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The cardiac pathophysiology and ultrastructure in dietary copper deficiency and repletion in young and older sedentary and exercise trained rats

Davidson, Jeannette, Ph.D.
The Ohio State University, 1992
THE CARDIAC PATHOPHYSIOLOGY AND ULTRASTRUCTURE
IN DIETARY COPPER DEFICIENCY AND REPLETION
IN YOUNG AND OLDER SEDENTARY AND EXERCISE TRAINED RATS

DISSERTATION

Presented in partial fulfillment of the requirements for
the degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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FIELD OF STUDY

Human Ecology

Studies in Human Nutrition
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CHAPTER I

INTRODUCTION

Background and setting

Copper is an essential nutrient for humans and animals (1-4). The current safe and adequate dietary intake set by the National Research Council (5) is in the range of 1.5 to 3 mg of copper/day. The average daily copper (Cu) intake of men and women between 1982 and 1984 was reported as 1.2 and 0.9 mg, respectively (6). Naturally occurring dietary Cu deficiency in "healthy" humans has not been documented, yet concern has been expressed that human Cu intakes may be marginal (7-10). Apart from the actual Cu levels in the diet (11,12), other dietary factors are known to interact with Cu uptake and/or utilization, and may further reduce Cu availability or increase needs. The interaction of Cu with these factors are not yet well understood, but the intake of high zinc supplements, ascorbic acid tablets, the increasing fructose consumption and the regular use of antacids may be of concern (11,12).

Young rats fed a Cu deficient diet consistently produce a well documented hypertrophic cardiomyopathy (13-25), characterized by functional, ultrastructural and biochemical changes. It is thought that most of the effect of Cu deficiency on the development of cardiac hypertrophy and pathology may be accomplished through the decreased activity of cuproenzymes, e.g. lysyl oxidase, cytochrome c oxidase, dopamine-ß-hydroxylase and super oxide dismutase.

The hypertrophic cardiomyopathy of experimental Cu deficiency is characterized by cardiac hypertrophy with prominent concentric
components, associated with a significantly thickened outer wall and septum and reduced chamber volume (13,20-25). Disturbed connective tissue, increased interstitial collagen, weakened aortal elastic fibers and early cardiac failure occur (14-18). Resting systolic blood pressure may be reduced in young rats (25-27), or increased in older rats (28,29). Electrocardiography demonstrates electrical disturbances, including abnormal PR and ST segments and increased QRS wave amplitude (19,20,24). Ultrastructural changes include hypertrophy of the myocytes, with increased mitochondrial volume density (22), increased mitochondrial:myofibrillar ratio (23), and fragmented cristae (24). Furthermore, quantitative increases in myofibrils have been reported with Cu deficient hypertrophy (24). Altered norepinephrine levels may be implicated (30). Functional and ultrastructural changes induced by dietary Cu restriction in older rats, or due to dietary Cu repletion in previously Cu depleted rats, have not been reported.

Pathological concentric cardiac hypertrophic conditions in humans due to pressure overload (31-35), induced by conditions such as chronic hypertension, bear a marked resemblance to the hypertrophy of dietary Cu deficiency. Functional abnormalities in pathological cardiac hypertrophy, include decreased contractility (power and velocity), decreased relaxation, and decreased capillary reserve (32). Ultrastructural changes include increased myofibrillar:mitochondrial ratio and decreased vascularization (32), fibrosis, and foci of interstitial collagen hyperplasia. ECG abnormalities include abnormal PR & ST segments, Q waves, and increased QRS wave amplitude (36).

Sub-maximal, aerobic, exercise training is associated with physiological cardiac hypertrophy in humans and animals, of the eccentric type (37-41). However, the hypertrophy is modest, and is characterized by improved function. Increases in mitochondria seem to parallel increases in fiber size, and an optimal oxygen diffusion distance is maintained by increased vascularization (32,37). A shift
towards the faster β-myosin isoenzyme of myosin heavy chain improves myocardial contractility (37,42,43). Some studies have investigated the effect of exercise training on various forms of existing or developing cardiac pathology. The results are equivocal. However, the bulk of evidence seems to support some positive effects (44-51). No studies have been documented as to the interaction of Cu depletion and exercise training.

Significance of the study

Although the cardiac ultrastructural and functional parameters of dietary induced Cu deficiency have been well documented in young rats, the effect in older rats is not clear. Previously, it has been thought that the cardiac hypertrophic and functional effects occurring in young rats do not develop when Cu deficiency is induced in older rats, primarily because cardiac mass has not been found to increase significantly, as in young rats. Additionally, Cu deficiency may take a long time to manifest if the rats had accumulated adequate tissue Cu reserves. The simultaneous study of ultrastructural and functional parameters in older rats has not been done. Similarly, ultrastructural and functional changes that may take place when previously Cu depleted rats are Cu repleted, are lacking. A concurrent investigation of both function and ultrastructure, could lead to a better understanding of the mechanisms of hypertrophic cardiomyopathy due to dietary Cu deficiency.

Aerobic exercise training has not been attempted for rats who are also Cu deficient. Some of the positive effects of exercise training on parameters of cardiac function and structure may be favorable to the development of Cu induced hypertrophy, such as improved myosin isoenzyme patterns, increased vascularization, decreased oxygen diffusion distances, and increased myofibril mass. Furthermore, the classic hypertrophic pattern with aerobic exercise training is mildly eccentric, while that of Cu deficiency is predominantly concentric. Interestingly, physiological hypertrophy is more difficult to induce in males than
females, with male rats often not developing any measurable absolute hypertrophy, whereas in Cu deficient rats, males show a more marked hypertrophy. There is some indication that exercise training may indeed be beneficial in some cases of existing cardiac pathology. The possibility that exercise training may have a beneficial effect on the development of cardiac hypertrophy in Cu deficiency, should be investigated.

**Statement of the problem**

Three questions are addressed in this research:

1. Can feeding a Cu deficient diet induce significant changes in cardiac function and ultrastructure in older rats?

2. What is the effect of repletion of dietary Cu in previously Cu deficient rats on cardiac function and ultrastructural abnormalities?

3. Does sub-maximal, aerobic, exercise training have a positive effect on the development of cardiac hypertrophic pathology in rats fed a Cu deficient diet?

The following hypotheses are designed to answer these questions:

1. There will be no significant changes in cardiac function and ultrastructure in rats fed a Cu deficient diet several weeks after weaning.

2. The existing cardiac pathology of dietary Cu induced hypertrophic cardiomyopathy will not significantly be improved through Cu repletion.

3. Sub-maximal aerobic exercise training will have no significant effect on the development of cardiac pathology in rats fed a Cu deficient diet.
CHAPTER II
REVIEW OF LITERATURE

The physiological, biochemical and metabolic role of Cu has been recently reviewed (1-4). The established functions of Cu are generally mediated through cuproenzymes. Key Cu-dependent enzymes are lysyl oxidase (cross-linking of elastin and collagen), cytochrome c oxidase (electron transport chain), ceruloplasmin (ferroxidase activity), superoxide dismutase (free radical detoxification) and dopamine-ß-hydroxylase (catechol production) (1-3).

Dietary copper deficiency in humans

Information as to the dietary intake of Cu by humans is limited and estimated daily intakes equivocal. Pennington et al (6,12) reported average Cu intakes of women to be 0.93 mg/day and men 1.24 mg/day, whereas Klevay estimated that the mean intake of many Americans is 1.55 mg/day (7).

There is no Recommended Dietary Allowance (RDA) for Cu, because of the uncertainties surrounding quantitative dietary Cu needs (5), but 1.5 to 3 mg/day has been established as a safe and adequate dietary intake range by the National Research Council in 1989. This is a wider range than the 2 to 3 mg/day established in 1980, because it was felt that there was a discrepancy between the intake estimated from balance studies and reported intakes, without obvious clinical signs of Cu deficiency, specifically anemia and neutropenia (5).

Naturally occurring Cu deficiency in "healthy" humans has not been reported. Some incidence of Cu deficiency in severely malnourished
young Peruvian and Chilean children, developing premature babies, adults on total parenteral nutrition and people with Menkes' Kinky Hair Syndrome, have been recorded (3,5). However, some concern has been expressed as to the adequacy of current Cu intakes by healthy people generally (7-9) and athletes (10). In a study by Reiser et al (9), 4 out of 23 humans developed heart related problems when the subjects were fed a typical American diet with a high sucrose, but not starch, content (diet Cu level was 1 mg/d). The bioavailability of Cu and extent of dietary interaction is not precisely known (11,12), but factors such as zinc, ascorbic acid, fructose or sucrose, iron deficient anemia, fiber and phytates, and antacids (11,12), may decrease absorption or increase needs of Cu.

Copper deficient cardiomyopathy

The cardiomyopathy of experimental dietary Cu deficiency in weaning rats have been well documented (2,3). Copper deficiency seems to act as stimulus for the development of compensatory cardiac hypertrophy, probably through its effect on cardiac cuproenzymes (13). The hypertrophy is characterized by gross, functional and ultrastructural changes, with a high morbidity and mortality (14,15).

Interstitial inflammation with focal necrosis, subendocardial fibroplasia and hemorrhage have been found in studies by Kelly et al (13) and Allen & Klevay (15). Abnormal connective tissue is associated with decreased activity of lysyl oxidase and inhibition of the cross-linking of lysyl-residues of collagen and elastin (16,17) and an increased ratio of Type III:Type I collagen (18). Aortal distortion and depletion of elastic tissue, rupture, and cardiac aneurysms, hemothorax, pleural effusion, rupture and hemopericardium are present (14-16). Low cytochrome c oxidase activity may contribute to cardiac lesions (13).

Blood pressures in different studies are more variable and may be specie and age dependent. Blood pressure seems to be decreased in
younger Cu deficient rats (25-27), while blood pressure may be increased in older rats (28,29).

Heart rate and electrical abnormalities have been reported in a number of studies. Prohaska & Heller (19) reported decreased heart rates in Cu deficient rats, while Kopp et al (20) found no significant change in heart rate, though it was somewhat lower in Cu deficient rats. Electrocardiographic (ECG) abnormalities included abnormal ST segments, increased PR intervals, R wave duration and amplitude (14,20,24), and ventricular and supraventricular beats (14). Kopp et al (20) reported increased H-V intervals in His Bundle Electrography, and suggested this was indicative of slow conductivity of the His-Purkinje system. Additionally, Medeiros et al (24) recorded increased QRS amplitude and QT duration.

Altered catecholamine levels and sensitivity in the heart and nervous tissue may contribute to the cardiac hypertrophy and functional changes in Cu deficiency. There is some indication that norepinephrine levels are decreased with increased dopamine levels in Cu deficient rats (19,30) which could involve the cuproenzyme, dopamine-B-hydroxylase.

Ultrastructural changes in the myocardium of young Cu deficient rats are well established. Hypertrophy of myocytes, due to increased size and possibly number, and mitochondria (20,21,23) have been documented. Abnormal mitochondrial DNA molecules, in association with megamitochondria, have been reported in rats treated with cuprizone, an established Cu chelator (51). Recently, Medeiros et al (23) reported that increased myofibrils content contributed to myocytal hypertrophy. Non-specific, sub-cellular, degenerative changes in myocytes have been reported (23), characterized by fragmented mitochondrial cristae and vacuolation of matrix (23), and distorted sparse myofibrils with poorly aligned Z-bands. Sarcomeres appeared separated at the Z-bands and unbanded myofibrils were observed (16).
The different response of mitochondria, observed in the increased mitochondrial volume density and mitochondrial:myofibril ratio in this and other studies on Cu deficiency, may be coupled to the role of Cu as a cofactor in the synthesis of cytochrome c oxidase (CCO). CCO, on the inner mitochondrial membrane, is essential to mitochondrial respiration and oxidative phosphorylation, catalyzing the transfer of electrons from ferry cytochrome c to oxygen (52). CCO is composed of several subunits encoded both nuclearly and mitochondrially (53). CCO is turned over cooperatively, with a $t_{1/2}$ of CCO of 4-5 d (54). When even one subunit is absent, the enzyme cannot be assembled, causing decreased respiration and breakdown. This includes degeneration of the inner mitochondrial membrane, loss of cristae and vacuolization of the matrix, loss of DNA, RNA and proteins, and disintegration of the outer mitochondrial membrane with swelling of the organelle (52,54). Once integrity of the mitochondria is breached, and respiratory function is compromised, mitochondria are rapidly degraded by lysosomal and cytoplasmic proteolytic enzymes. Disruption of these membranes may lead to increased phospholipid peroxidation and free radicals damage. It is thought possible that the mitochondrial inner membrane disruption may be a signal for early degeneration (52,54). Decreased CCO activity in the heart and skeletal muscle of rats (55) and mice (56) with Cu deficiency had been reported. This decrease in CCO need not be linked to changes in ATP concentration, since ATP may be augmented by the creatinine-phosphate kinase system, and anaerobic glycolysis of glucose and glycogen. The finding of increased glycogen granules in Cu deficient myocytes lends credence to this theory (23,24).

There are only three human studies reporting cardiovascular abnormalities with experimental Cu deficiency. Klevay et al (7), reported increased blood total cholesterol and LDL cholesterol and ECG abnormalities in a man after 15 weeks on a diet supplying 0.8 mg Cu/d. Reiser et al (9) reported increased LDL cholesterol and decreased HDL
cholesterol, with no change in total cholesterol in 24 men with Cu intakes of 1 mg/d. At 4 weeks, one man developed myocardial infarction, one developed tachycardia at 7 weeks and the study was terminated when one developed type II heart block at 11 weeks. However, Turnland (11) could find no differences in blood cholesterol or cardiac function in 11 men after 6 weeks on diets containing 0.8 mg Cu /d.

Pathological cardiac hypertrophy

Myocardial hypertrophy in humans is seen as an adaptive process, that takes place to enable the heart to compensate for conditions of overload (31). However, the hypertrophic myocardium is not normal and eventually cardiac failure will result. Meerson (32) described three phases of the hypertrophic process as compensatory hypertrophy following acute and chronic overload, characterized by stable hyperfunction but decreased cardiac reserve capacity, and overcompensation or hyperadaptation leading to cardiac failure. Hypertrophic cardiomyopathies have also been induced experimentally in rats, by banding of the aorta, aortic stenosis, and renal arterial constriction (32).

Cardiac function may fail through inadequate venous emptying or reduced venous ejection fraction against arterial pressure (31). The types and pathophysiology of pathological cardiac hypertrophy have been recently reviewed (31,33,34). Concentric hypertrophy is characterized by increased ventricular wall thickness, and is associated with pressure overload conditions, such as hypertension and aortic stenosis. Eccentric hypertrophy is characterized by increased left ventricular chamber volume and a relatively thin wall. Volume overload in aortic insufficiency, ischemia and reduced volume load of mitral stenosis, underlie eccentric hypertrophy. Congenital cardiomyopathies with hypertrophy may have increased left ventricular volume and symmetrical or asymmetrical increases in ventricular wall thickness (32-34).
The primary stimulus for cardiac hypertrophy seems to be chronic pressure or volume overload, associated with changes in workload and energy and oxygen demands, leading to activation of nucleic acid and protein synthesis (32). The precise signal is not yet understood, but there is some evidence (57) that suggests that the stimulus may be associated with the signal transduction pathways of myocyte protooncogenes (c-onc). It is possible that hemodynamic factors in the overloaded heart may trigger the expression of c-onc, e.g. c-fos and c-myc (31,57), that seems to precede the hypertrophic process. The expression of "fetal" forms of the contractile proteins, creatine kinase, myosin light and heavy chain, α-actin and β-tropomyosin, and atrial-natriuretic polypeptide (ANP) has been confirmed in the hypertrophic process. Changes in the sarcolemma and calcium transport processes have been noted (32). Myosin isoenzyme form 3 (V₃) in myosin heavy chains, is increased relative to α (V₁) in hypertrophic rat ventricles compared to mainly V₁ in normal ventricles (41). Since V₃ is associated with slow contractile myosin-ATP-ase, this would decrease myocardial ATP demand and subsequent oxygen consumption initially. However, increased V₃ may lead to decreased contractility and ultimately contribute to myocardial failure (32,41). The locus for familial hypertrophic cardiomyopathy has been shown to map on chromosome 14, closely linked to cardiac myosin heavy chain genes (58).

Functional abnormalities in hypertrophic cardiomyopathies include decreased contractility (power and velocity), decreased relaxation, and decreased cardiac reserve (32). Sasson et al (36) reported abnormal ECG patterns with atrial and ventricular arrhythmias. Abnormal ST segments were sometimes elevated, Q waves were prominent, R waves demonstrated increased amplitude, and PR intervals QRS waves were short. Previously, it was thought that catecholamine concentration was decreased in cardiac failure. More recently (59), it was shown that there is an increased cardiac catechol concentration, with selective down-regulation of β₁-
adrenergic receptors relative to $\beta_2$-receptors, that effectively reduces norepinephrine sensitivity and sympathetic adrenergic influence.

Ultrastructural changes in the myocyte in cardiac hypertrophy, appear to be dependent on the stage of hypertrophic development (32). In the early, acute stage, there is an increase in protein synthesis, with an early increase in mitochondria, followed by an increase in myofibrils. In the stable stage of hypertrophy there does not seem to be significant changes in the myocyte ultrastructure. The stable phase of compensated hypertrophy is associated with increased myofibrillar growth and an essentially normal ratio of mitochondrial to myofibrillar volume densities. The third stage of decompensated hypertrophy and myocardial failure has a variable response. Pressure overload, but not volume overload or physiological hypertrophy, is associated with early increased mitochondrial volume density (31,32,35,60). The increased width of the hypertrophied myocyte with pressure overload, associated with the lateral addition of sarcomeres, stimulates mitochondrial synthesis to comply with increased sarcomeres energy needs. However, when sarcomeres are added end-to-end at intercalated discs, as in volume overload eccentric hypertrophy, increased mitochondrial size and number is unnecessary. The needs of the increased cell length are met by endothelial hyperplasia and capillary length-wise growth. Furthermore, mitochondrial volume density may be disproportionally increased in response to relative anoxia in hypertrophy associated with anemia, hypoxia or ischemia (61,62).

With progression of the cardiomyopathy to the last stage of overcompensation and ultimately failure, there seems to be a decreased mitochondrial to myofibrillar ratio in the hypertrophied myocyte, with resultant imbalance between ATP production (mitochondria) and demand (myofibrils) (31,32). Transverse capillary density is decreased and arterial lumen diameter diminished with progressive myocardial failure, so that oxygen diffusion distance is increased (60-62), of especial
significance in the stressed heart. Cell mass has been reported to be increased relative to cell surface, thus suggesting reduced sarcolemmal surface and ion transport receptors (32). Fibrosis and foci of increased interstitial collagen deposition are increased (32-36), contributing to progressive myocardial stiffness and decreased contractility.

