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COPPER-MANGANESE INTERACTIONS CONCERNING RED-CELL AND PLASMA LIPID PEROXIDATION

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Manganese decreases the formation of methemoglobin and partially inhibits lipid peroxidation induced by copper in human erythrocytes. This is followed by delay in hemolysis. Manganese also reduces lipid peroxidation induced by copper in human plasma. these effects of manganese are stronger than those of zinc, a metal which is considered to have protective effects against free radical damage.

KEY WORDS: Copper, zinc, manganese, red blood cells, plasma, lipid peroxidation

INTRODUCTION

The induction of lipid peroxidation by copper in red blood cells[1] and plasma[2] has recently been investigated in our laboratory. Copper toxicity may be related to the prooxidative effects of this metal. However, other metals, such as zinc and manganese may have a protective effective as antioxidants. Manganese is a scavenger of the superoxide radical, especially in some aerotolerant bacteria such as *Lactobacillus plantarum* which is devoid of the enzyme superoxide dismutase[3,4] and it is also the cofactor of the mitochondrial superoxide dismutase.[5] Manganese inhibits lipid peroxidation *in vitro* in systems containing microsomes, lysomes and in rat brain homogenates.[6–10]

In the present paper we describe the protective effects of manganese against the damaging effects of copper in red blood cells and in plasma. These effects are compared with the effects of zinc.

MATERIALS AND METHODS

Erythrocytes and plasma were obtained from fresh adult human blood from healthy donors. Blood was collected in tubes containing ACD, in the proportion of 7.5 ml:1 ml. Erythrocytes were separated by centrifugation and washed three times with physiological saline and the suspension adjusted to a final concentration of 5% (v/v). Red cells were pre-incubated with 2 mM sodium azide as previously described.[1] For incubations containing plasma, the mixture of plasma-ACD was diluted with saline so that 0.3 ml of the mixture was present in a final volume of 5 ml.

Copper, manganese or zinc chloride of "Analar" grade were added diluted in saline.

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When two different salts were used in the same incubation, copper chloride was added 5 min later.

All the incubations were carried out in 25 ml Erlenmeyer flasks, in a shaking water bath at 37 °. The final volume of the incubation mixture in each flask was always 5 ml. Methemoglobin (metHb), thiobarbituric acid (TBA) reactive products (TBArp) and hemolysis were determined as described previously.[1,2]

In some experiments the precipitate obtained from the deproteinization step of the TBA method was washed further with 4 ml of 5% phosphotungstic acid. The washed precipitate was collected by centrifugation, 0.8 ml of 5% phosphotungstic acid and 0.8 ml of 0.8% TBA were added. The remaining steps of the assay were identical to those employed for the supernatant.[2] The determinatin of TBArp was considered as a measure of lipid peroxidation. Prelimianry experiments demonstrated that manganese and zinc chloride do not interfere with the employed method for TBArp determination.

The variability of the data is presented as mean ± standard deviation. Each graph corresponds to a representative of at least three experiments. The assays were carried out with blood from different donors. Student's test for paired values was used to determine the statistical significance. P values less than 0.05 were considered significant.

RESULTS

1) Influence of Manganese on the Effect of Copper on Erythrocytes

Copper chloride at 175 μM was found to induce methemoglobin and TBArp formation and cause hemolysis (Table I). However in the presence of 175 μM manganese chloride methemoglobin formation is inhibited by 70%, lipid peroxidation by 44% and hemolysis by 84%. The degree of inhibition is dependent on the manganese concentration up to when the concentration of the metal is higher than that of the copper then the variation becames smaller (Figure 1). Also small variations in TBArp formation corresponded to great variations in hemolysis in manganese concentration range 0–175 μM (Figure 2).

