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Copper-Deficient Rat Embryos Are Characterized by Low Superoxide Dismutase Activity and Elevated Superoxide Anions[1]

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ABSTRACT

The teratogenicity of copper (Cu) deficiency may result from increased oxidative stress and oxidative damage. Dams were fed either control (8.0 μg Cu/g) or Cu-deficient (0.5 μg Cu/g) diets. Embryos were collected on Gestational Day 12 for in vivo studies or on Gestational Day 10 and cultured for 48 h in Cu-deficient or Cu-adequate media for in vitro studies. Superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione reductase (GR) activities were measured in control and Cu-deficient embryos as markers of the oxidant defense system. Superoxide anions were measured as an index of exposure to reactive oxygen species (ROS). No differences were found in GPX or GR activities among treatment groups. However, SOD activity was lower and superoxide anion concentrations higher in Cu-deficient embryos cultured in Cu-deficient serum compared to control embryos cultured in control serum. Even so, Cu-deficient embryos had similar CuZnSOD protein levels as controls. In the in vitro system, Cu-deficient embryos had a higher frequency of malformations and increased staining for superoxide anions in the forebrain, heart, forelimb, and somites compared to controls. When assessed for lipid and DNA oxidative damage, conjugated diene concentrations were similar among the groups, but a tendency was observed for Cu-deficient embryos to have higher 8-hydroxy-2′-deoxyguanosine concentrations than controls. Thus, Cu deficiency resulted in embryos with malformations and reduced SOD enzyme activity. Increased ROS concentrations in the Cu-deficient embryo may cause oxidative damage and contribute to the occurrence of developmental defects.

conceptus, developmental biology, embryo, stress

INTRODUCTION

Copper (Cu) deficiency during pregnancy can result in early embryonic death and gross structural abnormalities in the embryo and fetus, including skeletal, neuronal, pulmonary, and cardiovascular defects [1–3]. Using an in vitro rat embryo culture model, we have shown that Cu deficiency-induced embryonic anomalies result, in part, from an impaired oxidant defense system [4]. Morphologically, the Cu-deficient embryos were characterized by blisters, blood pooling, heart anomalies, and swollen hindbrain. Superoxide dismutase (SOD) activity was decreased in Cu-deficient embryos cultured in Cu-deficient serum compared to controls. The teratogenicity of Cu deficiency was reduced by addition of the antioxidant enzymes CuZnSOD or glutathione peroxidase (GPX) to the culture medium. Collectively, these data suggest that oxidative stress, secondary to an impaired oxidant defense system, is a mechanism contributing to Cu deficiency-induced teratogenesis.

Copper deficiency can directly impact cuproproteins with antioxidant or putative antioxidant functions, such as CuZnSOD, ceruloplasmin, or metallothionein [5, 6], and can indirectly lower GPX activity [7]. The enzyme SOD catalyzes the dismutation of superoxide anions into less reactive hydrogen peroxides. Under conditions of compromised Cu status, the activities of extracellular and cytosolic CuZnSOD were decreased in developing embryos and in weanling or adult rats [4, 7–9]. Lynch et al. [10] examined the effects of dietary Cu restriction on oxidative stress and damage in weanling male rats, and they observed a 68% reduction in CuZnSOD activity, a 58% increase in superoxide anions, and an increase in lipid peroxidation. Other investigations have also reported high levels of oxidative damage to macromolecules in Cu-deficient rats [11–14].

Considerable research has focused on weanling animals, but the relationship between a Cu deficiency-induced compromise of the oxidant defense system and oxidative damage has not been well characterized during embryonic development. Studies have shown that targeted disruption of CuZnSOD results in female homozygous mice that ovulate and conceive normally but are characterized by a striking increase in embryonic lethality [15, 16]. In the present study, we tested the hypothesis that Cu deficiency results in a compromised oxidant defense system, an increase in ROS, and oxidative damage. We measured the activities of GPX, glutathione reductase (GR), and SOD, and we assessed CuZnSOD protein in Cu-deficient and control embryos. We also quantified superoxide anions and determined their anatomical localization in the embryos. In addition, we determined the extent of Cu deficiency-induced embryonic lipid and DNA oxidative damage by assessing conjugated diene and 8-hydroxy-2′-deoxyguanosine concentrations, respectively. Both in vivo and in vitro models of Cu deficiency were used.

MATERIALS AND METHODS

Animals and Diets

The protocols used in this study were approved by the Animal Use and Care Administrative Advisory Committee of the University of California at Davis. Virgin female Sprague-Dawley rats (Simonsen Labs, Gilroy, CA) were housed individually in suspended stainless-steel, wire-bottom cages at 22°C on a reverse 12L:12D photoperiod (lights-on at 2000 h). Females were fed one of two egg white-based, purified diets: Cu adequate (8.0 μg Cu/g diet) or Cu-deficient (<0.5 μg Cu/g diet). The concentration of Cu in the purified diets was verified by flame atomic absorption spectrophotometry [17]. During the first 3 wk of dietary treat-

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ment, the Cu-deficient diet was supplemented with the Cu chelator triethylenetetramine (TETA; 1% w/w), which has been shown to facilitate a rapid Cu deficiency without inducing a secondary iron deficiency [1, 18]. After 3 wk, the dams were fed a Cu-deficient, TETA-free diet for the remainder of the study. After 4 wk of dietary treatment, the rats were mated for 3 h (1000–1300 h) with breeder males that were fed a commercial rodent diet (no. 557; Ralston Purina, Belmont, CA). The appearance of a copulatory plug was designated as Gestational Day (GD) 0.

