PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 158, 279–282 (1978)

# Effects of Copper and Zinc Status of Rats on Erythrocyte Stability and Superoxide Dismutase Activity (40188)[1]

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Anemia is generally associated with copper deficiency in animals, and this is due in part to defective iron metabolism (1). In addition the erythrocyte survival time is decreased in copper deficient pigs (2). The explanation for this phenomenon is not known but decreased membrane stability is a possibility. Superoxide dismutase, a copper and zinc dependent enzyme, catalyzes dismutation of the oxygen free-radical, $O_2^-$, and its activity is depressed in red cells (3) and brains (4) of copper deficient animals. The superoxide anion has been implicated in the peroxidation of isolated lipids (5, 6), but removal of the anion by use of superoxide dismutase has been shown to both promote (7) and impair (8) peroxidation of biological membranes depending upon experimental conditions.

There is increasing evidence that zinc affects the structure and function of membranes (9). Zinc is incorporated into erythrocyte membranes and improves the filterability of sickle cells (10). High levels of dietary zinc decrease the osmotic fragility of red cells (11). In contrast to $Cu^{2+}$, which accelerates lipid peroxidation (12), $Zn^{2+}$ protects liver cells against $CCl_4$-induced lipid peroxidation (13). $Zn^{2+}$ confers increased thermal stability to model biomembranes while $Cu^{2+}$ has a small destablizing effect (14).

Plasma zinc and copper levels frequently vary inversely in disease and pathological conditions and there is abundant evidence that excess dietary zinc depresses plasma copper (15). There is less information regarding the effect of dietary deficiencies of one element on the plasma level of the other. O'Dell *et al.* (16) have shown that plasma copper is elevated in zinc deficiency where plasma zinc is depressed. Copper deficiency had no such reciprocal effect on plasma zinc levels when plasma copper was low.

The purpose of this investigation was to determine the respective effects of copper and zinc deficiencies and of extracellular concentration of the ions on the integrity of the red cell membrane. An ancillary objective was to determine the effects of dietary deficiencies on the superoxide dismutase activity in erythrocytes.

*Materials and methods. Animals and diets.* Wistar strain rats were maintained in suspended stainless steel cages and distilled water and the respective diets were supplied *ad libitum.*

Copper deficiency was induced in second generation rats by feeding the dams a low copper diet based on non-fat milk solids (4). The basal (<1.0 ppm Cu) and control (10 ppm Cu) diets were fed during gestation, lactation and the growth period after weaning. Animals of the same age from the two dietary regimens were selected randomly as regards sex and used experimentally when they were 6- to 8-weeks of age.

Zinc deficiency was induced in immature female rats, approximately 100 g bw, by feeding a low zinc diet for 3 weeks. The basal diet, which was based on EDTA-treated soybean protein (17), contained <1.0 ppm Zn and the control diet was supplemented with 100 ppm zinc. Depressed growth rate (16, 17) was the primary criterion of deficiency.

*Enzyme Assays.* Cytochrome C oxidase activity was determined by the method of Smith (18) using an initial concentration of reduced cytochrome C that had an absorbance of 0.5 at 550 nm. Liver homogenate (0.33 mg) was added to the reaction mixture (3 ml) and the rate of oxidation followed for 2 min. The rate constant was multiplied by a factor of 225 to convert to μmoles/min/g liver.

Ceruloplasmin was determined by the method of Rice (19) using *p*-phenylenediamine as substrate. The change in absorbance at 525 nm was multiplied by 349 to convert

[1] A contribution of the Missouri Agricultural Experiment Station, Journal Series No. 8038. Supported in part by NIH Grant No. HL11614.



0037-9727/78/1582-0279$01.00/0

to international units.

Superoxide dismutase (SOD) activity was determined by the method of Marklund and Marklund (20) which is based on inhibition of the autooxidation of pyrogallol at 25°. The reaction was monitored at 420 nm. Four concentrations of the enzyme source were used so that the reaction mixtures (3 ml) contained the hemolyzed equivalent of 0.8–3.0 μl of packed red cells. One unit of activity is defined as the amount of enzyme which inhibits the rate of reaction by 50%. In this assay pure SOD (Truett Laboratories, Dallas, TX) contained 2.1 units/μg. Units were converted to micrograms using this factor.

*Osmotic fragility of erythrocytes.* Fragility was based on the method of Cartwright (21) using one concentration of NaCl. The concentration was chosen by preliminary trials which showed the range of osmotic pressures at which the red cells were most sensitive. For the copper deficient blood, 0.34% NaCl and, for zinc deficient blood, 0.42% NaCl was used. Hemolysis in water was considered to be 100% and in 0.85% buffered saline, 0%. Assays were run in quadruplicate.

