

Hearts in adult offspring of copper-deficient dams exhibit decreased cytochrome *c* oxidase activity, increased mitochondrial hydrogen peroxide generation and enhanced formation of intracellular residual bodies[☆]

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Abstract

The long-term effects of low dietary copper (Cu) intake during pregnancy and lactation on cardiac mitochondria in first-generation adult rats was examined. Rat dams were fed diets containing either low (1 mg/kg Cu) or adequate (6 mg/kg Cu) levels of dietary Cu beginning 3 weeks before conception and ending 3 weeks after birth. Cytochrome *c* oxidase (CCO) activity was 51% lower in isolated cardiac mitochondria from 21-day-old offspring of Cu-deficient dams than in the offspring of Cu-adequate dams. CCO activities in the cardiac mitochondria of 63- and 290-day-old offspring were 22% lower and 14% lower, respectively, in the offspring of Cu-deficient dams after they had been repleted with adequate dietary Cu from the time they were 21 days old. Electron micrographs showed that the size of residual bodies and the cellular volume they occupied in cardiomyocytes rose significantly between 63 and 290 days in the Cu-repleted offspring of Cu-deficient dams, but not in the offspring of Cu-adequate dams. The rate of hydrogen peroxide generation by cardiac mitochondria also was 24% higher in the 290-day-old repleted offspring of Cu-deficient dams than in the offspring of Cu-adequate dams. The increase in hydrogen peroxide production by cardiac mitochondria and in the relative volume and size of dense deposits in cardiomyocytes is consistent with increased oxidative stress and damage resulting from prolonged reduction of CCO activity in the offspring of Cu-deficient dams.

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1. Introduction

Cytochrome *c* oxidase (CCO), the terminal respiratory complex (complex IV) of the mitochondrial electron transport chain, is composed of 13 subunits, three of which are mitochondrial DNA gene products. Four redox-active metal centers containing copper (Cu) and heme are embedded in the mitochondrially encoded subunits, which serve as the catalytic core of the complex [1]. A number of studies have shown that Cu deficiency decreases CCO activity in a variety of organs [2–4]. The role of Cu as an

essential cofactor is likely involved in the reduction of CCO activity during Cu deficiency, but other factors also contribute to the loss of activity. The contents of nuclear-encoded subunits IV and V [5–7], CCO protein and heme associated with cytochrome *aa3* [8] are reduced in the cardiac mitochondria of Cu-deficient rats. Together, these findings indicate that impaired CCO assembly and loss of Cu from the active site both contribute to the loss of CCO activity during Cu deficiency.

CCO activity in the hearts of newborns is sensitive to maternal dietary Cu intake. Prohaska et al. [9] showed an 80% reduction in cardiac CCO activity in the 1-month-old offspring of dams that began consumption of a severely Cu-deficient diet on the seventh day of pregnancy. Furthermore, cardiac CCO activity in the offspring of Cu-deficient dams was about 10% lower in females and 20% lower in males after 1 month of Cu repletion compared to that in the offspring of dams that consumed adequate dietary Cu during pregnancy and lactation. These results indicate that reduced

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cardiac CCO activity in the offspring of Cu-deficient dams is resistant to repair by Cu repletion. An earlier report by Dallman [10] also showed that the reduction in cardiac CCO activity caused by Cu deficiency is resistant to repair by long-term Cu repletion and that even 50 days of Cu repletion are not sufficient to fully restore CCO content in the hearts of Cu-deficient rats.

Although it is unlikely that partial small reductions in cardiac mitochondrial CCO activity remaining after prolonged Cu repletion of Cu-deficient rats would significantly impair mitochondrial energy production, such reductions may increase the mitochondrial production of reactive oxygen species (ROS). Mitochondrial generation of ROS is governed largely by the redox state of respiratory complexes and is highest when the complexes are highly reduced [11,12]. It has been shown that partial inhibition of CCO increases hydrogen peroxide production in housefly flight muscle mitochondria [13], and increased hydrogen peroxide production has been reported in hepatic mitochondria from Cu-deficient rats [14]. These findings suggest that partial inhibition of CCO activity by either exogenous inhibitors or Cu deficiency creates a blockage of electron flow at the terminus of the electron transport chain, which sufficiently increases the reducing potential of upstream respiratory complexes to promote ROS formation through single-electron transfer to molecular oxygen. Thus, the persistence of reduced cardiac CCO activity in the offspring of Cu-deficient dams may cause prolonged exposure of cardiac mitochondria to elevated levels of ROS.