In Vivo Experiments

Dams were anesthetized with CO_2 and killed on GD 12 via exsanguination by open-chest cardiac puncture. Copper status as assessed by plasma and liver Cu concentrations as well as maternal body weight and implantation sites were determined in a subset of dams. Maternal blood was collected into heparinized tubes and centrifuged at 1500 × *g* for 15 min. The liver was perfused with 0.9% (w/v) saline, excised, and weighed. Plasma and liver samples were stored at −80°C until analyzed for Cu concentrations. The uterus was removed from the dam, and the number of implantation sites was recorded. Embryos were dissected free of the uterus and placed in warmed (37°C) Tyrode buffer. The decidua and extraembryonic membranes were removed from the embryos under a dissecting microscope. All embryos were assessed for developmental abnormalities by light microscopy. A subset of embryos was then scored for somite pair numbers and measured for head and crown-rump lengths. One subset of the embryos was fixed overnight in 4% (w/v) paraformaldehyde (4°C) and processed for immunohistochemistry, whereas another subset of the embryos was used to determine superoxide anion localization. The remaining embryos were individually stored in 25 μl of Tyrode buffer, frozen in liquid nitrogen, and stored at −80°C until analyzed. The embryos in each of the assays included normal and malformed embryos.

In Vitro Experiments

Dams were killed on GD 10, and maternal tissues were collected as described above. Embryos were dissected free of their decidua and Reichert membrane, leaving their yolk sac intact. Embryos from control and Cu-deficient dams at the six- to eight-somite stage were placed into culture vials containing 4 ml of Cu-adequate (15.9 μM Cu) or Cu-deficient (0.9 μM Cu) rat sera. Culture vials containing two to four embryos were placed in a roller-incubator (30 rev/min; B.T.C. Engineering, Cambridge, U.K.) and incubated at 37°C for 48 h. Twice daily, the culture flasks were gassed with the appropriate O_2, CO_2, and N_2 gas mixtures [19]. At the end of culture, gross morphology of the embryos was examined by light microscopy. Embryo somite pair number was counted, and head and crown-rump lengths were measured in a subset of embryos. Embryos were then fixed, further cultured, or frozen as described above for analyses.

Serum Collection

Male Sprague-Dawley rats fed Cu-adequate or Cu-deficient diets provided serum for the embryo culture. Males were fed the Cu-deficient diet containing TETA for 3 wk and the Cu-deficient, TETA-free diet for 1 wk. The control males consumed the Cu-adequate diet for 1 wk. Males were anesthetized with ether and killed via exsanguination by open-chest cardiac puncture. Blood was collected in 15-ml syringes, pooled within a dietary treatment group, and centrifuged at 1500 × *g* for 15 min at 4°C. The serum was collected and heat inactivated at 56°C for 30 min, after which streptomycin and penicillin G were added to a final concentration of 100 U/ml and 100 μg/ml, respectively (Gibco BRL, Grand Island, NY). The Cu concentrations for the control and Cu-deficient sera were 15.9 and 0.9 μM, respectively, as determined by flame atomic absorption spectrophotometry. Serum was stored at −80°C in trace element-free conical tubes.

Protein Quantification

The protein content of embryos was determined according to the method of Bradford [20] using a commercial kit (Biorad, Hercules, CA).

GPX Activity

The GPX activity was determined on pooled embryos according to the method of Lawrence and Burk [21]. The GPX activity represented the change in absorbance resulting from H_2O_2-mediated oxidation of NADPH. Three embryos were pooled within a litter for the in vivo studies and within a treatment group for the in vitro studies. The embryos were sonicated in 300 μl of sucrose buffer (0.25 M sucrose and 10 mM Tris; pH 7.4). A 37.5-μl aliquot of the sonicated embryos was added to 675 μl of assay mixture (1.0 mM glutathione, 0.1 mM NADPH, and 1.0 U/ml of GR in 50 mM potassium phosphate, 1.5 mM EDTA, and 1 mM NaN_3; pH 7.0) and 37.5 μl of H_2O_2. Blanks consisted of 0.9% saline instead of the embryonic samples. The change in absorbance was measured at 340 nm on a Beckman DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA) on duplicate samples. The GPX activity is expressed as μmol NADPH oxidized/min/mg protein.

GR Activity

Embryos were analyzed for GR activity based on the change of absorbance from the GSSG (oxidized glutathione)-mediated oxidation of NADPH [22]. Embryos were pooled as described above and sonicated in sucrose buffer (0.25 M sucrose and 10 mM Tris; pH 7.4). A 37.5-μl aliquot of the pooled embryos was mixed with 637.5 μl of glutathione solution (oxidized form; 1.18 mM GSSG in 100 mM sodium phosphate and 1.5 mM EDTA buffer; pH 7.0), and 75 μl NADPH (1 mM sodium phosphate and 1.5 mM EDTA buffer; pH 7.0). An aliquot of 0.9% (w/v) saline was used for the blank. The change in absorbance was measured at 340 nm on a Beckman DU 640 spectrophotometer (Beckman Instruments). Samples were run in duplicate. The GR activity is expressed as μmol NADPH oxidized/min/mg protein.

