The Effects of Copper Deficiency on the Pyridinium Crosslinks of Mature Collagen in the Rat Skeleton and Cardiovascular System (42973)

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Abstract. **The 3-hydroxypyridinium crosslinks of collagen were quantified in tissues of the skeleton and cardiovascular system of normal and copper-deficient rats. The copper-deficient rats used in this study displayed retarded growth, cardiac hypertrophy, anemia, and lowered liver copper concentrations. Quantification of the crosslinks by high performance liquid chromatography indicated that there were lower concentrations of collagen crosslinks in the hearts of copper-deficient animals, a finding that was manifest in both right and left ventricles. This was in contrast to the collagen of the aorta where no alteration in crosslink concentration was observed. The femoral diaphysis of copper-deficient rats also had lower amounts of collagen crosslinks than copper-supplemented animals, whereas crosslinking in the tibial diaphysis and articular cartilage was relatively unaffected by copper deficiency. These results are discussed with reference to the cardiac and skeletal abnormalities that occur in copper-deficient animals.**

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Copper is a cofactor of the enzyme lysyl oxidase which is involved in the initiation and regulation of collagen and elastin crosslinking (1). Crosslinks provide these connective tissue proteins with the tensile strength necessary for the maintenance of proper function. A decrease in the number of collagen crosslinks is thought to be involved in the skeletal abnormalities, such as bone fragility, observed in copper deficiency (2–4). The lesions induced by copper deficiency in the cardiovascular system, namely, cardiac hypertrophy (CH), aortic and ventricular aneurysms, and rupture (5–7), are also thought to be influenced by a decrease in collagen and elastin crosslinking (8–10). The evidence for a decrease in collagen crosslinking is based either on collagen solubility (3, 4, 9) or on direct measurement of the borohydride-reducible (3) or mature crosslinks (11).

The nonreducible crosslinks of mature collagen are formed from the reducible, intermediate bifunctional compounds which are essentially of two chemical types depending on whether lysine or hydroxylysine in the telopeptides acts as the substrate for lysyl oxidase (12). The trifunctional crosslink pyridinoline is a 3-hydroxypyridinium derivitive (13) that is a maturation product of the ketoamine bifunctional crosslink (14). It incorporates three hydroxylysine residues and is found in large amounts in cartilage (15, 16) and to a lesser extent in bone (17). A deoxy derivitive which incorporates a lysine rather than a hydroxylysine residue in the helical region of the neighboring collagen molecule has also been identified (18). Bone and dentin collagen appear to be the only appreciable sources of this analogue (19).

The aim of this study was to determine whether a link could be established between the concentrations of pyridinoline and deoxypyridinoline and abnormalities of the skeleton and cardiovascular system that are associated with copper deficiency.

Materials and Methods

The animals used in this study have been described previously (20). Briefly, the animals comprised 12 male and 12 female rats of the Hooded Lister strain that were obtained from dams who had been receiving a copper deficient diet from 12 days post partum. At weaning, six rats of each sex were fed a diet based on purified amino acids (20) having a basal copper content of <0.2 μg copper/g of diet (−copper group) or the same diet supplemented with copper sulfate to provide

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10 μg of copper/g of diet (+copper control group). Rats were housed in groups of six according to sex and diet, and food and water were available *ad libitum*. After 7 weeks on the diet, the rats were weighed, anesthetized with diethyl ether, and blood was collected in heparinized tubes by cardiac puncture. The liver, tibia, femur, thoracic aorta, and heart were dissected out and, after recording the heart weight, stored at −20°C.

