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Lysyl oxidase activity, collagen cross-links and connective tissue ultrastructure in the heart of copper-deficient male rats

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Copper deficiency is characterized by multiple connective tissue manifestations, such as skeletal and joint abnormalities, and vascular lesions that lead to aneurysms and aortic rupture. However, the rupture of the heart observed in cooper-deficient male rats is not fully understood. We demonstrated the effect of copper deficiency on cardiac collagen content and solubility, regional differences in cardiac lysyl oxidase activity, collagen cross-links, and on the ultrastructural morphology of collagen in the myocardium. Weaned male rats fed copper-deficient or copper-adequate diets were examined. Copper-deficient rats died prematurely of heart rupture at the apex, and those who survived exhibited heart enlargement, decreased cardiac lysyl oxidase activity with increasing heart soluble collagen content. Mature collagen cross-links, as assessed by the concentrations of pyridinoline and deoxypyridinoline, were lower both in the apex and in the right and left ventricles of copper-deficient rats as compared with copper-adequate controls. However, in copper-deficient rats, cross-links levels were significantly lower at the apex than at the left and right ventricles. In addition, severe ultrastructural abnormalities in the size and shape of endo-and epimysium collagen fibers were observed in the apex of copper-deficient rats. Transmission electron microscopy showed giant, spiralled or frayed, and spiny collagen fibers. These findings allow us to postulate that the reduced cardiac lysyl oxidase activity accompanied by altered collagen cross-links and an abnormal connective tissue ultrastructure play a significant role in the manifestation of copper deficiency in the male rat. (J. Nutr. Biochem. 7:437–444, 1996.)

Keywords: heart; collagen; connective tissue; copper deficiency; lysyl oxidase; cross-links

Introduction

Copper is a nutritionally essential element that exerts a key catalytic function in the maturation of collagen, particularly in the formation of lysine-derived cross-links. Copper is important for the activation of lysyl oxidase through mechanisms that appear to involve a role as a cofactor[1] and perhaps regulation of lysyl oxidase synthesis.[2] Lysyl oxidase catalyses the oxidative diminution of selected lysyl and hydroxylysyl residues in collagen to form divalent cross-links between adjacent collagen polypeptide chains and subunits.[3] These intermediate divalent cross-links are converted to stable trivalent cross-links that accumulate in the tissue as the turnover decreases during maturation.[4] The major mature cross-link in collagens of various tissues is the trifunctional cross-link pyridinoline. It is a 3-hydroxypyridinium derivative that incorporates three hydroxylysine residues. A less abundant deoxy analog incorporates two hydroxylysine residues and one lysine residue.[5,6]

The connective tissue of cardiac muscle provides a rather complex distributed skeleton composed of components diverse in size and shape, and reflects the considerable dynamic requirements of the heart. Collagen, the major component of the extracellular matrix, is responsible for the

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functional integrity of the myocardium. Collagen fibrils, situated between myocytes and vascular elements, are effective in transducing the contractile force of myocytes to ventricular chamber, and play a major role in the stress-strain relation within the left ventricle.[7] The cross-linking of the fibrils is especially evident in cardiac tissue, probably because of the continuous mechanical stress imposed on it.[8]

Nutritional copper deficiency results in collagen and elastin abnormalities that affect the integrity of connective tissues in several young animal species. Deformed bones and tendons are documented in the skeletal matrix of copper-deficient rabbits, chicks, pigs, and dogs.[9] Myocardial atrophy with replacement fibrosis and cardiac failure was first observed in cattle and was termed "falling disease."[10] Spontaneous aortic aneurysm leading to aortic rupture have been described in the copper-deficient chick[11] and swine.[12] In male rats, dietary copper deficiency causes enlarged hearts with necrosis of myofibers and inflammatory tissue with or without fibrosis.[13] These abnormalities eventually result in premature death due to heart rupture.[14,15] However, the rupture of the heart at the apex in these rats is still ill-defined. Cardiac pathology observed in copper-deficient male rats probably has a multiple etiology associated with the decreased activity of some copper-dependent enzymes. Most of the studies have emphasized the nutritional, biochemical, and gross organ effects of the deficiency. Unfortunately, there have been only a few publications concerning cardiac histopathological aspects of copper deficiency in weaning male rats. Studies conducted by Kopp et al.[16] showed that copper-deficient rats developed electrocardiographic abnormalities, alterations in cardiac high-energy phosphatic metabolites, and disruption of mitochondrial fine structure in heart tissue. Borg et al.[17] who studied the cardiac connective tissue network of copper-deficient rats, observed numerous unbanded microfibrils, single-collagen fibrils, a few bundles of loosely associated bundles of banded collagen fibrils, and large amounts of electron-dense material that coated the collagen fibrils. These changes were assumed to result from decreased lysyl oxidase activity, but no attempt was made to measure the activity in the heart.

