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Cytochrome *c* Oxidase, Cu,Zn-Superoxide Dismutase, and Ceruloplasmin Activities in Copper-Deficient Bovines

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ABSTRACT

The activity of several cuproenzymes in relation to the immune system was examined in serum and blood cells from bovines with molybdenum-induced copper deficiency. Five female cattle were given molybdenum (30 ppm) and sulfate (225 ppm) to induce experimental secondary copper deficiency. Ceruloplasmin activity was determined in serum. The Cu,Zn-superoxide dismutase and cytochrome *c* oxidase activities were measured in peripheral blood lymphocytes, neutrophils, and monocyte-derived macrophages. Copper deficiency was confirmed from decreased serum copper levels and the animals with values less than 5.6 μmol/L were considered deficient. The content of intracellular copper decreased between 40% and 70% in deficient cells compared with the controls. In copper-deficient animals, the serum ceruloplasmin activity decreased to half of the control value. Both of them, the Cu,Zn-superoxide dismutase and the cytochrome *c* oxidase activities, undergo a significant reduction in leukocytes, showing differences among diverse cell populations. We concluded that the copper deficiency alters the activity of several enzymes, which mediate antioxidant defenses and ATP formation. These effects may impair the cell immune functionality, affecting the bactericidal capacity and making the animals more susceptible to infection.

Index Entries: Bovine; ceruloplasmin; cytochrome *c* oxidase; copper; Cu,Zn-superoxide dismutase; leukocytes.

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INTRODUCTION

Copper is an essential trace element that has an important role in many physiological functions in nervous, hematological, cardiovascular, reproduction and immune systems *(1)*. Copper plays a significant role, being associated with specific proteins. The majority of the biological functions of copper are believed to be associated with copper's role as a ligand in the active site of metalloenzymes. Among the principal enzymes, it can be mentioned that the ceruloplasmin, a plasma glycoprotein, may function as a copper transport and as an antioxidant. The amine oxidases, isolated from plasma and organs, are responsible of oxidative deamination. Dopamine-β-monooxygenase located in noradrenergic neurons and involved in conversion of dopamine to norepinephrine. Cytochrome *c* oxidase (C*c*O) is the terminal mitochondrial electron carrier. Lysyl oxidase is reponsible for oxidative deamination of peptidyllysine. Cu,Zn-Superoxide dismutase is a cytosolic protein that speeds up the dismutation of superoxide at neutral pH. Tyrosinase, located in melanocytes, is involved in the conversion of tyrosine into melanin.

Some of these enzymes such as superoxide dismutase and cytochrome *c* oxidase are ubiquitous enzymes underscoring the importance of Cu in all animal cells *(1)*. In this article, we fix our attention to copper enzymes related with the immune response either humoral or cellular and the impact of copper deficiency on this system.

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal member of the respiratory chain, catalyzing the reaction $4H^+ + 4\ e^- + O_2 \rightarrow 2H_2O$ and coupling this exergonic reaction to the translocation of a proton across a membrane barrier, generating a proton electrochemical gradient that is then used to synthesize ATP. Eukaryote C*c*O contain two heme prosthetic groups and three copper atoms *(2)*.

Cu,Zn-superoxide dismutase (Cu,Zn-SOD, EC 1.15.1.1) *(3)* is a cuproenzyme that catalyzes the dismutation of O_2^- to H_2O_2. Cu,Zn-SOD is located in the cytosol of most tissues and is an integral part of the body's defense mechanism against the consequences of reactive oxygen intermediates, which are deleterious to cell metabolism.

Ceruloplasmin (EC 1.16.3.1) is a single chain α_2-glycoprotein that binds up to 95% of serum copper. Its most important functions are the transport of copper from liver to peripheral, nonhepatic tissues, catalysis of the oxidation of ferrous ions, and action as an extracellular antioxidant *(4)*.

Immunoglobulins, synthesized by lymphocytes, plays an important role in host immunity because the $F(ab')_2$ portion of the Ig mediates antigen binding and is reponsible for the specific immune response, whereas the Fc portion of it determines the effector function of the antibody as Fc receptor binding.