SOD Activity

Total SOD activity was measured in individual embryo homogenates using a modification of a chemiluminescence assay [23]. The SOD activity was detected by the inhibition of the chemiluminescence of luminol (5-amino-2,3-dihydro-1,4-pthalazinedione) in the presence of superoxide anions generated from the reaction of xanthine oxidase with xanthine. Whole embryos were sonicated in 425 μl of 100 mM NaH_2PO_4 and 0.1 mM EDTA buffer (pH 7.8). The embryo homogenate (25 μl) was mixed with 600 μl of a luminol mixture (1.0 mM luminol, 50 mM $NaHCO_3$, 0.25 mM xanthine, and 0.1 mM EDTA; pH 10.1). Luminescence was initiated by the addition of 40 μl of xanthine oxidase (11 mU/ml in 50 mM $NaHCO_3$; pH 10.1) to the embryo homogenate/luminol mixture. The samples were read for luminescence at steady-state conditions on a TD-20E luminometer (Turner Designs, Sunnyvale, CA). The SOD activity was determined based on a standard curve generated using bovine erythrocyte CuZnSOD (Sigma, St. Louis, MO). To ensure that these values reflected embryonic SOD activity and not residual SOD from the culture serum that may have adhered to the embryo, SOD activity was measured in the Tyrode buffer that was used to rinse the embryo after culture. No SOD activity was detected in the buffer (data not shown).

Superoxide Anion Assay (Quantitative)

A spectrophotometric assay was used to quantify superoxide anions in Cu-adequate and Cu-deficient embryos [24]. The assay is based on the reduction of nitroblue tetrazolium (NBT) to formazan by superoxide anions. Two embryos were pooled within a litter and within a treatment group for in vitro studies and sonicated for 5 sec in 200 μl of sucrose buffer (0.25 M sucrose and 10 mM Tris; pH 7.4). A 30-μl aliquot of the embryo homogenate was mixed with 10 μl of NADH (1% [w/v]), 10 μl of NADPH (1% [w/v]), 600 μl of sodium carbonate buffer (50 nM; pH 10), and NBT (3.3 mg/ml). Each sample was run in duplicate. To determine the specificity of this assay for superoxide anions, each sample was also run in the presence of 50 μl of CuZnSOD (875 U; Sigma). Differences in the rates of NBT reduction were determined between samples with NBT alone and samples containing NBT and CuZnSOD. The change in absorbance was measured at 560 nm on a Beckman DU 640 spectrophotometer (Beckman Instruments). Superoxide anion concentration is expressed as μmol NBT reduced/min/mg protein.

Superoxide Anion Redox-Potential in Embryos (Qualitative)

The localization of superoxide anions was determined in both in vivo and in vitro embryos based on a modification of the method of Hobson et al. [25]. Immediately after dissection (in vivo) or 48 h culture (in vitro), intact, viable Cu-adequate and Cu-deficient embryos were incubated for 30 min in Hanks Balanced Salt Solution (HBSS) containing 2 mg/ml of NBT in a shaking water bath at 37°C. The embryos were rinsed in HBSS and examined by light microscopy for dark-blue, stippled staining, indicating the reduction of NBT to formazan by superoxide anions.

TABLE 1. Influence of copper deficiency on maternal weight, implantation sites, and copper status of GD 12 (in vivo) and GD 10 (in vitro) rat dams.[a]

	In vivo experiments		In vitro experiments	
Diet	Control	Cu-deficient	Control	Cu-deficient
No. of dams	12	11	14	16
GD 0 body weight (g)	219.3 ± 8.1	239.2 ± 5.3	234.1 ± 4.9	235.8 ± 3.8
Final body weight (g)	264.4 ± 6.5	273.1 ± 3.9	266.4 ± 4.5	268.9 ± 3.8
Implantation sites	11.1 ± 1.4	11.8 ± 1.1	14.1 ± 0.9	15.3 ± 0.7
Maternal plasma Cu (μM)	23.3 ± 0.8[b]	5.7 ± 1.4[c]	16.5 ± 1.5[d]	5.1 ± 1.1[e]
Maternal liver Cu (nmol/g)	71.0 ± 7.6[b]	43.3 ± 4.9[c]	72.6 ± 2.6[d]	22.1 ± 2.1[e]

[a] Values are expressed as mean ± SEM.
[b–e] Values within in vivo or in vitro experiments with differing superscripts are significantly different at $P < 0.05$.

CuZnSOD Protein

For immunohistochemistry, embryos were dehydrated through a graded series of ethanol washes, cleared in xylene, and embedded in molten paraffin at 56°C. The embryos were sectioned (thickness, 8 μm) on an '820' Spencer Microtome (American Optical Corporation, Buffalo, NY) and collected on microscope slides. The embryo sections were deparaffinized in xylene and rehydrated through an alcohol series to PBS. The sections were incubated in H_2O_2 for 30 min to block endogenous peroxidase activity and in 5% (v/v) serum to reduce nonspecific antigen binding. The sections were incubated overnight at 4°C with a CuZnSOD primary antibody (1:500; Chemicon, Temecula, CA). The following day, the embryo sections were incubated at room temperature with a biotinylated secondary antibody (1:500; Vector Laboratories, Burlingame, CA), and the antigen-antibody complex was visualized by the avidin-biotin-horseradish peroxidase staining system (ABC kit; Vector Laboratories) in the presence of 3-3′-diaminobenzidine tetrahydrochloride and H_2O_2. Embryos were counterstained with toluidine blue and mounted for evaluation by light microscopy.