*Red blood cell stability and lipid peroxidation.* This procedure was used to assess the effect of $Cu^{2+}$ and $Zn^{2+}$, added *in vitro*, on the stability of red cells in an isotonic buffer containing chelating amino acids. Amino acids known to complex $Cu^{2+}$ and $Zn^{2+}$ were added in attempt to simulate the free ion concentrations in plasma. The buffer contained 0.85% NaCl, 0.57 m*M* glutamine, 0.074 m*M* histidine and 0.033 m*M* cysteine, pH 6.2. A 0.5 ml aliquot of pooled blood from stock colony rats was added to 5 ml of buffer and incubated on a rotating wheel at 25° for 10 hr. The suspension was centrifuged and hemolysis determined by absorbance (540 nm) of the supernate. Peroxidation was measured by determination of malondialdehyde (22).

*Results.* The effects of zinc and copper deficiencies on the osmotic fragility of red cells are shown in Table I. The concentration of plasma zinc was markedly decreased in the zinc deficient animals while plasma copper was elevated above control levels. Red cells from zinc deficient rats showed greatly increased fragility in 0.42% NaCl. Zinc therapy for 2 days improved the stability significantly but did not restore it to normal. In contrast, the red cells from copper deficient animals, which had low plasma copper and normal plasma zinc, were much less fragile in 0.34% NaCl than those from controls fed adequate copper. Treatment of the deficient animals with copper for 2 days had no tendency to decrease osmotic stability to the control level.

To determine whether or not the effect on osmotic stability was due primarily to the change in concentrations of $Cu^{2+}$ and $Zn^{2+}$ in the extracellular environment of the cells or to cellular changes *per se*, red cells from stock control animals were incubated *in vitro* with buffers containing different concentrations of these cations. As may be seen in Table II, $Cu^{2+}$ increased the rate of hemolysis of normal cells in proportion to concentration. Addition of $Zn^{2+}$ alone had no effect as observed in this system and $Zn^{2+}$ added along with $Cu^{2+}$ had an antagonistic effect. Membrane peroxidation as measured by malondialdehyde production was also progressively increased by $Cu^{2+}$. In this case $Zn^{2+}$ did not counteract the effect of $Cu^{2+}$. Thus a corre-

TABLE I. OSMOTIC FRAGILITY OF ERYTHROCYTES FROM COPPER AND ZINC DEFICIENT RATS.

| Dietary status | Plasma Zn (ppm) | Plasma Cu (ppm) | Hemolysis in 0.42% NaCl (%) | Hemolysis in 0.34% NaCl (%) |
|---|---|---|---|---|
| Zinc deficient (10)* | 0.5 ± 0.1 | 1.3 ± 0.1 | 52** ± 1[a] | — |
| Zinc control (10) | 1.1 ± 0.1 | 1.0 ± 0.1 | 22 ± 1[b] | — |
| Zinc deficient + Zn*** (8) | — | — | 40 ± 4[c] | — |
| Copper deficient (9) | 1.1 ± 0.1 | 0.3 ± 0.1 | — | 37 ± 4[a] |
| Copper control (9) | 1.1 ± 0.1 | 1.0 ± 0.1 | — | 90 ± 1[b] |
| Copper deficient + Cu**** (6) | — | — | — | 38 ± 2[a] |

* Number of samples.

** Mean ± SEM. Values with different superscripts are statistically different by Student's *t* test, $P < 0.01$.

*** Received 100 μg Zn/day subcutaneously for 2 days prior to sampling.

**** Received 50 μg Cu/day subcutaneously for 2 days prior to sampling.

TABLE II. EFFECT OF *in Vitro* $Cu^{2+}$ AND $Zn^{2+}$ ON ERYTHROCYTE STABILITY AND PEROXIDATION.

| Ions added to buffer (ppm) | Hemolysis* (%) | Malondialdehyde** (μmoles/mlRBC) |
|---|---|---|
| Cu-0.25 | 1.3*** ± 0.1[a] | 73 ± 4[a] |
| Zn-0.25 | 0 | 1 ± 1[b] |
| Cu-1.0 | 1.7 ± 0.1[b] | 241 ± 4[c] |
| Zn-1.0 | 0 | 1 ± 1[b] |
| Cu-5.0 | 2.9 ± 0.1[c] | 371 ± 9[d] |
| Zn-5.0 | 0 | 4 ± 2[b] |
| Cu-1.0 + 1.0 ppm Zn | 0.7 ± 0.1[d] | 238 ± 4[c] |

* The buffer with no added ions served as the blank and thus showed no hemolysis. The zero values tabulated were equal to or less than the blank.

** Malondialdehyde produced during the 10-hr incubation.

*** See footnote 2, Table I; 10 analyses per group.

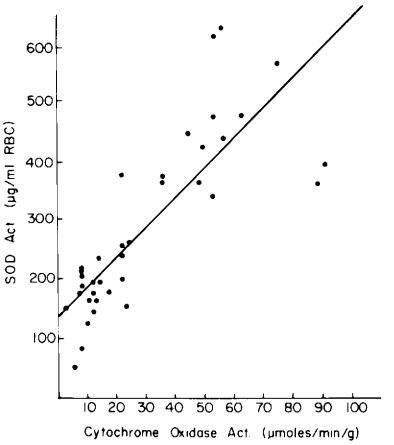


FIG. 1. Correlation of liver cytochrome oxidase and erythrocyte superoxide dismutase activities. Equation of the best fit line is $y = 4.89x + 136.9$ $r = 0.805$.

lation between hemolysis and malondialdehyde production does not exist under all conditions.