**Physiological cardiac hypertrophy**

In contrast to pathological myocardial hypertrophy (63), physiological hypertrophy induced by chronic, repetitive, physical activity, is characterized by improved neuro-endocrine responses, stable myocardial structure and improved work capacity (32,36,37) and vasculature (64). The stages of hypertrophic development is essentially the same as for pathological hypertrophy, but is quantitatively less (32,36,37), due to the intermittent and mild nature of the exercise stimulus. The primary stimulus for physiological hypertrophy is an increased energy and oxygen demand created by increased cardiac work output. The resulting structural changes are regarded as favorable and represent balanced increases in contractile, mitochondrial, sarcolemmal proteins and vascularity (36,61).

Exercise training is associated with two types of myocardial hypertrophy. Dynamic (isotonic or aerobic) exercise training, such as running and swimming, results in eccentric type hypertrophy in response to volume overload, whereas isometric exercise (static) training, such as weight lifting and wrestling, results in a concentric hypertrophic pattern associated with pressure overloading (36, 38-40). Endurance athletes develop dilated ventricular volumes, with modest or no increase in ventricular wall thickness and increased end-diastolic volume, whereas weight lifters develop markedly increased left ventricular wall thickness, with normal ventricular cavities (38,39).

Myocardial hypertrophy in rats is modified by age and sex of the rats, and type, intensity and duration of the exercise regime (36,65).
Swimming appears to have a more pronounced hypertrophic effect, while treadmill running appears to be more controversial (36-38,66). Absolute heart wt does not always increase with treadmill training, although heart wt: body wt ratio seems to be increased more consistently. This could be due to a failure of male rats to increase food intake to compensate for increased energy needs with exercise, resulting in decreased in body wt in exercising compared to sedentary rats. Exercise training may reduce growth rate in young rats, and the hyperplasia of early neonatal life may confound the hypertrophic effect of exercise training (36). In contrast to male rats, female rats are reported (64) to increase their food intake proportionately with exercise, so that their body wt is comparable to that of sedentary rats. Absolute cardiac hypertrophy is more pronounced in female rats, with male rats often showing no measurable hypertrophy (64). The opposite has been found to occur in Cu deficient rats, where the males develop more pronounced hypertrophy. Reported exercise protocols are variable (65), but it seems that a period of adjustment (2 to 4 weeks) may be needed to attain the full exercise training protocol, with a further 4 weeks to reach a training effect. Exercise regimes at high intensities are more likely to produce hypertrophy, but heart functionality may be reduced, due to a hyperadaptation effect (32).

The major advantages of aerobic exercise training are related to structural changes that improve the velocity and amplitude of contractions, and increase myocardial relaxation (32,36). A shift to the faster isoenzyme, \( \beta(V_1) \), of myosin heavy chain has been shown to occur (47), with resultant increase in myosin ATPase activity and increased contractility. Improved contractile capacity of the myocardium after aerobic exercise training has been reported (37,67). In contrast to skeletal muscle, cardiac mitochondrial number and size do not seem to be increased per se (36). Rather mitochondrial: myofibrillar ratio seems to be maintained in the hypertrophied myocyte,
allowing ATP production to keep pace with increased energy demands \((32,36,68,69)\). The mitochondrial increase seems to precede the myofibrillar increase, due to the shorter mitochondrial \(t_m\). Increased coronary vascularization, due to both neoformation of capillaries, and increased arteriolar luminal diameter, decreases oxygen diffusion distances and preserves coronary reserve, thereby increasing myocardial functionality \((36,64,62,69)\). Myocardial myoglobin concentration is increased \((36)\) and myocyte mass: volume ratio seems to be maintained, thereby allowing normal sarcolemmal and calcium ion dynamics \((68,69)\). Improved myocardial calcium uptake has been reported \((70)\). Absolute catecholamine concentration may be decreased, but myocardial sensitivity to norepinephrine is increased, so that sympathetic adrenergic effectivity is improved \((71)\).

**Myocardial response to aerobic exercise training in cardiac pathology**

Aerobic exercise training has been advocated to reduce the risk of developing ischemic atherosclerotic heart disease by improving cardiac functionality. Although the potential benefits of improved myocardial contractility, energetics and vascularization, would deem this advantageous, the question remains whether exercise training would be of use in existing pathological conditions.

Experimental rat models that have been used, include ischemia induced by banding of coronary arteries, hypoxia by e.g. inhalation of carbon dioxide, hypertension by renal artery constriction or aortic banding \((32)\). More recently the spontaneously hypertensive rat (SHR) model and diabetic rats, using streptozotocin have been used \((47,48)\). In 1978, McElroy et al \((72)\), reported that exercise training reduced infarct size after coronary occlusion. Scheuer & Bahn \((67)\) induced hypertension in swim trained rats and reported that blood pressure development per se was not affected, but there was a favorable shift from isoenzyme \(\beta\) \((V_3)\) induced by hypertension, to \(\alpha\) \((V_1)\). When exercise training was combined with induced hypertension, the hypertrophic effect
was significantly increased, but cardiac flow was enhanced (49). Cardiac contractile protein abnormalities of pathological hypertrophy were corrected by exercise training (44). Similarly, isoenzyme patterns were shifted toward normal in the swim trained SHR (43).

Rats trained on a treadmill are better able to withstand hypovolemic shock (45), and conditions of hypoxia and ischemia (46), than untrained rats. Treadmill training for 8 weeks appeared to improve myocardial contractility, ATPase activity and increase coronary vessel density in streptozotocin induced diabetic rats (47,48). Kanter et al (50) reported improved antioxidant defense against the cardio-toxicity of doxorubicin in trained rats. Although investigation into the effect of exercise training on existing pathology is not conclusive, results of studies are somewhat positive.

No studies have been published addressing the effect of aerobic exercise training on the development of Cu deficient hypertrophic cardiomyopathy.
List of references


CHAPTER III

ULTRASTRUCTURAL AND PATHOPHYSIOLOGICAL CARDIAC PARAMETERS

IN COPPER DEPLETED AND REPLETED YOUNGER AND OLDER RATS

Abstract

Ultrastructural and functional cardiac parameters were determined simultaneously in copper depleted and repleted younger and older rats. Five groups of rats were fed either copper adequate or copper deficient diet from weaning. After 5 wk, 1 group from each diet was sacrificed to obtain baseline values. One copper adequate group was crossed to the copper deficient diet to evaluate the effect of copper depletion in the older rat, the remaining copper deficient rats were fed the copper adequate diet to evaluate copper repletion, and 1 group continued to receive the copper adequate diet as a control. Copper depleted rats of all ages exhibited significant ultrastructural pathology in myocytes despite lack of hypertrophy in older rats. Specifically increased mitochondrial volume densities, disarranged cristae, and non-aligned myofibrils with disturbances at Z-bands were observed. Significant pathology in all copper depleted rats at capillary-myocyte interface was demonstrated by increased distance between capillary endothelium and myocyte mitochondria, and fragmented basal laminae underlying the sarcolemma. All copper depleted rats displayed abnormal electrocardiograms, especially increased QRS amplitude and notching, and greater QT intervals, even without hypertrophy and anemia. These results suggest that capillary-myocyte interface changes may play a significant role in the developing pathology of copper depletion.
Introduction

A number of publications have reported numerous cardiovascular alterations in rats fed copper (Cu) deficient diets prior to or from weaning until 5 to 8 weeks thereafter. Cardiac hypertrophy and increased mortality (1-3), hemothorax and aneurysms (2-4), and abnormal ECG patterns (3, 5, 6) have been reported.

Similarly, several studies have reported increased area occupied by mitochondria in the myocyte of the Cu deficient rat heart (5-11). Our laboratory recently reported that the hypertrophied Cu deficient rat heart not only has an increase in this organelle component, but also the myofibril volume density appears to be greater as the hypertrophy or heart mass increases (6). Additionally, disruption of the mitochondrial fine structure and the presence of increased glycogen granules and lipid droplets have been reported by our laboratory and others (5, 8, 11) concomitantly with cardiac hypertrophy observed in the Cu deficient state. Distorted myofibrils, with poorly organized and non-aligned Z-bands have been described (9-11). These changes are largely similar to changes reported in cardiac hypertrophy associated with pathological cardiomyopathies and congestive heart failure in humans (12-16).

Reports on rats raised on a Cu adequate diet, followed by the feeding of a Cu deficient diet, have been limited. Additionally, cardiac histopathology and deleterious changes in cardiac function in the absence of cardiac hypertrophy have not been reported. Conversely, there appears to be a paucity of information on the reversibility of the cardiopathology in Cu deficient rats, when repleted with a Cu adequate diet. Viestenz & Klevay (3) did report a decrease in the electrocardiogram (ECG) abnormalities present in rats fed a Cu deficient diet from weaning, after being repleted for several weeks.

Studies on the histopathology at the level of the capillary-myocyte interface have been limited. Coronary vessels and the aorta have deleterious alterations in the Cu deficient state. Farquharson &
Robins (18) reported abnormal distribution of Type IV collagen in the endo- and perimysium of Cu deficient rat hearts, and fragmentation and disorganization of myocyte basal laminae, in the absence of vascular pathology and seemingly independent of fibrosis.

The objectives of the present study were to determine if rats raised on a Cu adequate diet from weaning for 5 wk, and then switched to a Cu deficient diet for another 6 wk would develop cardiac pathophysiology and abnormal ultrastructure. Similarly, we investigated whether rats fed a Cu deficient diet from weaning for 5 wk and then replened with a Cu adequate diet for another 6 wk would demonstrate a decrease in the occurrence of abnormal cardiac pathophysiology.

The results reported here revealed that older rats raised on a Cu adequate diet followed by a Cu deficient diet, develop abnormal ECG's and ultrastructure in the myocytes, as well as at capillary-myocyte interface, even in the absence of overt cardiac hypertrophy. Conversely, feeding a Cu adequate diet to rats raised on a Cu deficient diet, did reverse the hypertrophy and some of the ECG abnormalities, but did not reverse the ultrastructural damage. Finally, these results suggest that, in all conditions, one of the pertinent cardiac abnormalities in Cu deficiency resides in changes at capillary-myocyte interface, as demonstrated by increased distances between the capillary lumen and myocyte mitochondria and distortion of the myocyte basal laminae.

Materials and methods

Animals, diets and experimental protocol. The protocol for the study was approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

Thirty-one male weanling Long-Evans rats (Harlan Sprague-Dawley, Indianapolis, IN) were weighed on arrival, and randomly divided into 5 groups, so that mean group weights were similar. The groups were randomly assigned to either Cu adequate (AC, 3 groups, n=18) or Cu
deficient (NC, 2 groups, n=13) diets. At the end of 5 weeks, 1 group of 6 rats from each of the AC and NC groups were sacrificed to obtain baseline values and to confirm cardiac pathophysiology with Cu deficiency. The remaining 2 groups of rats fed the AC diet were randomly assigned to the AC control diet (ACC, n=6) or crossed over to the NC diet (NCD, n=6), to evaluate the effect of Cu depletion on cardiac function and ultrastructure in older rats. The remaining group of rats fed the NC diet from weaning was crossed over to the AC diet (NCR, n=7), to establish the effect of Cu repletion in previously Cu deficient rats. These latter 3 groups were maintained for a further 6 weeks on their respective diets.

All rats were fed a basal diet (US Biochemical Corp., Cleveland, OH), following the recommendations of the American Institute of Nutrition (19), consisting of 500 g sucrose, 150 g cornstarch, 200 g casein and 50 g corn oil/kg diet. The NC diet had no added Cu, while the AC diet had Cu added in the form of cupric carbonate, at a level of 6 mg/kg feed. The diets contained 0.4 mg Cu/kg diet (NC) and 5.88 mg Cu/kg diet (AC) as determined by flame atomic absorption spectrophotometry. This diet has been used consistently by our laboratory and has produced Cu deficiency among rats consuming the Cu deficient diet.

Rats were singly housed in stainless steel cages, in a room with a 12 h light-dark cycle and mean temperature of 21.7 ºC, with free access to deionized-distilled water, and fed ad-libitum. Rats were weighed weekly.

ECG's were performed on the AC and NC groups at the end of 5 weeks, and ECG's and tail cuff blood pressures on the ACC, NCD and NCR groups at the end of 11 weeks of the study. The following day rats were anesthetized by CO2 inhalation and the thoracic cavities opened for the necessary measurements as described below.
Electrocardiograms. Rats were anesthetized with ketamine (85 mg/kg) and xylazine (15 mg/kg) intraperitoneally. The ECG's were recorded, using leads I, aVF and V₃, at 100 mm/s paper speed on a photographic oscillograph with frequency response flat to over 1 000 Hz, using subcutaneous needle electrodes, as described in Medeiros et al (6). The ECG parameters measured and statistically analyzed from the V₃ lead were heart rate (beats/min), duration of P, PQ, QRS and QT waves (msec), amplitudes of QRS, R and S waves (mm), and the ratio of R to S waves (R:S). Subjective interpretation of ECG patterns was done on all 3 leads, with respect to gross indications of disturbances in electrical conduction (abnormalities in P waves and PR intervals, notching in the QRS complex), and evidence of left ventricular hypertrophy and/or intraventricular conduction disturbance (large QRS and R wave amplitude, QRS wave prolongation and notching in the QRS complex).

Preparation of samples for TEM. After the thoracic cavities were opened, <0.5 mL blood was removed by cardiac puncture and placed in heparinized tubes for hematocrit analysis. The hearts were arrested in diastole by injection of 1 mol/L KCl into the right ventricle, removed, rinsed in Dulbecco's phosphate-buffered saline (Gibco Laboratories Life Technologies, Grand Island, NY) at 37°C, weighed, and then perfused in 2.5% glutaraldehyde in 0.1 mol/L Na cacodylate in .1 mol/L sucrose (Electron Microscopy Sciences, Fort Washington, PA) at pH 7.4 and 22°C. Samples of the left ventricular free wall were cut tangentially to the outer wall of the left ventricle to obtain fibers in the longitudinal plane. Processing of tissue into blocks for TEM was based on the method described by Medeiros et al (6). Briefly, pieces less than 1 mm were fixed in 2.5% glutaraldehyde in 1 mol/L Na cacodylate in .1 mol/L sucrose, post-fixed in 1% osmium tetroxide (Polysciences Inc., Warrington, PA), dehydrated in a series of increasing concentrations of acetone, and embedded in Spurr resin (Ladd Research Industries Inc., Burlington, VT) in embedding molds, orientated so that outer muscle wall
could be sectioned. Samples in resin blocks were first thick-sectioned (± 900 nm) by an ultramicrotome (Sorball MT2B, Ivan Sorball Inc., Norwalk, CT) with glass knives, and orientated under a light microscope to ensure the presence of muscle fibers in a longitudinal plane. Samples were then thin-sectioned (± 90 nm) with a Diatome diamond knife (Fort Washington, PA) and the sections caught on 75 X 300 mesh Cu grids. Random sections from each Cu grid were examined by TEM (Philips EM300, Eindover, the Netherlands, at 60 kV), and negatives were developed and printed.

**Morphometric analysis.** Electron micrographic prints (12 500 X) were analyzed morphometrically by overlay of a plastic grid with 1 cm square divisions. Mitochondrial, myofibrillar and "other" volume densities (μm³/μm³) were determined by a point system as described by Weibel (20) and Steer (21). By definition, "other" included volume density occupied by intracellular material other than clearly defined mitochondria and myofibrils. Mean score for each rat was calculated from mean scores of 2 blocks/rat. Orientation of the muscle fibers in a longitudinal plane allowed for decreased variability in the measurements.

A four point qualitative scale was used for analysis of the capillary-myocyte interface in a blind manner by examination of electron micrographic prints (27 000 X). The myocyte sarcolemma, capillary endothelial cell membrane and basal laminae were evaluated. The normal appearance of these structures includes membranes with minimal convolutions, intact basal laminae, narrow intermembrane areas, and normally arrayed myofibrillar and mitochondrial ultrastructure, with minimal accumulated debris. Samples with these characteristics were assigned a maximum value of 4. Samples exhibiting gross pathology, convoluted membranes, broad intermembrane areas, obviously damaged basal laminae, disarrayed mitochondria and accumulated intermembrane and subsarcolemmal debris, were assigned values approaching 1. The 1-4 point scale had increments of 0.5. Mean values were obtained by averaging 2
prints. To check for accuracy, a subsample of prints were scored several weeks later and scores were compared.

**Superoxide dismutase activity.** Liver SOD activity, to assess relative Cu status of rats in the different treatment groups, was determined spectrophotometrically, based on the autoxidation of pyrogallol, as described by Marklund (22) and modified by Prohaska (23). SOD was extracted from approximately 1 g of homogenized liver tissue by chloroform-ethanol. The auto-oxidation of pyrogallol was determined in TAPS buffer, without added liver sample, to serve as reference. The amount of pyrogallol added to give an absorbance of 0.016 dA/30 s was determined, followed by determination of the amount of liver sample to reduce the absorbance to 0.008 dA/30 s. One unit of SOD activity was defined as the amount of activity to inhibit the auto-oxidation of pyrogallol by 50%, expressed as U/g.

**Hematocrit.** Blood obtained by cardiac puncture was drawn into heparinized tubes and hematocrit determined using a microhematocrit centrifuge.

**Statistical analysis.** The dependent variables of body weight, heart weight, heart to body weight ratio, hematocrit, SOD, ECG measurements, myocardial, myofibril and other volume densities, mitochondrial to myofibrillar ratio, and capillary-myocyte interface scores for all groups, and blood pressure for the latter 3 groups, were analyzed, using the Statistical Analysis System (SAS Institute, Cary, NC). General Linear Models Procedure (GLM) was used to determine significant differences between means by ANOVA. Where significant F values were obtained the least significant difference (LSD) procedure to determine which group means differed from one another was used. A repeated measures analysis of variance was performed to determine significant differences in means of body weight by treatment and weeks of treatment. The alpha level was set a priori at 0.05.
**Results**

Body wt were significantly lower (*P*<0.05) in NC than in AC rats, as well as in NCD than in ACC rats, with no significant difference (*P*>0.05) between NCD and NCR, and NCR and ACC rats (Table 1). The lower body wt in the Cu deficient rats, could be due to decreased growth rates. Liver SOD analyses showed a highly significant difference (*P*<0.0001) in activity between rats fed a Cu adequate and deficient diet, as well as between the 2 older experimental groups. Liver SOD activity of rats in the AC and ACC groups was not significantly different (*P*>0.05, 20,000 - 24,000 U/g), as opposed to 4,000 - 5,000 U/g in the NC rats; 9,000 - 12,000 U/g in the NCD rats; and 16,000 - 19,000 U/g in the NCR rats. Hematocrit of NC rats was significantly lower (*P*<0.05) than that of all other groups. However, there was no statistical difference (*P*>0.05) in hematocrit between any of the older groups.

Cardiac hypertrophy was clearly established in the younger Cu deficient rats. NC rats had significantly greater absolute HW (1.61 g) than AC (1.06 g) and NCD (1.32 g) rats, as well as greater H:BW (*P*<0.001) than all other experimental and control rats. In the older rats, absolute HW was significantly greater only in the NCR (1.54 g) than in NCD rats, while H:BW was not significantly different (*P*>0.05) between any of the older rat groups (Table 1).