Our goal was to further characterize the metabolic and histological manifestations of copper deficiency. The study was conducted to determine whether the lesions observed in the myocardium of copper-deprived male rats were related to chemical and structural alterations in the cardiac connective tissue network. We focused on changes in some biochemical parameters observed in the cardiac extracellular matrix as well as on fine apical morphological changes. Biochemical studies included cardiac collagen solubility, levels of collagen cross-links (pyridinoline and deoxypyridinoline), and the activity of lysyl oxidase at the apex and left and right ventricles. Fine cardiac collagen ultrastructural pathology was assessed in the apical area of copper-deficient male rats.

Methods and materials

Eighty weanling male Sprague Dawley rats weighing 40 to 45 g each were obtained from the animal colony of the Department of Food Engineering and Biotechnology, Technion-Israel Institute of Technology, Haifa, Israel. The rats were randomly divided into two groups and housed individually in stainless-steel cages with wire-mesh bottoms in a temperature-controlled room with 12-hr light/dark periods. The animals were treated according to the Ethics Committee of the Technion for experimentation in animals. The rats were all fed the basal diet that contained fructose as the sole carbohydrate source. The diets were either deficient (0.6 mg Cu/Kg diet) or adequate (6 mg Cu/Kg diet) in copper. The composition of the diets was as follows (g/Kg of diet): fructose, 627; egg white, 200; soybean oil, 95; cellulose, 30; AIN-93[18] mineral mixture (without copper), 35; AIN-93[18] vitamin mixture, 10; choline bitartrate, 2.7; and biotin, 0.002. Copper was added to the appropriate diet as copper carbonate. All animals were allowed free access to the diet and to distilled deionized drinking water.

After 5 weeks on their respective diets, rats were fasted for 14 hr and decapitated. Blood was kept on ice, and serum was separated by centrifugation at 2500 × g for 25 min at 4°C and stored at −70°C. Eight rats from each dietary group were used for the following analysis. Hearts, livers, pancreases, and testes were quickly removed, blotted, and weighed. Liver samples, 1.0 to 1.5 g each, were used for copper and iron analysis. The apical area of the heart was removed and sliced to small pieces, 1-mm cubes each, for electron microscopy. The remaining cardiac tissue was further sliced into 2-mm cubes each, for measuring copper levels, lysyl oxidase activity, and collagen content and solubility. Dorsal skin was shaved, cleaned of adherent tissue, and pieces, 2-mm square each, were used for measuring copper content and lysyl oxidase activity.

Serum ceruloplasmin activity was measured as described by Schosinsky et al.[19] Serum cholesterol, triglycerides, uric acid, and blood urea nitrogen levels were measured enzymatically using the appropriate kits obtained from Sigma Chemical Corp., St. Louis, Missouri, USA.

Soluble collagen was extracted from heart samples with 0.5 M acetic acid as previously described.[20] Hydroxyproline (HyPro) content in the soluble and insoluble fractions was determined as described by Grant,[21] and was used as an index for collagen content. Total collagen levels were calculated as the sum of the soluble and insoluble collagenic fractions.

The sliced apical samples were fixed in 0.05 M phosphate buffer, pH 7.2, containing 2.5% glutaraldehyde, washed in phosphate buffer, and post-fixed in 1% osmium tetroxide. After dehydration in graded alcohols, the tissue was put through propylene oxide and embedded in Epoxy resin (Policed 812). Blocks were sectioned into 1-micron-thick sections stained with toluidine blue and examined by a light microscope. Selected areas were thin sectioned using an LKB ultramicrotome. These thin sections, up to 0.1 micron thick each, were double stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope.

Diets, liver, heart, and skin samples were digested by dry heat and acid.[22] Trace minerals were measured by using an atomic absorption spectrophotometer. Reference bovine liver, obtained from the National Institute of Standards and Technology (Gaithersburg, Maryland, USA) was digested and analyzed along with the samples to verify accuracy.