The persistence of reduced cardiac CCO activity in the offspring of Cu-deficient dams and the potential for cardiac mitochondria to experience prolonged exposure to elevated levels of ROS production in the offspring of Cu-deficient dams are relevant to human nutrition. The recommended daily allowance (RDA) and the estimated average requirement (EAR) for Cu are 1000 and 800 $\mu\text{g/day}$ Cu, respectively, during pregnancy, and 1300 and 1000 $\mu\text{g/day}$ Cu, respectively, during lactation [15]. Data on usual Cu intakes from food compiled in the National Health and Nutrition Survey II, the Continuing Survey of Food Intakes by Individuals II and the Total Diet Studies indicate that substantial numbers of women who are pregnant, lactating and aged 19–50 years do not meet either the EAR or the RDA for Cu (see Tables C-15, D-2 and E-3 in Ref. [15]). Although these surveys provide widely varying estimates of Cu intake from food, they indicate that Cu intakes below the EAR and the RDA are not uncommon for pregnant and lactating women and for women of reproductive age in general. However, it is not clear whether prolonged low Cu intakes by pregnant and lactating women can produce heart defects related to mitochondrial dysfunction in their adult offspring. The objective of the current study was to utilize a rat model to examine the potential of low Cu intake to alter CCO activity, ROS production and structure in the cardiac mitochondria of first-generation adults before conception and throughout pregnancy and lactation.

2. Materials and methods

2.1. Animals and diets

Adult (145–150 g) female Sprague–Dawley rats (Charles River, Wilmington, MA, USA) were housed in a room maintained at $22\pm 2^\circ\text{C}$ and $50\pm 10\%$ humidity with a 12-h light/dark cycle. The female rats were placed into two groups (12 rats/group) and fed AIN-93 G diet [16] formulated with $\text{CuSO}_4\cdot\text{H}_2\text{O}$ to contain either 1 mg/kg Cu (CuD diet) or 6 mg/kg Cu (CuA diet). The analyzed Cu contents of CuD and CuA diets were 1.06 and 6.02 mg/kg Cu, respectively. After 3 weeks of dietary treatment, the rats were mated with male Sprague–Dawley rats maintained on commercial rat chow. The pregnant dams were maintained on CuD and CuA diets throughout pregnancy and lactation. All litters were adjusted to eight pups and cross-fostered to dams within the same dietary treatment group, when necessary, to have four female and four male pups in each litter. Hearts and livers were harvested from two males and two females from each litter on Postnatal Day 21. One male and one female from each litter were then fed CuA diet for 6 weeks before organs were harvested. The remaining male and female from each litter were fed commercial rat chow (Purina; 13.1 mg/kg Cu) for 9 months before organs were harvested. The study was approved by the Animal Care and Use Committee of the Grand Forks Human Nutrition Research Center, and the rats were maintained in accordance with the National Research Council Guidelines for the care and use of laboratory rats.

2.2. Analyses

Adult rats were anesthetized with ketamine/xylazine, and the livers, hearts and blood were removed for analysis. Liver Cu and iron concentrations were measured by atomic absorption spectrophotometry [17]. Plasma ceruloplasmin was assayed in serum by its amine oxidase activity [18]. An electronic cell counter (Cell-Dyne 3500; Abbott Diagnostics, Abbott Park, IL, USA) was used to measure hemoglobin concentration and hematocrit. The livers and hearts were harvested from neonates on Postnatal Day 21 following decapitation.

