Hyperbaric Hyperoxia Exaggerates Respiratory Membrane Defects in the Copper-Deficient Rat Lung

THOMAS K. AKERS¹ AND JACK T. SAARI²

¹Department of Physiology, School of Medicine, University of North Dakota, Grand Forks, ND 58202; and ²United States Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202

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ABSTRACT

Scanning (SEM) and transmission electron microscopy (TEM) were used to examine the effect of dietary copper deficiency and hyperbaric hyperoxia, alone and in combination, on lung structure. Male, weanling Sprague-Dawley¹ rats were fed a copper-deficient (CuD, 0.2 μg/g) or copper-adequate diet (CuA, 5.1 μg/g). After 35–41 d on their respective diets, rats from each group were placed inside a pressure vessel kept at 27°C under one of two pressure protocols. Air controls were maintained at 1 atm for 75 min. Rats exposed to oxygen were maintained at 1 atm of air plus 3 atm of oxygen for 1 h and then decompressed for 15 min. Under SEM, none of the treated lungs (CuD, CuA-O₂ exposed, or CuD-O₂ exposed) showed abnormal lung morphology from the conducting bronchioles down to the alveoli. Copper-deficient red blood cells were abnormally shaped. Under TEM, CuA-O₂-exposed lungs showed thicker respiratory membranes, especially basement membranes and endothelial cells, and alveolar Type II cells having more than the usual number of surfac-

*Author to whom all correspondence and reprint requests should be addressed.

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tant vacuoles. CuD lungs also showed thicker endothelial and basement membrane components of the respiratory membrane, but normal looking Type II cells. CuD-O₂-exposed lungs showed greatly thickened respiratory membranes and severe disruption of both endothelium and basement membrane and, judging by the increased number of nuclei per field, an increase in the number of both Type I and Type II cells. We conclude that copper deficiency enhances the damage caused by O₂ toxicity, an effect that may be caused by reduced antioxidant status.

**Index Entries:** Copper deficiency; hyperbaria; hyperoxia; lung; electron microscopy; peroxidation; free radicals.

## INTRODUCTION

The morphological effects of copper deficiency on the lung of the young rat have been likened to those of developmental emphysema (I), a condition characterized by structural deterioration of the alveolar wall. Although the etiology of this defect is not understood for emphysema, the defect in copper-deficient lungs, as well as structural defects in other organs, has been attributed to a deficit in the copper-dependent enzyme, lysyl oxidase, and a resultant inability to crosslink connective tissue proteins (2–5).

Evidence is accumulating that indicates that oxygen-derived free radicals and other reactive species may also contribute to organ damage in copper deficiency. This evidence includes the reduction of antioxidant enzyme activity in copper deficiency (6–9), the inhibition of effects of copper deficiency by supplementation with exogenous antioxidants (10,11), as well as the enhanced formation of products of lipid peroxidation in copper deficiency (12–16).

Given the above general evidence for oxidative damage in copper deficiency, it is appropriate to ask whether CuD lungs are more susceptible to such damage than normal lungs. A common method of applying an oxidative stress to the lungs is by placing animals in a hyperoxic or hyperbaric hyperoxic environment. Hyperoxia has been shown to enhance production of breath ethane (17) and thiobarbituric acid reactive compounds in lungs of experimental animals (18); both are evidence of increased lipid peroxidation. Hyperbaria and/or hyperoxia are known to cause changes in lung morphology and biochemistry in proportion to the length or magnitude of exposure (18–21).

Copper deficiency enhances breath ethane production in normoxic and hyperoxic rats (16). Mortality and the lung edema associated with hyperoxia are enhanced by copper deficiency (22). The purpose of this study was to determine, by scanning and transmission electron microscopy, whether copper deficiency enhances any of the morphological changes caused by hyperbaric hyperoxia in the rat lung.
MATERIALS AND METHODS

Animals and Diets

Twenty-eight male, weanling, Sprague-Dawley rats were fed (ad libitum) a purified diet that was either deficient in copper (CuD, \( n = 15 \)) or was supplemented with 5 \( \mu g \) of copper/g of diet (CuA, \( n = 13 \)). The purified diets were based on casein (20%), sucrose (39%), cornstarch (29%), and safflower oil (5%). Mineral and vitamin compositions were those described by Johnson and Kramer (23). All animals received deionized water ad libitum. They were housed in quarters maintained at 22–24°C with a 12 h light-dark cycle. After 35–41 d on their respective diets, the rats were transported from the USDA Human Nutrition Research Center to the University of North Dakota Physiology Department High Pressure Life Laboratory.