Conjugated Dienes

The oxidation of fatty acids on phosphotidylserine, phosphotidylethanolamine, and phosphotidylcholine was assessed in embryos by measuring conjugated diene formation using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [26]. Fat was isolated from pooled embryos (3–4 embryos per litter for in vivo studies and 3–4 embryos within a treatment group for in vitro studies) using a standard Folch extraction technique [27] with a chloroform:methanol:water ratio of 8:4:3 (v/v/v). All reagents were HPLC grade, and both chloroform and methanol contained 0.002% (w/v) butylated hydroxytoluene to minimize oxidation during the extraction procedure. The lipid-rich organic layer was dried under nitrogen and resuspended in the working mobile phase. Samples were analyzed by HPLC using a normal-phase column (Supelcosil LC-SI, 25 cm × 2.1 mm, 5 μm; Supelco, Bellefonte, PA) with a LC-SI guard column. The mobile phase consisted of acetonitrile:hexane:phosphoric acid (95:3.1:1.9, v/v/v). Samples were eluted at a flow rate of 0.1 ml/min for the first 10 min. The flow rate was then increased over the next 0.5 min to 0.45 ml/min for the duration of the run. Samples were analyzed with an UV detector (1100 series diode array detector; Hewlett-Packard, Waldbronn, Germany) at 210 and 234 nm (4-nm bandwidth; reference of 550 nm with a 50-nm bandwidth). The ratio of oxidized (234 nm) to nonoxidized (210 nm) fatty acids was calculated for each class of phospholipids.

8-Hydroxy-2′-Deoxyguanosine

The DNA was extracted by a modification of the procedures of Nakae et al. [28] and Shigenaga et al. [29]. Embryos were pooled within a litter for in vivo studies and within a treatment group for in vitro studies. The embryos were homogenized on ice in 1 ml of homogenization buffer (10 mM Tris-HCl [pH 7.5], 0.32 M sucrose, 0.1 mM desferoxamine mesylate, and 5 mM $MgCl_2$) with a Teflon glass homogenizer and stored at −80°C until analyzed. Homogenates were centrifuged for 20 sec at 4°C and 10 000 × *g*. The pellets were resuspended in a lysis solution (10 mM Tris-HCl, 1% [v/v] Triton X-100, 0.32 M sucrose, and 5 mM $MgCl_2$; pH 7.5) and then vortexed. The above centrifugation and lysis steps were repeated on each sample. To the resulting pellets, 200 μl of 1% (w/v) sodium dodecyl sulfate and 5 mM Na_2-EDTA in 10 mM Tris-HCl (pH 8.0) were added. To each sample, 6 μl of solution containing 0.3 U of RNase A and 0.6 U of RNase T (Boehringer Mannheim, Indianapolis, IN) were added. Following a 10-min incubation at 56°C, 12 μl of proteinase K (20 mg/ml; Boehringer Mannheim) were added to the samples and incubated for 1 h at 50°C. Samples were centrifuged for 5 min at room temperature and 10 000 × *g*. Then, 300 μl of NaI solution (7.6 M NaI, 40 mM Tris-HCl [pH 8.0], and 20 mM EDTA-Na_2; pH 8.0) were added to the samples. Samples were mixed by inversion, and 500 μl of 2-propanol were added. Samples were mixed and the pellets collected following centrifugation for 10 min at room temperature and 10 000 × *g*. The pellets were washed sequentially with 1 ml each of 40% (v/v) 2-propanol and 70% (v/v) ethanol, briefly air-dried, and resuspended in 200 μl of 20 mM sodium acetate (pH 4.8) containing 0.1 mM desferoxamine mesylate. Samples were stored overnight at −20°C, and DNA hydrolysis occurred the following day as described by Shigenaga et al. [29] but with desferoxamine mesylate at a concentration of 0.1 mM.

Samples were analyzed using an 1100 HPLC system (Hewlett-Packard) equipped with a diode-array detector and an ESA Coulochem II electrochemical detector (ESA Inc., Chelmsford, MA). The chromatographic separation utilized a Supelco Supelcosil LC-18-DB 3-μm column (4.6 × 150 mm) with a Supelco Supelguard LC-18-DB guard column. The 8-hydroxy-2′-deoxyguanosine was eluted using 100 mM sodium acetate (pH 5.2) and 7% (v/v) methanol at a flow rate of 1.0 ml/min. The quantity of 8-hydroxy-2′-deoxyguanosine was determined using electrochemical detection and expressed relative to the quantity of 2′-deoxyguanosine in the sample. External standards (Sigma) were used to quantitate both forms of deoxyguanosine.

Statistical Analyses

Data are expressed as mean ± SEM. Data generated from in vitro experiments were analyzed independently of the in vivo data. Differences between Cu-adequate and Cu-deficient groups were determined by one-way analysis of variance (version 1.04; Statview, Berkeley, CA). Binomial statistics were used to analyze differences in the incidence of malformations. All statistical tests with a P value of <0.05 were considered to be significant.

RESULTS

Maternal Outcome

Dams fed the Cu-deficient diet had similar body weight and number of implantation sites as dams fed the control diet (Table 1). However, maternal Cu status was affected by dietary Cu deficiency, as shown by lower plasma and liver Cu concentrations in these dams compared to controls (Table 1).

Embryo Outcome

In vivo. Embryos removed from control dams at GD 12 typically developed normally. The Cu-deficient embryos were developmentally smaller than controls as assessed by head and crown-rump lengths and somite number (Table 2). A higher incidence of swollen hindbrain was found in Cu-deficient embryos than in controls (Table 2).