Subjective evaluation of cardiac tissue consistency suggested that rats fed Cu adequate diets had firm hearts with regular shapes. However, the cardiac texture of Cu deficient rats differed between the various experimental groups. Young NC rat hearts felt soft and flabby with no evidence of gross fibrosis or ischemia. The older NCR rat hearts seemed mainly soft, with areas of toughness and damage, whereas the older NCD rat hearts displayed a tougher, rubbery texture, with some evidence of gross ischemia.
TABLE 1

Body and heart wt, heart:body wt, liver SOD and hematocrit in rats fed Cu adequate and deficient diets

<table>
<thead>
<tr>
<th>Indices</th>
<th>AC</th>
<th>NC</th>
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<td>n = 6</td>
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<td>n = 6</td>
<td>n = 7</td>
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</tr>
<tr>
<td>Body wt²,g</td>
<td>221±10</td>
<td>166±6</td>
<td>415±11</td>
<td>396±23</td>
<td>369±14</td>
</tr>
<tr>
<td>Heart wt³,g</td>
<td>1.1±0.08</td>
<td>1.6±0.11</td>
<td>1.5±0.04</td>
<td>1.6±0.08</td>
<td>1.3±0.05</td>
</tr>
<tr>
<td>Heart:body wt⁴, X 10⁻³³</td>
<td>4.8±0.19</td>
<td>9.8±0.89</td>
<td>3.5±0.13</td>
<td>3.9±0.12</td>
<td>3.6±0.09</td>
</tr>
<tr>
<td>Liver SOD⁵, U/g</td>
<td>21 857±442</td>
<td>4 333±140</td>
<td>21 800±390</td>
<td>17 522±315</td>
<td>10 133±390</td>
</tr>
<tr>
<td>Hematocrit⁶</td>
<td>0.49±0.008</td>
<td>0.36±0.035</td>
<td>0.42±0.008</td>
<td>0.42±0.014</td>
<td>0.43±0.012</td>
</tr>
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</table>

¹Values are expressed as means ± SEM. Data were analyzed by ANOVA and LSD where applicable.

²Body wt was significantly less (P<0.05) in NC than AC rats; and NCD than ACC rats. NCR body wt was not significantly different (P>0.05) than that of ACC or NCD rats.

³Younger NC hearts weighed significantly more (P<0.05) than AC and NCD hearts; older NCR hearts weighed significantly (P<0.05) more than NCD hearts; there was no significant difference (P>0.05) between NCR, ACC and NC heart wt.

⁴Heart:body wt was significantly greater (P<0.05) in NC than all other groups; heart:body wt was not significantly different (P>0.05) between any of the older groups.

⁵Liver SOD was significantly lower (P<0.05) in rats fed Cu deficient (NC, NCR, NCD) than Cu adequate (AC, ACC) diets; and in the NCR than NCD group.

⁶Hematocrit was significantly lower (P<0.05) in the NC than all other groups; there was no significant difference (P>0.05) between any of the older groups.
**Histopathology.** Results from TEM disclosed significant cardiac pathology in both younger and older rats fed Cu deficient diets (Table 2). Morphometric analysis revealed all rats fed Cu deficient diets had significantly higher ($P<0.0001$) mitochondrial (VMITO) and "other" (VOTHER) volume densities and mitochondrial:myofibrillar volume density (VMITO:VMYO) of left ventricular myocytes compared to those of rats fed Cu adequate diets. There were no significant differences ($P>0.05$) in VMITO and VOTHER between older or younger groups on similar diets, suggesting that there was no age, but only a treatment, effect. However, VMITO of NCD rats (0.508±0.022) was significantly greater ($P<0.05$) than VMITO of NCR rats (0.462±0.009), while VMITO of NCR rats was not significantly different ($P>0.05$) from that of NC rats. VOTHER of NCR myocytes (0.142±0.007) was significantly greater ($P<0.05$) than that of AC (0.045 ± 0.003), ACC (0.042 ± 0.001), and NCD (0.100±0.010) rats, with no difference between NC (0.112±0.008) and NCD rats, suggesting differences between Cu depleted, repleted and adequate rats. VMYO of NC and NCR rats was similar ($P>0.05$), but significantly lower ($P<0.05$) than that of ACC and AC rats, while VMYO of NCD rats was not significantly different ($P>0.05$) from other groups, illustrating differences between depletion stages.
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<tr>
<td>VMITO$^2$ $\mu m^3/\mu m^3$</td>
<td>0.318 ± 0.010</td>
<td>0.482 ± 0.013</td>
<td>0.322 ± 0.020</td>
<td>0.462 ± 0.009</td>
<td>0.508 ± 0.022</td>
</tr>
<tr>
<td>VMYO$^3$ $\mu m^3/\mu m^3$</td>
<td>0.590 ± 0.009</td>
<td>0.375 ± 0.018</td>
<td>0.650 ± 0.021</td>
<td>0.396 ± 0.007</td>
<td>0.528 ± 0.132</td>
</tr>
<tr>
<td>VOTHER$^2$ $\mu m^3/\mu m^3$</td>
<td>0.045 ± 0.003</td>
<td>0.112 ± 0.008</td>
<td>0.042 ± 0.001</td>
<td>0.142 ± 0.007</td>
<td>0.100 ± 0.010</td>
</tr>
<tr>
<td>VMITO:VMYO$^2$</td>
<td>0.541 ± 0.025</td>
<td>1.375 ± 0.107</td>
<td>0.502 ± 0.044</td>
<td>1.170 ± 0.038</td>
<td>1.164 ± 0.195</td>
</tr>
<tr>
<td>Cap-myoc interface$^4$</td>
<td>3.33 ± 0.20</td>
<td>1.67 ± 0.25</td>
<td>2.88 ± 0.17</td>
<td>1.71 ± 0.16</td>
<td>1.79 ± 0.16</td>
</tr>
</tbody>
</table>

VMITO = volume density of mitochondria; VMYO = volume density of myofibrils; VOTHER = volume density of material other than clearly defined mitochondria and myofibrils; VMITO:VMYO = ratio of mitochondria to myofibrils.

1Values are expressed as means ± SEM. Data were analyzed by ANOVA and LSD where applicable.
2Volume densities of mitochondria, "other" material and mitochondria:myofibrils were significantly greater ($P<0.0001$) in both younger and older rats fed Cu deficient diets compared to rats fed Cu adequate diets. VMITO in NCD was significantly greater ($P<0.05$) in NCD than NCR, with no difference ($P>0.05$) between NC and NCR myocytes. VOTHER of NCR was significantly greater ($P<0.05$) than that of NCD, with no difference ($P>0.05$) between NCD and NC. There was no significant difference ($P>0.05$) in VMITO:MYO between any of the experimental groups.
3Volume density of myofibrils was significantly lower in NC than AC, and in NCR than ACC ($P<0.05$) rat myocytes, but there were no significant differences ($P>0.05$) between NCD and any other group.
4Capillary/myocyte interface integrity was significantly lower ($P<0.05$) in all experimental groups. There was no significant difference ($P>0.05$) between NCD and NCR groups.
The increased volume density of mitochondria was clearly illustrated in the micrographs of individual myocytes. (Plate I). Additionally, there was obvious disarray of myofibrillar and mitochondrial ultrastructure among NC, NCR and NCD rats, compared to AC and ACC rats (Plate I). Mitochondrial cristae Cu depleted younger and older rats appeared fragmented and disorganized, with disturbed inner and outer membranes, and translucent matrices. Myofibrils, in more of the Cu deficient compared to the Cu adequate myocytes, were sparse with considerable loss of normal organization, highly convoluted intercalated discs, accumulation of Z-band material and some splitting of Z-bands. In all experimental myocytes, higher concentrations of glycogen granules were visible in myocytes, and nuclei were often contorted, sometimes with bizarre shapes.
Transmission electron micrographs of cardiac myocytes from Cu adequate (a) young and (c) older, and Cu deficient (b) younger and older Cu (d) repleted and (e) depleted rats. Both younger and older Cu deficient rats displayed increased volume densities of mitochondria, sparse and disorganized mitochondrial cristae, and reduced and disorganized myofibrils, with Z-band splitting. M = mitochondria; my = myofibrils; G = glycogen granules; L = lipid droplet; o = other. Bar = 1 μm.
PLATE I

Volume densities of mitochondria and myofibrils
PLATE I (continued)

Volume densities of mitochondria and myofibrils
PLATE I (continued)

Volume densities of mitochondria and myofibrils
PLATE I (continued)

Volume densities of mitochondria and myofibrils
PLATE I (continued)

Volume densities of mitochondria and myofibrils
Evaluation of the capillary-myocyte interface evidenced a significantly higher ($P<0.001$) amount of pathology in the myocytes of rats fed Cu deficient, compared to rats fed Cu adequate diets (Table 2 and Plate II). Thesarcolemmal membranes of myocytes in experimental rats seemed more convoluted and asymmetrical, with seemingly fragmented and thickened basal laminae evident on some of the Cu deficient rat micrographs. Sarcolemmal ballooning with spherical particles in close proximity to the basal laminae was evident in more of the experimental compared to the control rats. Subsarcolemmal accumulation of debris was evident, intermembrane space was significantly increased, and pericapillary collagen deposition appeared increased in most cases in experimental compared to control rats. Capillary membranes seemed less affected than myocyte membranes. Significant distortion of mitochondria and myofibrillar disarray were more frequently observed in the subsarcolemmal area in myocytes of NC, NCR and NCD rats, compared to control rats. However, there were no significant ($P>0.05$) differences in pathology at the capillary-myocyte interface between NC, NCR and NCD rats (Table 2 and Plate II).
Transmission electron micrographs of the capillary-myocyte interface of left ventricles from Cu adequate (a) young and (c) older, and Cu deficient (b) younger and (d) older Cu repleted and (e) depleted rats. Histopathology is evident in all Cu depleted rats, regardless of age. The sarcolemma is convoluted and asymmetrical, with broad inter-membrane spaces and subsarcolemmal debris and disorganized myofibrils and mitochondrial disarray. Basal laminae appear focally fragmented and thickened, and pericapillary collagen appears increased. S = sarcolemma; BL = basal laminae; A = intermembrane area; C = capillary. Bar = 1 μm.
PLATE II

Histopathology
PLATE II (continued)

Histopathology
PLATE II (continued)

Histopathology
PLATE II (continued)

Histopathology
PLATE II (continued)

Histopathology
Electrocardiograms. There was clearly no significant difference (P>0.05) in blood pressure between the experimental and control groups (Table 3). Heart rate was significantly lower (P<0.05) in the older compared to the younger rats. However, experimentally, heart rate was significantly lower only in the NC, compared to the AC rats (P<0.05).

There was no statistical difference (P>0.05) in PR interval, S wave amplitude and R:S between any of the groups. NCR rats had a longer P wave duration (P<0.01) than all other groups (Table 3). The patterns displayed by QRS wave duration and R wave amplitude were similar. There were no differences (P>0.05) in QRS wave duration or R wave amplitude between the younger deficient (NC) and older depleted (NCD) rats, while they were both significantly greater than in the repleted (NCR), ACC and AC rats. Similarly, there was no significant difference (P>0.05) in either QRS wave duration or R wave amplitude between NCR, ACC and AC rats. NC rats had significantly higher (P>0.05) QRS wave amplitude and greater QT interval duration than all other rats. NCD rats also had significantly higher (P>0.05) QRS wave amplitude than NCR, ACC and AC rats, while that of NCR, ACC and AC was similar. However, there was no significant difference (P>0.05) between QT interval duration of NCD and NCR rats, while that of NCD was still greater than AC and ACC rats. There were no significant differences (P>0.05) between younger (AC) and older (ACC) rats in QRS wave duration or amplitude, R wave amplitude, or QT interval duration, suggesting that the differences observed were not due to differences in age, but may be related to stage of Cu depletion.
# TABLE 3

## ECG parameters and blood pressure in rats fed Cu adequate and deficient diets

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<td>Systolic blood pressure&lt;sup&gt;2&lt;/sup&gt;, mm Hg</td>
<td>-</td>
<td>-</td>
<td>111</td>
<td>120</td>
<td>109</td>
</tr>
<tr>
<td>Heart rate&lt;sup&gt;3&lt;/sup&gt;, beats/min</td>
<td>351 ±16.2</td>
<td>320 ±8.3</td>
<td>291 ±9.2</td>
<td>295 ±5.9</td>
<td>277 ±10.6</td>
</tr>
<tr>
<td>P wave&lt;sup&gt;4&lt;/sup&gt;, ms</td>
<td>15±1.8</td>
<td>15±0.2</td>
<td>14±0.8</td>
<td>19±1.3</td>
<td>16±0.8</td>
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<tr>
<td>P-R interval&lt;sup&gt;2&lt;/sup&gt;, ms</td>
<td>48±1.1</td>
<td>48±1.7</td>
<td>52±2.5</td>
<td>52±1.8</td>
<td>47±3.1</td>
</tr>
<tr>
<td>QRS duration&lt;sup&gt;5&lt;/sup&gt;, ms</td>
<td>12.5 ±1.12</td>
<td>17.7 ±2.0</td>
<td>13.3 ±1.05</td>
<td>12.3 ±0.97</td>
<td>15 ±0.00</td>
</tr>
<tr>
<td>QRS amplitude&lt;sup&gt;6&lt;/sup&gt;, mV</td>
<td>1.88 ±0.087</td>
<td>2.65 ±0.313</td>
<td>1.38 ±0.095</td>
<td>1.37 ±0.157</td>
<td>2.02 ±0.236</td>
</tr>
<tr>
<td>R amplitude&lt;sup&gt;5&lt;/sup&gt;, mV</td>
<td>1.6±0.10</td>
<td>1.9±0.19</td>
<td>1.1±0.12</td>
<td>1.2±0.22</td>
<td>1.8±0.24</td>
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<td>S amplitude&lt;sup&gt;2&lt;/sup&gt;, mV</td>
<td>0.3±0.07</td>
<td>0.7±0.16</td>
<td>0.3±0.20</td>
<td>0.8±0.71</td>
<td>0.2±0.03</td>
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<tr>
<td>R:S&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.6±1.6</td>
<td>4.0±1.1</td>
<td>9.6±3.3</td>
<td>14.2±4.1</td>
<td>11.6±1.9</td>
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<tr>
<td>QT duration&lt;sup&gt;7&lt;/sup&gt;, ms</td>
<td>67±3.3</td>
<td>100±4.5</td>
<td>66±3.0</td>
<td>68±2.4</td>
<td>77±3.6</td>
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</table>

<sup>1</sup> Values are expressed as means ± SEM. Data were analyzed by ANOVA and LSD where appropriate.
<sup>2</sup> There were no significant differences (P>0.05) in systolic blood pressure, P-R interval, S heights or R:S between any of the groups.
<sup>3</sup> Heart rate of NC rats was significantly lower (P<0.05) in NC than AC groups, and in older than younger rats.
<sup>4</sup> P wave duration was significantly (P<0.01) greater in NCR than all other groups. P intervals were not significantly different (P>0.05) between any other groups.
<sup>5</sup> QRS duration and R heights were not significantly different (P>0.05) between NC and NCD, or NCR, AC and ACC; while both NC and NCD were significantly greater (P<0.05) than the others.
<sup>6</sup> QRS amplitude was significantly higher (P<0.05) in NC than in all others, and in NCD than in AC, ACC and NCR rats. There was no significant difference (P>0.05) between NCR, AC and ACC.
<sup>7</sup> Q-T intervals were significantly longer in NC than all other groups (P<0.05) and in NCD than ACC rats (P<0.05), however, there was no significant difference (P>0.05) between NCD and NCR groups.
Subjective evaluation of ECG patterns indicated the same pattern as suggested by the quantitative ECG measurements (Figure 1). NC rats displayed disturbances in the QRS complex, as compared to AC rats. Notching was present in the QRS complex of all 6 NC rats, as compared to 2 of AC rats in lead aVF. The most notable differences between the experimental and control groups were greater QRS amplitudes and notching of the QRS complex in leads aVF and V3, compared to control rats. Three of the NCR rats displayed notching in lead V3, compared to none in the ACC group. NCD rats tended to exhibit more frequent notching in the QRS complex in lead V3 compared to the ACC rats.
Representative ECG tracings from Cu adequate younger (AC) and older (ACC), and Cu repleted (NCR) and Cu depleted (NCD) older rats. There is more notching (arrows) in leads aVF and V3 in the QRS complex in rats fed a Cu deficient than in rats fed a Cu adequate diet. QRS amplitude is increased in the NC and NCD rats.
<table>
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<tr>
<th>Leads</th>
<th>AC 642</th>
<th>NC 645</th>
<th>ACC 658</th>
<th>NCR 628</th>
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<tr>
<td>I</td>
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<tr>
<td>V₃</td>
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<td><img src="image" alt="V₃ lead tracing" /></td>
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</table>

10 mm = 1 mV
Discussion

The functional and ultrastructural pathophysiology of Cu deficient cardiomyopathy has been primarily focussed on feeding rats a Cu deficient diet prior to or at weaning age for varying lengths of time. There is a paucity of information pertaining to the effects of Cu restriction in post weaning and older rats, as well as the effects of Cu repletion in previously Cu deficient rats. Reports of the simultaneous investigation of biochemical, functional and ultrastructural factors are limited.

*Superoxide dismutase.* In this study, liver SOD activity was found to vary with dietary Cu manipulation. There were clear differences between the SOD activity in Cu adequate and deficient rats, while the SOD activity was not different between younger and older rats (5 and 14 wk of age) fed Cu adequate diets, suggesting that liver SOD activity was not significantly affected by age. The younger NC rats had significantly lower SOD activity than all other groups and may reflect severity of depletion. The group in which Cu restriction was induced at 8 wk of age (NCD), had significantly lower SOD activity than the 6 wk repleted rats (NCR). This may reflect the magnitude of repletion, and indicates that liver Cu SOD activity may be a sensitive measure of both Cu depletion and repletion. Although cardiac SOD activity was not measured in this study, others have reported significant decreases in SOD activity in Cu deficient rats (24) and mice (23). Since the cytoplasmic Cu,Zn-superoxide dismutase is important in the cellular defense against free radical and superoxide damage it is conceivable that lowered SOD activity may be a factor in the ultrastructural pathology. In a study by Saari & Medeiros (25), administration of a powerful antioxidant, dimethyl sulfoxide, reduced heart wt and mitochondrial and myofibrillar pathology in Cu restricted rats, even though decreased free radical production as measured by thiobarbital could not be shown.
Hematology. As previously reported in young rats (2,5,11), hematocrit (HCT) was significantly lower in NC compared to AC rats. However, no such difference was found among any of the older (14 wk of age) control (ACC) or experimental (NCD, NCR) groups. Even in the younger rats, the decline in HCT, although statistically significant, did not nearly approach the values of anemia. It is therefore unlikely that anemia was primarily involved in the development of the cardiac pathology observed in these rats. This is in agreement with the suggestions of Medeiros et al (11) and others (1).