Hearts from the remaining rats were dissected into three regions, namely the apex and 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. The dissected regions were further sliced into 2-mm cubes and identical regions from four rats were pooled. Half of the pooled samples were used for the analysis of lysyl oxidase activity and the remaining tissue for the analysis of collagen cross-links.

Lysyl oxidase activity was determined radioactively as previously described.[20] Protein content in enzyme preparations was determined according to Lowry et al.[23] with bovine serum albumin as a standard.

Collagen cross-links were determined according to Black et

al.[24] Weighed samples were hydrolyzed in 6 M HCl at 108°C for 24 hr, after which time the acid was removed by evaporation under reduced pressure at room temperature. After dissolving in water, an aliquot was analyzed for the collagen cross-links, pyridinoline, and deoxypyridinoline, by reverse-phase high-performance liquid-chromatography (HPLC). To increase the recovery of the pyridinium cross-links, the hydrolyzates were subjected to a preliminary fractionation using cellulose CF1 partition column chromatography[24] before the analysis by HPLC. An aliquot of the hydrolyzate was also assayed for HyPro,[21] and the values obtained were used to calculate the molar ratio of the pyridinium cross-links in collagen, assuming 300 HyPro residues per collagen molecule.

Data were analyzed by the Student's *t*-test,[25] and P value < 0.05 was considered statistically significant.

Results

One rat fed the copper-deficient diet died after 5 weeks, and the study was terminated. Autopsy of the deceased animal revealed hemothorax and cardiac rupture at the area of the apex. All copper-deficient animals were thin, with roughened, dry, dull coats. The data in *Table 1* illustrate the changes in body weight and relative organ sizes in copper-deficient and adequate rats. Copper-deficient rats gained less body weight compared with copper-adequate controls. Copper-deficient rats exhibited pale and enlarged hearts along with an increased in relative liver and testicular weights and atrophied pancreas.

Table 2 summarizes data pertaining to copper indices and serum metabolites. Copper deficiency was verified by undetectable serum ceruloplasmin activity and low hepatic, cardiac, and skin copper levels compared with copper-adequate rats. Copper-deficient rats were severely anemic and exhibited a 70% increase in hepatic iron concentrations compared with the controls. Determination of serum parameters demonstrated a significant elevation in cholesterol, triglycerides, and blood urea nitrogen levels in copper-deficient rats, whereas the content of serum uric acid was slightly increased but was not statistically different from the controls (*Table 2*).

Copper-deficient rats exhibited a significant elevation in soluble cardiac collagen and a reduction in insoluble cardiac collagen (*Table 3*). Our data indicate that the increased soluble cardiac collagen content was probably due to a reduction in the activity of cardiac lysyl oxidase. A reduction of 46.5% in cardiac lysyl oxidase activity was observed in the copper-deficient rats. We also assayed skin lysyl oxidase activity and found a 39% decrease in the copper-deficient rats (*Table 3*).

Table 4 summarizes the levels of HyPro, lysyl oxidase activity, and the content of collagen cross-links, pyridinoline, and deoxypyridinoline in three heart regions. HyPro level, calculated on a wet basis, was lower in the apex and the left ventricle than in the right ventricle, whereas there was no significant difference between the copper-deficient and copper-adequate rats.

Lysyl oxidase activity in the examined sites was significantly decreased by copper deficiency. However, lysyl oxidase was inhibited to a greater extent in the apex and the left ventricle than in the right ventricle (40% versus 25%, respectively).

In the examined regions, the covalent cross-link pyridinoline predominantes, whereas very low levels of deoxypyridinoline were detected. In copper-adequate rats, there were no significant differences in the two cross-links concentrations at the various heart sites. Regardless of the cardiac region, copper deficiency significantly decreased the total amount of pyridinoline and deoxypyridinoline. Furthermore, within the cardiac regions of copper-deficient

Table 1 Body weight and relative organ sizes of rats fed a diet either adequate or deficient in copper

Parameter	Copper-adequate	Copper-deficient
Number of rats	8	8
Body weight (g)	250 ± 13	223 ± 16*
Relative weight of organs (g/100 g BW)		
Liver	4.23 ± 0.29	5.24 ± 0.45*
Pancreas	0.62 ± 0.10	0.27 ± 0.09*
Testes	1.18 ± 0.07	1.40 ± 0.11*
Heart	0.42 ± 0.02	0.65 ± 0.09*

Values are means ± SD. BW, body weight.
*P < 0.05 versus copper-adequate controls.