Liver and heart mitochondria were prepared as previously described [19]. The two hearts harvested from male pups in each litter on Postnatal Day 21 were pooled as were the two hearts harvested from female pups to provide single samples for the isolation and biochemical analysis of mitochondria. Heart and liver samples from older rats were not pooled. In brief, liver and heart samples were weighed and homogenized in 10 vol of either liver homogenizing buffer (0.25 M sucrose, 10 mM HEPES and 0.1 mM EGTA, pH 7.4) or heart homogenizing buffer (0.225 M mannitol, 0.075 M sucrose, 20 mM HEPES and 1 mM EGTA). The homogenates were centrifuged at $600\times g$ for 10 min, and the resulting pellets were discarded. The supernatants were centrifuged at $7700\times g$ for 10 min, and the resulting mitochondrial pellets were washed once and resuspended in either liver homogenizing buffer or heart homogenizing buffer (1 ml/g tissue).

CCO activity in isolated mitochondria was assayed by monitoring the oxidation of ferrocytochrome *c* at 550 nm [20]. The rate of hydrogen peroxide production by heart mitochondria was determined by measuring the increase in fluorescence caused by the conversion of 10-acetyl-3,7-dihydrophenoxazine (Amplex Red; Molecular Probes, Eugene, OR, USA) to resorufin in the presence of horseradish peroxidase [21,22]. The assay was conducted with either glutamate/malate (10 mM glutamate and 5 mM malate) or succinate (7 mM) as substrate. The rate of hydrogen peroxide production with glutamate/malate as substrate was measured in the absence and presence of rotenone (2 μ M).

Protein concentrations in the mitochondrial preparations used for enzyme assays and measurements of hydrogen peroxide production were determined with bicinchoninic acid (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL, USA) using bovine serum albumin as standard.

2.3. Electron microscopy and morphometry

Heart tissue was taken as an oblique slice from the external lateral aspect of the left ventricle and diced to small blocks (~1.0 mm) in 4% glutaraldehyde in 0.1 M cacodylate buffer with 1 mM calcium chloride at pH 7.4. Following storage in a fixative for several days, the tissue was washed with 0.1 M cacodylate buffer containing 13.3% sucrose and 1 mM calcium chloride at pH 7.4. The tissue was then treated with 1% osmium tetroxide in buffer (pH 7.4), with sucrose adjusted to maintain osmolarity. After washing in the above buffer, the tissue was dehydrated in a graded series of ethanol concentrations and flat-embedded in an epoxy resin mixture (Embed-812; Electron Microscopy Sciences, Fort Washington, PA,

USA). Tissue samples were sawn from flat embedments and mounted, without orientation, onto resin cylinders. Sections were cut at a thickness of 70–90 nm with an LKB Ultratome V (LKB Produkter AB, Bromma, Sweden) equipped with a diamond knife (RMC, Inc., Tucson, AZ, USA). The sections were mounted on 200-mesh Cu grids (Ted Pella, Inc., Redding, CA, USA), stained for 10 min in an alcoholic uranyl acetate solution and prepared from equal volumes of absolute ethanol and a saturated solution of uranyl acetate in 50% ethanol followed by lead citrate for 3–5 min [23].

Tissue sites for morphometry micrographs were selected by orienting the electron microscope camera to grid bars at the corners of the grid opening. Sites were examined beginning with the leftmost corner of the grid square, proceeding clockwise around the square then moving to the adjacent square. A micrograph was recorded when the area examined was free of staining and sectioning artifacts, and was estimated to be composed of a minimum (in terms of area) of 50% cardiomyocytes, thus avoiding blood vessels, connective tissues and intercellular spaces. Four micrographs per animal were recorded on the negative at a magnification of $\times 5200$.

Negative images were enlarged 2.65 \times to produce prints for morphometric analysis. A transparent overlay grid, comprised of 1.0-cm squares, was used to derive the estimated relative cellular volumes of various structural elements and organelles and to assess the incidence of occurrence of some cytoplasmic inclusions [24]. Grid dimensions were based on the average area of the measured mitochondrial profile. As applied to four micrographs from each animal, there is potential to sample 1900 squares and/or 1728 intercept points. For an estimation of the relative

Table 1

The effect of maternal Cu intake on CCO activity in isolated heart and liver mitochondria in 21-day-old offspring and adult offspring consuming adequate dietary Cu for 6 weeks and 9 months after weaning