Analysis of Collagen Crosslinks. *Cardiovascular tissue.* The heart was dissected into the right and left ventricles, with the latter including the free wall and interventricular septum. Care was taken not to include any valvular tissue in the samples. In some cases the apex of the left ventricle (lower 30%) was dissected from the rest of the left ventricle. The thoracic aorta from the aortic arch to the diaphragm was also analyzed. Weighed samples were hydrolyzed in 6 *M* HCl at 107°C for 24 hr, after which time the acid was removed by evaporation *in vacuo*. After dissolving in water, an aliquot was analyzed for pyridinoline and deoxypyridinoline by reversed phase high-performance liquid chromatography (21). An aliquot of the hydrolysate was also assayed for hydroxyproline (22) and the values obtained were used to calculate the molar proportions of pyridinium crosslinks in collagen, assuming 300 hydroxyproline residues/molecule. Before analysis by HPLC (21), the hydrolyzates were subjected to a preliminary fractionation using CF1 partition chromatography (21). With the use of this protocol, it has previously been shown that recoveries of the pyridinium crosslinks is approximately 95% (21).

Skeletal tissue. The diaphyses of the tibias and femurs (midshaft point ± 1 cm) were decalcified in several changes of 0.5 *M* EDTA (pH 7.2) at 4°C for 2 weeks and washed with distilled water. Articular cartilage of the femoral head and the decalcified bone samples were hydrolyzed in 6 *M* HCl at 107°C for 24 hr. Samples were washed free of acid by rotary evaporation in several changes of water and, as for the cardiovascular tissue, aliquots were taken for collagen crosslink and hydroxyproline determination. The skeletal tissue was not subjected to preliminary fractionation before analysis by high-performance liquid chromatography.

Other Analyses. Copper contents of duplicate samples of diet and livers were measured by atomic absorption spectrophotometry (23). Hematocrits were determined on blood that had been centrifuged in microhematocrit tubes on a Beckman microfuge. Hemoglobins were determined by the cyanomethemoglobin technique. The results were analyzed using Student's *t* test.

Results

All of the rats fed the −copper diet had characteristic signs of copper deficiency, these being impaired growth, decreased liver copper concentrations, CH, and anemia (Table I). Two of the male copper-deficient rats died 2 days before the end of the experiment but these animals displayed no evidence of cardiac rupture.

As a measure of the collagen content of each tissue, the hydroxyproline concentration was calculated on a wet weight basis (Table II). Due to the cardiac hypertrophy seen in the −copper animals, the total hydroxyproline contents of the left and right ventricles were not significantly altered by copper deficiency (Table II). The hydroxyproline concentration was higher in the right than in the left ventricle, whereas there was no significant difference between the control and copper-deficient groups for the left ventricle of the female rats, but the hydroxyproline in the right ventricle was significantly lower ($P < 0.01$) by approximately 34% in the male and female −copper animals. In comparison to their respective controls, the hydroxyproline concentration of the apex and the "remainder" of the left ventricle of the male -copper rats was 24% ($P < 0.01$) and 16.5% ($P < 0.05$) lower, respectively. In contrast, the hydroxyproline concentrations of the aorta and the tibia and femur diaphyseal bone were unchanged by copper deficiency (Table III).

In the heart and the aorta, pyridinoline predominates with very low amounts of deoxypyridinoline detected (Table IV). For the male -copper rats, there were

Table I. Body and Heart Weights, Hematologic Parameters, and Liver Copper Concentrations in Control and Copper-deficient Rats[a]

Copper status	Sex	Body weight (g)	Heart weight/ 100 g body wt	Hemoglobin (g/dl)	Hematocrit (%)	Liver Copper (μg/g wet wt)
+Copper	M	257.4 ± 23.0 (***)	0.35 ± 0.02 (***)	12.52 ± 0.83[b] (***)	44.29 ± 1.15[b] (***)	4.95 ± 1.84 (***)
−Copper	M	172.1 ± 19.6	0.71 ± 0.07	6.94 ± 1.83	24.38 ± 3.06	0.73 ± 0.14
+Copper	F	181.9 ± 8.48 (***)	0.39 ± 0.01 (***)	13.48 ± 0.83 (**)	39.58 ± 1.13 (**)	5.38 ± 0.44 (***)
−Copper	F	143.6 ± 13.5	0.59 ± 0.05	10.16 ± 2.35	31.00 ± 5.42	0.82 ± 0.24

[a] Expressed as mean ± SD of six animals/group unless otherwise indicated.
[b] Mean ± SD of four animals/group. Significance of difference to corresponding group of control rats is given in parentheses: ** $P < 0.01$; *** $P < 0.001$.