In vitro. The Cu-deficient embryos cultured in Cu-deficient sera were the same developmental stage as the in vitro controls when assessed by somite number; however, Cu-deficient embryos had smaller head and crown-rump lengths than controls (Table 2). Control embryos removed

TABLE 2. Influence of copper deficiency on morphology and percentage of developmental abnormalities in GD 12 rat embryos (in vivo) and GD 10 rat embryos cultured for 48 h (in vitro).[a]

	In vivo experiments		In vitro experiments	
	Control embryos	Cu-deficient embryos	Control embryos cultured in control sera	Cu-deficient embryos cultured in Cu-deficient sera
Morphological indices				
No. of embryos examined	107	115	127	93
Head length (mm)	2.40 ± 0.02[b]	2.22 ± 0.03[c]	2.06 ± 0.03[d]	1.71 ± 0.05[e]
Crown-rump length (mm)	4.66 ± 0.04[b]	4.32 ± 0.05[c]	3.77 ± 0.03[d]	2.99 ± 0.12[e]
Somite number	30.2 ± 0.1[b]	29.1 ± 0.2[c]	27.8 ± 0.1	28.1 ± 0.2
Developmental abnormalities (%)				
No. of embryos examined	238	183	135	140
Swollen hindbrain	0[b]	4.9[c]	8.9[d]	48.6[e]
Swollen forebrain	0	0	0.7[d]	36.4[e]
Blisters	0	0.6	3.7[d]	35.0[e]
Blood pools	0	0	0.7[d]	30.0[e]

[a] Values are expressed as mean ± SEM or as percentage as indicated.
[b–e] Values within in vivo or in vitro experiments with differing superscripts are significantly different at $P < 0.05$.

from dams at GD 10 and cultured for 48 h in control sera typically developed normally and were morphologically similar to in vivo control embryos (Fig. 1A). The Cu-deficient embryos cultured in Cu-deficient sera developed abnormally; these embryos had a high incidence of swollen hindbrain (48.6%), swollen forebrain (36.4%), blisters on the forelimb (35.0%), and extensive hemorrhaging in the dorsal aorta, pharyngeal arches, and caudal regions (30.0%) (Fig. 1B and Table 2).

GPX and GR Activities

The GPX and GR activities were similar in the Cu-deficient and control embryos in both the in vivo and in vitro experimental groups (Table 3).

SOD Activity

In vivo Cu-deficient embryos had lower SOD activity than control embryos (Table 3). The Cu-deficient embryos removed at GD 10 and cultured in vitro for 48 h in Cu-deficient sera had lower SOD activity than in vitro controls and even lower SOD activity than in vivo Cu-deficient embryos.

CuZnSOD Protein

As depicted by substrate staining, CuZnSOD protein was widely expressed in both control and Cu-deficient embryos, with lower expression in the neuroepithelium and foregut (Fig. 1, C and D). Based on detection in situ, CuZnSOD protein expression was not affected by Cu deficiency, as evidenced by the comparable pattern and intensity of staining in the Cu-deficient embryos and their respective controls.

Superoxide Anions (Quantitative)

No differences in superoxide anion concentration were found in embryos removed from Cu-deficient and control dams at GD 12 in vivo (Table 3). With regard to embryos removed from dams on GD 10 and cultured in vitro for 48 h, control embryos exhibited the same degree of superoxide anion concentrations as in vivo controls. Embryos from Cu-deficient dams cultured in Cu-deficient sera were characterized by an elevated concentration of superoxide anions compared to controls.

Superoxide Anion Redox-Potential in Embryos (Qualitative)

The Cu-deficient embryos from both the in vivo and in vitro experiments had increased areas of superoxide anion localization compared to controls as determined by formazan staining (purple stipple) (Fig. 1, E and F). Specifically, the prosencephalon, pharyngeal arches, forelimb, somites, and optic and heart regions of Cu-deficient embryos stained positively for superoxide anions. Addition of CuZnSOD in the presence of NBT effectively prevented the staining (data not shown).

Conjugated Dienes

No differences were found in fatty acid oxidation as assessed by the ratio of oxidized to nonoxidized phosphotidylserine, phosphotidylethanolamine, and phosphotidylcholine between the in vivo control and Cu-deficient embryos (Table 4). With respect to embryos cultured for 48 h in vitro, no differences were found in the oxidation of phosphotidylethanolamine or phosphotidylcholine between the treatment groups. However, in vitro control embryos had more oxidized phosphotidylserine than the Cu-deficient embryos.

8-Hydroxy-2′-Deoxyguanosine

The Cu-deficient embryos, both in vivo and in vitro, tended to have higher concentrations of 8-hydroxy-2′-deoxyguanosine than control embryos, although this did not reach statistical significance (Table 4). In vitro embryos had higher concentrations of 8-hydroxy-2′-deoxyguanosine than in vivo embryos.

DISCUSSION

Similar to our previous findings, no differences were observed in maternal outcome with respect to body weight or number of implantation sites between Cu-deficient and control dams. Overall, the in vitro embryos were developmentally less mature than the in vivo embryos. However, in both the in vivo and in vitro experiments, Cu-deficient embryos were smaller than controls as assessed by head and crown-rump lengths. Consistent with our previous report [4], the control embryos in vivo taken on GD 12 were morphologically similar to the in vitro controls. We observed a small number of abnormalities in the in vivo Cu-