Age (days)	Maternal diet	Heart [μ mol cytochrome <i>c</i> oxidized/(min mg protein)]		Liver [μ mol cytochrome <i>c</i> oxidized/(min mg protein)]	
		Male	Female	Male	Female
21	CuA	2.20 \pm 0.24 (8)	2.10 \pm 0.13 (8)	1.10 \pm 0.13 (8)	1.12 \pm 0.14 (8)
	CuD	1.04 \pm 0.30 (8)	1.08 \pm 0.29 (8)	0.76 \pm 0.16 (8)	0.75 \pm 0.14 (8)
63 ^a	CuA	2.04 \pm 0.40 (10)	1.92 \pm 0.32 (10)	1.42 \pm 0.20 (10)	1.54 \pm 0.21 (10)
	CuD	1.55 \pm 0.27 (10)	1.54 \pm 0.34 (10)	1.32 \pm 0.16 (10)	1.48 \pm 0.12 (10)
290 ^b	CuA	2.86 \pm 0.65 (6)	3.02 \pm 0.38 (9)	0.37 \pm 0.07 (6)	0.47 \pm 0.08 (9)
	CuD	2.51 \pm 0.19 (8)	2.52 \pm 0.69 (8)	0.36 \pm 0.04 (8)	0.46 \pm 0.05 (8)
Age (days)	Variable	ANOVA			
		Heart		Liver	
21	Diet	<i>P</i> <.05		<i>P</i> <.05	
	Sex	NS ^c		NS	
	Diet \times Sex	NS		NS	
63	Diet	<i>P</i> <.05		NS	
	Sex	NS		<i>P</i> <.05	
	Diet \times Sex	NS		NS	
290	Diet	<i>P</i> <.05		NS	
	Sex	NS		<i>P</i> <.05	
	Diet \times Sex	NS		NS	

^a Fed purified diet containing 6 mg/kg Cu for 6 weeks postweaning.

^b Fed commercial rat chow containing 13 mg/kg Cu for 9 months postweaning.

^c NS, not significant.

sizes of specific organelles and inclusions among treatment groups, a 3-mm grid was applied to randomly encountered examples. Points over the residual bodies were used to estimate myocardium profiles and areas of the residual body [24]. The relative volumes of dense bodies were represented per volume of myocardium.

2.4. Statistics

Data were analyzed by two-way analysis of variance (ANOVA) to determine the effects of maternal diet, sex and Maternal Diet×Sex interaction followed by Tukey's multiple comparison to detect significant differences between means when the Maternal Diet×Sex interaction was significant (SAS/STAT Version 9.1; SAS Institute, Inc., Cary, NC, USA). Unless otherwise indicated, values in the text, table and graphs are presented as mean±S.D. Data regarding the Cu status of the dams were evaluated by Student's *t* test for unequal variance. Differences were considered significant at $P<.05$.

3. Results

Dams that began consuming a CuD diet beginning 3 weeks before conception exhibited signs of Cu deficiency 3 weeks

after parturition. Liver Cu concentration in dams that consumed a CuD diet was 5.8 ± 1.9 µg/g dry liver compared to 12.0 ± 1.1 µg/g dry liver in dams that consumed a CuA diet ($P<.05$, Student's *t* test). Plasma ceruloplasmin activity in dams that consumed a CuD diet was 12 ± 19 U/L compared to 116 ± 16 U/L in dams that consumed a CuA diet ($P<.05$, Student's *t* test). However, anemia was absent in the dams that consumed a CuD diet, and their hepatic Fe was not significantly elevated ($P>.05$, *t* test). Hemoglobin concentration and hematocrit were 156 ± 2 g/L and 0.45 ± 0.02 , respectively, in dams that consumed a CuD diet compared to 160 ± 2 g/L and 0.47 ± 0.03 , respectively, in dams that consumed a CuA diet. Liver Fe concentration in dams that consumed a CuD diet was 1.30 ± 0.32 mg/g dry liver compared to 1.09 ± 0.42 mg/g dry liver in dams that consumed a CuA diet.

Liver Cu concentrations indicate that the Cu status of the 21-day-old offspring of Cu-deficient dams was poor. Liver Cu concentrations were 17.8 ± 10.8 and 24.0 ± 14.1 µg/g dry liver in the 21-day-old male and female offspring, respectively, of Cu-adequate dams compared to 3.4 ± 2.2 and 2.9 ± 1.7 µg/g dry liver in the 21-day-old male and female offspring, respectively, of Cu-deficient dams ($P<.05$ for the effect of maternal diet, ANOVA). Neither the effect of sex nor the effect of Maternal Diet×Sex interaction on liver Cu was statistically significant.