Table II. Total Hydroxyproline and Hydroxyproline Concentration in the Heart of Control and Copper-Deficient Rats[a]

	Sex	Copper status	Total hydroxyproline[b] (mg)	Hydroxyproline concentration (mg hydroxyproline/g wet wt)
Left ventricle (apex)	M	+Copper	NA	0.341 ± 0.025 (**)
	M	−Copper	NA	0.259 ± 0.037
Left ventricle (apex removed)	M	+Copper	NA	0.453 ± 0.035 (*)
	M	−Copper	NA	0.378 ± 0.060
Left ventricle	F	+Copper	0.196 ± 0.032 (NS)	0.504 ± 0.080 (NS)
	F	−Copper	0.172 ± 0.040	0.393 ± 0.110
Right ventricle	M	+Copper	0.073 ± 0.022 (NS)	0.941 ± 0.220 (**)
	M	−Copper	0.059 ± 0.010	0.607 ± 0.083
Right ventricle	F	+Copper	0.061 ± 0.016 (NS)	0.791 ± 0.112 (**)
	F	−Copper	0.048 ± 0.014	0.528 ± 0.103

[a] Expressed as mean ± SD of six animals/group.
[b] NA, not applicable due to inconsistencies in amount of starting material; however, with the two fractions combined (apex + remainder), the total hydroxyproline contents of the left ventricles of the control animals (0.224 ± 0.016) and −Copper animals (0.203 ± 0.028) was not significantly different. Significance of difference between control and copper-deficient rats is given in parentheses: NS, not significant; * $P < 0.05$; ** $P < 0.01$.

Table III. Hydroxyproline Concentration in the Skeleton and Aorta of Control and Copper-Deficient Rats[a]

	Sex	Copper status	Hydroxyproline concentration[b] (mg hydroxyproline/g wet wt)
Femur	M	+Copper	14.93 ± 1.93 (NS)
	M	−Copper	15.01 ± 2.79
Femur	F	+Copper	16.09 ± 1.40 (NS)
	F	−Copper	14.32 ± 2.41
Tibia	M	+Copper	16.07 ± 1.52 (NS)
	M	−Copper	15.74 ± 1.37
Tibia	F	+Copper	18.30 ± 0.71 (NS)
	F	−Copper	17.33 ± 4.84
Thoracic aorta	M	+Copper	12.71 ± 1.56 (NS)
	M	−Copper	14.49 ± 1.24
Thoracic aorta	F	+Copper	11.09 ± 2.00 (NS)
	F	−Copper	10.42 ± 3.04

[a] Expressed as mean ± SD of six animals/group.
[b] Significance of difference between control and copper-deficient rats is given in parentheses: NS, not significant.

significantly lower levels of pyridinoline ($P < 0.001$) and deoxypyridinoline ($P < 0.01$) at both sample sites of the left ventricles compared with the copper-supplemented controls. In contrast, the crosslinks of the aortas of the same copper-deficient rats were not significantly different from those of the copper-supplemented animals. Similarly, copper deficiency had no effect on the crosslink concentration of the female aorta. In male and female copper-deficient rats, significantly lower concentrations of both crosslinks were observed in the left and right ventricles. Both ventricles of the control animals had similar concentrations of both crosslinks (Table IV).

Bone had a higher concentration of deoxypyridinoline than pyridinoline; the deoxy analogue was, however, not detected in cartilage (Table V). The response of the collagen crosslinks to copper deficiency was not consistent in the two bones studied; the crosslinks of the femur were generally more severely affected than those of the tibia. In the articular cartilage, pyridinoline levels were the same in both sexes and were not affected by copper status.