Gross pathology and myocardial hypertrophy. In human and animal models of cardiomyopathies, and physiological hypertrophy, myocardial hypertrophy is secondary to increased hemodynamic demand, due to pressure (concentric hypertrophy) or volume (eccentric hypertrophy) overload (12-14,17,26). This hypertrophy is associated with adaptive growth, increased muscle fiber thickness and length, and connective tissue hyperplasia. The hypertrophy enters a stable phase where the myocardial enlargement allows for continued function, with stable metabolism, but failure ensues when the metabolic and functional reserve of the heart becomes exhausted (12, 17). The gross pathology of Cu deficient cardiomyopathy in young rats is mixed, but has predominantly concentric hypertrophy similar to that produced by pressure overload. The ventricular outer wall and septum are thickened due mainly to lateral myocyte hypertrophy, with greatly reduced ventricular cavities (2, 3, 5, 6, 11).

As reported by others, there was a marked myocardial hypertrophy in this study in young rats fed Cu deficient diets. The HW in the NC group was significantly greater than all other groups, even than older control ACC, and NCD and NCR groups. Interestingly, HW was significantly lower in NCD than in NCR and ACC rats, demonstrating the absence of hypertrophy in the group that was Cu depleted from 8 weeks of age, after being fed an adequate diet from weaning. Similarly, no
cardiac hypertrophy in the older Cu depleted rats was apparent when hypertrophy was expressed as H:BW. This indicates that the functional and ultrastructural changes discussed below, developed despite the absence of overt myocardial hypertrophy in these rats.

**Electrocardiograms.** Reported blood pressure response to dietary Cu deficiency is variable and may be specie and age dependent. Blood pressure appears to be decreased in younger Cu deficient rats (27-29), while blood pressure may be increased in older rats (30,31). In this study, blood pressure was not measured for the young Cu deficient rats, but there was no significant difference in blood pressure between any of the older groups. Apparently, in this instance, increased blood pressure was not a prerequisite for the developing myocardial pathology observed in these rats.

ECG abnormalities and changes in heart rate in Cu deficiency have been reported in a number of studies. Prohaska & Heller (8) reported decreased heart rates in Cu deficient rats, while Kopp et al (5) found no significant change in heart rate, although heart rate was somewhat lower in Cu deficient rats. Abnormal ST segments, increased PR intervals, increased R wave amplitude and duration (3, 5, 6), some ventricular and supraventricular beats have been found. Kopp et al (5) reported increased H-V intervals underlying the prolonged PR interval, in His Bundle electrogram, suggesting slow conductivity within the His-Purkinje system, while Medeiros et al (6) also reported increased QT interval duration and QRS wave amplitude.

In the present study, except for the young NC rats who had a lower heart rate than AC rats, there was no experimental difference in heart rate between any of the groups. The predominant ECG abnormalities were centered in the QRS complex and QT interval. The deviations from normal were more explicit in the younger Cu deficient (NC) rats than in any other group, except in older Cu depleted (NCD) rats where some deviations appeared. NCD rats had the same QRS wave duration and R wave
amplitude as NC rats, and lower QRS wave amplitude and QT interval duration than that of NC rats, but these measurements were consistently greater than that of NCR and ACC rats. Subjective evaluation revealed a higher incidence of notching in the QRS complex in Cu depleted rats compared to rats fed Cu adequate diets. In many instances NCR rats showed responses similar to ACC rats. These results indicate that, despite the absence of myocardial hypertrophy, anemia or hypertension, ECG changes associated with functional pathology were found in rats that were Cu depleted after 8 weeks of age. Also, it would appear that the repleted rats were regressing toward normal, although some damage was still evident.

**Morphometrical and ultrastructural changes in myocytes.** Changes in volume density of myocellular components in Cu deficient cardiomyopathy in young rats fed Cu deficient diets prior to, or from, weaning have been shown in many studies. Increased volume density of mitochondria (VMITO) due to increased size and possibly number of mitochondria have been documented (5, 10, 11). Recently, Medeiros et al (11) indicated that increased myofibrils also contribute to the hypertrophy of myocytes in Cu deficient rats. Ultrastructural changes indicated deterioration of mitochondrial and myofibrillar integrity, and the presence of "other" material in abnormal proportion. In young Cu deficient rats disruption of mitochondrial fine structure, with fragmented cristae, vacuolation, translucent matrix (11), and damage to inner and outer mitochondrial membranes (5) have been reported, with distorted myofibrils and non-aligned Z-bands.

This study revealed similar changes in volume density and organelle abnormalities in both younger and older Cu depleted rat myocytes. Specifically, the VMITO and VMITO:VMYO and VOTHER of Cu depleted myocytes of all age groups were significantly greater than that of Cu adequate controls, although NCR VMITO was significantly lower than that of NCD rats, indicating a difference between depletion and
repletion stages. VMYO was significantly decreased only in NC and NCR rat myocytes, signifying a myofibrillar contribution to ventricular hypertrophy in young Cu deficient rats, and rats repleted after Cu deficiency, respectively.

These findings are similar to those in other models of hypertrophic cardiomyopathies in both animals and humans with the onset of myocardial failure, in ultrastructure, but not volume densities. Typically, there is a transient increase in VMITO, followed by increased sarcomerogenesis, and essentially normal VMITO:MYO and ultrastructure of organelles, until the onset of failure, when there may be a relative increase in VMITO due to degeneration of the contractile units, and degenerative changes in mitochondrial and myofibrillar ultrastructure similar to the changes in Cu deficiency (13-15). Diffuse interstitial fibrosis, distortion of myocyte shape, increased size and basophilia of nuclei, expansion and splitting of Z-bands and accumulation of Z-band material, myofibrillar disarray and increased intercalated disc size and tortuosity of shape have been reported in these other models (32, 33). In severe cardiac overload and excessive exercise, degenerative changes may become apparent much earlier (33).

**Histopathology of capillary-myocyte interface.** Studies reporting histopathological changes of the capillary-myocyte interface in Cu deficiency are limited. Of importance in the developing cardiomyopathy would be the type and amount of change in the collagen surrounding the myocytes and capillaries, the integrity of the membranes and supporting organelles subcellularly.

Capillary insufficiency had been reported in myocardial pathology in humans (35), the spontaneously hypertensive rat (36), and other rat models (37), as well as disorientation of capillaries, due to increased interstitial collagen, and myocytes with mitochondria being shifted away from the capillary interface (15). The ratio of capillary:myocyte number is thought to remain unchanged at 1:1 after weaning in rats and
humans. As the myocyte hypertrophies with cardiomyopathy, the distance between the capillary lumen and myocytal mitochondria will increase. Similarly, distortion of the normal arrangement of mitochondria in the myocyte relative to capillary location, may serve to increase the relative distance of individual mitochondria to capillary lumen. Both of these factors may serve to increase oxygen diffusion distance, thereby creating relative ischemia. There is little possibility that the process of oxygen diffusion per se may be interfered with due to capillary-myocyte pathology, since oxygen diffuses freely and quickly, not only from capillaries, but also from arterioles. Previously, increased epi- and endomysial collagen deposition in Cu deficiency had been reported (9, 38) and a change in the relative synthetic and degradative rates of Types III and I collagen was suggested by Dawson et al (39). The diminished strength of the collagen in Cu deficiency was linked to insufficient 3-OH pyridinium crosslinking of collagen, due to reduced activity of the cuproenzyme, lysyl oxidase (38).

Thickening of the basal laminae surrounding the myocyte sarcolemma, with cellular distortion, and the presence of subsarcolemmal spherical particles in close proximity to basal laminae, with the appearance of "budding" were illustrated in progressive myocardial failure without Cu deficiency (13). Leigh (40) first described abnormalities in the boundaries between capillaries and adjacent myocytes in Cu deficient steers compared to steers fed adequate Cu diets, specifically, ballooning of the sarcoplasma and sarcoplasmic reticulum, expanded intercellular space, presenting a scalloped appearance. Recently, Farquharson & Robins (18), using immunohistochemical methods in Cu deficient rat hearts, confirmed the presence of abnormal Types III and I collagen in focal areas, associated with necrosis and fibrosis. Type IV collagen was found exclusively in the basal laminae surrounding the capillaries and myocytes. Focal areas of distortion, fragmentation, and disorganization of a sometimes
thickened or collapsed basal laminae supporting myocytes were indicated, whereas capillaries were less affected.

In the present study, a significant increase in pathology at the capillary–myocyte interface was found in all groups fed a Cu restricted diet, compared to control groups. No significant age difference was indicated, suggesting that differences observed were due to depletion of Cu. The pathology revealed both apparent increased distances between capillary lumen and sarcolemmal surface, and disruption of mitochondria with shifts in normal mitochondrial distribution. In agreement with Farquharson & Robins (18) it seemed as if the capillary wall was less affected than that of the sarcolemma. These changes suggest it would be possible for increased oxygen distances to exist, and raise the question whether it may be involved in the development of myocardial pathology. Could the swelling of the mitochondria be an attempt to increase surface area to maximize uptake of both oxygen and Cu to increase mitochondrial respiration?

It was more difficult to evaluate the actual pathology of the basal laminae by investigation of our micrographs. Areas of distortion and fragmentation of the basement membrane underlying the sarcolemma were more noticeable in the Cu deficient as compared to the Cu adequate groups, while there was no age difference between any of the treatment and control groups. The basement membrane underlying the capillary endothelium seemed relatively unaffected by Cu deficiency. The basal lamina forms an extremely tough, thin mat underlying endothelial and cellular membranes, composed of 3 layers. It consists mainly of Type IV collagen, with the adhesive proteoaminoglycan laminin, and fibronectin to connect it to underlying and surrounding collagen. The functions of the basal laminae include structural support of the membranes, adhesion of cellular array, filtering of metabolites, cell polarity, membrane organization and cell behavior (41). The constituents of basal laminae are stabilized by disulfide and other co-valent crosslinks, but the main
strength of Type IV collagen is derived from hydroxy-lysine and lysine residue deaminative crosslinking catalyzed by the cuproenzyme, lysyl oxidase (41). The connection between dietary Cu deficiency, decreased lysyl oxidase activity and collagen strength had been illustrated (42).

Should alterations in basal laminae due to defective deaminative crosslinking prove to be an early response to Cu deficiency, it may be one of the mechanisms in the etiology of functional and ultrastructural changes in Cu deficiency. It is conceivable that the extensive changes observed in the sarcolemma and subsarcolemmal area may have profound cellular effects.
Summary.

It is clear that dietary Cu deficiency induces a cardiac pathology similar to that seen in other animal models and humans. Despite lack of overt myocardial hypertrophy, increased blood pressure and anemia, typical functional and ultrastructural pathology was found when Cu depletion was induced in rats that had been fed a Cu adequate diet from weaning to 8 weeks of age. Rats that were Cu repleted after being fed a Cu deficient diet from weaning for 5 weeks, showed significant signs of regression of pathology, however after 6 weeks some abnormalities, both in function and ultrastructure, remained. Although Cu deficiency mimics other models of cardiomyopathies, the rapidity of onset of pathology and the response of mitochondrial volume densities are characteristic of Cu deficiency. The question remains as to what is the primary stimulus in the development of Cu deficient cardiomyopathies? It is clear that the etiology is complex and multifactorial, involving decreased activity of several key cuproenzymes and their influence on collagen crosslinking and cellular respiration. Other enzymes, e.g. SOD, may contribute to the developing pathology. The role of the basal laminae, underlying capillary endothelium and myocyte sarcolemma, warrants further investigation, since our data suggest a defect in this substance could initiate the onset of pathology observed in Cu deficient rats.
List of references


CHAPTER IV
DIETARY INDUCED COPPER DEFICIENT CARDIOMYOPATHY IN OLDER RATS: INFLUENCE OF SUB-MAXIMAL, AEROBIC EXERCISE TRAINING

Abstract
The dual effects of copper depletion and aerobic sub-maximal exercise training on myocardial function and ultrastructure, and the oxidative capacity of soleus muscle, were investigated in older rats. Rats were fed either copper adequate or deficient diets and were either trained or sedentary for 8 wk. Results support the development of characteristic myocyte degeneration and increased myocardial mitochondrial volume density of copper depletion cardiomyopathy in older rats, despite lack of overt cardiac hypertrophy. Training combined with copper depletion induced mild left ventricular hypertrophy. Electrocardiographic tracings revealed increased QRS and QT duration and notching in the QRS complex with copper depletion, consistent with ventricular conductance disturbances. Capillary-myocyte pathology displayed sub-sarcoplasmic fragmented basal laminae, foci of interstitial and peri-capillary collagen hyperplasia, accumulation of debris, and infiltration of macrophages, in the absence of overt fibrosis, with copper depletion. The oxidative capacity of soleus muscle increased with training in copper adequate rats, but was reduced with progressive copper depletion. These data suggest that copper depletion and training interact synergistically to effect hyper-induction of collagen hyperplasia, with deleterious effect on exercise
capacity. Early involvement of type IV collagen in basal laminae integrity and myocyte pathology is suggested.

Introduction

Hypertrophic cardiomyopathy of copper (Cu) deficiency has been well documented in young sedentary rats (1-4). The ultrastructural and functional cardiac pathology is characterized by cardiac hypertrophy with a significantly thickened outer wall and septum and reduced chamber volume (2,3), increased interstitial collagen (5-7), early myocardial failure and death, often by aneurysms or hemothorax (8,9). In our laboratory it has been demonstrated that the pathology associated with the hypertrophy in young rats, also occurs in older sedentary rats despite absence of overt cardiac hypertrophy (Chapter III). The type of hypertrophy, when present, is mixed, with predominantly concentric characteristics (9). Systolic blood pressure may be reduced in young rats (10,11), or increased in older rats (12,13). Electrocardiographic (ECG) disturbances include abnormal PR and ST segments and QRS wave amplitude (14-16). Ultrastructural changes include increased mitochondrial volume density (3,4,17), increased mitochondria:myofibril ratio (3), and fragmented cristae (3,4,14). In addition, myofibril volume density in young Cu deficient rat hearts increases as the heart wt increases (3). These changes are thought to be associated with decreased functionality/activity of the cupro-enzymes, lysyl oxidase (collagen cross-linking), cytochrome c oxidase (respiratory chain), superoxide dismutase (free radical scavenging) and dopamine-β-hydroxylase (norepinephrine synthesis).

The cardiac functional disturbances and ultrastructural changes induced by dietary Cu deficiency appear similar to that of patients with cardiomyopathies and congestive heart failure induced by conditions such as chronic hypertension (18-20). The precise role of Cu in the pathology of human heart disease is unknown. Concern has been expressed that human Cu status may be marginal, due to a borderline intake of Cu
per se (21,22), together with high intakes of dietary factors known to reduce Cu availability or increase needs, such as zinc supplements, ascorbic acid tablets, high fructose consumption and the regular use of antacids (23).

Sub-maximal, aerobic exercise training has been advocated to reduce the risk of developing heart disease by improving cardiac functionality. Although the potential benefits of improved myocardial contractility, energetics and vascularization, would deem this advantageous, the question remains whether exercise training would be of use in the prevention of pathologies similar to those caused by a dietary Cu deficiency.

The effect of aerobic exercise training on cardiac ultrastructure and function is equivocal. However, some of the beneficial effects of exercise training upon cardiac function and structure that could modify the development of Cu deficiency induced cardiomyopathy, are improved myosin isoenzyme patterns (24, 25), increased vascularization (26,27), decreased oxygen diffusion distances (24,28) increased activity of mitochondrial enzymes (29,30) and enhanced contractile performance (24,25). Furthermore, the classic hypertrophic pattern associated with aerobic exercise training is mildly eccentric in rats (24) and athletes (31), and may compliment the predominantly concentric pattern of Cu deficiency. Similarly, aerobic exercise training has been shown to be beneficial in some cases of existing cardiac pathology when cardiac function had been compromised (32-35).

In this study we used the older rat model to investigate the dual effect of Cu depletion and sub-maximal aerobic exercise training on the development of cardiac pathophysiology and ultrastructure, by simultaneously measuring functional, enzymatic and ultrastructural parameters in rats on controlled exercise programs and Cu intakes. Results of this study support the development of cardiac pathophysiology and histopathology in older rats (as opposed to rats fed Cu deficient
diets from weaning) fed Cu deficient diets, in the absence of overt cardiac hypertrophy. Sub-maximal, aerobic exercise training, combined with Cu deficiency, induced mild cardiac hypertrophy. Fragmentation and disruption of the basal lamina in Cu depletion suggest early involvement of type IV collagen. ECG patterns and end diastolic pressure development (EDP) subscribe to the functional pathology of left ventricular conduction and cation flux disturbances. Exercise training and Cu deficient diets appear to act synergistically to lead to hyper-induction of collagen synthesis, with deleterious effect on exercise capability and survival.

Material and methods

Animals and diets. Twenty-eight male weanling Long-Evans rats (Harlan Sprague-Dawley, Indianapolis, IN) were maintained for 6 wk on a basal diet (US Biochemical Corp., Cleveland, OH), following the recommendations of the American Institute of Nutrition (36) consisting of 500 g sucrose, 150 g cornstarch, 200 g casein, and 50 g corn oil/kg diet. After 6 wk, the rats were randomly divided into four treatment groups (n = 7 per group), so that mean group weights were similar: 1) sedentary group fed an adequate Cu diet (AC Sed) 2) exercise trained group fed a Cu adequate diet (AC Tr) 3) sedentary group fed a Cu deficient diet (NC Sed), and 4) exercise trained group fed a Cu deficient diet (NC Tr). Cu was added to the adequate Cu diet (AC) as 6 mg Cu/kg feed cupric carbonate, while no Cu was added to the deficient Cu diet (NC). The diets contained 0.4 Cu mg/kg diet (NC) and 5.9 mg Cu/kg diet (AC) as determined by flame atomic absorption spectrophotometry. These diets had been used consistently by our laboratory, and the NC diet effectively elicits Cu deficiency in rats (3,16).

The exercise program was designed to train the rats sub-maximally and aerobically on a rodent motor-driven treadmill. The rats were adapted to training by gradually increasing speeds, duration and grade
over a 4 wk period, then trained at 8% grade, for 60 min/day, at 27 m/min, for a further 4 wk. Due to the increasing pathology of dietary induced Cu depletion, the NC rats were unable to complete the last 3 daily exercise sessions. The two sedentary groups did not participate in exercise, but were handled in a similar manner. The rats were group housed until the initiation of treatment, and then singly, in stainless steel cages in quarters with a 12 h light dark cycle and mean temperature of 21.7°C, and free access to deionized-distilled water and diet. Food intake and body weights were recorded weekly for the 8 wk duration. The protocol for this study was approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

Experimental protocol and procedures. The parameters of measurement were selected to reflect cardiac function, ultrastructure and biochemistry concomitantly. Measurements were conducted at the end of 8 wk of treatment (17 wk of age), 24 h after the last bout of exercise to allow the effect of training, and not acute exercise, to be measured. Tail cuff blood pressure was performed on all rats. ECG and hemodynamic measurements were performed on 5 rats from each group, while samples for transmission electron microscopy (TEM) were obtained from 2 rats from each group. For all rats the thoracic cavities were opened, and 0.5 mL blood was removed by cardiac puncture and placed in heparinized tubes for hematocrit determination. The hearts selected for TEM were arrested in diastole by injection of 1 mmol/mL KCL into the left ventricle, excised, rinsed by perfusing Dulbecco's phosphate-buffered saline supplemented with 14 mmol/L glucose through the aorta (Gibco Laboratories Life technologies, Grand Island, NY), blotted and weighed. The hearts for the ECG and hemodynamic measurements were halved by a median sagittal cut at right angles to the intraventricular septum. One half was stored in 10 % formalin for later gross morphometry and light microscopy. The other half was frozen for later analysis of superoxide dismutase (SOD) activity. Soleus muscles from the right hind-limbs of
the 5 rats selected for ECG and hemodynamic studies were quickly excised, frozen in liquid nitrogen and stored at -70°C until later analysis of cytochrome c oxidase (CCO) activity. Livers were removed from all rats and frozen until later analysis of SOD activity.