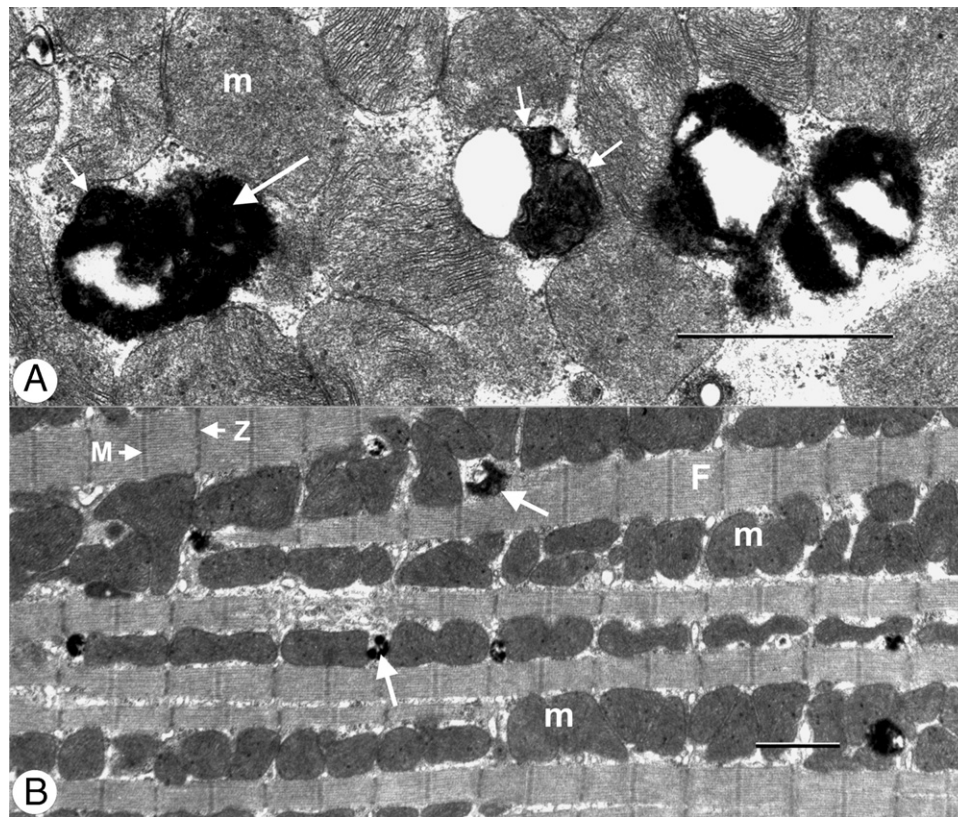


Fig. 1. Transmission electron micrographs from cardiomyocytes of a 290-day-old Cu-repleted female offspring of a Cu-deficient dam. (A) Details of perinuclear cytoplasmic inclusions. Residual bodies (large arrow) are interspersed among mitochondria (m) of normal morphology and configuration. A limiting membrane is apparent at points (small arrowheads) along the periphery of the residual bodies. (B) Lower magnification of cardiomyocyte organization. The myofibrillae (F) with very regular M and Z lines are separated by linear accumulations of mitochondria (m). Residual bodies (large arrows) are within the rows of mitochondria. Bars=1.0 µm.