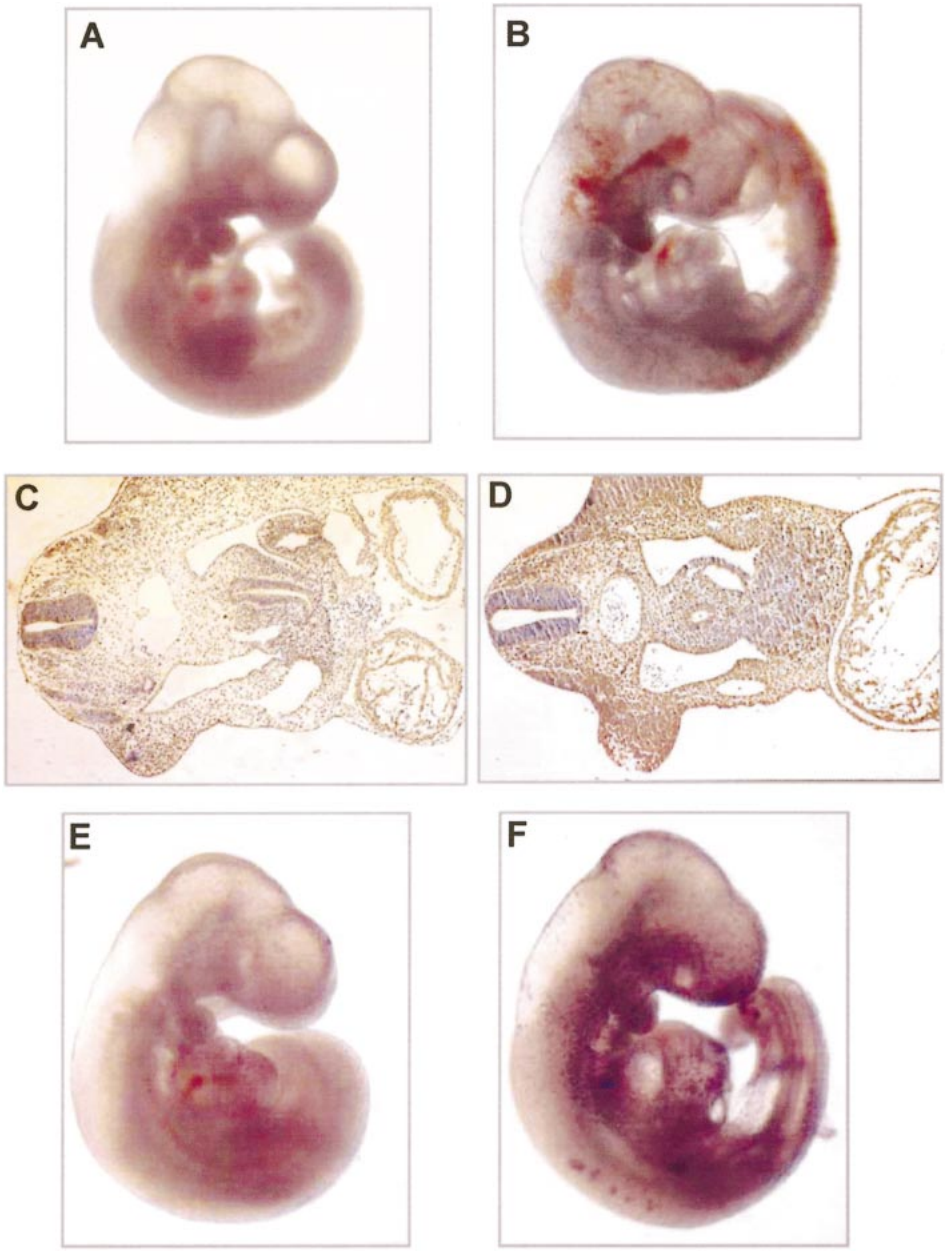

FIG. 1. Representative photomicrographs of rat embryos explanted on GD 10 and cultured for 48 h. **A**) Control embryo cultured in control serum shows normal development. **B**) Embryo explanted from Cu-deficient dam and cultured in Cu-deficient serum shows hindbrain swelling, forebrain hypoplasia with blebbing, hemorrhages in the pharyngeal arches and caudal regions, and abnormal heart with swelling of the pericardium. **C** and **D**) Transverse sections (×10) of CuZnSOD protein expression in control embryo cultured in control serum (**C**) and in Cu-deficient embryo cultured in Cu-deficient serum (**D**). **E** and **F**) Formazan staining (purple stipple) shows little superoxide anions in the control embryo cultured in control serum (**E**), whereas the Cu-deficient embryo cultured in Cu-deficient serum (**F**) shows superoxide anions localized in the somites, forebrain, pharyngeal arches, and optic and heart regions.

deficient embryos, which is consistent with previous reports showing that in vivo Cu deficiency occasionally results in swollen hindbrains [30]. In contrast, Cu-deficient embryos cultured in vitro were characterized by blisters, hemorrhaging, swollen hindbrain, and swollen forebrain. The difference in the developmental outcome of the Cu-deficient embryos from the in vivo and in vitro studies may be caused by the teratogenic nature of culture sera, which was more severely depleted in Cu (0.9 μM) than in the sera to which the embryos were exposed in utero (≈5.0 μM). We have shown that when Cu-deficient embryos are cultured in Cu-deficient culture media that has been repleted with Cu or are cultured in serum from Cu-adequate animals, the embryonic malformations are decreased [4, 31]. These data demonstrate the sensitivity of postimplantation rat embryos to changes in culture Cu concentrations.