**Electrocardiograms.** Rats were anesthetized by injection with ketamine (85 mg/kg) and xylazine (15 mg/kg) intraperitoneally. ECG measurements were recorded with the rats in right lateral recumbency, with small needle electrodes placed subcutaneously into 4 limbs (leads I and aVF) and precordially (lead V3), as described by Medeiros et al (16). Heart rate (beats/min), the duration of P wave, and P-R, QRS and QT intervals (ms), the amplitudes of QRS, R, and S waves (mV), and peak to peak ratio of R:S, were derived from tracings of lead aVF for statistical analysis. The regularity and pattern of the ECG, with specific attention to notching in the P wave and QRS complex, were analyzed subjectively on leads I, aVF and V3.

**Hemodynamics.** The rate of maximum left ventricular positive and negative pressure development (±dP/dt, mm Hg/s), end diastolic volume (EDP) (mm Hg) and total developed left ventricular pressure (Pd, mm Hg), were measured in the same anesthetized rats, using a modification of the procedure of Saragoca and Tarazi (37) as described by Medeiros et al (16). A short-beveled 21-gauge hypodermic needle attached directly to a pressure transducer, was thrust through the left thoracic wall into the left ventricle. The variables were derived from tracings and statistically analyzed.

**Gross morphometry.** Median sagittal sections of hearts stored in formalin were photographed. Cardiac dimensions were obtained measuring the left free ventricular free wall thickness (LVFW), left ventricular lesser dimension (LVLD), left ventricular major length (LVML), intraventricular septum (IVS) thickness and apical dimension (AD). Cross-sections were stained with H & E and Masson's trichrome for light
microscopy, and analyzed for the presence of fibrosis in a blind manner by a board certified veterinary pathologist.

**Preparation of samples for TEM.** After weighing, hearts were perfused with 2.5% glutaraldehyde in 0.1 mmol/mL Na cacodylate in 0.1 mmol/mL sucrose (Electron Microscopy Sciences, Fort Washington, PA). Samples < 1 mm were cut from the left ventricle, tangentially to the outer wall to obtain fibers in the longitudinal plane and fixed in the perfusion buffer. Preparation of the samples for TEM followed the procedure of Medeiros et al (16), using acetone dehydrations and Spurr resin embedment. Cu grids were selected from 2 blocks of each rat. Random sections from each Cu grid were examined by TEM (Philips EM300, Eindhoven, The Netherlands), at 60 keV, and negatives were developed and printed.

**Morphometric analysis.** Electron micrograph prints were analyzed morphometrically as reported elsewhere by Medeiros et al (3, 16). Prints with enlargement of 12 500 X were used to determine volume densities of mitochondria (VMITO), myofibrils (VMYO) and volume density occupied by intracellular material other than clearly defined mitochondria and myofibrils (VOTHER) in $\mu m^3/\mu m^3$, using a point system as described by Weibel (38) and Steer (39). VMITO:VMYO was calculated. Mean score for each rat was calculated from mean scores of 2 blocks/rat. To evaluate the pathology at capillary-myocyte interface micrographs (25 000 X) were scored on a 1-4 point scale (with 0.5 increments) in a blind manner. The normal appearance of the capillary-myocyte interface included a clear well-defined sarcolemma and capillary endothelium with a continuous, even line of basal lamina underlying these membranes, the absence of phagocytes and normally appearing myofibrillar and mitochondrial array and fine structures. Samples with these characteristics were assigned a maximum value of 4. Samples exhibiting gross pathology, tortuous membranes, broad inter-membrane areas, fragmented basal laminae, disarrayed mitochondria and accumulated inter-
membrane and debris, increased pericapillary interstitium, and the presence of phagocytes, were assigned values approaching 1. Mean values were obtained by averaging 2 prints. Micrographs (50,000 X) were used to investigate the integrity of the basal laminae underlying the individual myocyte sarcolemma and capillary endothelium, and to demonstrate collagen accumulation interstitially and in areas surrounding the capillaries.

**Superoxide dismutase activity.** Liver and heart SOD activity was determined spectrophotometrically, based on the autoxidation of pyrogallol, as described by Marklund (40) and modified by Prohaska (41). SOD was extracted from approximately 1 g of homogenized liver tissue by chloroform-ethanol. The auto-oxidation of pyrogallol was determined in TAPS buffer, without added liver sample, to serve as the reference. The amount of pyrogallol added to give an absorbance of 0.016 dA/30 s was determined, followed by determination of the amount of liver sample to reduce the absorbance to 0.008 dA/30 s. One unit of SOD activity was defined as the amount of activity to inhibit the auto-oxidation of pyrogallol by 50%, expressed as U/g.

**Cytochrome c oxidase activity.** The activity of CCO was measured in soleus muscle by the decrease in optical density of reduced cytochrome c as it is oxidized by CCO at 550 nm, by the method of Prohaska & Wells (42). 1 U CCO activity was expressed as nmol reduced cytochrome c oxidized/ min. In the soleus muscle, non-myofibril protein (NMPROT) containing predominantly mitochondria (MITO protein) was separated from myofibrils, as described previously (4). Briefly, tissue was homogenized in 10 volumes of 0.1 mmol/mL KCL and 1.5% Triton X-100 and centrifuged at 1100 X g and supernatant was removed. The concentration of protein was determined by the Lowry method (43) photometrically at 500 nm after acetone precipitation and redissolving in dd H2O to remove interfering substances. The specific activity of CCO (U/mg NMPROT or MITO protein), was used as an indication of mitochondrial functionality.
and for the measurement of changes in CCO activity (U/g wet wt T) concomitant with changes in mitochondrial protein due to exercise training. Similarly, concentration of MITO protein in the soleus (mg/g wet wt T) was considered to be an index of exercise training.

**Statistical analysis.** Differences by Cu treatment and exercise training for dependent variables were analyzed by ANOVA for a 2 X 2 design, using the Statistical Analysis System (SAS Institute, Cary, NC). General Linear Models Procedure (GLM) was used to determine significant differences between group means. Where significant F values for interaction effects occurred, the least significant difference (LSD) procedure to determine differences among group means, was used. A repeated measures analysis of variance was performed to determine significant differences among body weight and food intake means by treatment and weeks of treatment. The alpha level was set a priori at 0.05. All variables are reported as means ± SEM, except TEM variables, reported as means ± SD to compensate for the small sample size.

**Results**

Body wt and hematocrit did not differ \((P>0.05)\) between groups (Table 4). Food intake (FI) was similar for the groups over all weeks, except the last week when the Tr groups of both dietary treatments showed a slightly higher FI than Sed groups \((P<0.01)\). Dietary Cu depletion or exercise training per se did not induce cardiac hypertrophy \((P>0.05)\). Heart wt and heart:body wt were greater \((P<0.05)\) in the NC Tr than AC Tr groups. Liver SOD activity demonstrated significant differences in main and interaction effects, suggesting different levels of liver Cu (Figure 2). Liver SOD was highest in the AC Sed group \((P<0.0001)\), followed by the AC Tr group \((P<0.0001)\), the NC Sed group \((P<0.0001)\) and the NC Tr group \((P<0.05)\). Heart SOD activity varied only with dietary Cu level, with the AC groups significantly higher \((P<0.0001)\) than the NC groups (Figure 2). There was no difference \((P>0.05)\) in heart SOD activity between Tr and Sed groups (Figure 2).
<table>
<thead>
<tr>
<th>Indices</th>
<th>n</th>
<th>AC Sed</th>
<th>AC Tr</th>
<th>NC Sed</th>
<th>NC Tr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt(^2), g</td>
<td>7</td>
<td>437±20</td>
<td>416±12</td>
<td>444±23</td>
<td>423±8</td>
</tr>
<tr>
<td>Heart wt(^3), g</td>
<td>7</td>
<td>1.69±0.098</td>
<td>1.52±0.046</td>
<td>1.61±0.035</td>
<td>1.72±0.050</td>
</tr>
<tr>
<td>Heart:body wt(^3) X 10(^{-3})</td>
<td>7</td>
<td>3.9±0.16</td>
<td>3.7±0.07</td>
<td>3.7±0.19</td>
<td>4.1±0.09</td>
</tr>
<tr>
<td>Hematocrit(^2)</td>
<td>6</td>
<td>0.44±0.019</td>
<td>0.46±0.018</td>
<td>0.42±0.011</td>
<td>0.44±0.017</td>
</tr>
<tr>
<td>Food intake(^4), wk 8, g</td>
<td>7</td>
<td>17.9±0.70</td>
<td>22.6±0.85</td>
<td>20.9±1.35</td>
<td>22.9±0.72</td>
</tr>
</tbody>
</table>

\(^1\)Values are expressed as means ± SEM. Data were analyzed by 2 X 2 ANOVA and LSD where appropriate.
\(^2\)Body wt and hematocrit were not significantly different (P>0.05) between any of the groups.
\(^3\)Heart wt and heart:body wt of NC Tr were significantly greater (P<0.05) than that of AC Tr.
\(^4\)Food intake of wk 8 was significantly greater (P<0.01) in Tr than Sed groups irrespective of dietary treatment.
SOD activity in liver and heart

SOD activity in (a) liver and (b) heart of sedentary and exercise trained rats fed Cu adequate and deficient diets. SOD activity (U/g) was significantly different \((P<0.0001)\) in liver between all groups, with activity of AC Sed 31 903 ± 555, AC Tr 23 753 ± 555, NC Sed 9 964 ± 685 and 7 929 ± 273. SOD activity in the heart was significantly lower \((P<0.0001)\) in Cu depleted (NC Sed 5146 ± 297; NC Tr 4985 ± 156) compared to Cu adequate (AC Sed 8242 ± 89; AC Tr 8168 ± 200) groups. Values are means ± SEM.

1 U SOD activity = amount of activity to inhibit the auto-oxidation of pyragallol by 50 %.
FIGURE 2

SOD activity in liver and heart
All rats appeared healthy with no differences in appearance or color evident. There was no apparent difference between rats fed Cu adequate or Cu deficient diets in compliance to the training program, except for the final 3 days, when the NC Tr rats were unable to complete the exercise runs.

CCO specific activity in the soleus (U/mg MITO protein) was significantly lower ($P<0.0001$) in both groups of rats fed a Cu deficient diet compared to rats fed a Cu adequate diet; and significantly higher ($P<0.05$) in Tr compared to Sed rats of the same dietary treatment. CCO activity in the soleus (U/g wet wt T) was more than doubled in the AC Tr rats ($P<0.0001$) compared to the AC Sed rats, confirming the efficacy of the training program (Figure 3). Additionally, CCO activity was significantly higher ($P<0.0001$) in the AC Tr than in NC Sed and NC Tr rats. One U of CCO activity expresses the nmol of reduced cytochrome c oxidized per min.

NMPROT concentration in the soleus, reflecting predominantly MITO protein concentration, (mg/g wet wt T) was significantly higher in the AC Tr compared to the AC Sed ($P<0.0005$) rats; and in NC Sed compared to the AC Sed ($P<0.0001$) and AC Tr ($P<0.005$) rats. There was no significant difference ($P>0.05$) between the AC Tr and NC Tr, or NC Sed and NC Tr rats (Figure 3).
FIGURE 3

Soleus CCO activity and MITO protein concentration

(a) CCO specific activity (U/mg MITO protein), (b) CCO activity (U/g wet wt T) and (c) MITO protein concentration (mg/ wet wt T) in rat soleus muscle of sedentary and trained rats fed Cu adequate and deficient diets. CCO specific activity was significantly lower (P<0.0001) in NC rats (NC Sed 62.0 ± 2.67; NC Tr 88.5 ± 7.95) compared to AC rats (AC Sed 184.1 ± 27.15; AC Tr 234.2 ± 10.46); and higher (P<0.05) in Tr than Sed groups of the same diet. CCO activity was significantly higher (P<0.0001) in AC Tr (AC Tr 21.4 ± 2.01) than AC Sed (AC Sed 8.8 ± 1.13) and both NC groups (NC Sed 8.0 ± 0.62; NC Tr 9.1 ± 0.51). MITO protein concentration was significantly higher in the AC Tr (87.8 ± 5.89) compared to the AC Sed rats (44.6 ± 8.13) (P<0.0005); and in the NC Sed (126.4 ± 8.07) than the AC Sed (P<0.0001) and AC Tr (P<0.005) rats. There was no significant difference (P>0.05) between NC Tr and NC Sed rats. 1 U CCO activity = nmol reduced cytochrome c oxidized/ min.
FIGURE 3

Soleus CCO activity and MITO protein concentration
Gross morphometry. Despite the lack of overt cardiac hypertrophy as revealed by heart wt and heart:body wt (Table 4), gross morphology of sagittal heart sections revealed the presence of some degree of left ventricular hypertrophy (Table 5 and Plate III). IVS (P<0.005) and AD (P<0.05) were increased in Cu depleted rats, whereas there were no statistical differences (P>0.05) in IVS and AD between Sed and Tr rats. LVLD was significantly decreased (P<0.0001) in Cu depleted compared to Cu adequate rat hearts; and significantly increased (P<0.005) in Tr compared to Sed rat hearts. LVFW was significantly increased (P<0.0001) in rats fed a Cu deficient diet compared to rats fed a Cu adequate diet; and was significantly decreased (P<0.005) in Tr compared to Sed rats. LVML was not affected by dietary Cu levels (P>0.05). However, LVML was significantly increased (P<0.01) in Tr compared to Sed rats (Table 5, Plate III).

Light microscopy revealed minimal fibrosis in Sed compared to Tr rat hearts. Only 1 heart in each of the Tr groups had evidence of minimal fibrosis, compared to all the hearts in the Sed groups.
Plate III

Gross Morphometry

Median sagittal sections of sedentary and trained rat hearts fed Cu adequate and deficient diets. NC hearts display predominantly concentric hypertrophy of the left ventricle compared to AC hearts, by significantly increased IVS (P<0.0005), LVFW (P<0.0001) and decreased LVLD (P<0.0001). Trained hearts illustrate evidence of mixed hypertrophy with strong eccentric component, by significantly increased LVLD (P<0.005) and LVML (P<0.05), and decreased LVFW (P<0.005) compared to Sed hearts.
Plate III

Gross morphometry
TABLE 5
Ventricular dimensions of sedentary and trained rats fed Cu adequate and deficient diets

<table>
<thead>
<tr>
<th>Measure</th>
<th>n</th>
<th>AC Sed</th>
<th>AC Tr</th>
<th>NC Sed</th>
<th>NC Tr</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS squared</td>
<td>5</td>
<td>3.4±0.25</td>
<td>3.2±0.12</td>
<td>4.4±0.29</td>
<td>4.0±0.00</td>
</tr>
<tr>
<td>LVLD squared</td>
<td>5</td>
<td>4.0±0.45</td>
<td>5.0±0.32</td>
<td>1.9±0.33</td>
<td>3.3±0.20</td>
</tr>
<tr>
<td>LVFW squared</td>
<td>5</td>
<td>3.7±0.30</td>
<td>2.6±0.19</td>
<td>5.1±0.25</td>
<td>4.4±0.19</td>
</tr>
<tr>
<td>LVML squared</td>
<td>5</td>
<td>11.5±0.22</td>
<td>12.7±0.30</td>
<td>11.5±0.32</td>
<td>12.0±0.32</td>
</tr>
<tr>
<td>AD squaured</td>
<td>5</td>
<td>2.0±0.27</td>
<td>1.8±0.20</td>
<td>2.3±0.20</td>
<td>2.5±0.22</td>
</tr>
</tbody>
</table>

IVS = intraventricular septum
LVLD = left ventricular lesser diameter
LVFW = left ventricular free wall
LVML = left ventricular major length
AD = apical dimension.

Values are expressed as means ± SEM. Data were analyzed by 2 X 2 ANOVA and LSD where appropriate.

IVS (P<0.0005) and AD (P<0.05) were significantly greater in NC compared to AC rats; there were no statistical differences (P>0.05) between Tr and Sed rats.

LVLD was significantly less in NC than AC rats (P<0.0001), and significantly greater in Tr than Sed rats (P<0.005).

LVFW was significantly greater (P<0.0001) in Cu depleted compared to Cu adequate hearts; and was significantly less (P<0.005) in Tr compared to Sed hearts of the same dietary treatment.

LVML was significantly increased (P<0.01) in Tr compared to Sed groups; there was no significant difference (P>0.05) between rats fed Cu deficient and Cu adequate diets.
Electrocardiograms and hemodynamics. Heart function was affected by both Cu and Tr (Table 6). Systolic blood pressure was significantly lower \((P<0.005)\) in Tr compared to Sed rats, while dietary treatment had no effect \((P>0.05)\) on blood pressure. Blood pressure of the AC rats was higher in this study \((149 \pm 5.2 \text{ mm Hg})\) than in our previous study \((111 \pm 6.2 \text{ mm Hg}, \text{Chapter III})\). This could be due to the high sucrose content of the basal diet and the age of the rats. There was a significant interaction effect in QRS duration between Cu and Tr. QRS duration was significantly greater in AC Tr than AC Sed \((P<0.05)\) rats; in NC Sed than AC Sed \((P<0.0005)\) rats; and in NC Tr than AC Sed \((P<0.05)\) rats. There was no statistically significant difference \((P>0.05)\) between NC Sed and NC Tr rats. Although the QT duration was significantly increased \((P<0.0001)\) in NC compared to AC rats, it was significantly decreased \((P<0.01)\) in Tr compared to Sed groups. No statistical differences \((P>0.05)\) were evident in any of the other ECG variables. ECG tracings revealed a greater degree of notching in Cu depleted than Cu adequate groups, regardless whether Sed or Tr (Figure 4). The only significant difference in hemodynamic measurement between the treatment groups was an increase in EDP \((P<0.05)\) in the Tr compared to the Sed groups.