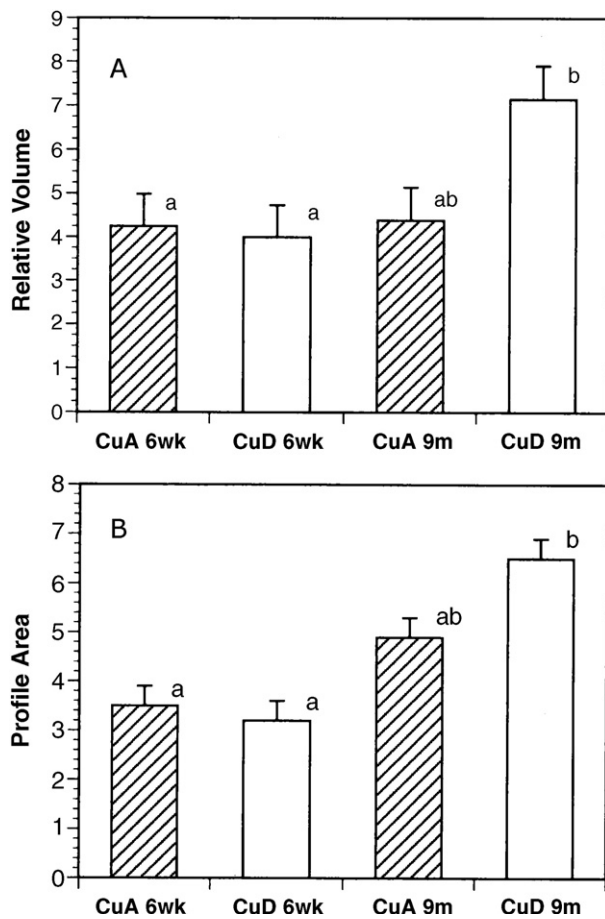


Fig. 2. The relative cellular volume (A) and area (B) of the residual bodies present in cardiomyocytes from the 290-day-old offspring of Cu-adequate dams (CuA; cross-hatched bars) and the 290-day-old Cu-repleted offspring of Cu-deficient dams (CuD; open bars). The relative cellular volume of the residual bodies is defined as: (total residual body area/myocardium area)×100. Profile areas are in square micrometers. The relative cellular volume of the residual bodies was significantly affected by age and Maternal Diet×Age interaction ($P<.05$, ANOVA). The areas of the residual body profiles were significantly affected by age, maternal Cu status and Age×Maternal Cu Status interaction ($P<.05$, ANOVA). Bars with different labels represent means that are significantly different ($P<.05$, Tukey's multiple comparison test). Values are mean±S.E.M. for combined male and female progeny.

Table 1 shows the effects of maternal diet, sex and Maternal Diet×Sex interaction on CCO activity in heart and liver mitochondria isolated from 21-, 63- and 290-day-old offspring of Cu-adequate and Cu-deficient dams. At all ages, CCO activity in heart mitochondria was significantly lower in the offspring of Cu-deficient dams even though the 63- and 290-day-old offspring had consumed adequate dietary Cu from the time they were weaned. Although CCO activity in hepatic mitochondria was significantly lower in the 21-day-old offspring of Cu-deficient dams, Cu supplementation fully restored CCO activity in the hepatic mitochondria of the 63- and 290-day-old offspring. CCO activities in the hepatic mitochondria of the 63- and 290-day-old offspring were not affected by maternal Cu

intake, but were slightly lower in males compared to females, regardless of maternal Cu intake.

Very few abnormalities were observed in the electron micrographs of cardiomyocytes from the Cu-repleted offspring of Cu-deficient dams. A representative transmission electron micrograph of cardiomyocytes from a 290-day-old offspring of a Cu-deficient dam is shown in Fig. 1. Conspicuous changes in mitochondrial size and matrix density were not observed. Structural alterations in Z-line alignment and myofibril bundles also were not observed. However, maternal diet affected the size of residual bodies interspersed among the mitochondria and the volume of the residual bodies relative to other cellular components in the cardiomyocytes. As shown in Fig. 2, the size of the residual bodies and the relative volume they occupy within the cardiomyocyte were significantly higher in the 290-day-old offspring than in the 63-day-old offspring of Cu-deficient dams. Differences in the size and relative volume of the residual bodies between the 63-day-old and the 290-day-old offspring of Cu-adequate dams were not statistically significant.

The effect of maternal Cu intake on hydrogen peroxide generation by heart mitochondria isolated from 290-day-old offspring is shown in Fig. 3. The rate of hydrogen peroxide production with glutamate/malate was significantly higher in the offspring of Cu-deficient dams than in the offspring of Cu-adequate dams ($P<.05$ for the effect of maternal diet). The rates of hydrogen peroxide production, with glutamate/malate as substrate in the presence of rotenone or with succinate as substrate, were not affected by maternal diet, sex or Maternal Diet×Sex interaction ($P>.05$, ANOVA).