Table 2 Copper indices and serum metabolites in rats fed a diet either adequate or deficient in copper

Parameter	Copper-adequate	Copper-deficient
Number of rats	8	8
Hematocrit (%)	42 ± 2	21 ± 2*
Ceruloplasmin activity (U/L)	124 ± 25	ND
Hepatic copper (μg/g WT)	6.18 ± 0.41	1.59 ± 0.19*
Cardiac copper (μg/g WT)	5.48 ± 0.52	1.07 ± 0.21*
Skin copper (μg/g WT)	1.48 ± 0.27	0.95 ± 0.12*
Hepatic iron (μg/g WT)	81.2 ± 17.1	139.0 ± 22.1*
Cholesterol (mmol/L)	2.16 ± 0.16	2.53 ± 0.15*
Triglycerides (mmol/L)	0.37 ± 0.10	0.71 ± 0.10*
Blood urea nitrogen (mmol/L)	4.22 ± 0.64	8.45 ± 1.70*
Uric acid (mmol/L)	120.8 ± 20.6	136.8 ± 27.8

Values are means ± SD. ND, non-detectable; WT, wet weight.
*P < 0.05 versus copper-adequate controls.

Table 3 Cardiac collagenic fractions and heart and skin lysyl oxidase activity in copper-adequate and copper-deficient male rats

Parameter	Copper-adequate	Copper-deficient
Number of rats	8	8
Heart collagenic fractions (mg HyPro/g WT)		
Total	322 ± 11	324 ± 20
Insoluble	89 ± 12	28 ± 5*
Soluble	233 ± 14	292 ± 23*
Lysyl oxidase activity (dpm/mg protein)		
Heart	3525 ± 205	1885 ± 225*
Skin	4150 ± 305	2530 ± 225*

Values are means ± SD. WT, wet tissue.
*P < 0.05 versus copper-adequate controls.

Table 4 Hydroxyproline content, lysyl oxidase activity and levels of the collagen cross-links, pyridinoline and deoxypyridinoline, in the ventricles and the apex of rats fed a diet either adequate or deficient in copper

Parameter	Copper-adequate	Copper-deficient
Number of samples[1]	8	7
Hydroxyproline (mg/g WT)		
left ventricle	273 ± 20	284 ± 22
apex	249 ± 27	258 ± 29
right ventricle	433 ± 24	428 ± 23
Lysyl oxidase activity (dpm/mg protein)		
left ventricle	3800 ± 205	2285 ± 225*
apex	3650 ± 205	2170 ± 295*
right ventricle	3505 ± 240	2610 ± 235*
Pyridinoline (residues/collagen)		
left ventricle	0.254 ± 0.047	0.158 ± 0.026*
apex	0.235 ± 0.021	0.112 ± 0.018*#
right ventricle	0.206 ± 0.052	0.148 ± 0.023*
Deoxypyridinoline (residues/collagen)		
left ventricle	0.021 ± 0.005	0.012 ± 0.002*
apex	0.017 ± 0.003	0.008 ± 0.002*#
right ventricle	0.018 ± 0.007	0.013 ± 0.003*

Values are means ± SD. WT, wet tissue.
[1]Each sample contains a pool of tissues from four rats.
*$P < 0.05$ versus copper-adequate controls.
#Within the copper-deficient group, $P < 0.05$ versus left and right ventricles.

rats, the levels of both cross-links are significantly lower in the apex compared with the right and left ventricles.

Light microscopy of the 1-micron-thick apical sections of all copper-deficient rats revealed focal necrosis of myofibers accompanied by severe interstitial edema and inflammatory cells appeared in both the myocyte compartment and the connective tissue space. Low magnification transmission electron microscopy (TEM) of the inter- and pericellular collagen revealed that 30 to 40% of the collagen fibers appeared abnormal (Figure 1). The abnormal collagen fibers were usually present adjacent to the necrotic myofibers. High magnification TEM of the myocytes showed ruptured cell membranes, large mitochondria and some of the necrotic myocytes detached at the intercalated disc. Macrophages and connective tissue cells (CTCs) were present in the interstitial tissue. Collagen fibrils, at higher power TEM, were systematically observed in cross and longitudinal sections. Cross-sectioned fibers displayed a wide range of diameters (*Figure 2*) and great variability in shape, sometimes circular, or with a "flower-like" or "stellate" appearance (*Figure 3*), and with a moth-eaten pattern (*Figure 4*). Longitudinally-sectioned fibers also showed a variety of diameters, ranging from 20 to 230 nm, with the characteristic periodic cross-banded pattern (*Figure 5*). Very few collagen fibers displayed irregular granules of dense material adhering to the band, forming the classical spiny collagen (*Figure 5*). Many of the fibers appeared frayed into very thin fibrils separated from each other. The great majority of the thickest collagen fibers, "hyperfibrils," appeared to split into smaller fibers, thus suggesting that the thick fibers are the result of apposition of thinner fibers (*Figure 6*). The control