Neutrophils and macrophages defend against bacterial infection by various effector mechanisms, including the production of a group of powerful oxidizing species (H_2O_2, ClO^-, and others) that derive from a

single, relatively less reactive precursor: the superoxide radical (O_2^- *(5)*. Following activation, these O_2^- radicals are generated in large quantities by the membrane-bound NADPH oxidase systems. Although production of O_2^- and the metabolites such as H_2O_2 and HO^- are key steps in the oxidative damage of the invading pathogens, overproduction of these radicals can damage the host tissues *(6)*. In order to protect the host cells from any unwarranted oxidative destruction, the cells are provided with antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. The Cu,Zn-SOD catalyses the dismutation of O_2^- to H_2O_2 and catalase dismutates hydrogen peroxide to water and oxygen. Both soluble and particulate agents stimulate neutrophils and macrophages to produce a high flux of O_2^- through the reduction of molecular oxygen, known as the respiratory burst *(7)*.

In this study, we examined the effect of molybdenum-induced Cu deficiency of several cuproenzyme activities in serum, lymphocytes, neutrophils, and macrophages.

MATERIALS AND METHODS

Animals

Eight female Aberdeen Angus calves were separated into control (n = 3, group 1) or Cu-deficient (n = 5, group 2) groups. All animals were fed with basal diet containing alfalfa hay and concentrate ration with 6.8 and 3.6 mg of Cu and Mo per gram of dry matter, respectively, in a small pen at the experimental farm of our university. The animals had free access to water. Additionally, the heifers in the Cu-deficient group were supplemented orally with 30 ppm of Mo as ammonium molybdate and 225 ppm sulfate as sodium sulfate, 5 days a week. This supplementation was provided to induce a low Cu status *(8)*, for 120 d, controling the copper status every 30 d. The decrease of serum copper level was used as indicator of body copper status.

Blood samples were taken from the jugular vein. When the animals had a low copper status, peripheral blood leukocyte cells were isolated as described in the following.

Copper Levels

In both serum and blood cells, copper levels were determined by atomic absorption spectrophotometry (AAS) with a Perkin-Elmer spectrophotometer (Norwalk, CT). In serum, the copper level was determined following 1:1 dilution with trichloroacetic acid (TCA) (200 g/L). Cells were washed three times in saline solution (9 g NaCl/L), resuspended in deionized water at 6×10^7 cells/mL, lysed by freezing-thawing, and diluted 1:1 with TCA.

Ceruloplasmin

Ceruloplasmin (Cp) activity was assayed by the method of Ravin *(9)* using 100 µL of serum, 2 mL of acetate buffer, pH 6.4, and 1 mL of 0.5% *p*-phenylenediamine in 42 m*M* sodium hydroxide as substrate. The reaction was stopped by addition of 1 mL 0.11% sodium azide solution. The absorbance was measured at 530 nm and results are expressed as in units of IU/mL.

Cell Isolations

Blood from each cow was drawn into acid citrate dextrose anticoagulant at a ratio of 10:1. The anticoagulated blood was centrifuged. The plasma and top one-third of the red blood cell pellet were discarded. Neutrophils were recovered from the packed erythrocytes after erythrocyte lysing. The cells recovered were rinsed and suspended at 2×10^6 cells/mL in Hanks balanced salt solution (HBSS). The mononuclear cell layer was removed and layered over Ficoll–Hypaque 1.083 *(10)*. The mononuclear cell layer was harvested and placed into a tissue-culture Petri dish with RPMI 1640 media supplemented with 10% autologous serum. After incubation at 37°C with 5% CO_2 for 60 min, nonadherent cells were recovered (lymphocytes) and fresh media was added to the flasks. Monocytes were incubated for an additional 36 h in order to obtain a more uniform and pure population of cells. Then, the adherent cells were gently scraped from the bottom of the flasks *(11)*. The scraped cells were washed in phosphate-buffer solution–calcium magnesium-free (PBS-CMF) and resuspended to 5×10^7 cells/mL in serum-free RPMI 1640 medium. Recovered cells were identified as macrophages based on morphological features, adherence to plastic, and nonspecific esterase staining; 85–90% were viable as determined by exclusion of 0.2% Trypan Blue.