Pressure Protocol

Ten CuD rats and eight CuA rats were placed in a hyperbaric chamber containing 1 atmosphere absolute (ATA) of air plus 3 ATA of 100% oxygen that was maintained for 1 h; the chamber was then decompressed over 15 min. Five CuD and five CuA rats were placed in the chamber and exposed to air at 1 ATA for 1.25 h.

Electron Microscopy

Rats were removed from the chamber and immediately anesthetized (pentobarbital sodium, 65 mg/kg, ip). Each rat’s trachea was exposed by glottal incision and cannulated. Lungs were fixed in vivo (2% glutaraldehyde in Hanks saline at 4°C) by dripping fixative into the tracheal cannula from a reservoir placed 80 cm above each rat. Fixation proceeded for 20–30 min with continuous thorax massage to ensure complete fixation. One lung was removed from each rat and placed in an ice bath-cooled beaker containing fixative for 1–2 h. Heart, liver, kidneys, and the remaining lung were also removed for subsequent mineral analysis.

Apex, cardiac, and basal regions of each lung were dissected and postfixed with 2% OsO\(_4\) in 0.072N sodium cacodylate buffer for 30 min; tissues were then rinsed four times with sodium cacodylate buffer (4°C) at 15-min intervals, the last rinse being held overnight.

Acetone gradients were used for dehydration of scanning electron microscope (SEM) samples; ethanol gradients were used for transmission electron microscope (TEM) samples. All tissues were placed in the initial 30% gradient for 30 min–1 hr (4°C) and then treated with 50%, 70%, 90%, and two changes of 100% dried-gradient for 6 min/step.

SEM tissues were brought to room temperature and critical-point dried with a Balzers Union drier (Hudson, NH) or Peldri (Ted Pella, Inc,
Redding, CA). Tissues were sputter coated for 3–5 min with a gold/palladium deposition by use of a Technics Hummer I (Anatech, Ltd, Alexandria, VA) sputter coater. Specimens were viewed on a Hitachi S-800 SEM (Mountain View, CA) and preserved in a glass desiccation jar.

After dehydration, TEM tissues were treated with two changes of propylene oxide at 15-min intervals. Embedding into beam capsules was completed by using an EMS Araldite #6005 Kit (Fort Washington, PA). Thin sections were cut on a Sorval Porter-Blum MT-2 (Dupont, Newtown, CT) ultra microtome with a MJO-Diatome diamond knife (Fort Washington, PA). Sections were stained on copper grids with uranylacetate and lead citrate solutions and viewed on a JEOL 100-S TEM (Peabody, MA).

**Blood, Organ, and Diet Analysis**

Serum was analyzed for ceruloplasmin (24) by using a Cobas Fara automated analyzer (Roche Diagnostics Systems, Nutley, NJ).

The lung, liver, heart, and kidney, as well as diet samples, were assayed for copper and iron content. Organs were lyophilized, digested with nitric acid and hydrogen peroxide, diluted in hydrochloric acid and analyzed for mineral content by flame atomic absorption spectrophotometry (Perkin Elmer, Model 503, Norwalk, CT) (25). The same procedure, except for lyophilization, was followed for diet samples. Analysis of three samples of each diet indicated that the CuA diet contained 5.1 ± 0.2 (SD) μg copper/g diet and the CuD diet contained 0.2 ± 0.1 (SD) μg copper/g diet. Analytical assurance was accomplished by using National Institute Standards and Technology (NIST) reference standard #1577a (bovine liver) for organ analysis and NIST standard #1572 (citrus leaves) for diet analyses. Measured mineral contents of reference standards were within the ranges specified.