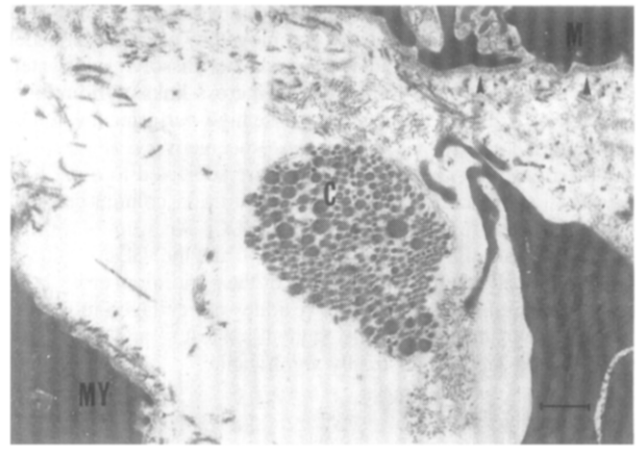

Figure 1 *Electron micrograph of the apical region exhibiting a* bundle of cross-sectioned collagen fibers with 30 to 40% abnromal collagen fibers (C). At the lower left corner note a necrotic myocyte (MY). At the upper right corner note a necrotic myocyte with intact basal lamina (arrow) and the invading macrophages (M). Magnification ×24,750 (sclae marker = 500 nm).

copper-adequate rats did not show any ultrastructural evidence of collagen abnormalities.

Discussion

Induced dietary copper deficiency in rats caused some abnormalities including depressed weight gain, anemia, enlarged heart and liver, pancreatic atrophy, elevated hepatic iron, serum cholesterol, triglycerides, and blood urea nitrogen levels. These observations confirm previous results reported by other investigators and us.[14,16,26–30]

Copper deficiency was verified by reduced cardiac, hepatic, and skin copper levels and undetectable serum ceruloplasmin activity. The hepatic accumulation of iron observed in copper-deficient rats is consistent with the early work of Elvehjem,[31] and reflects an impaired iron absorption and transport. The inactive ceruloplasmin affects the copper-sensitive iron metabolism, which result in reduced erythropoiesis[9] and might account for the severe anemia.

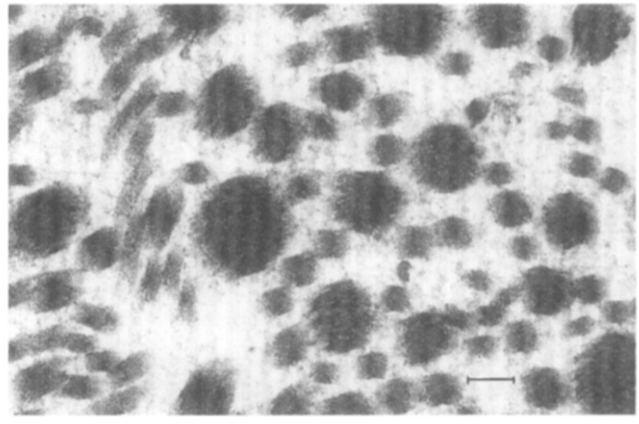

Figure 2 Electron micrograph of the apical collagenic network in copper-deficient male rats demonstrating cross-sectioned collagen fibers of different sizes. Magnification × 122,000 (scale marker = 100 nm).