Discussion

The decrease in collagen concentration of the copper-deficient heart shown in this study is not typical of CH; depending on the stimulus to the hypertrophy, the collagen concentration can increase, decrease, or remain unchanged (24). This lack of collagen proliferation suggests that the CH seen in copper deficiency is probably not associated with work overload or ischemic conditions (25). Total amounts of collagen in all tissues studied was found to be relatively constant in copper deficiency, which is consistent with the findings for bone (4), heart (9, 24), and lung (26). The higher collagen concentration seen in the right compared with the left ventricle is in agreement with previous observations (24, 27, 28).

The impaired collagen crosslinking in the heart shown in this study is consistent with the results of

Table IV. Pyridinium Crosslinks in the Heart and Aorta of Control and Copper-Deficient Rats

	Sex	Copper status	Residues/collagen molecule[a]	
			Pyridinoline	Deoxypyridinoline
Left ventricle (apex removed)	M	+Copper	0.214 ± 0.025 (***)	0.014 ± 0.003 (**)
	M	−Copper	0.116 ± 0.021	0.008 ± 0.002
Left ventricle (apex)	M	+Copper	0.113 ± 0.024 (***)	0.010 ± 0.002 (**)
	M	−Copper	0.048 ± 0.008	0.006 ± 0.002
Left ventricle	F	+Copper	0.176 ± 0.018 (***)	0.012 ± 0.002 (***)
	F	−Copper	0.102 ± 0.031	0.005 ± 0.002
Right ventricle	M	+Copper	0.186 ± 0.043 (**)	0.016 ± 0.006 (**)
	M	−Copper	0.121 ± 0.012	0.007 ± 0.002
Right ventricle	F	+Copper	0.170 ± 0.020 (***)	0.015 ± 0.002 (***)
	F	−Copper	0.095 ± 0.022	0.009 ± 0.002
Thoracic aorta	M	+Copper	0.070 ± 0.013 (NS)	0.008 ± 0.003 (NS)
	M	−Copper	0.068 ± 0.011	0.010 ± 0.004
Thoracic aorta	F	+Copper	0.087 ± 0.014 (NS)	0.006 ± 0.001 (NS)
	F	−Copper	0.071 ± 0.014	0.005 ± 0.002

[a] Expressed as mean ± SD of six animals/group. Significance of difference between control and copper-deficient rats is given in parentheses: NS, not significant; ** $P < 0.01$; *** $P < 0.001$.

Table V. Pyridinium Crosslinks in the Skeleton of Control and Copper-Deficient Rats

	Sex	Copper status	Residues/collagen molecule[a]	
			Pyridinoline	Deoxypyridinoline
Tibia diaphysis	M	+Copper	0.045 ± 0.004 (*)	0.063 ± 0.007 (NS)
	M	−Copper	0.038 ± 0.005	0.055 ± 0.008
Tibia diaphysis	F	+Copper	0.065 ± 0.008 (NS)	0.104 ± 0.010 (NS)
	F	−Copper	0.076 ± 0.009	0.108 ± 0.014
Femur diaphysis	M	+Copper	0.063 ± 0.016 (*)	0.068 ± 0.016 (**)
	M	−Copper	0.041 ± 0.007	0.042 ± 0.008
Femur diaphysis	F	+Copper	0.059 ± 0.006 (NS)	0.076 ± 0.002 (***)
	F	−Copper	0.054 ± 0.005	0.051 ± 0.007
Articular cartilage	M	+Copper	0.399 ± 0.018 (NS)	ND
	M	−Copper	0.423 ± 0.027	ND
Articular cartilage	F	+Copper	0.430 ± 0.040 (NS)	ND
	F	−Copper	0.407 ± 0.027	ND

[a] Expressed as mean ± SD of six animals/group.
[b] ND, not detected. Significance of difference between control and copper-deficient rats is given in parentheses: NS, not significant; * $P < 0.05$; ** $P < 0.01$.