**Statistical Analysis**

Two-way analysis of variance (ANOVA) (26) was used to assess the effects of copper deficiency and hyperbaria on animal characteristics related to copper status.

**RESULTS**

**Copper Status**

Characteristics of rats fed CuA and CuD diets are shown in Table 1. Direct evidence that copper status was influenced in CuD rats was the decreased copper content in liver and lung. Also found were the characteristically higher liver iron content and lower lung iron content in CuD rats when compared to CuA rats. Rats fed the CuD diet had lower body
<table>
<thead>
<tr>
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<th>CuA</th>
<th>CuD</th>
<th>ANOVA, p values</th>
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<tr>
<td></td>
<td>Air</td>
<td>O₂</td>
<td>Air</td>
</tr>
<tr>
<td>Liver Cu (μg/g dry wt)</td>
<td>11.9 ± 1.2*</td>
<td>12.3 ± 0.8</td>
<td>1.8 ± 0.8</td>
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<td>Liver Fe (μg/g dry wt)</td>
<td>323 ± 40</td>
<td>366 ± 37</td>
<td>505 ± 108</td>
</tr>
<tr>
<td>Lung Cu (μg/g dry wt)</td>
<td>5.9 ± 1.0</td>
<td>5.1 ± 1.3</td>
<td>3.8 ± 1.1</td>
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<tr>
<td>Lung Fe (μg/g dry wt)</td>
<td>636 ± 384</td>
<td>899 ± 383</td>
<td>305 ± 96</td>
</tr>
<tr>
<td>Ceruloplasmin (mg/dL)</td>
<td>29.8 ± 11.0</td>
<td>23.9 ± 5.2</td>
<td>4.0 ± 0.3</td>
</tr>
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<td>Hematocrit (%)</td>
<td>44.0 ± 2.0</td>
<td>45.0 ± 3.0</td>
<td>25.0 ± 7.0</td>
</tr>
<tr>
<td>Heart weight/body wt (mg/g)</td>
<td>3.6 ± 0.2</td>
<td>3.7 ± 0.3</td>
<td>6.6 ± 1.2</td>
</tr>
<tr>
<td>Body wt (g)</td>
<td>269 ± 18</td>
<td>252 ± 29</td>
<td>196 ± 41</td>
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*Values are mean ± SD.
Fig. 1. Terminal bronchiolar wall in CuA (A), CuD (B), CuA-O₂-exposed (C), and CuD-O₂-exposed (D) lungs. From this view, none of the structures, including cilia (Ci), microvilli (Mv) and mucus caps (MC), were affected by either treatment. Bar = 10 μm.

weights, higher heart weights and heart weight/body weight ratios, lower hematocrits, and lower serum ceruloplasmin than CuA rats; all are signs previously associated with copper deficiency (27,28).

**Scanning Electron Microscopy**

SEM micrographs (Fig. 1) revealed no qualitative differences between the four treatment groups with regard to appearance of terminal bronchiolar structures, including mucous cells, cilia and microvilli. Similarly, no qualitative differences between the treatment groups were ob-
Fig. 2. Alveolar surface in CuA (A), CuD (B), CuA-O₂-exposed (C), and CuD-O₂-exposed (D) lungs. In this qualitative view neither treatment affected size or shape of alveoli. Bar = 40 μm.

served under SEM (Fig. 2) regarding shape, relative size, or structure of alveoli.

SEM micrographs of erythrocytes in pulmonary capillaries (Fig. 3) revealed no effect of hyperoxia on cell shape, but copper deficiency caused erythrocyte shape changes that may be described as cupped (stomatocytic), crenated (echinocytic), or triconcave (knizocytic) (29).
Fig. 3. Erythrocytes trapped in lungs from CuA (A), CuD (B), CuA-O₂-exposed (C), and CuD-O₂-exposed (D) lungs. Erythrocytes from CuA lungs, (A), and (C), have a normal biconcave shape. Erythrocytes from CuD lungs, (B) and (D), may be cupped (Cp), triconcave (Tr), or crenated (Cr). Bar = 2 μm.