Copper deficiency has been shown to result in low activities of components of the oxidant defense system, including GPX and CuZnSOD [7, 32]. No effect of dietary Cu or culture media Cu on embryonic GPX or GR activities was observed. Whereas these data show that the Cu-deficient and control embryos have a similar capacity for converting H_2O_2 to more innocuous compounds, the specific fate of the H_2O_2 in the embryos is unknown. The H_2O_2 may be converted to water by either GPX or catalase or to more reactive intermediates, such as the hydroxyl radical, via the Fenton reaction, which can damage macromolecules. We previously reported an improved developmental outcome of Cu-deficient embryos cultured in sera supplemented with GPX as assessed by a decreased incidence of blisters, hemorrhages, and heart abnormalities [4]. The protective effect of GPX may be the result of directing H_2O_2, which has a high diffusion constant, to form water and, thereby, to protect certain components of the embryo from hydroxyl radical attack. Alternatively, GPX may be acting as an antioxidant at the yolk sac, thereby reducing the teratogenicity of Cu deficiency. Given our findings of improved development in Cu-deficient embryos with addition of the oxidant defense enzymes GPX or CuZnSOD [4], we

TABLE 3. Oxidant defense enzyme activities and superoxide anion concentrations in GD 12 rat embryos (in vivo) and GD 10 rat embryos cultured for 48 h (in vitro).[a]

	In vivo experiments		In vitro experiments	
	Control embryos	Cu-deficient embryos	Control embryos cultured in control sera	Cu-deficient embryos cultured in Cu-deficient sera
Glutathione peroxidase activity (μmol NADPH oxidized/min/mg protein)	22.0 ± 0.6	22.4 ± 0.9	16.7 ± 1.3	12.9 ± 3.6
Glutathione reductase activity (μmol NADPH oxidized/min/mg protein)	10.8 ± 0.7	11.4 ± 0.06	23.4 ± 5.7	11.5 ± 4.7
SOD activity (U/mg protein)	3.90 ± 0.58[b]	2.14 ± 0.41[c]	6.69 ± 1.77[d]	1.37 ± 0.16[e]
Superoxide anions (nmol NBT reduced/min/mg protein)	3.8 ± 0.9	3.6 ± 0.9	3.9 ± 0.9[d]	112.7 ± 29.9[e]

[a] Values are expressed as mean ± SEM.
[b–e] Values within in vivo or in vitro experiments with differing superscripts are significantly different at $P < 0.05$.

suggest that Cu deficiency results in an oxidative stress that is teratogenic.

Elevated reactive oxygen species (ROS) can occur when ROS production is increased and/or the oxidant defense system is decreased. High levels of ROS have been implicated as a causative agent in the pathologies of a number of teratogens, including ethanol, cocaine, thalidomide, and diabetes [33–36]. Importantly, antioxidant addition has been correlated with decreased ROS concentrations and modulation of the teratogenicity of some agents [24, 36, 37]. For example, SOD addition to culture media diminished the teratogenesis, superoxide anion generation, lipid peroxidation, and free radical damage in ethanol-exposed mouse embryos [24].

Of particular interest to us is the primary oxidant defense enzyme CuZnSOD, which catalyzes the dismutation of superoxide anions to H_2O_2 and oxygen. The CuZnSOD is expressed in developing rat embryos [38] and is responsive to changes in dietary Cu [4]. In both the in vivo and in vitro experiments, Cu-deficient embryos had lower SOD activity than controls. We hypothesized that the low SOD activity in Cu-deficient embryos compromises the ability of the embryo to dismutate superoxide anions and, thereby, exposes the embryo to excessive ROS, resulting in subsequent ROS-induced embryonic damage. Our results show a differential response between in vivo and in vitro Cu deficiency. With regard to in vivo experiments, Cu-deficient embryos at GD 12 had lower SOD activity but similar superoxide anion concentrations to controls as well as few structural defects. In contrast, Cu-deficient embryos cultured in Cu-deficient sera were characterized by an even lower SOD activity, a marked increase in the concentration of superoxide anions, and a high incidence of structural anomalies. The Cu status is more severely compromised in the in vitro (0.9 μM Cu) than in the in vivo model (≈5 μM Cu), a situation that is reflected by the lower SOD activity of the cultured Cu-deficient embryos compared to in vivo Cu-deficient embryos. The timing and severity of maternal Cu deficiency can affect the incidence of malformations. Maternal Cu deficiency secondary to chelating drugs such as D-penicillamine or TETA results in a high incidence of malformations [39–41]. Similarly, Cu deficiency secondary to defects in Cu transporters, such as in human patients with Menkes disease (i.e., defect in the Cu-transporter ATP7A) or in mice with a disruption of the metallochaperone Atox1 locus, has also been shown to be teratogenic [42, 43]. It has been reported that the embryo culture technique results in an increased oxidative stress condition relative to that occurring in vivo [44]. However, our findings of similar superoxide anion concentrations in the in vivo and in vitro control embryos suggest that either the culture environment itself may not result in an excessively high oxygen tension or that the control embryos are better able than the Cu-deficient embryos to adapt to an increased oxygen load. These data also suggest that the embryo culture condition may amplify the developmental risk of Cu deficiency, or that embryos with suboptimal Cu nutrition may be more susceptible to additional oxidative insults.

The large increase in superoxide anions in Cu-deficient embryos cultured in vitro relative to controls in both experimental groups may be a consequence of an increase in membrane permeation of oxygen caused by the Cu-deficient culture conditions, low SOD activity, or low cytochrome *c* oxidase activity [4], which may result in leakage of electrons from the electron-transport chain to form superoxide anions. In the case of cocaine teratogenicity, electron-transport particles prepared from malformed limb buds spontaneously leaked electrons and reduced molecular oxygen to superoxide, in contrast to other nonmalformed re-

TABLE 4. Conjugated dienes[a] as an index of lipid peroxidation and 8-hydroxy-2′-deoxyguanosine[b] as an index of DNA oxidation in GD 12 rat embryos (in vivo) and GD 10 rat embryos cultured for 48 h (in vitro).