Determination of Cu,Zn-SOD Activity

Cells isolated or harvested were washed twice in saline solution and lysed by sonication. Following centrifugation of the lysate, the supernatant fraction was used to measure the SOD activity, by a modification of the method of Beauchamp and Fridovich *(12)*. The assay involves the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide, which is generated by the reaction of photoreduced riboflavin and oxygen. For each sample, six tubes were set up containing 0.1 mol/L EDTA buffer, 0.15 mol/L phosphate buffer, 1.5 mmol/L NBT, 0.12 m*M* riboflavin, and various quantities of cell extract. The tubes were placed in a light box containing an 18-W fluorescent tube and received uniform illumination for 12 min. Optical densities were measured at 560 nm. Mn-SOD activity was measured by the addition of 5 m*M* NaCN to the reaction mixture and Cu,Zn-SOD was calculated by subtraction of the Mn-SOD value from the total. A unit of SOD enzyme activity is defined

as the amount of enzyme that inhibits the reaction by 50%. Cellular SOD activity was expressed as U/10^7 cells.

Determination of Cytochrome c Oxidase Activity

In the supernatant, the fraction of lysate was assayed for CcO activity by the method of Cooperstein and Lazarow *(13)*. Briefly, 3 mL of reduced cytochrome *c* solution and 80 µL of the lysate were added into the spectrophotometer cuvet. Readings were taken every 30 s at 550 nm for 3 min, until a few grains of potassium ferricyanide were added (to oxidize the cytochrome *c* completely) and the extinction redetermined. Results are expressed as U/mg cellular protein.

Statistical Analysis

All values are expressed as means ± SD. The results were analyzed using the Student's *t*-test. A *p*-value of <0.05 was taken to indicate statistical significance.

RESULTS

Bovines supplemented with molybdate and sulfate had significantly lower levels of Cu in serum than those not supplemented, as determined by atomic absorption spectrophotometry, decreasing from 11.8 to 5.6 ± 0.7 µmol/L at 120 d after starting the trial, whereas the control group maintained the initial values (11.8 ± 0.5 µmol/L). When the animals from group 2 reached those values, the blood samples were processed and the cells cultivated, as mentioned earlier (Table 1).

The levels of intracellular copper decreased notably in neutrophils from deficient bovines in relation to the controls, reaching values of 0.032 µg Cu/6×10^7 cells for deficient bovines and 0.116 µg Cu/6×10^7 cells for the control group; this is a decrease of 72% of intracellular metal element content. The quantity of intracellular copper was 35–40% less in monocyte-derived macrophages from deficient bovines than that in control cells, being 0.028 ± 0.005 µg/6×10^7 cell and 0.045 ± 0.004 µg/6×10^7 cell, respectively. Data are shown in Table 1.

Before the treatment started, the ceruloplasmin activity in the serum of control animals was $46 \pm 6 \times 10^{-2}$ IU/mL. Copper deficiency decreased the enzyme activity by approximately 50%, attaining values of $22 \pm 5 \times 10^{-2}$ IU/mL (Table 1).

Results of the intracellular Cu,Zn-SOD activity measurement are shown in Fig. 1. The addition of cyanide to the reaction mixture abolished the Cu,Z,-SOD activity and allowed the determination of its activity by subtraction from the total value. Cellular Cu,Zn-SOD activity was

Table 1
Copper Content in Serum and White Blood Cells, and Ceruloplasmin Activity

	Control Group	Deficient Group
Serum Cu μmol.l^{-1}	11.8 ± 0.5	5.6 ± 0.7[a]
Neutrophil Cu content μg/6 x 10^7 cells	0.116 ± 0.009	0.032 ± 0.006[b]
Macrophage Cu content μg/6 x 10^7 cells	0.045 ± 0.004	0.028 ± 0.005[c]
Cp activitiy IU 10^{-2}.ml^{-1}	46 ± 6	22 ± 5[d]

Note: Values represent mean ± SD. Significant differences ($p < 0,05$) are indicated by footnotes a–d.