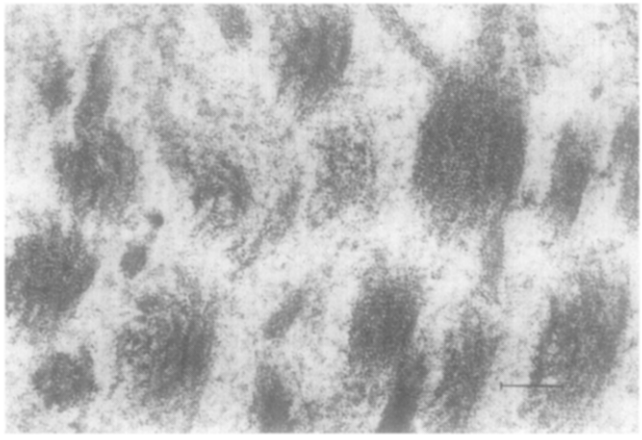

Figure 3 Electron micrograph of apical collagenic matrix of copper-deficient male rats demonstrating cross-sectioned collagen fibers showing the "flower-like" pattern. Magnification × 165,000 (scale marker = 100 nm).

Elevated fasting blood cholesterol, triglycerides, and uric acid levels, are metabolic risk factors associated with ischemic heart disease,[32] the primary cause of death in western industrial societies. The same blood risk factors were observed in copper-deficient rats in the present study. At this point it should be emphasized that the daily diets in Western societies may be marginal in copper,[33] and considered to be below the estimated adequate and safe intake of 1.5 to 3 mg Cu/day.[34] Thus, copper deficiency might be a prime important risk factor in the etiology of ischemic heart disease.[15]

Elevated blood urea nitrogen level along with growth retardation, as observed in the present study, suggests that copper-deficient rats are in a negative protein balance. One may consider weight loss as a contributing factor to the clinical alterations and heart damage observed in copper-deficient rats. However, we have previously demonstrated that per-weight controls showed no sign of altered serum metabolites nor gross cardiac pathology.[30]

The most dramatic effects in this study were on the heart enlargement, pathology, and mortality. Cardiac enlargement that accompanies copper deficiency, may be a result of several metabolic alterations characteristic to the deficiency. Shields et al[12] found a good correlation between the degree of anemia and the relative heart weight, suggesting that cardiac hypertrophy is a response to increase cardiac output consequent upon anemia.[12] In contrast, according to Gubler et al.[35] cardiac hypertrophy cannot be explained entirely by the presence of anemia, because they showed that iron-deficient pigs are equally anemia as copper-deficient pigs, but did not have as great a degree of cardiac hypertrophy. Tissue anoxia, a result of possible reduction in blood oxygen-carrying capacity,[36] as well as decreased cardiac cytochrome oxidase activity and norepinephrine levels[37] have been also suggested as the cause of cardiac enlargement in copper deficiency. In addition, TEM studies showed enlargement of the myocardial mitochondria in both copper- and iron-deficiency anemia, but with considerably larger mitochondria occurring with iron deficiency.[38] However, the mechanisms by which copper deficiency causes cardiac pathology are not completely understood. The involvement of some copper-dependent enzymes with a key metabolic role, whose activities are altered by the deficiency,[39] cannot be ruled out. Ceruloplasmin is important in hemoglobin metabolism; dopamine-β-hydroxylase is essential in the synthesis of norepinephrine; cytochrome oxidase is needed for oxidative phosphorylation; superoxide dismutase affects cellular integrity, and lysyl oxidase is crucial for the cross-linking of collagen and elastin.

Figure 4 Electron micrograph of apical collagenic matrix of copper-deficient male rats demonstrating cross-sectioned collagen fibers showing a moth-eaten appearance (arrow). Magnification × 165,000 (scale marker = 100 nm).

The development of copper deficiency had no effect on myocardial collagen production as observed by measuring total cardiac HyPro concentrations in whole hearts homogenates (*Table 3*) and directly in the right and left ventricles and the apex (*Table 4*). The higher HyPro level seen in the right compared with the left ventricle is in agreement with previous observations.[40]

The present experiment demonstrated that copper-deficient rats exhibited impaired cardiac collagen metabolism characterized by reduced lysyl oxidase activity, and a decrease in the concentration of the mature collagen cross-links, pyridinoline and deoxypyridinoline. These changes eventually resulted in an increase in cardiac-soluble collagen content. The increase in cardiac collagen solubility observed in the present study is in agreement with our previ-