**Transmission Electron Microscopy—Type I Cells**

Figure 4 illustrates respiratory membranes in the region of the alveolar type I cell. In the normoxic, CuA (control) rats, the distance between the alveolar space and the blood was narrow and included intact endothelial, basement, and epithelial (alveolar Type I cell) membranes.
Fig. 4. Blood-air barrier in the region of the Type I alveolar epithelial cell in CuA (A), CuD (B), CuA-O₂-exposed (C), and CuD-O₂-exposed (D) lungs. Capillary is on the left side of each micrograph, alveolus is on the right. The three components of the membrane are (left to right) capillary endothelium (En), basement membrane (BM), and Type I epithelium (Ep). Thickening of the respiratory membrane, in particular endothelium and basement membrane, is evident in CuD (B) and CuA-O₂-exposed (C) lungs. Combined treatment caused greater thickening of all membrane components and disruption of endothelium and basement membrane. Bar = 1 μm.
mally occurring vesicles were apparent in both endothelial and epithelial cells. Hyperoxia in CuA rats caused a widening of the basement membrane, some breakdown of the endothelial membrane, and sometimes-observed enhancement of vesiculation in the epithelial cells. In normoxic CuD lungs the distance between the alveolar space and the blood was increased mainly by an increase in the thickness of the endothelial cell and possibly by some increase in the thickness of the basement membrane. In lungs with combined oxygen toxicity and copper deficiency, the epithelial cell appeared somewhat wider but relatively normal in structure. However, both basement and endothelial membranes were much wider than normal and severely disrupted.

Figure 5 illustrates some additional features associated with combined oxygen toxicity and copper deficiency. A capillary and three associated alveoli are shown. Red blood cells were abnormally shaped. By qualitative assessment, a greater number of more prominent nuclei were present per representative field in both endothelium and Type I epithelium. Some disruption of endothelium was apparent and severe destruction of basement membrane occurred.

**Transmission Electron Microscopy—Type II Cells**

Alveolar type II cells are illustrated in Fig. 6. Type II cells of normoxic, CuA lungs typically contained five to six surfactant-containing lamellar bodies. They had microvilli on the alveolar surface and normal-looking mitochondria. The hyperoxic, CuA type II cells typically contained more than twice as many lamellar bodies per cell. The appearance of the normoxic CuD Type II cell was very similar to that of the corresponding CuA cell in terms of lamellar body numbers, microvilli, and mitochondria. The primary feature of hyperoxic, CuD Type II cells was the prominent nuclei, a characteristic shared with both endothelium and Type I cells (Fig. 5) under this condition. The cells otherwise looked normal, both in terms of number of lamellar bodies and structure of the cell.

**DISCUSSION**

The primary findings of this study are that copper deficiency caused an enhancement of respiratory membrane disruption by hyperbaric hypoxia, in particular of the endothelium and basement membrane, and an apparent proliferation of endothelium and Type I and Type II epithelial cells in response to hyperbaric hypoxia.

Prior studies have indicated that copper deficiency, whether induced by diet (1,30,31), chelation (32), or genetics (33), causes morphological changes in the lung. Those prior studies indicated an enlargement of alveoli and disruption of connective tissue, primarily elastin. Although the present study showed evidence of damaged connective tissue (Figs. 4
Fig. 5. Generalized membrane structure of a CuD-O_{2}-exposed lung in the vicinity of a capillary (Cap) and three alveoli (A). Note misshapen erythrocytes (E), prominent multiple nuclei (N), indicating cellular proliferation of both endothelium and Type I cells, and thickened, disrupted, and vacuolated intercellular basement membrane space (arrows). Bar = 2 μm.