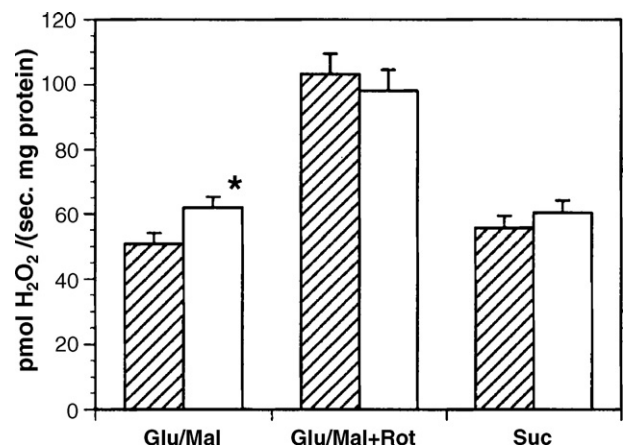


Fig. 3. The rate of hydrogen peroxide production by the heart mitochondria of the 290-day-old offspring of Cu-adequate dams (cross-hatched bars) and the 290-day-old Cu-repleted offspring of Cu-deficient dams (open bars). Hydrogen peroxide production was measured using glutamate/malate (10 mM glutamate and 5 mM malate) in the absence or presence of rotenone (2 μ M) or succinate (7 mM). Bars labeled with an asterisk indicate that the rate of hydrogen peroxide was affected by maternal Cu status ($P<.05$, ANOVA). Values are mean±S.E.M. for combined male and female progeny.

4. Discussion

In the present study, Cu deficiency in dams during pregnancy and lactation produced Cu deficiency in their 21-day-old offspring that was marked by reduced hepatic Cu concentration and reduced CCO activity in cardiac and hepatic mitochondria. Six weeks of repletion with adequate dietary Cu did not restore CCO activity in cardiac mitochondria from the offspring of Cu-deficient dams even though the activity was normalized in hepatic mitochondria. Furthermore, CCO activity in cardiac mitochondria from the 290-day-old offspring of Cu-deficient dams was lower than the activity in the 290-day-old offspring of the control dams after 9 months of Cu repletion. These findings agree with previous findings showing that CCO activity in the hearts of offspring of Cu-deficient dams is resistant to short-term (4–6 weeks) Cu repletion [9]. They also agree with an earlier study showing that the reduction of CCO activity in the heart mitochondria of Cu-deficient rats was not completely repaired by 50 days of Cu repletion [10]. The duration of Cu repletion was longer in our study than in earlier studies. Our results, together with those of earlier studies, confirm that the reduction of cardiac CCO activity caused by Cu deficiency is resistant to repair by Cu repletion and is prolonged well into adulthood in the offspring of Cu-deficient dams.

As suggested by Dallman [10], the rate of CCO repair in Cu-deficient rats following Cu repletion may be related to the rate of mitochondrial biogenesis. The half-life of heart mitochondria is about 18 days compared to about 9 days for liver mitochondria [25]. Thus, the resistance of cardiac CCO repair to short-term (4–6 weeks) Cu repletion compared to the relative ease of hepatic CCO repair may reflect the relatively slower turnover of the affected cardiac mitochondria. However, the reduction in cardiac mitochondrial CCO activity that occurred in the 290-day-old offspring of Cu-deficient dams after 9 months of Cu repletion suggests either that the affected mitochondria turn over more slowly than normal or that newly formed mitochondria have lower CCO activity resulting from a defect propagated during mitochondrial biogenesis. It is also possible that Cu deficiency during pregnancy and lactation irreversibly damaged Cu transport mechanisms in the developing cardiomyocytes of the fetus/neonate. Such damage has the potential to permanently impair the delivery of Cu to cardiomyocytes and to prevent the full restoration of normal intracellular Cu concentration and Cu-dependent functions in the heart after dietary Cu repletion.