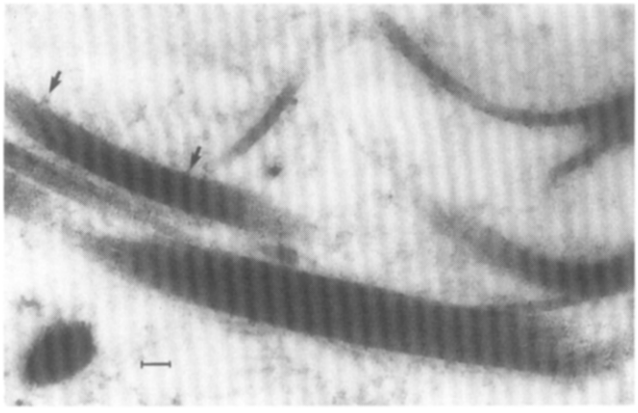

Figure 5 Electron micrograph of apical collagenic network in copper-deficient male rats demonstrating longitudinally sectioned collagen fibers measuring up to 200 nm in diameter. Note the spiny collagen appearance (arrow). Magnification x 75,000 (scale marker = 100 nm).

ous results,[41] and the results of Dawson et al.,[42] who demonstrated an increase in pepsin-soluble collagen with no change in net collagen synthesis, in the heart of copper-deficient rats. Fluorescent immunohistochemical staining, conducted by Borg et al.,[17] indicated the morphological presence of the antigen of lysyl oxidase both in copper deficient and control rats. However, our present data revealed that the activity of lysyl oxidase was decreased exclusively in copper-deficient rats. Within the heart regions, lysyl oxidase activity was reduced in both the apex and the two ventricles. However, the reduction was more pronounced in the apex and the left ventricle (*Table 4*). Despite the similar relative reduction in lysyl oxidase activity (40%) at the apex and the left ventricle, the apex demonstrated a 52% decrease in the pyridinoline cross-link, whereas the left ventricle only 38%. On the other hand, lysyl oxidase activity in the right ventricle was reduced by 25%, but pyridinoline content only by 28%. Previous studies have shown

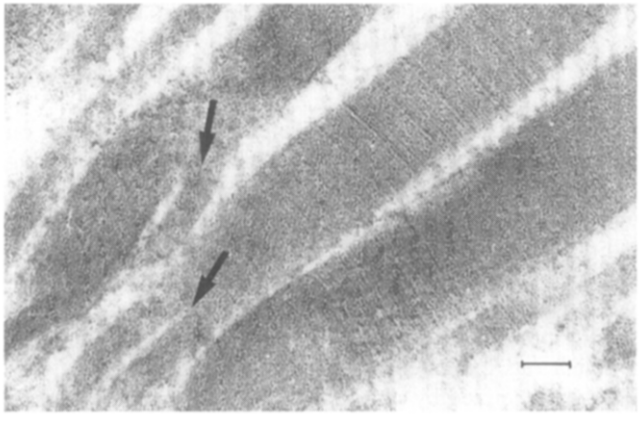

Figure 6 Electron micrograph of apical collagenic network in copper-deficient male rats demonstrating longitudinally sectioned collagen fiber. The width of the fibers is about 260 to 300 nm in diameter. Note the apparent splitting of collagen fibrils (arrow) from larger fibrils. Magnification x 115,000 (scale marker = 100 nm).

that the effect of dietary copper deprivation on lysyl oxidase activity seems to be tissue specific.[43–45] We showed that decreased lysyl oxidase activity was not specific to the heart, but was also observed in the skin of the copper-deficient rats (*Table 3*). In contrast, copper levels and lysyl oxidase activity in the brain of the copper-deficient rats remained unchanged as compared with the copper-supplemented controls (Werman, M.J., unpublished data). The present study, however, raises the possibility that a region-specific effects of reduced lysyl oxidase activity with regard to collagen crosslinking may exist.

Opsahl et al.[43] showed that chicks fed a copper-deficient ration exhibited a reduction in reducible divalent cross-links in bone collagen that was coincident with a decrease in bone mechanical strength. In this regard, our findings indicate that copper deficiency may have caused an impairment in the maturation of cardiac collagen cross-links and fibrillogenesis and thus, affected the viscoelastic properties of cardiac collagen. Changes in cardiac collagen cross-links and solubility may have altered the strength and stiffness of the myocardial connective tissue matrix resulting in impaired mechanical properties of the epimysial and endomysial weave network. This could explain some of the impaired mechanical properties such as lower spontaneous heart rates, decreased coronary resistance, and less systolic pressure observed in vitro, in copper-deficient rats.[46] Reduction of contractile function, tissue anoxia, depletion of norepinephrine, and cytochrome oxidase activity may contribute to focal cardiac necrosis[17] and to an end-stage cardiomyopathy that lead to heart failure. Rupture of the ventricular wall, composed of an abnormal collagen crosslinked network may occur. Indeed, with regard to lysyl oxidase activity and collagen cross-links levels, the apex, where stress and strain forces are concentrated, was the most affected cardiac region and thus, appears to be the most susceptible site to rupture. In species where aortic rupture due to copper deficiency has been reported, such as in pig[47] and the chick,[48] an increase in the solubility of aortic collagen, which indicates of decrease crosslinking, was observed.