Morphometrics. Transmission electron micrographs revealed that VMITO of the cardiac myocyte was significantly increased \((P<0.001)\), while VMYO was significantly decreased \((P<0.001)\), in Cu deficient compared to Cu adequate rats, with a consequent increase in the ratio of VMITO:VMYO \((P<0.001)\) (Table 7). Volume densities of mitochondria, myofibrils and the ratio of VMITO:VMYO did not appear different between Sed and Tr groups of the same dietary treatments.
### Table 6

Variables of cardiac function in sedentary and trained rats fed Cu adequate and deficient diets

<table>
<thead>
<tr>
<th>Variable</th>
<th>AC Sed n = 5</th>
<th>AC Tr n = 5</th>
<th>NC Sed n = 5</th>
<th>NC Tr n = 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure^2 mm Hg</td>
<td>149±5.2</td>
<td>117±8.3</td>
<td>143±7.9</td>
<td>129±5.9</td>
</tr>
<tr>
<td>Heart rate^3 beats/min</td>
<td>335±9</td>
<td>322±15</td>
<td>321±10</td>
<td>327±4</td>
</tr>
<tr>
<td>ECG components:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P wave^3, ms</td>
<td>19.0±1.0</td>
<td>17.2±1.0</td>
<td>15.8±0.5</td>
<td>17.3±0.8</td>
</tr>
<tr>
<td>P-R interval^3, ms</td>
<td>46±1.9</td>
<td>42±1.2</td>
<td>36±9.1</td>
<td>46±3.8</td>
</tr>
<tr>
<td>QRS duration^4, ms</td>
<td>10.8±0.37</td>
<td>15.4±1.29</td>
<td>18.6±0.98</td>
<td>16.0±2.31</td>
</tr>
<tr>
<td>QRS amplitude^3, mV</td>
<td>1.28±0.10</td>
<td>0.87±0.19</td>
<td>1.02±0.04</td>
<td>1.15±0.17</td>
</tr>
<tr>
<td>R wave^3, mV</td>
<td>0.7±0.21</td>
<td>0.6±0.13</td>
<td>0.5±0.07</td>
<td>0.8±0.27</td>
</tr>
<tr>
<td>S wave^3, mV</td>
<td>0.7±0.13</td>
<td>0.5±0.18</td>
<td>0.5±0.08</td>
<td>0.4±0.17</td>
</tr>
<tr>
<td>R:S^3</td>
<td>2.0±1.38</td>
<td>1.7±0.52</td>
<td>1.1±0.17</td>
<td>3.8±1.98</td>
</tr>
<tr>
<td>QT duration^5, ms</td>
<td>67±2.0</td>
<td>59±3.3</td>
<td>80±1.6</td>
<td>71±3.0</td>
</tr>
<tr>
<td>Hemodynamics:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dP/dt^3, mm Hg/s</td>
<td>12 072±2893</td>
<td>12 938±687</td>
<td>11 536±2171</td>
<td>12 834±2140</td>
</tr>
<tr>
<td>EDP^6, mm Hg</td>
<td>12.5±1.55</td>
<td>19.7±2.07</td>
<td>14.7±3.19</td>
<td>19.7±4.76</td>
</tr>
<tr>
<td>Pd^3, mm Hg</td>
<td>113±24.2</td>
<td>126±6.7</td>
<td>118±22.9</td>
<td>120±10.7</td>
</tr>
</tbody>
</table>

dP/dt = rate of pressure development;  EDP = end diastolic pressure;  Pd = total left ventricular pressure development

^1Values are expressed as means ± SEM. Data were analyzed by 2 X 2 ANOVA and LSD where appropriate.

^2Blood pressure was significantly lower (P<0.005) in the Tr compared to the Sed groups.

^3There were no significant differences between any of the groups at P>0.05.

^4QRS duration was significantly greater in AC Tr than in AC Sed (P<0.05); in NC Sed than AC Sed (P<0.0005); and in NC Tr than Ac Sed (P<0.05).

^5QT duration was significantly greater (P<0.0001) for rats fed NC than AC diets and significantly less (P<0.01) in Tr compared to Sed rats.

^6EDP was significantly greater (P<0.05) in ACTr than Sed rats.
Representative tracings of (a) ECG and (b) hemodynamics (±dP/dt), illustrating differences with treatment. AC Tr rats had significantly increased QRS duration in leads aVF and V₃ compared to AC Sed rats (P<0.05); in NC Sed than AC Sed (P<0.0005); and in NC Tr than AC Sed rats (P<0.05). QT duration was significantly greater (P<0.0001) in NC compared to AC rats; and significantly less (P<0.01) in Tr compared to Sed rats. There is a higher incidence of notching in the QRS complex in NC compared to AC rats. Tr rats had significantly higher (P<0.05) EDP compared to Sed rats. No other differences were significant at P>0.05).
FIGURE 4

ECG and hemodynamic tracings
VOTHER was significantly increased ($P<0.0005$) in the NC compared to the AC groups, due mainly to increased sarcoplasmic reticulae, enlarged T-tubules, accumulation of Z-band material, and disrupted fibers at intercalated discs. Lipid droplets and glycogen granules were increased (Table 7). Additionally, VOTHER was significantly increased ($P<0.05$) in Tr compared to Sed groups, possibly due to the residual effect of the last exercise. Mitochondrial cristae seemed mostly normal, even in the NC and Tr groups, except for the area closest to the capillary-myocyte interface and sarcolemma.

Histopathological development evident at the capillary-myocyte interface of NC rats (Plate IV) was increased tortuosity of the sarcolemma, increased amounts of debris and widened inter-membrane areas (Plate IV). The basal lamina underlying the sarcolemma was fragmented in areas with variable thickness (Plate V) in NC compared to AC rats. There was a large increase in interstitial collagen and accumulation of debris between cells and in pericapillary areas in Cu deficient rats. Rats in the NC Tr group demonstrated the most deposition of collagen (Plate VI). The presence of phagocytes (Plate V, VI) were evident in intra- and pericapillary areas in many of the NC rats and none of the AC rats. These phagocytes were concentrated in areas of collagen induction and basal lamina pathology.
### TABLE 7

Histopathological cardiac indices in sedentary and trained rats fed Cu adequate and deficient diets

<table>
<thead>
<tr>
<th>Variable</th>
<th>AC Sed</th>
<th>AC Tr</th>
<th>NC Sed</th>
<th>NC Tr</th>
</tr>
</thead>
<tbody>
<tr>
<td>VMITO(^2)</td>
<td>0.313 ±0.0207</td>
<td>0.338 ±0.0076</td>
<td>0.475 ±0.0109</td>
<td>0.467 ±0.0359</td>
</tr>
<tr>
<td>VMYO(^3)</td>
<td>0.646 ±0.0218</td>
<td>0.608 ±0.0022</td>
<td>0.453 ±0.0054</td>
<td>0.455 ±0.0337</td>
</tr>
<tr>
<td>VOTHER(^4)</td>
<td>0.042 ±0.0011</td>
<td>0.052 ±0.0044</td>
<td>0.072 ±0.0044</td>
<td>0.080 ±0.0022</td>
</tr>
<tr>
<td>VMITO:VMYO</td>
<td>0.485 ±0.0483</td>
<td>0.558 ±0.0105</td>
<td>1.049 ±0.0366</td>
<td>1.0328 ±0.1556</td>
</tr>
<tr>
<td>CAP-MYOC(^2)</td>
<td>3.7±0.27</td>
<td>3.3±0.47</td>
<td>1.5±0.01</td>
<td>1.6±0.11</td>
</tr>
</tbody>
</table>

VMITO = volume density of mitochondria  
VMYO = volume density of myofibrils  
VOTHER = volume density of intracellular material other than clearly defined mitochondria and myofibrils  
VMITO:VMYO = ratio of volume density of mitochondria to myofibrils  
CAP-MYOC = capillary-myocyte pathology rated on a 1-4 point scale

\(^1\)Values are expressed as means ± SD. Data were analyzed by 2 X 2 ANOVA and LSD where appropriate.  
\(^2\)VMITO, VMITO:VMYO and CAP-MYOC interface were significantly more (\(P<0.001\)) in NC than AC groups.  
\(^3\)VMYO was significantly less (\(P<0.001\)) in NC compared to AC groups.  
\(^4\)VOTHER was significantly more (\(P<0.0005\)) in NC than AC groups and significantly more (\(P<0.05\)) in Tr compared to Sed groups.
PLATE IV

Capillary–myocyte interface

Transmission electron micrographs of the capillary–myocyte interface in left ventricles of (a) rats fed AC diets and (b) NC Sed and (c,d) NC Tr rats. The sarcolemma of NC rats were more convoluted, there was more sub-sarcolemmal debris, and inter-membrane space was increased in the Cu deficient groups. Note in (d) the presence of a phagocyte at the myocyte sarcolemma in close proximity to basal lamina fragmentation and collagen deposition. C = capillary; BL = basal lamina; S = sarcolemma; A = intermembrane area; P = phagocyte. Bar = 1 μm.
PLATE IV

Capillary-myocyte interface
PLATE IV (continued)

Capillary-myocyte interface
PLATE IV (continued)

Capillary–myocyte interface
PLATE IV (continued)

Capillary-myocyte interface
PLATE V

Basal laminae

Transmission electron micrographs demonstrating fragmentation and focal distortion of the basal laminae underlying the sarcolemma, together with disorganization of myofibrils and mitochondria in (b) NC Sed and (c) NC Tr compared to (a) AC rats. Mitochondrial cristae did not seem grossly affected. BL = basal laminae; M = mitochondria my = myofibrils.

Bar = 1 μm.
PLATE V

Basal laminae
PLATE V (continued)

Basal laminae
PLATE V (continued)

Basal laminae
PLATE VI

Interstitial collagen

Transmission electron micrographs illustrating interstitial collagen between myocytes and pericapillary in (a) Cu depleted sedentary and (b,c) trained rats. Collagen seems to be present to a greater degree in NC Tr than in NC Sed rats. co = collagen; mt = myocyte; C = capillary. Bar = 1 μm.
PLATE VI

Interstitial collagen
PLATE VI (continued)

Interstitial collagen
PLATE VI (continued)

Interstitial collagen
Discussion

Studies on the cardiac pathology of dietary Cu deficiency have primarily focussed on sedentary rats fed Cu deficient diets prior to or from weaning for varying lengths of time. It has been generally accepted that older rats remain largely unaffected by dietary Cu deficiency. Similarly, the interaction of an aerobic exercise training program with a developing Cu deficiency has not been explored. In a previous study in this laboratory (Chapter III), it has been revealed that a Cu deficient diet fed to 8 wk old rats elicited a similar Cu deficient cardiomyopathy to that in younger rats, despite the absence of overt cardiac hypertrophy. The purpose of this study was to assess the influence of sub-maximal exercise training on the development of Cu deficient cardiomyopathy in older rats. Simultaneous measurement of cardiac function and ultrastructure helped to narrow the possible primary mechanisms responsible for the pathological changes.

General response to the training program. Rats fed Cu deficient diets were able to adhere to the sub-maximal, aerobic exercise training program with ease until the last 3 d. At that time they were unable to complete training sessions despite outwardly healthy appearance. Various studies have reported the beneficial effects of sub-maximal, aerobic exercise training upon cardiac function in healthy rats (24). Improved vascularization (26,27), oxygenation distances (28) and contractile capacity (25) have been reported in rats that are exercise trained, in contrast to patients with hypertrophic cardiomyopathies. Additionally, exercise training has been reported to reduce cardiac pathology. Paulson (34) reported decreased effect of myocardial dysfunction in trained diabetic rats, Crisman (32) demonstrated increased capillarity in spontaneously hypertensive (SHR) trained rats and Rupp & Jacob (44) reported a reversal from V3 to V1 in trained SHR.
However, decreased cardiac reserve, insufficient capillarity and oxygenation, and compromised contractile function have been reported in patients with progressive congestive heart failure (18,20) and rat models with compromised heart function (19).

In this study it appeared that when rats were simultaneously fed Cu deficient diets and exercise trained, the cumulative effects were detrimental. This suggests that as the cardiac pathology in Cu deficiency progresses myocardial reserve may be depleted resulting in compromised function during physiological stress. Furthermore, it appears likely that the inability of Cu depleted rats to maintain their exercise regime could involve other systems, such as skeletal muscle.

**Body weight, food intake and hematology.** Several studies have reported decreased body wt, food intake, hematocrit and early mortality in young rats (1,2,4) fed Cu deficient diets. Similarly, body wt and food intake of exercise trained male rats have been reported significantly lower than sedentary rats (24,25,45). In contrast, reported body wt, hematocrit and mortality of older rats fed Cu a deficient diet did not differ from rats fed a Cu adequate diet (Chapter III).

In this study, there were no differences between any of the groups in body wt, food intake, hematocrit or mortality. It would appear that differences in age of initiation of treatment or species may partly explain our results.

**Cu,Zn-Superoxide dismutase.** Decreased SOD activity in rats fed Cu deficient diets has been reported in liver (46,47, Chapter III), heart (47) and other organs (47). Interestingly, in this study, liver SOD activity was not only reduced in rats fed Cu deficient diets, but also in Tr compared to Sed rats, while heart SOD activity was only reduced by Cu depletion and not exercise training. This suggests that the myocardial degenerative changes (discussed below) varied with Cu status.
but not exercise training. This would appear to suggest that cardiac cytoplasmic antioxidant defenses could be reduced in Cu deficiency and contribute to the degenerative process.

Cytochrome c oxidase and mitochondrial changes in skeletal muscle. The oxidative respiratory capacity of skeletal muscle, as represented by mitochondrial mass and associative oxidative enzyme systems, undergo an adaptive increase with regular repeated exercise. The soleus, an intermediate oxidative muscle consisting mainly of slow-twitch red fibers (29,30,48), is considered a good model to measure oxidative changes with training. Increased CCO activity (30,48,49), concentration (29), and mitochondrial protein (48,49) with sub-maximal aerobic training have been reported. Conversely, decreased CCO activity has been reported in rats with denervated skeletal muscle (50), in patients with various neuromuscular diseases and secondary to a mitochondrially induced defect (51). Furthermore, decreased CCO activity has been reported in Cu deficient rats (8).

In this study, soleus muscle CCO specific activity (U/mg MITO protein) was significantly decreased in Cu depleted compared to Cu adequate rats, suggesting decreased functionality of the enzyme. Since the mitochondrially expressed subunit II of CCO is Cu dependent (52), the increase in mitochondrial mass (represented by increased NMPROT concentration) in the Cu deficient rats could demonstrate an attempt to increase respiratory capacity. The significant increase in CCO activity (U/g wet wt T) and mitochondrial mass (mg/g wet wt T) in rats fed Cu adequate diets with training, reflect an increased oxidative capacity response and efficacy of the training program. These adaptive oxidative mechanisms seemed to have failed with progressive Cu depletion, as reflected by the decrease in CCO activity and mitochondrial protein in the exercise trained Cu depleted rats. The combination of decreased
oxidative capacity of Cu depletion and increased oxygen needs with exercise may have been partially responsible for the reduced exercise capacity towards the end of the study period. The low diet Cu levels may have been partially responsible for the severity of the observed effects. Marginal Cu intakes may more closely resemble the human model.

**Gross myocardial and left ventricular hypertrophy.** Marked cardiac hypertrophy with predominantly concentric components, associated with increased transverse thickness of muscle fibers, has been consistently reported in young Cu deficient rats (14-16) and patients with hypertrophic cardiomyopathies (53). Conversely, significant cardiac hypertrophy was not reported when Cu deficient diets were introduced in older rats (Chapter III). A more eccentric hypertrophy with increased chamber volume and a lengthwise increase in muscle fibers, is associated with congestive dilated cardiomyopathies in humans (54) and aerobic exercise training in rats (24) and humans (31). However, the effect of the intermittent stimulus of mild volume overloading in exercise training, is equivocal. Generally, absolute cardiac hypertrophy is not associated with moderate exercise training in male rats and humans (24,55,56). However, heart:body wt may be increased due to the failure of exercised male rats to adapt food intake to higher energy needs (24,55,56).

In this study, no overt cardiac hypertrophy due to either Cu depletion or exercise training per se as measured by heart wt or heart:body wt was revealed. However, both heart wt and heart:body wt were somewhat increased in NC Tr compared to AC Tr groups. Additionally, gross morphometry revealed that, despite this seeming lack of cardiac hypertrophy, cardiac dimensions were consistent with some left ventricular hypertrophy in both Cu deficient compared to Cu adequate, and Tr compared to Sed hearts. The increased left ventricular
free wall and intraventricular septum, with concomitant decreased left ventricular chamber in Cu depleted compared to Cu adequate rats, suggest a concentric component to the hypertrophy. Furthermore, the increased left ventricular diameter, together with a slight increase in left ventricular free wall width in trained compared to sedentary rats, suggest a more eccentric component. The actual changes measured in gross morphometry were small, but were statistically significant due to the small variability in each group. Since the overall heart wt and heart:body wt were not consistent with overt cardiac hypertrophy, left ventricular remodelling due to Cu depletion and exercise training may have been responsible for the left ventricular changes.

**Cardiac morphometrics.** Increased volume density of mitochondria have been reported in Cu deficient young (3,4,16,17) and older (Chapter III) rats. In contrast, mitochondrial volume density is decreased in patients with progressive congestive and hypertrophic cardiomyopathies and myocardial failure (18,19,27,53,56). The myocardial mitochondrial response to aerobic exercise training, in contrast to that of skeletal muscle (24,48,57) remains equivocal (24). Generally, no changes in mitochondrial protein and mitochondrial enzymes have been reported as a function of training (24,48,55). However, a shift towards smaller mitochondrial size, associated with an increase in surface:volume ratio has been suggested (58).

Morphometric analysis of TEM micrographs in this study revealed increased mitochondrial volume density and VMITO:VMYO in left ventricular Cu depleted rat myocytes, with no difference in mitochondrial volume density between Tr compared to Sed rats. This change in the myocardium that seems unique to Cu depletion, is consistent with the increased mitochondrial mass demonstrated in skeletal muscle. Since decreased CCO activity was indicated in skeletal
muscle in this study, and decreased cardiac COO activity reported by others (46,47) in Cu deficiency, it is conceivable that the increase in mitochondrial volume density may be a specific attempt to enlarge mitochondrial surface area to increase cellular respiration by maximizing Cu and oxygen uptake. The increased presence of glycogen granules tends to support this mechanism. Increased cytoplasmic glycolysis could be attempted by the cell to increase ATP production with reduced efficiency of mitochondrial oxidative pathways.

Ultrastructural changes. Degenerative changes in myocardial myocytes and interstitium have been reported by others in patients with congestive and hypertrophic cardiomyopathies (18,19,20,53,54). These changes characteristically include convoluted myocytes with invaginated tortuous sarcoplasm, disarranged intercalated discs, bizarre shaped nuclei with prominent nucleoli, increased sarcoplasmic reticulae and T-tubules, disarrayed sparse myofibrils with changed Z band material, swollen mitochondria with decreased cristae and electron lucent matrix and the accumulation of subcellular particles and myelin figures. In contrast, the ultrastructure is normally preserved in rats that are moderately exercise trained (24,48). Immediately after an exercise bout (57) and with arduous exercise programs (48,49), degenerative changes are observed.