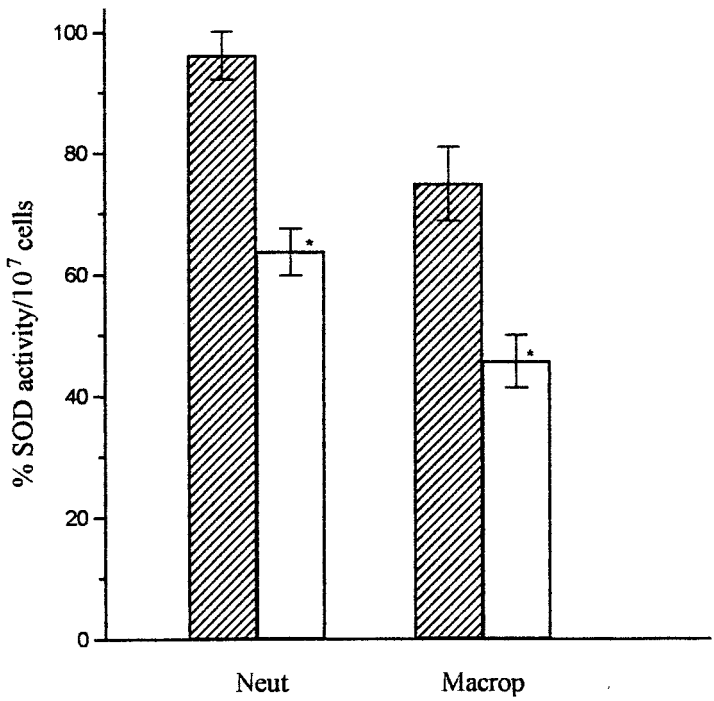

Fig. 1. Cu,Zn-SOD activity in neutrophils and macrophages, expressed as percentage/10^7 cells (control group [striped bars, $n = 3$] and deficient group [open bars, $n = 5$]). Values represent mean ± SD. Significant diferences ($p < 0.05$) between groups are indicated by an asterisk.

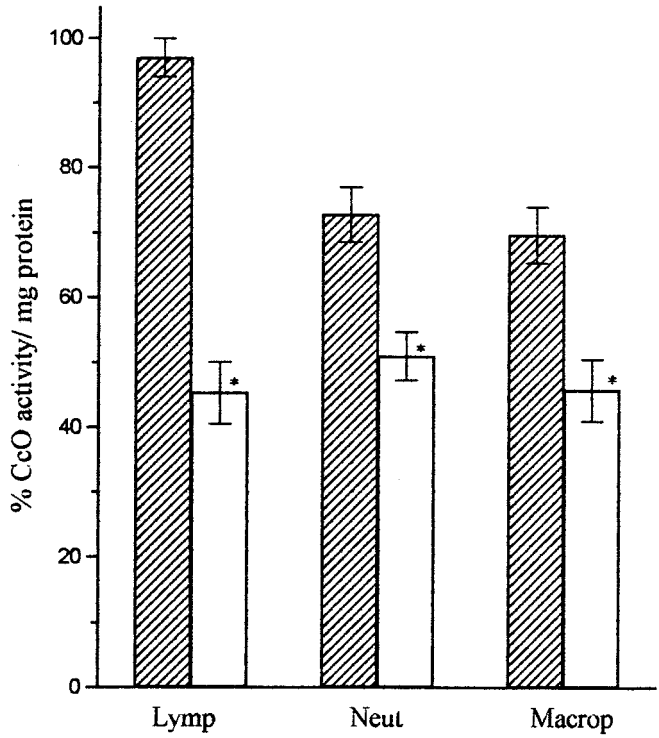

Fig. 2. C*c*O activity in lymphocytes, neutrophils, and macrophages, expressed as percentage/mg protein cell (control group [striped bars, $n = 3$] and deficient group [open bars, $n = 5$]). Values represent mean ± SD. Significant diferences ($p < 0.05$) between groups are indicated by an asterisk.

significantly reduced, to 31% of the value in control neutrophils, whereas this reduction reached 39% in macrophages, expressed as $U/10^7$ cells.