In addition to having reduced CCO activity, cardiac mitochondria from the Cu-repleted 290-day-old offspring of Cu-deficient dams also exhibited slightly elevated rates of hydrogen peroxide production when glutamate/malate was used as substrate. The increase in the rate of hydrogen peroxide production is consistent with previous studies showing that inhibition of CCO by cyanide [13] or severe Cu deficiency [14] raises mitochondrial hydrogen peroxide

production. Although the major sites for mitochondrial ROS generation are complexes I and III [26,27], significant differences in hydrogen peroxide production by cardiac mitochondria from the offspring of Cu-deficient and Cu-adequate dams occurred when glutamate/malate (a complex-I-linked substrate) was used, but not when succinate (a complex-II-linked substrate) was used. This suggests that the source of ROS contributing to the increased rate of hydrogen peroxide production was upstream of complex III and was most likely complex I.

Electrons derived from NADH are conducted by complex I through flavin mononucleotide through a series of iron–sulfur centers that reduce ubiquinone, which then shuttles the electrons to complex III. Complex I also has two quinone or semiquinone binding sites that are likely sources of the superoxide that contributes to the mitochondrial formation of hydrogen peroxide [26]. Rotenone binds to the 20-kDa subunit of complex I and inhibits its activity by blocking the electron flow from the iron–sulfur center N_2 to quinone [26,28]. Rotenone causes an increased mitochondrial generation of superoxide and hydrogen peroxide at this site by inhibiting electron transfer to quinone and by promoting electron transfer to molecular oxygen. In our study, rotenone increased hydrogen peroxide generation by cardiac mitochondria from the 290-day-old offspring of Cu-deficient and control dams, but eliminated the difference in hydrogen peroxide production observed between these groups in the absence of rotenone. This suggests that rotenone maximized the production of hydrogen peroxide by the cardiac mitochondria of Cu-deficient dams by replacing partial blockage with a more complete blockage of electron transfer between the iron–sulfur center N_2 and the quinone. Although our findings only provide indirect evidence for partial blocking of electron transfer within complex I as a cause of increased hydrogen peroxide production, taken together with the reduction observed in CCO activity, they indicate that cardiomyocytes of the 290-day-old Cu-repleted offspring of Cu-deficient dams contain a population of mitochondria that have functional alterations in complexes I and IV.

Although mitochondrial biochemistry was altered in the adult offspring of Cu-deficient dams, the mitochondria did not exhibit gross swelling, cristae fragmentation and lipid droplet accumulation typically observed in Cu-deficient rats [29–32]. This is not surprising because these animals were subjected to Cu repletion and were not Cu-deficient. However, maternal Cu status had a subtle effect on residual bodies in cardiomyocytes. Significant differences in mean sizes and relative volumes of the residual bodies occurred between the 63-day-old and the 290-day-old offspring of Cu-deficient dams, but not between the 63-day-old and the 290-day-old offspring of Cu-adequate dams or between same-age offspring of either Cu-deficient or Cu-adequate dams. This indicates that age was a key determinant of the effect of maternal Cu status on the size and the relative volume of the residual bodies and suggests that normal aging processes that contribute to the formation of the

residual bodies in cardiomyocytes may be slightly accelerated in the offspring of Cu-deficient dams. Although the components of the residual bodies were not identified in the present study, the residual bodies we observed are visually similar to the residual bodies that accumulate in cardiomyocytes with age and whose accumulation correlates well with age-related increases in lipofuscin inclusions [33]. Proteins, including some of mitochondrial origin, that become highly cross-linked as a result of the oxidative modification of their side chains are a major component of lipofuscin [34,35]. Thus, it is possible that persistent reduction of CCO activity and elevation of ROS production in cardiac mitochondria may have promoted oxidative modifications of mitochondrial proteins that, in turn, accelerated the formation of residual bodies in the cardiomyocytes of the offspring of Cu-deficient dams. However, given the relatively small reductions in CCO activity and the increases in ROS production in cardiac mitochondria from the 290-day-old offspring of Cu-deficient dams, it cannot be conclusively stated that these changes in mitochondrial biochemistry were the sole cause of the accelerated formation of residual bodies.

Further research is required to determine if protein oxidation is accelerated in cardiac mitochondria in the offspring of Cu-deficient dams and to identify oxidized proteins. However, our findings indicate that exposure to maternal Cu deficiency during fetal and postnatal development may cause long-term biochemical changes in cardiac mitochondria that promote age-related heart pathologies in the first generation.

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