and 5), SEM micrographs indicated no obvious alveolar enlargement (Fig. 2). We suspect that this latter difference resulted from the lower severity or duration of our deficiency as compared to that of other studies. Despite the lack of a visual change of alveoli under SEM, it is quite apparent under TEM that an alteration of the respiratory membrane did occur in CuD animals (Fig. 4). It is important to recognize that the observed effect of copper deficiency on the basement membrane structure of the lung is paralleled by similar effects on kidney (34,35), pancreas (36), and heart (37,38). This indicates that basement membrane compro-
Fig. 6. Type II cells in CuA (A), CuD (B), CuA-O₂-exposed (C), and CuD-O₂-exposed (D) lungs. Copper deficiency alone (B) causes no apparent change in these cells. O₂ exposure (C) enhances the number of surfactant-producing lamellar bodies (LB). Combined treatment (D) shows no apparent increase in lamellar bodies, but nuclei (N) are more numerous and prominent, suggesting cellular proliferation and possible remodeling. Bar = 2 μm.

mise is a general occurrence in organs subjected to copper deficiency and may be expected to lead to common functional deficits (for instance, alteration of membrane permeability) in all organs.

Whereas interpretation of most findings in CuD lungs has focused on the impairment of activity of the copper-dependent enzyme lysyl
oxidase, further work has indicated that CuD lungs may be susceptible to damage by hyperoxia, thus suggesting the importance of copper-dependent antioxidant enzymes in lung function. Jenkinson et al. (22) found that lung edema and mortality were increased by dietary copper deficiency in rats exposed to hyperoxia (85% O₂ for 4 d); further, survival time in a hyperbaric hyperoxic environment (100% O₂, 4 ATA) was severely reduced in CuD rats. These findings were associated with a reduction of lung superoxide dismutase (SOD) activity in CuD animals. The observation of edema in hyperoxic, CuD rats (22) was reinforced by the present study, which indicated an enhanced disruption and thickening of the blood-air barrier, in particular of the endothelium and basement membrane, by exposing CuD animals to hyperbaric hyperoxia (Fig. 4). The physiological consequence of this disruption and the resultant edema would be reduced gas transport.

A recent study by Taylor and Bray (39) noted changes in wet lung weight similar to those of Jenkinson et al. (22), but, in contrast, found that hyperoxia (85% O₂ for 7 d) enhanced lung superoxide dismutase activity and produced no evidence of lung damage by proton magnetic resonance imaging in CuD animals. Reasons for the discrepant data of the above two studies (22, 39) at atmospheric pressure are not obvious, but they suggest that the conditions of the Taylor and Bray study were less severe, thus enabling an induction of SOD activity by hyperoxia that protected against severe lung damage. Regardless of the absence of exact agreement between these studies, their results tend to emphasize the interplay between antioxidant enzyme (SOD) and hyperoxic damage in copper deficiency.

The apparent increase in the number of more prominent nuclei per field in CuD hyperoxic lungs could be interpreted as an increase in number of cells, that is, cellular hyperplasia. Because we did not count cells, this cannot be an absolute conclusion. Another interpretation for the apparently prominent nuclei might be a form of dysplasia, that is, cellular alteration and distortion involving the nuclei. Hyperplasia and dysplasia are both common responses to cellular injury (40). Either type of remodeling, in particular that involving type II cells, would tend to thicken the respiratory membrane and impair gas transport. Surfactant-producing lamellae in type II cells of CuD hyperoxic lungs apparently were not increased as they were in CuD hyperoxic lungs; this suggests that surfactant production is copper dependent or that energy is spent on cell proliferation over surfactant production in response to the combined insult of copper deficiency and hyperoxia.

A finding not related to the main focus of this study is our observation of altered erythrocyte shapes in copper deficiency. Depressed hematocrit is a common sign of dietary copper deficiency (6) and the question regarding its mechanism has not been answered. The relative rapidity of the development of anemia following copper deprivation (41) argues for a destructive mechanism, as opposed to reduced production of cells. The
altered erythrocyte shapes observed in this study are all consistent with the predisposition toward and occurrence of anemia (29).

In summary, this study confirms damage to the respiratory membrane, in particular, the endothelium and basement membrane, in dietary copper deficiency and supports the hypothesis that copper deficiency enhances vulnerability of the respiratory membrane to oxidative damage.

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