Although the erythrocytes of copper deficient pigs have a shorter survival time than normal, there is no evidence in the rat that this is due to greater fragility or peroxidative damage. On the other hand zinc deficiency increases red cell fragility and this may be related to the higher plasma $Cu^{2+}$ concentration. As shown in Table III, the zinc effect is not due to a decrease in superoxide dismutase activity. Whereas copper deficiency decreased erythrocyte SOD activity and plasma ceruloplasmin concentrations approximately threefold, zinc deficiency had no effect on SOD but increased ceruloplasmin significantly.

Superoxide dismutase activity in erythrocytes may well serve as a useful clinical index of copper status in as much as blood is easily sampled and the activity in red cells correlates well with another copper-dependent intracellular enzyme, cytochrome oxidase (Fig. 1). As copper deficient animals were repleted, both liver cytochrome oxidase and erythrocyte SOD activities increased, reaching control levels in approximately 3 weeks. The correlation coefficient $r$ was 0.80. As a clinical index of copper status, red blood cell SOD has the distinct advantage of being present in a tissue that is easily sampled. Furthermore tissue concentration of the enzyme is not as transient as plasma copper and ceruloplasmin (23).

*Discussion.* The increase in ceruloplasmin activity observed during zinc deficiency confirms earlier observation (16) that serum copper increases during zinc deficiency. A corresponding increase of SOD activity was not found during zinc deficiency suggesting that excess plasma copper has no regulatory effect on erythrocyte SOD activity. This observation is substantiated by the gradual return of SOD activity to control levels during copper therapy in experimental copper deficiency.

The fact that low plasma zinc is associated with decreased membrane integrity explains the common observation in this laboratory that it is difficult to obtain hemoglobin-free plasma from severely zinc deficient animals. These observations support the hypothesis (9) that excess zinc increases membrane integrity. However, the fact that osmotic fragility is decreased during copper deficiency cannot be explained by increased plasma zinc because plasma zinc does not rise in copper deficiency (16).

The *in vitro* studies, involving addition of copper and zinc ions, indicate that copper exerts a detrimental effect in terms of spontaneous hemolysis and membrane peroxidation. Zinc provides a protective effect against copper induced spontaneous hemolysis but does not protect against copper induced peroxidation. The results suggest that zinc can stabilize red cell membranes after peroxidative damage has occurred. This may serve to elucidate Chvapil's hypothesis (9) that zinc can "interfere with metal catalyzed peroxi-

TABLE III. SUPEROXIDE DISMUTASE AND CERULOPLASMIN ACTIVITIES IN BLOOD OF COPPER AND ZINC DEFICIENT RATS.

| Dietary status | Body weight* (g) | Superoxide dismutase** (μg/ml RBC) | Ceruloplasmin** (IU/ml Plasma) |
|---|---|---|---|
| Zinc deficient | 114 | 468 ± 16[a] (10) | 45 ± 1[a] (16) |
| Zinc control | 189 | 467 ± 11[a] (10) | 35 ± 1[b] (16) |
| Copper deficient | 69 | 166 ± 10[b](30) | 9 ± 1[c] (10) |
| Copper control | 141 | 450 ± 16[a] (21) | 33 ± 1[b] (10) |

* Mean weights of 100 g females fed the zinc deficient and control diets for 3 weeks and of mixed sex rats fed the copper deficient and control diets to 7 weeks of age.

** See footnotes 1 and 2, Table I.

dation". Zinc does not diminish Cu-induced lipid peroxidation, but it does diminish the weakening effect on the membrane caused by the peroxidative damage.

There is increasing evidence for antagonistic interactions of $Cu^{2+}$ and $Zn^{2+}$, both physiological and chemical. The present results confirm earlier observations (16) of physiological interaction and the *in vitro* observations suggest a direct effect on membrane structure. The latter effect could occur by way of changes in conformation of proteins or fluidity of lipids in the membrane (14).

*Summary.* Erythrocytes from copper deficient rats, which had normal plasma zinc but low plasma copper (high Zn:Cu), were more stable to osmotic stress than those from controls. Erythrocytes from zinc deficient rats, which had low plasma zinc and high plasma copper (low Zn:Cu), were more fragile than those from controls. Addition of $Cu^{2+}$ and $Zn^{2+}$ to normal red cells *in vitro* to provide a low Zn:Cu ratio increased hemolysis and peroxidation. In general erythrocyte membranes are sensitive to the concentrations of extracellular Cu and Zn. The effects are inversely related, zinc exerting a stabilizing and copper a labilizing effect. Ceruloplasmin levels increased in zinc deficiency but RBC superoxide dismutase activity was unaffected. SOD activity is suggested as a clinical index of tissue copper status.

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Received January 30, 1978. P.S.E.B.M. 1978, Vol. 158.