In this study, as reported by others in Cu deficiency (1-4, 17, Chapter III), these degenerative changes were obvious. The increased VOTHER in Cu depletion revealed by morphometric analysis indicates accumulation of intracellular debris. Additionally, VOTHER was also somewhat increased in Tr compared to Sed rats. This effect may be related to a residual effect of the last exercise bout, less than 24 h before the measurements were made.
Capillary-myocyte interface histopathology. Thickening and disruption of the basal lamina (BL), associated with increased collagen fibrils, has been reported in patients with cardiomyopathies (53). This was associated with myocyte degeneration and cellular remodelling. Furthermore, when myocyte pathology in rats was induced by ischemia, freeze-thawing and isoprotenerol, degenerative changes at myocyte-collagen stroma level were demonstrated (59). Although the BL in these studies remained largely intact and continued to delineate the sarcolemma and underlying collagen stroma, it was collapsed and patchy in areas. Additionally, Farquharson & Robins (7), using antibodies and immunolocalization, described focal areas of basal lamina fragmentation, distortion and thickening in Cu deficient rats.

In accordance with these studies, significant pathology at capillary-myocyte interface was demonstrated with Cu depletion in this study. No differences were apparent in Tr compared to Sed rats. The distortion and fragmentation of the BL, in association with inflammatory cells in close proximity to tortuous areas of sarcoplasm, considerable extracellular and intracellular debris, increased areas of interstitial collagen fibrils with fibroblasts were demonstrated in TEM micrographs. Type IV collagen, found predominantly in the BL (7,60), is covalently stabilized at cysteine residues by the formation of disulfide (60) and at lysine and hydroxylysine residues by non-reducible covalent cross-links (60). Since the enzyme, lysyl oxidase, that catalyzes the deaminative cross-linking is Cu dependent, it is conceivable that BL integrity may be compromised in Cu deficiency (6).

The pathology at capillary-myocyte interface with Cu depletion, may be associated with decreased oxygenation and could be one mechanism that contributes to increased mitochondrial volume density. The presence of this pathology, sometimes in the absence of sub-sarcolemmal
mitochondrial cristae fragmentation and overt myofibril degeneration, seemingly suggests early involvement. If the alteration of basal lamina underlying cell membranes occurs in other organs in Cu deficiency, what effect could it have on membrane integrity and functions, such as receptor coupling and intracellular signalling systems?

**Pericapillary interstitial collagen deposition.** Progressive foci of subendocardial fibrosis and necrosis, associated with increased interstitial and pericapillary collagen, has been shown by others in patients with congestive and hypertrophic cardiomyopathies (18,20,53,54) and in young (5,6,7) and older (Chapter III) Cu deficient rats. However, exercise training is associated with minimal collagen hyperplasia (24). Additionally, increased interstitial collagen deposition in Cu deficiency has been reported (5,6,7). The decreased 3-hydroxypyridinium cross-linking of type I and III collagen by lysyl oxidase (6), results in reduced strength of mature collagen fibrils.

The presence of focal interstitial collagen deposition in this study was clearly revealed in TEM micrographs. Exercise training and Cu depletion seemed to act synergistically to effect hyper-induction of collagen in focal areas. Conversely, light microscopy analysis revealed no more than minimal fibrosis in Cu deficient and trained rats. Since interstitial collagen hyperplasia is a progressive process, overt fibrosis may not have developed yet. This underscores the importance of using higher magnifications for analysis of collagen deposition. The focal collagen hyperplasia, together with inter- and intracellular debris, could contribute to increased oxygen diffusion distances from capillary lumen to myocyte mitochondria, and decreased oxidative capacity. This would be particularly relevant in exercise training, since oxygen needs would be increased.
**Electrocardiograms and hemodynamics.** Blood pressure has been reported to be decreased in young (10,11) and increased in older Cu deficient rats (12,13). ECG abnormalities have been reported in young (14-16) and older (Chapter III) Cu deficient rats and in Cu deficient men (61). These include supraventricular and ventricular arrhythmias, abnormalities in P wave and P-Q interval, increased QRS wave amplitude and duration, and ST-T changes. Similarly, the predominant abnormalities in patients with congestive heart failure are arrhythmia, prolonged QRS duration, ST-T wave abnormalities, and notching in QRS (18,20,62). Late potential has been reported even in the absence of left ventricular hypertrophy (63). Athletes (24) and exercise trained rats (24,48) generally have decreased resting heart rates. Resting systolic blood pressure changes associated with exercise training are equivocal, but generally decreased (48). Increased amplitude of R wave in leads V₅ and V₆ and increased EDP have been reported in trained rats (24).

In this study, the only ECG abnormalities of statistical significance were increased QRS and QT duration in Cu deficient compared to Cu adequate rats. Furthermore, ECG tracings revealed an increased appearance of notching in the QRS complex of Cu deficient rats. These changes may be related to left ventricular hypertrophy and fibrosis (64). In this instance, they are more likely associated with disturbances of the electrical conductance system, reflecting alterations in cation flux. High individual variability and the variations in ECG leads may have caused lack of sensitivity to detect increased amplitude. EDP was increased, and blood pressure decreased, with exercise Tr compared to Sed rats. However, blood pressure was not affected by Cu depletion, in agreement with our previous data (Chapter III). Differences in age, dietary composition and species may contribute to these equivocal findings.
Summary.

The development of myocardial pathology in older Cu depleted rats, in the absence of overt cardiac hypertrophy, has been confirmed by this study. ECG measurements, light microscopy and TEM data support these findings. The marked induction of myocardial mitochondrial protein could be an attempt to increase mitochondrial respiration by maximizing Cu uptake through increased surface area of the organelle. Decreased oxygenation associated with capillary-myocyte pathology and interstitial collagen induction may have profound influences upon cardiac respiration. Reduced cardiac SOD activity may exacerbate myocytal degenerative changes. Basal lamina fragmentation and reduced sarcolemmal integrity may be an early mechanism for the pathological development. Initially, rats fed a Cu deficient diet responded well to sub-maximal aerobic exercise training. However, with progressive Cu depletion, cardiac reserve seemed to become depleted with resultant inability to exercise. Training and Cu depletion appeared to have acted synergistically in the focal super-induction of interstitial collagen. Further studies concerning the role of interstitial collagen and the effect of basal laminae on the development of Cu deficient cardiomyopathy may serve to elucidate pathological mechanisms and processes. Investigation of basal laminae underlying cell membranes in other organs and its effect on membrane receptors and signalling systems may be timeous. To more closely approximate human conditions, the inclusion of diets with marginal Cu levels over longer periods of time is recommended.
List of references


CHAPTER V
SUMMARY AND RECOMMENDATIONS

The cardiac pathophysiology and ultrastructure of dietary Cu deficiency has been well defined in young rats. However, it has been accepted that this cardiomyopathy is not of concern in the older rat, primarily because hypertrophy was absent. Additionally, little work has been reported on the effect of Cu repletion in previously Cu deficient rats. Furthermore, aerobic sub-maximal exercise training is often advocated to improve cardiac function, yet no studies reported the interaction of training and Cu depletion on developing cardiomyopathy.

The objectives of this research were to investigate:

1. The effect of dietary Cu deficiency on cardiac pathophysiology and ultrastructural changes in older rats.
2. The effect of dietary Cu repletion on cardiac pathophysiology and ultrastructure in previously Cu deficient rats.
3. The dual effect of aerobic sub-maximal exercise training and Cu depletion on the developing pathology.

Two studies were conducted. In Study 1, 5 groups of weaning rats were fed either a Cu adequate or deficient diet. After 5 weeks, 1 group from each diet was used to obtain baseline values; 1 Cu adequate group was crossed to a Cu deficient diet; 1 Cu deficient group was crossed to a Cu adequate diet; and 1 Cu adequate remained as control for a further 6 weeks of study. In Study 2, 4 groups of 9 week old rats were fed...
either a Cu adequate or deficient diet, and were either trained or sedentary for 8 weeks.

The results of these studies support the following:

1. Both young and older rats developed similar pathophysiological and ultrastructural changes. These were increased mitochondrial volume densities, degenerative changes such as sparse disarrayed myofibrils, decreased mitochondrial cristae and increased intracellular debris, with ECG abnormalities. However, older Cu depleted rats did not display overt cardiac hypertrophy, as encountered in young rats.

2. When previously Cu deficient rats were Cu repleted, some ECG abnormalities and degenerative changes were regressed, but there was no difference in ultrastructure after 6 weeks on the Cu adequate diet.

3. Histopathological abnormalities at the capillary-myocyte interface of Cu depleted rats demonstrated significant foci of basal laminae fragmentation and interstitial collagen hyperplasia, with invasion of phagocytes. It seems likely to be an early mechanism for the pathological development.

4. Trained rats were unable to complete their usual exercise regime with progressive Cu depletion, despite their healthy appearance. Our data suggest that Cu depletion and training interact synergistically to effect hyper-induction of collagen hyperplasia with deleterious effect on exercise capability.

5. Decreased activity of cardiac superoxide dismutase suggests contribution of increased free radical damage to subcellular degeneration. Decreased activity of cytochrome c oxidase in
skeletal muscle with Cu depletion, suggests complicity of cellular respiration.

It is recommended that the role of basal laminae in the histopathology of various organs with Cu depletion be pursued. The possible involvement of collagen type IV in basal laminae damage and the associative decreased functionality of cellular membranes and receptors may be one mechanism for the pathology of Cu deficiency. Furthermore, the focal nature of collagen hyperplasia, and the involvement of collagen type I interstitially and pericapillary, despite lack of overt fibrosis, could effect both cellular respiration through decreased oxygenation, and electrical conduction. These aspects warrant further investigation.

The observation that mitochondrial cytochrome c oxidase activity is diminished with Cu depletion, while mitochondrial protein is greatly increased in skeletal muscle, together with demonstrated increased cardiac mitochondrial volume density, warrants closer scrutiny. Subunit II of mitochondrial cytochrome c oxidase is thought to be Cu-dependent. The increase in mitochondria may be an attempt to increase surface to volume ratio, in order to maximize Cu intake.

To more closely approximate human conditions, the employment of marginally Cu deficient diets, over longer periods of time, is highly recommended. Very low levels of dietary Cu are unlikely to be encountered among humans on mixed diets, and are not compatible with life. Marginal dietary Cu levels could allow a more gradual development of the pathology.
APPENDIX A

Tissue embedment data sheet
Tissue embedment data sheet

Date............

Rat & vial number
#....................................
#....................................
#....................................
#....................................
#....................................
#....................................

Schedule

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<td>10</td>
</tr>
<tr>
<td>cacodylate rinse buffer</td>
<td>10</td>
</tr>
<tr>
<td>cacodylate rinse buffer</td>
<td>10</td>
</tr>
<tr>
<td>osmium tetroxide fixative</td>
<td>45–60</td>
</tr>
<tr>
<td>cacodylate rinse buffer</td>
<td>10</td>
</tr>
<tr>
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Dehydration sequence (2 changes each time)

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Spurr resin infiltration

<table>
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<tr>
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<tr>
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<td>spurr resin</td>
<td>60</td>
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</tbody>
</table>

Embedment and orientation

Polymerization, 60°C, overnight or till hard.
APPENDIX B

Reagents for tissue embedment
Reagents for tissue embedment

Rinse buffer

0.1 M cacodylate in sucrose.
Make up 0.2 M: Weigh 4.28 g Na-cacodylate and bring up to 80 mL with dd H_2O, pH to 7.4 and bring up to 100 mL. Pour into bottle, label and store closed in refrigerator.
Dilute buffer with 100 mL dd H_2O and add 7.2 g solid sucrose (MW sucrose = 360 g) to make 0.1 M cacodylate rinse buffer in 0.1 M sucrose. Label and store closed in refrigerator.

Fixative #1

2.5 % glutaraldehyde in 0.1 M cacodylate in sucrose.
Make up 25 % glutaraldehyde stock and keep sealed in refrigerator.
To 5 mL 25 % glutaraldehyde, add 45 mL 0.1 M cacodylate buffer in sucrose, to make 50 mL fixative. Label, seal and keep in dark bottle in refrigerator.

Fixative #2

1 % osmium tetroxide.
Always keep OsO_4 under double seal in refrigerator.
To make up 4 % OsO_4: Take 1 g vial, wash and rinse while sealed. Score with file, plunge into LN, using tongs, to allow crystals to fall free. Snap neck of vial, while holding with paper towels, and shake crystals immediately into already measured 25 mL dd H_2O in special bottle. Double seal and label. Leave at least 3 days in fume hood to dissolve. Sonicate or shake periodically.
Dilute stock 1:3 with rinse buffer to get 1 % OsO_4.

Acetone series

Dilute with dd H_2O: 30 %-50 %-70 %-80 %-85 %-95 %-100 %

Spurr resin

To make 250 mL: Use tripour beaker, tare weight, add sequentially and tare after each addition: 60 g VCD, 36 g DER 736, 156 g NSA. Stir with wooden applicator to mix well. Add 2 g DMAE. Mix very well (about 45 s) with slow strokes, to prevent formation of air bubbles. Distribute into 50 cc polypropylene tubes, label, seal and store in the freezer. Before use, warm to room temperature (about 1 h) and make sure outside is thoroughly dry before opening. Unused resin may be used again, after refreezing. To discard resin, polymerize at 60 °C overnight and through away when hard.
APPENDIX C

Tissue preparation for electron microscopy
Tissue preparation for electron microscopy

Procedure

Oven temperature must be set to maintain 60°C, with vent open.

1. Preparation

Rats are sacrificed by inhalation of CO₂. Make sure weights have been recorded. Thoracic cavities are opened and hearts exposed, then blood for hematocrit is obtained by cardiac stick. Immediately afterwards 1 M KCl is injected by cardiac stick. Hearts are removed and immediately perfused with Delbucco's to remove the blood, and weighed in Delbucco's, then fixed in glutaraldehyde in 0.1 M cacodylate rinse buffer in 0.1 M sucrose.

2. Obtaining samples

Using doubled edged EM razor blades, slice thin (< 1 mm) doughnut from apex of ventricles, slice to obtain left ventricle outer wall and trim to ≤ 0.5 mm. Keep under fixative. Slice tissue into thin slivers (< 0.5 mm), 10 per heart, drop into vials with fixative, and cover. Total time in fixative should be 1 - 1.5 h.

3. Rinse samples

Place wooden stand with vials in fume hood. Rinse samples 3 times in rinse buffer, using plastic disposable pipettes, mix well and let stand for 10 min each. Make absolutely sure that samples are never exposed to air. Use timer and set when first vial has been completed. See that vials are tightly covered. Glutaraldehyde waste may be thrown down the drain.

4. Post-fix in 1 % osmium tetroxide

Draw off rinse buffer with pipette and add OsO₄. Mix and leave covered for 45-60 min. Rotate occasionally.

If fresh Spurr is used, it should be made up at this stage, or if frozen Spurr should be taken out of freezer and placed in fume hood to thaw.

5. Rinse samples

Rinse 3 times as before. All OsO₄-containing waste must be poured into a disposable beaker to which 95%-100% ethanol has been added. Leave waste until liquid turns black, after which it may be poured down the drain.

6. Dehydrate samples in acetone series

Suction off the rinse buffer into the ethanol beaker and add the acetone sequentially, starting with 30 %. Do 2 changes each sample and leave covered for 10 min total per concentration. Use fresh 100 % acetone and a new pipette for the last change. (Clean molds by soaking in acetone for 5-10 min, and leave to dry in fume hood).

7. Infiltrate with Spurr
Dilute first resin 1:1 in 100 % acetone. Leave 45-60 min with mixing. Change to 100 % spurr resin and leave 1 h. If samples cannot be placed in a rotator, mix frequently but do not shake, to avoid the formation of bubbles. Keep tightly covered at all times, to avoid entrance of moisture. Repeat 2 more times. Samples may be left overnight at room temperature at any of these stages. Freeze sealed left-over resin. Place all spurr waste in one container.

Make labels for individual molds, using pencil on index cards.

8. Embedding

Embed in fresh spurr resin in embedding molds. Fill molds partially with fresh Spurr. Remove samples from vials onto glass slides, using a cut-off pipette and place samples in separate molds with wooden applicator sticks. Make sure to mark the molds according to rat and vial numbers. Position samples under dissecting microscope, using toothpicks. Orientate so that the outer free wall edge is at the top of the mold. Label.

9. Polymerize

Make sure samples are in position and not covered by labels. Move samples to incubator on rigid tray, to prevent shifting of the samples and labels, and polymerize at 60°C overnight. Also polymerize the container with spurr waste - this may be thrown away in the contaminated waste container when hard.

10. Store

Remove molds from oven and let cool. Remove blocks from molds and store on double-sided tape in cardboard boxes. Label clearly.
APPENDIX D

Thick sectioning for electron microscopy
Thick sectioning for electron microscopy

Switch hot plate on. Setting 4 or ± 60°C.

1. Rough trim

Place section in chuck (block holder), tighten with allen wrench. Screw chuck onto block facer. Mount on microtome by lifting left side lever and sliding block facer over mount. Adjust orientation by lifting side lever and turning block facer.

Focus microscope. Clean EM razor blade with 70% EtOH, dry with kimwipe. Use each blade only once. Trim away most of the Spurr, to expose shape of sample and to be in trapezoid shape. Be careful to slice away and down. Carefully slice empty Spurr on end point horizontally, parallel to microtome surface.

2. Thick section

Remove facing mount from microtome and unscrew chuck and sample. Mount chuck into receptacle on neck of microtome, make sure that face of sample is level and broad side is down.

Insert glass knife into knife holder by lifting little lever and sliding knife under. All screws must be loose. Make sure that back of knife is flush with back of holder and cutting edge is even with blade when raised. Screw side lever tight, set front screw till snug. Set locking lever. Mount onto knife holder, secure, set thickness to 2½ lines on side.

Mount knife holder onto microtome, align one side with "0". Secure with left lever. Make sure block is in forward and downward position of cutting swing, so that it is as far forward as it can get, by turning the large wheel at the right side of the microtome. Always turn wheel ONLY clockwise. Adjust position of glass knife edge to sample block and secure with front lever. Press reset button. Loosen screw on left, so that knife can be moved forward with coarse nut, for gross adjustment. Move the knife closer to the block, without touching. Tighten left screw to set fine adjustment.

Fill glass knife's boat with dd H₂O. Use advance drum to position glass knife edge very close to block edge. Move till the shadow on the block face JUST disappears. Make sure the speed, height and duration of swing passage settings are correct and switch on the motor (upper control 10, bottom 15). Use advance drum to make small advances until block is properly engaged.

Section until block surface is perfectly smooth and there are many good sections in the water. Clean boat out with absorbent EM paper. Section ± 6 good sections. Pull knife back from block and remove the sections with shaved wooden applicator stick, with rolling motion, into drop of water onto glass slide (cleaned with 70% EtOH).
Dry the water by placing the glass slide on heated hot plate. Keep one end off the plate to prevent burning. Add drop of 1% Toluidine Blue to the slide and replace on the hot plate until silver edge forms round the sides (± 15-30 s). Rinse dye with dd H₂O, dry bottom of slide and place back on hot plate to dry. Check under compound light microscope for desired qualities (longitudinal, quality). If good, label and reserve for thin sectioning. Label.
APPENDIX E

Daily alignment procedure for the electron microscope
Daily alignment procedure for the electron microscope

1. Spec holder out.
   Holy grid at 12 o 'clock.
   Focus at 12, MAG at Tap 1, C2 at 26.

2. KV on, uA, wait for needle to jump, slowly turn filament clockwise to give some illumination.
   C2 to crossover (smallest circle).
   Turn filament to see image of filament (if not able, take OBJ aperture out first (top large, anti-clockwise till it stops).