Results of the mitochondrial C*c*O activity measurements are shown in Fig. 2. It can be seen that the enzyme activity was more intense in control lymphocytes than in control neutrophils and macrophages. Depletion of Cu in animals from group 2 caused a significant reduction of C*c*O activity in all cell populations. This decrease was more evident in lymphocytes than in polymorphonuclear neutrophils and macrophages, as the same quantity of cellular protein from deficient group was able to transform only 50% of reduced cytochrome *c* to oxidized cytochrome *c* compared to the control group, whereas in neutrophils and macrophages, this reduction reached 32% and 27% of the control values, respectively.

DISCUSSION

The concept that nutritional status influences the susceptibility of a host to infectious disease is well established. Copper is an essential trace element that has an important role in the immune response; nonetheless,

the precise mechanism by which Cu deficiency alters host inmunity is still uncertain.

Active oxygen species, including the O_2^-, H_2O_2 and the OH^-, are produced through a number of reaction mechanisms as part of physiological enzyme functions. The species that escape being scavenged can induce oxidative stress in vivo. Antioxidant enzymes are thought to play an important role in providing protection from the deleterious effects of active oxygen species, both by decreasing their levels and by preventing the destructive propagation of radical chain reactions *(14)*.

It is assumed that SOD protects the cells from uncontrolled and damaging reactions of O_2^- through catalyzing its dismutation to molecular oxygen and hydrogen peroxide. The dismutation of the superoxide anion to hydrogen peroxide and molecular oxygen by SOD is often called the primary defense *(15)*, as it prevents radicals derived from O_2^- such as hydroxyl, peroxinitrite, and the perferyl ion complex, which are more oxidant and able to initiate lipid peroxidation by extraction of hydrogen from unsaturated fatty acid, thereby destroying the membrane integrity *(16)*. Inadequate copper levels have adverse effects on the synthesis and activity of cuproenzymes. It is probable that the less SOD activity determined in Cu-deficient neutrophils and macrophages compared with control group impaired not only the primary defense but also induce the lipid peroxidation.

The lipid peroxidation in a localizated membrane segment may perturb the microenvironment of membrane-bound proteins, altering their function *(17)*. Oxidative damage to lipid components of leukocyte membranes initiate the process of prostaglandin and leukotriene synthesis leads to a loss of fluidity as well as alteration of the receptor function *(18)*. These alterations in receptor structure because of liperoxidation can be the responsible for the lower activity in the burst respiratory in copper deficient neutrophils *(19)* and macrophages when they were stimulated.

Copper, through its cofactor role in C*c*O, is directly involved in oxidative phosphorylation. The observed reduction in C*c*O levels in copper-deficient lymphocytes may affect intracellular ATP levels. It is known that the synthesis of macromolecules formation of antibody molecules requires energy. When the C*c*O activity falls enough to reduce the electron flux to molecular oxygen, the mitochondrial energy metabolism is impaired and this can be the cause of lower antibody synthesis, as was demonstrated previously *(20)*. The morphological appearance of abnormal mitochondria in lymphoid tissue of copper-deficient mice *(21)* adds further credence to this hypothesis.

In addition, bovine IgG through Fc induces superoxide production in bovine neutrophils and is one of the mayor components of serum stimulatory factor, playing an important role in the antimicrobial function of them in tissue *(22)*. If the IgG production is less than normal, it is probably an impaired stimulatory effect on polymorphonuclear monocytes with diminished activity.

Ceruloplasmin is among those enzymes involved in the acute-phase reaction of inflammation and in the scavenging of oxygen radicals to protect cells against oxidative damage. Copper deficiency, associated with a low level of plasma Cp, leads to a decrease of the antimicrobial activity of phagocytes *(23)*. This enzyme may be needed in inflammation because it functions as a copper-transport protein, delivering copper to Cu-dependent enzymes, such as lysyl oxidase, which is involved in the repair of damaged tissues and Cu,Zn-SOD. The copper-transporting function of Cp is important for the maintenance of the activity of leukocyte enzymes involved in the respiratory burst.

We concluded that the limitation of a single nutrient, copper, can adversely affect immunity and host resistance. The immunocompetent cells could suffer a functional defect and can, therefore, neither synthesize enough antibody nor undergo an adequate respiratory burst. As a consequence, the animals suffer severe bacterial infections, because the decreased function of phagocytic cells compromises the nonspecific immune defense system of Cu-deficient animals and contribute to their greater susceptibility to infections.

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