Group (*n*)	Ca	P	CuX10^{-5}
NØN (9)	4.78 ± 0.13	2.21 ± 0.36	4.77 ± 0.41*
NON (8)	5.12 ± 0.22	1.62 ± 0.29^{a}	3.91 ± 0.16^{a}
NOL (8)	4.46 ± 0.57	3.25 ± 0.13^{b}	4.83 ± 0.10^{b}
LON (8)	4.61 ± 0.19	2.97 ± 0.24^{ab}	6.08 ± 0.50^{c}
LOL (9)	4.81 ± 0.09	3.31 ± 0.22^{b}	4.57 ± 0.33^{ab}

*Indicates a significant difference ($p < 0.05$) for NØN vs NON.
Within a column, means not sharing a common superscript are significantly different ($p < 0.05$).

DISCUSSION

The present study supports data reviewed by Kalu *(22)* that the ovariectomized rat is a suitable model for studying problems that are relevant to postmenopausal bone loss. Kalu proposed that the most appropriate "aged rat model" is a 12-mo-old rat in which all the bone parameters (length, weight, density, and calcium content of the femur) have reached plateau levels. However, owing to the cost and availability of aged rats, he proposed that an approx 3-mo-old "mature rat model" is adequate to display the effects of ovariectomy on her skeleton within a month or less. Kalu found that at 3 mo of age, the rate of increase of femur length, weight, density, and Ca content was more gradual than the rate measured from rats 1–3 mo of age. The rats in the present study were ovariectomized at 11 wk of age and were maintained on either a normal or low Cu diet for an additional 11 wk, thus approximating the criteria set forth by Kalu in developing an appropriate mature rat model for a postmenopausal bone loss study.

Ovariectomy of the rats in this project resulted in a major decrease in serum estradiol concentrations and in an increase in body weight. These rats also exhibited a significant decrease in the percentage of trabecular bone and in the percentage of trabecular bone of trabecular area. There was no significant change in cortical thickness index or cortical area resulting from ovariectomy. This is especially important since loss of trabecular bone is observed in the pathophysiology of osteoporosis *(23)*. Furthermore, the percent mineral in the bones of ovariectomized animals was significantly reduced when measured relative to bone dry weight. Specific gravity and ash weight per unit volume took into account bone mass relative to bone volume, which remained unchanged, as did the other morphological characteristics, indicating that the osteopenia observed was similar to osteoporosis of humans since bone was lost without a major change in bone composition.

The effects of ovariectomy and of a low Cu diet on altering serum Cu appeared to be additive. They both resulted in lowering serum Cu,

with the lowest serum Cu values being in those animals receiving a Cu deficient diet late in the project (NOL and LOL), followed by those that had either a low Cu diet early in the project or ovariectomy alone. The highest serum Cu concentration was in the control animals (NϕN). Ovariectomy alone reduced serum Cu, which supports the suggestion that estrogens stimulate ceruloplasmin synthesis. It has been reported that increased estrogen levels in pregnancy and/or in users of oral contraceptives result in elevated serum Cu and ceruloplasmin *(6,24,25)*, and that the postmenopausal estrogen deficient-state causes a significantly lower serum Cu concentration *(25,26)*.

Cu is apparently necessary for normal bone development since Cu deficient lambs and calves develop osteoporosis even though they have normal serum Ca and P *(6)*. Our studies indicate that low copper diet causes a reduction in bone mass, a slight change in bone volume and alteration in bone composition. Ovariectomized animals fed a normal diet had slightly decreased bone mass, compared to nonovariectomized animals and further significant reduction was observed in bone mass when a low Cu diet was given either before or after ovariectomy but not both (LOL). These animals possibly adapted to the low Cu diet, so the low Cu diet did not adversely affect bone density beyond the effect of ovariectomy.

Macroscopically, neither Cu deficiency nor ovariectomy affected femur length, diameter, or cortical thickness, although estrogen and Cu deficiency have been shown in our experiments and in others *(14,27)* to alter trabecular bone. Suttle et al. *(14)* found that mature trabeculae were often absent from the central metaphyseal region but with a Cu deficiency, rather than an estrogen deficiency. Other investigators have also found fewer trabeculae in the primary and secondary spongiosa *(27)* with a Cu deficiency. In our studies, a low Cu diet did not appear to affect the trabecular bone, but it is quite apparent that estrogen deficiency caused lesions similar to that seen by others with Cu deficiency. Perhaps if the Cu deficiency in our diets had been severe, further differences might have been observed in trabecular bone loss.

Ovariectomy increased fat content of bone and decreased the mineral content. Cu deficiency following ovariectomy also significantly increased the fat content in bone. Other researchers have not cited an observation regarding altered bone fat content and the authors have no logical physiological or biochemical explanation to adequately explain why Cu deficiency or estrogen deficiency increased the fat content of bone. Although a specific mechanism cannot be offered for the explanation regarding Cu deficiency-induced bone mineral depletion, many researchers have observed the same phenomena *(28,29)*. Decreased matrix deposition and decreased mineralization is commonly observed in Cu-deficient animals *(12,14,30)*.

In summary, the female Sprague-Dawley rats appeared to be an adequate postmenopausal model when successfully ovariectomized. Fur-

thermore, Cu deficiency superimposed at different points of the estrogen deficient time periods have the potential to increase the changes observed in the bone. No definite trends can be attributed to a long term Cu-deficiency (LOL) vs a Cu-deficiency only before (LON) or after (NOL) ovariectomy; more research is indicated. Future studies should consider a longer term Cu-deficiency and various degrees of Cu-deficiency both before and/or after menopause to delineate the effects on the bone and in the development of postmenopausal osteoporosis.

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