	In vivo experiments		In vitro experiments	
	Control embryos	Cu-deficient embryos	Control embryos cultured in control sera	Cu-deficient embryos cultured in Cu-deficient sera
No. of embryos examined	9	9	7	8
Phosphotidylserine[a]	12.11 ± 0.81	11.87 ± 1.05	12.97 ± 0.58[c]	10.41 ± 0.71[d]
Phosphotidylethanolamine[a]	9.14 ± 0.77	9.00 ± 0.39	9.93 ± 0.29	10.34 ± 0.67
Phosphotidylcholine[a]	18.81 ± 1.32	17.59 ± 0.61	20.73 ± 1.69	21.27 ± 2.77
8-Hydroxy-2′-deoxyguanosine[b]	0.76 ± 0.15	0.93 ± 0.17	1.54 ± 0.33	2.43 ± 1.11

[a] Conjugated diene values are expressed as mean ± SEM of the ratio of oxidized to nonoxidized fatty acid.
[b] 8-Hydroxy-2′-deoxyguanosine concentrations are expressed as 8-hydroxy-2′-deoxyguanosine/10^5 2-deoxyguanosine.
[c, d] Values within in vivo or in vitro experiments with differing superscripts are significantly different at $P < 0.05$.

gions of the embryo, that would only favor leakage of electrons to molecular oxygen if the electron-transport chain was artificially inhibited at cytochrome c oxidase with the addition of sodium azide [34].

To determine if the structural anomalies in the Cu-deficient embryos occurred in regions with high ROS, the spatial localization of superoxide anions was determined. In both in vivo and in vitro Cu-deficient embryos, the regions with the highest level of superoxide anions were the prosencephalon, heart, forelimbs, and somites. These areas corresponded to regions of Cu deficiency-induced abnormal development (e.g., blisters develop in the prosencephalon and forelimb region, and the heart is distended). Excessive superoxide anion accumulation in select areas of embryos exposed to teratogens has also been noted with ethanol toxicosis, a condition of increased oxidative stress [24].

The findings reported in the literature concerning the effects of Cu deficiency on CuZnSOD protein expression have been equivocal. Some investigators have reported that Cu-deficient rats have a low expression of CuZnSOD protein [45, 46], whereas others have reported that the protein level is unaffected [5]. Our data show that in both in vivo and in vitro experiments, Cu-deficient embryos have a level of CuZnSOD expression that is similar to that found in control embryos. Thus, the lower SOD activities in Cu-deficient embryos are likely caused by a decreased availability of the Cu catalytic cofactor. The importance of a functioning oxidative defense system in embryo development has been described by Ho et al. [15], who reported that female mice lacking CuZnSOD are characterized by an increase in embryo lethality. Given that CuZnSOD can also alleviate the teratogenicity of a broad number of embryotoxic agents, including ethanol [24] and hyperglycemia [23], CuZnSOD has considerable relevance to the developing embryo.

We have previously shown that addition of antioxidants to the culture media reduces some, but not all, of the Cu deficiency-induced malformations, indicating that other mechanisms in addition to increased oxidative stress may underlie the teratogenesis of Cu deficiency [4]. For example, cytochrome *c* oxidase activity is decreased in Cu-deficient embryos, with a tendency for lower ATP concentrations in these embryos [4]. The activity of lysyl oxidase, a cuproenzyme involved in the cross-linking of extracellular matrix proteins, is also low under Cu-deficient conditions [47]. Thus, in addition to increased ROS, altered energy metabolism as well as compromised vessel integrity may account, in part, for the abnormalities observed in the Cu-deficient embryos.

The lack of apparent oxidation to lipid membranes of Cu-deficient embryos may be attributed to a variety of factors. Given that for the conjugated diene measurements, the embryos were pooled in batches of three and included malformed as well as normal embryos, subtle differences in oxidation may not have been detected. In addition, the Cu-deficient embryos may have experienced membrane damage, but because of changes in the fatty acid profile of the membrane, such changes may have gone undetected. Alterations in the fatty acid profile of select tissues in response to oxidative insults in adult tissues is well recognized [48]. What has not been determined is whether the type or amount of embryonic polyunsaturated fatty acid can be influenced by elevated oxidative pressure. It is noteworthy that a change in the fatty acid profile alone may result in dysmorphogenesis. Mouse embryo culture studies have shown that addition of certain fatty acids, particularly polyunsaturated fatty acids, to culture media inhibited the development of pronuclear and 2-cell embryos [49]. The addition of antioxidants such as SOD to the culture media reversed the inhibitory effects of the polyunsaturated fatty acids.

Our data show a trend toward a higher concentration of oxidized DNA in Cu-deficient, whole-embryo homogenates compared to controls. Others have reported an increased susceptibility to DNA oxidative damage with Cu deficiency [50]. In future studies, the use of immunohistochemistry and specific antibodies to oxidized lipid and DNA should further define and localize the oxidative damage in Cu-deficient embryos. We are in the process of determining whether the areas of increased superoxide anion concentrations (i.e., prosencephalon, forelimb, somites, and heart region) are also areas of increased oxidative damage to macromolecules in Cu-deficient embryos.

Overall, embryos cultured in vitro for 48 h had higher concentrations of oxidized DNA than in vivo embryos, indicating that the culture conditions may induce some oxidative stress. The Cu-deficient embryos cultured in Cu-deficient serum had the highest concentrations of 8-hydroxy-2′-deoxyguanosine, highest concentrations of superoxide anions, lowest SOD activity, and highest frequency of malformations. These data indicate that embryos with suboptimal Cu nutrition are more susceptible to an oxidative insult. In conclusion, the low SOD activity, elevated concentrations of superoxide anions, and correlation of spatial localization of the superoxide anions with the abnormal morphometry of the Cu-deficient embryos support the hypothesis that oxidative damage, occurring secondary to impaired oxidant defenses, is one mechanism underlying Cu deficiency-induced abnormal development.

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