Dawson *et al.* (9) who found an increase in pepsin-soluble collagen from hearts of copper-deficient rats. The inhibition in collagen crosslinking is likely to alter the strength of the collagen network, resulting in an inability to transmit and distribute the force of muscular contraction. This could explain some of the altered mechanical properties seen in the copper-deficient heart such as reduced systolic pressure development (29) and also possibly CH and aneurysm formation (30). However, the possibility that there is a decrease in the maturation of crosslink intermediates and not a decrease in collagen crosslinks per se cannot be excluded.

Our results indicate that there were no significant alterations in the amounts of collagen crosslinks in the aortas of copper-deficient rats, which may explain why

copper-deficient rats are not susceptible to aortic rupture (2). In species where aortic rupture due to copper deficiency has been reported, such as in the pig (31) and the chick (32–34), there is an increase in the solubility of aortic collagen, which is indicative of decreased crosslinking. It would therefore be of particular interest to determine the pyridinium crosslink concentrations in the aortas of copper-deficient pigs and chicks. The impairment of elastin crosslinking in copper deficiency, not investigated in this study, undoubtedly plays a role in the weakening of the major blood vessels, as degenerative changes have been reported in the aorta of many copper-deficient species including the rat (5, 10).

Collagen crosslink concentration was found to be lower in the femur of copper-deficient rats but relatively normal in the tibia and articular cartilage. The lower concentration seen in the femur supports the preliminary findings of Robins *et al.* (11) and extends those of Opsahl *et al.* (3) who reported lower amounts of the mature crosslink precursor dihydroxylysinonorleucine in the copper-deficient chick. The lower concentration of collagen crosslinks in some bones of copper-deficient animals, combined with a decrease in osteoblastic activity (35, 36), could be responsible for the skeletal defects observed in copper-deficient pigs (35), chickens (3, 4), dogs (36), and rats (37).

The changes seen in the concentration of crosslinks are probably dependent on the activity of lysyl oxidase. Although not measured in this study, the enzyme activity was diminished by copper deficiency in the rat aorta (38) and chicken cartilage (39). Lysyl oxidase activity has also been shown to vary over a wide range of dietary copper intakes in bone and tendon of the growing chick (3). There is strong evidence, however, to suggest that normal collagen crosslinking takes place even when lysyl oxidase activity is less than optimal (3) and a reduction of at least 50% of normal activity was considered necessary to impair crosslink formation in order to produce clinically demonstrable disorders in the aneurysm-prone mottled mouse (40). Thus, a decrease in lysyl oxidase activity cannot always be regarded as equivalent to a decrease in crosslink formation.

Although direct evidence is lacking, it is possible that tissue-specific lysyl oxidase enzymes may exist, each with a different copper requirement for activity. This would help to explain the tissue differences in response to copper deficiency seen in this study and in other work (3, 41) in which the tensile strength and viscoelastic properties of bone and aorta but not tendon were severely decreased. Different forms of the enzyme have been detected within the human placenta (42), bovine lung (43), and bovine aorta (44), with those in the latter tissue shown to be immunochemically distinct. Lysyl oxidase from bovine lung, in contrast to the enzyme from other sources, was shown to be more resistant to inhibition by β-aminoproprionitrile (43) but this finding has been disputed by others (45).

It is conceivable that the greater decrease in the amount of both collagen crosslinks in the heart compared with other tissues of copper-deficient rats is, in part, secondary to the CH. There is evidence to suggest that the process of CH involves increased synthesis and degradation of collagen during the remodeling of the connective tissue matrix (28). This process results in increased synthesis of type I collagen (46) or type III collagen (27, 47). Either situation would lead to a higher proportion of less mature and consequently less crosslinked collagen fibers.

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