The changes observed by light microscopy at the thick sections are in agreement with previous observations.[13,14,17,49] The interstitial edema accompanied with the infiltration of CTCs and macrophages in the extracellular space possibly led to the separation of the myocardial cells, not only morphologically, but functionally as well. Thus, the electric coupling of the myocytes is inhibited, the diffusion distance for oxygen and substrates is enhanced, and the myocardial compliance is reduced.[50] In addition, changes in myocyte distribution may further alter the distribution of force of muscular contraction to the ventricular wall. Furthermore, TEM of copper-deficient rats demonstrated several ultrastructural abnormalities in the size and shape of cardiac collagen fibers. In the present study, abnormally large collagen fibrils, also termed giant, composite, or hyperfibrils were observed in copper-deficient rats. These fibrils were defined by Ghadially[51] and have been described widely in various pathological conditions such as inherited collagen disorders, where they might result from the packing of altered collagen fibrils.[52] They have been also observed in fibrotic tissues and can reflect proteolytic breakdown states of collagen fibrils.[53] The heterogeneity of

altered fibrils observed in the apical extracellular matrix of the copper-deficient rats may reflect a varied susceptibility of these fibrils to proteolysis. These hyperfibrils were not evenly distributed in the myocardium, but rather were observed in foci of abnormal connective tissue regions, in which increased proteolytic activity may occur. The higher fragility of some fibrils may be explained by altered fibrillogenesis resulting from the inhibition of lysyl oxidase activity and reduced collagen cross-links levels. However, abnormalities in lysyl hydroxylase activity cannot be ruled out. Copper deficiency has been shown to result in hepatic iron accumulation (*Table 1*) and to alter iron metabolism.[54] Iron is known as one of the cofactors of lysyl hydroxylase.[55] Altered lysyl hydroxylase activity results in underhydroxylated type I and III collagens in the skin of patients with Ehlers-Danlos syndrome.[52] Hydroxylysyl residues are sites of crosslinking as well as of glycosylation, and thus underhydroxylation and underglycosylation may each interfere with the formation of intramolecular cross-links and to decrease collagen stability. Decreased glycosylation of the collagen molecule can also alter the surface charge and modify the interaction of collagen with proteoglycans, which play a significant role in collagen fibril assembly[56] and act as a protective barrier against proteolytic attack.

TEM of the apical myocardium of copper-deficient rats exhibited another abnormality of collagen fibers (*Figures 3* and *4*), referred to as spiralled or frayed[51] and as hieroglyphic collagen.[53] These fibrils were previously found in the skin and tendons of sheep[57] and cattle[58] with dermatosparaxis. The abnormal fibrils differ from composite fibrils, however, in that they are small, twisted ribbons or sheets that in cross-section bear no resemblance to the normal round fibril.[52] A defective aggregation of collagen filaments or dissociation of previously normal fibrils are probably the two ways in which the appearance of spiralled collagen can develop.[51] These frayed fibrils have a greater diameter than normal fibrils, but in some instances they appear large enough to have been derived from two or more fibrils that assembled and expanded in diameter (*Figure 6*).

Another form of abnormal collagen, termed spiny collagen, was observed in the hearts of copper-deficient rats (*Figure 5*). This consists of mature striated collagen fibrils with irregular granules of relatively dense material adhering to the band periodically. Spiny collagen has been seen in aorta of lathyritic chick embryo in various pathological states.[59] Little is known about the nature or composition of the electron-dense granules in the matrix, and the significance of this change is also obscure.[51]

Our findings may provide an explanation for the harmful influence of dietary copper deficiency on the heart. The marked decrease in lysyl oxidase activity, observed at several heart regions, is a contributing factor in the impair formation and maturation of collagen cross-links, increased collagen solubility, and altered connective tissue morphology seen in the hearts of dietary copper-depleted rats. The most significant changes however, were observed in the area of the apex and would undoubtedly have an adverse effect on the cardiac connective tissue matrix influencing cardiac performance, and finally lead to cardiac rupture and death.

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