3. Use tilt knobs (top large L & middle) to make point of light concentric within circle of illumination.
   Use COND stigmata (each one) to get image of filament at crossover at sharpest focus. Use C2 to spread beam conc.

4. Spread beam w C2 (to reduce illumination on screen).
   Place spec holder in scope. Use translation knobs to get in field.
   Come to crossover w C2.
   Insert OBJ aperture (top large L, 2 clicks clockwise).
   Top of MAG knob to D+I (2 clicks to right).
   Use C2 to focus image & get pin point light. Center image with tilt knobs.

5. Saturate filament (image just disappears). MAG to 15 slowly.
   C2 to increase illumination. Use wobble for coarse & med focus.
   Find small hole in film on lollipop, focus med & fine focus, without wobble. Find fresnel fringe, using the black line and OBJ stigmata. The fringe must blend concentrically.

6. Spread the beam.
   C2 to 26, MAG to 1, holy grid out.

7. Put copper grid in spec holder and place in scope.
APPENDIX F

Operation of the electron microscope
Operation of the electron microscope

1. Put specimen in

C2 to 26, MAG to tap 1, pull empty spec holder out, load with copper grid and put into scope:
1 o'clock, PV light off
12 o'clock, PV lights off
anti-clockwise to 6 o'clock
let go all the way in, push button, pull out
let go button & turn to 12 o'clock

2. Increase illumination

Increase C2 slowly to 20 to 17/16 w large knob.
Use C2 to spread the beam. When tissue is brittle and likely to shatter, keep illumination low, find the right spot and increase just enough to enable focus and photo's. Use translation knobs to move grid to find right spot. Use lollipop & lever to focus binoculars to each eye. Focus with spec with coarse and medium knobs. Set with wobble button.

3. To take negatives

Position area needed between end points of small lines. Set MAG as needed, focus again, using wobble & coarse & med knobs, reduce light with C2 to 11 on meter remove lollipop, push toggle button, remove large screen, press EXP replace large screen & push toggle again.

4. To remove grid

C2 to 26, MAG to 1
turn anti-clockwise to 6
push button in, push all the way in
let go button, pull all the way out
turn to 12, pull out of scope

5. To finish session & take out film at end of session

Put empty spec holder back and turn to 12 o'clock.
C2 to 26, MAG to tap 1, focus to 12 o'clock, KV off position (out).
Filament turned anti-clockwise to off, current to zero.

6. To take out film

- pull center knob between drawers out 2 mm to click.
- pull straight out to vent camera. Leave ≥ 1½ min.
- vent vacuum oven by lifting lever, remove loaded cassette (with open bottom) and film, and take empty cassette (with smooth bottom) from shelf. Close oven, close lever, turn top lever and switch on to pressure of ± 20.
- right drawer: turn large knob so that flat side faces forward, open spring lever, open drawer and take out empty cassette and replace with loaded cassette. Close drawer with lever and turn large knob with flat side towards scope.
- left drawer: repeat, but replace loaded receiver cassette from drawer with empty smooth bottom cassette.
- turn center knob clockwise to stop, wait for PV light to go out.
- push center knob all the way in and turn clockwise to click.
  Pumping lights must be all out before filament can be turned on again.

7. To log off

Record date, name, negative number & number on back of scope.

8. To take negatives out

Take to dark room, replace used film with unused film from film box, put
used negatives in personal box for later development or develop straight
away. When done with the negatives, put full cassette and film box back
into vacuum oven and let pressure go to 30. Place empty cassette on
shelf.

9. To develop negatives

- open tap and let water bath fill up
- load negatives into plastic racks, careful not to touch
- remove lids on fixer & developer
- check temperature of developer, read time off chart
- turn water down low when bath is full, reduce temperature to ± 70°F
- turn on gaseous nitrogen
- lower plastic rack into developer with N switch down, then switch up
  when it is in
- set timer to specific time, remove rack and put into water
- set timer (1½ min) and move rack to developer (3½ min)
- place rack in water bath for 20 min
  Lights can be switched on.
- turn tap off, close N tank & vent nitrogen
- remove plastic racks from water bath, rinse off w dd H₂O & place in
  oven, switch on & set timer for 10 min
- place negatives into small envelopes and label.
APPENDIX G

Morphometric program
Morphometric program

To count volume densities

Start up computer, with BASICA DISK
WORK IN UPPER CASE
C:\menu> A:
A:/> BASICA
Insert MORPHO DISK
F3 to load MORPHO.BAS
F2 to run
Keywords: MITO

MYO

OTHER

ENTER

ENTER

Keys MITO = Z

MYO = X

OTHER = C

Using plastic counting sheet, do every 4th square.
Do 2 micrographs for each rat and average.
Do the first run, then punch 'Q' and write down the totals.
Press 'R' to restart.
APPENDIX H

Superoxide dismutase assay
Superoxide dismutase assay

Preparation of tissue

1. Thaw tissue samples and have bucket of ice and test tubes ready. Weigh out 1 g of tissue and place in test tube. Keep on ice. Be sure to record the weight of tissue samples.
2. To each test tube add 5 mL phosphate buffered saline (PBS) and homogenize in polytron. Dry the polytron between each sample, but don't rinse. Any tissue caught in the blades can be removed with forceps. Throw connective tissue away, but put muscle/organ tissue back in test tube and re-homogenize. Homogenize all samples of one type diet/treatment first, then rinse polytron, dry before doing the next treatment.
3. Place 1 mL of each sample homogenate into microfuge tube. Add 0.4 mL ethanol-chloroform to each tube and mix by inversion. Spin samples in microfuge for 5 min.
4. Remove precipitate and discard. Spin supernatant and ethanol-chloroform for another 2 min. Transfer supernatant to clean microfuge tubes. Use immediately, or store in freezer no longer than 1 month.

Prepare the spectrophotometer

1. Set to 320 nm no delay time, at room temperature, and read every 30 s for 2 min.
2. Stir TAPS buffer for 3-5 min to oxygenate. If it is stirred any longer, the mix will be unstable.

Determination of PG amount to add to TAPS

1. Place 1 mL TAPS buffer into each cuvette. Vary the amount of PG to add to TAPS buffer. Decide whether to use high or low blanks. High blanks are used for AC diets, and up to 5 wk for NC diets (PG ± 15-30 µL, dA 0.016); low blanks are used for NC rats > 5 wk (PG ± 10-15 µL, dA 0.008). Be sure to mix PG and TAPS well in cuvette (use parafilm).
2. Example: dA 30 s 0.016

<table>
<thead>
<tr>
<th>Cuvette</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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</thead>
<tbody>
<tr>
<td>PG</td>
<td>16</td>
<td>17</td>
<td>18</td>
<td>19</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>dA, 30 s</td>
<td>0.0117</td>
<td>0.012</td>
<td>0.0133</td>
<td>0.0147</td>
<td>0.0153</td>
<td>0.0171</td>
</tr>
</tbody>
</table>

The correct volume for PG is between 20 and 21, so try:

<table>
<thead>
<tr>
<th>Cuvette</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>20</td>
<td>20</td>
<td>20.5</td>
<td>20.5</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>dA, 30 s</td>
<td>0.013</td>
<td>0.015</td>
<td>0.016</td>
<td>0.016</td>
<td>0.0171</td>
<td>0.0176</td>
</tr>
</tbody>
</table>

The correct volume to add to TAPS this day, will be 20.5 µL PG

Preparation of sample

1. Dilute sample with PBS, 1:50 (for AC) or 1:25 (for NC)
2. Determine sample volume that will reduce dA by 50 % (from 0.016 to 0.008, high blank; or from 0.008 to 0.004, low blank)
3. To each cuvette, add: 1 mL TAPS (some cuvettes use 2 mL) correct volume PBS as determined ? diluted sample
(± 20-30 µL for AC; 40-100 µL for NC)
Calculations

\[ V_c \times D_s \times \frac{W_s + 5}{W_s} = U_{SOD} \]

where \( V_c = \) volume in cuvette
e.g. 1000 mL

\( V_s \)

\( W_s \)

\( V_s = \) volume of sample to
give 50% reduction

\( W_s = \) weight of sample

** low blanks will give \( \frac{1}{2} \) units, so multiply \( V_s \) by 2

Reagents

1. **TAPS buffer, 200 mL**
   2.43 g TAPS
   75 mg Detapac
   pH to 8.2 using 3 N NaOH and most of the water
   Bring up to volume with dd H₂O. Store at room temperature, good
   for about 30 d.

2. **3 N NaOH, 1 L**
   120 g Na pellets
   Bring up to volume with dd H₂O.

3. **PG, 40 mL**
   20 mg PG
   40 mL 0.01 N HCl
   Mix well, store sealed with parafilm in dark bottle, no > 3 d.

4. **0.01 N HCl, 1 L**
   86 mL stock HCl
   Bring up to volume with dd H₂O.

5. **Ethanol-chloroform, 40 mL**
   25 mL 100 % ETOH
   15 mL chloroform
   Store in freezer.
APPENDIX I

Cytochrome c oxidase assay
Cytochrome c oxidase assay

Method


Principle

The activity of cytochrome c oxidase (CCO) is measured by the decrease in optical density of the reduced cytochrome c as it is oxidized by the enzyme at 550 nm.

Reagents

Potassium phosphate dibasic (MW 174.2 g/mol)
Potassium phosphate monobasic (MW 136.1 g/mol)
Cytochrome c (horse heart, type IV, Sigma Chem. Co.)
Sodium hydrosulfate (sodium dithionate, Sigma Chem. Co.)
10 % Triton X-100 (Pierce)
Sucrose (used for liver preparation)

Phosphate stock buffers:
1. 1 M Potassium phosphate dibasic
   Weigh out 43.6 g
   Bring to 250 mL with S.Q.
2. 1 M Potassium phosphate monobasic
   Weigh out 34.025 g 1 M
   Bring to 250 mL volume with S.Q.
3. Titrate dibasic with monobasic to pH 7.0 using pH meter.
   Start by adding 100 mL of monobasic. Do at room temperature. Can be stored at 4°C up to 3 months.

Working phosphate buffer:
Dilute 200 mL of stock to 2 L (0.1 M).
Degas 1 L of this buffer to be used with the column.

Preparing the column

1. Swell 10 g Sephadex (G-25-80) with 100 mL of 0.1 M degassed phosphate buffer (pH 7.0) overnight.
2. Use the column dimensions of 1.5 X 27 cm.
3. Mix swelled gel in equal volume of buffer and pour into column.
   Allow to settle. Remove excess gel to the 27 cm mark and wash the column with 2 bed volumes of buffer at a flow rate of 0.5 mL/min.

Cytochrome c

Dissolve 23-35 mg CC in 0.1 mL of 0.1 M degassed phosphate buffer (40-80 mg/2 mL). Add excess sodium hydrosulfate (20-40 mg) and vortex. The CC will change color from dark red (oxidized) to dark orange (reduced).

Load the column with CC and start pump flow (flow rate 0.5 mL/min). Collect the pink fraction as it comes off the column. Watch carefully as it does not take long to collect.

Continue running the column to wash out the sodium hydrosulfate with at least 200 mL of 0.1 M phosphate buffer.

Set-up for working cytochrome c
Run absorbance reading on CC collected:
1.9 mL of 0.1 M phosphate buffer
0.1 mL of CC collected
Use dual wavelength function key on DU70
Program # 21
Wavelength A = 550
Wavelength B = 565
Factor = 1.00
Read average = 12
Calculation = (Abs 550/565)
**calculation must equal ≥ 6 - the ratio is usually 12-18.

**Working CC solution**

Use 50 μmole CC per test tube (MEQ = 19 600 mol/L/cm).

Calculate:
\[
\frac{\text{abs} @ 550 \text{ nm} \times \text{DF} (20)}{19 600 \times 1 000 \text{ mmol} / \text{ mol}} = Y \text{ μmol}
\]

\[
Z \text{ μL CC/ sample} = \frac{50 \text{ μmol} \times 2000 \text{ μL}}{Y \text{ μmol}}
\]

Into each sample tube:
Liver analysis: 2 000 mL in cuvette - [100 μL sample + Z μL CC]
Platelet analysis: 2 000 mL - [200 μL sample + Z μL CC]

Working CC solution can be made by multiplying the number of total samples plus several extras, times the volume of the calculated CC and the phosphate buffer respectively into a beaker.

Pipet 1.9 mL (liver preparation) or 1.8 mL (platelet preparation) of this working CC solution into a tube. Add 100 μL liver preparation or 200 μL platelet preparation. Vortex and immediately run sample through DU70 sipper.
APPENDIX J

Protein determination with folin phenol reagent
Protein determination with the folin phenol reagent

Principle
This procedure involves two color forming reactions to assay protein concentration photometrically. A complex is formed between protein and copper in alkaline solution. This complex reduces the folin reagent to form a colored compound that is measured photometrically. The reagent selectively reacts with aromatic amino acids. In the first reaction alkaline copper ions yield a deep bluish color in the presence of peptide bonds. In the second reaction, a complex inorganic salt mix yields an intense blue-green color in the presence of free or peptide bound tryptophan and tyrosine. The color formed is indicative of the protein concentration, since the combined levels of these amino acids are usually constant insoluble protein.

Sample
Any substance containing 40-300 mg protein. For this assay, 1 g of liver or 0.5 g of muscle sample will be homogenized in 5 mL dd H2O. Measure final volume of homogenate—this is necessary for the calculations.

Reagents
1. Alkaline copper solution: Make 500 mL at one time (at least 100 mL solution is needed for 6 standards and 3 samples plus doubles). Make a fresh solution each day.
   Dissolve 0.01 % CuSO4·5H2O in some dd H2O. Sequentially add 0.02 % sodium or potassium tartrate, 2 % Na2CO3 in 0.1 N NaOH.

2. 2 N Folin-phenol reagent: Normally made from 2 N reagent, diluted 1:1 with dd H2O. Store in refrigerator.

3. Protein standard (usually bovine serum albumin): 1 mg/mL.

Standard curve
1. Prepare a series of standards, in test tubes, from 0-0.6 mL standard solution, made up to 1 mL dd H2O.
<table>
<thead>
<tr>
<th>Test tube #</th>
<th>Standard, mL</th>
<th>H₂O, mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
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</tr>
<tr>
<td>4</td>
<td>0.3</td>
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<tr>
<td>5</td>
<td>0.4</td>
<td>0.6</td>
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<tr>
<td>6</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>0.6</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Procedure**

1. Add 100 μL sample homogenate to 4.9 mL dd H₂O (DF = 50) for liver, and heart, and 100 μL of sample homogenate to 2.4 mL of dd H₂O (DF = 25) for skeletal muscle. Use 1 mL diluted sample for assay. Use duplicate of samples.

2. To all tubes add 5 mL of alkaline copper reagent. Mix well and allow to stand for 10 min at room temperature.

3. Add 0.5 mL of folin-phenol reagent rapidly and vortex to mix immediately after adding. Let stand 30 min.

4. Read absorbance in spectrophotometer 20 at 500 nm. Plot a standard curve using prepared blank and standards to determine the amount of protein in the test tubes.
APPENDIX K

Animal use protocol approval request
Animal use protocol approval request

December 18, 1990
Memorandum
To: Dr. Margaret Duber Snyder  
Chairperson, ILACUC  
From: Dr. Denis M Medeiros  
Re: 89A0257  

Purpose  
To request permission to add an exercise protocol to the approved animal use protocol.

Rationale  
Copper deficient rats develop compromised cardiac function through ischemia associated with hypertrophy, necrosis and changes in blood pressure. There are no data that characterize the effect of exercise on the development of copper-induced cardiac pathology. Exercise is often proposed to improve cardiac function and to protect against the development of ischemic heart disease. This research will allow us to study the effects of endurance exercise on cardiac characteristics in copper deficient rats.

The inclusion of an exercise protocol may have human application, since endurance exercise is often recommended to reduce the risk for ischemic heart disease.

Number of rats: 34  
There will be two exercising groups and two sedentary control groups. Six animals per group are necessary to control variability and to allow meaningful statistical analysis. Four extra rats are included for each exercising group to allow for non-compliance and two extra rats for the copper deficient sedentary group to allow for higher mortality, as follows:

- No copper + exercise = 10 rats
- No copper, sedentary = 8 rats
- + copper + exercise = 10 rats
- + copper, sedentary = 6 rats

Duration of study: 4 to 5 weeks  
This will be sufficient for the development of measurable pathology, without undue mortality risk.

Anesthesia  
CO₂ will be used.

Parameters  
Cardiac function will be measured by electrocardiograph, rate of blood pressure increase, blood pressure and electron microscopy, as previously described. Erythrocyte superoxide dismutase and hematocrit will be measured to establish copper deficiency.

Pain or distress  
Rats in the exercise groups will be on a well established treadmill exercise protocol that is used internationally. An
avoidable electric shock grid is located at the rear of the treadmill to ensure that the animals maintain the required running intensity. The maximal electric output of the shocker control unit is less than 5 mA, but only the minimal stimulation to maintain running activity will be used. This is usually less than 3 mA and the exposure time to the shock is no less than the reflex time of the animal. Currents of this magnitude cause minimal discomfort. Experience indicates that a rat typically experiences one to three shocks per minute. Thus, if the rat contacts the shock grid 2 times/minute during a 120 minute training session, and the reflex time of the rat is 500 ms, the total duration of shock exposure will be 2 shocks/min x 120 min x .5 s = 120 s.

Normally the rat will resume running after contact with the shock grid. If it does not resume running and receives continuous repetitive shock for 10 s, it will be placed back on the treadmill to run. If it still refuses to run after five placements, it will be retired from running. If it is obvious that the rat is experiencing difficulty maintaining the pace of the treadmill and is receiving repetitive shocks of more than 15 shocks/minute, the rat will be removed from the treadmill, allowed to recover, and the exercise intensity reduced. If the rat still refuses to run even at reduced intensity, it will be removed from the study.

**Exercise protocol**

The rats will be randomly divided into four groups at 3 weeks of age. Treatment and diet will be assigned on a random basis. The ten rats in each of the two exercise groups will be exercised on the rodent motor-driven treadmill at 10 to 30 m/min, 8 to 18% grade, for 60 to 120 minutes. Speed, grade and duration of exercise will be gradually increased until the desired regime is reached to accommodate their ability and cardiac function. The animals will rest in their cages for 20 minutes after the first 60 minutes of running. This protocol will not cause undue duress. The two sedentary groups will experience a similar amount of handling. Exercise will continue from weaning to time of sacrifice.
LIST OF REFERENCES