When the effect of copper on erythrocytes was investigated as a function of time, in the presence and in the absence of manganese, it can be ovserved that in both cases TBArp formation precedes hemolysis (Figure 3). In the presence of manganese there is a delay in the appearance of TBArp and this remains at a lower concentration to

TABLE I

Influence of manganese on the effects of copper in the red blood cells (rbc). Incubations with 175 μM $CuCl_2$ and 175 μM $MnCl_2$ during 75 min. Number of experiments – 7

Incubation	MetHb (%)	TBArp A_{532}	Hemolysis (%)
rbc	0	0.020 ± 0.004	1.4 ± 0.5
rbc + $MnCl_2$	0	0.020 ± 0.004	1.3 ± 0.6
rbc + $CuCl_2$	37.5 ± 21.6	0.507 ± 0.052	78.2 ± 13.3
rbc + $MnCl_2$ + $CuCl_2$	11.3 ± 6.2	0.283 ± 0.056	12.7 ± 5.0

The protective effect of manganese is significant: metHB – $p < 0.005$; TBArp – $p < 0.001$; hemolysis – $p < 0.001$

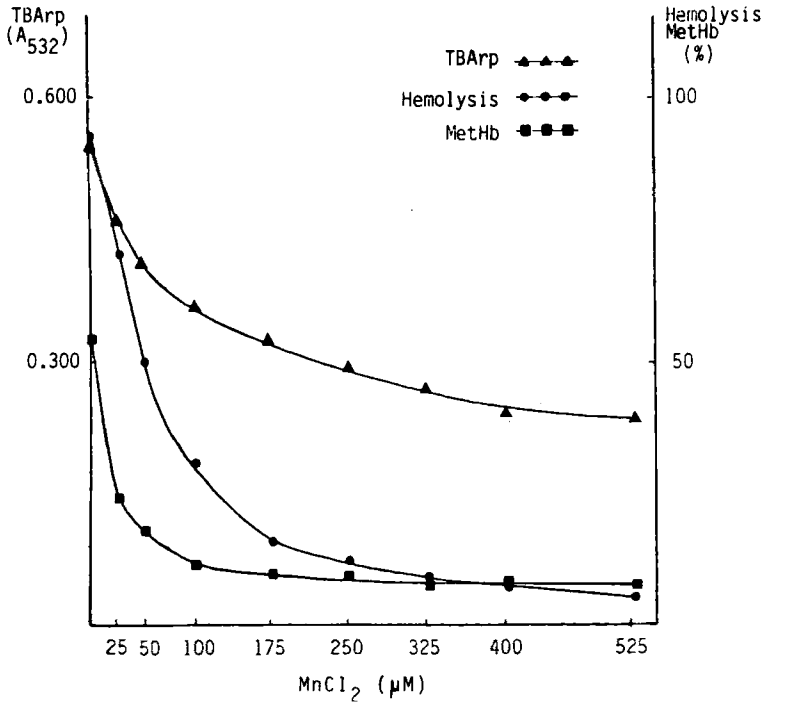

FIGURE 1 Protection against the effects of 175 μM $CuCl_2$ in the erythrocytes by varying concentrations of $MnCl_2$. Incubation time – 75 min.

when copper is solely present. There is also a delay in the appearance of hemolysis. Increasing concentrations of manganese (50, 175, 500 μM) have an increasing protective effect against the hemolytic effect of 175 μM $CuCl_2$ towards the end of a 5 h incubation period.

2) Influence of Manganese on Lipid Peroxidation Induced in Plasma by Copper

The presence of manganese chloride during the incubation of plasma with 125 μM copper chloride was found to cause a decrease in lipid peroxidation (Table II). This effect is observed both in the supernatant and in the precipitate obtained at the deproteinization step of the TBA method. The protective effect of manganese was found to be dependent on the concentration of the metal (Figure 4).

3) Comparison of the Influence of Manganese and of Zinc on the Effect of Copper on Erythrocytes

Only small concentrations of zinc can be used since this metal may cause hemolysis. When 50 μM manganese chloride was added, the TBArp formation is inhibited by 25% and hemolysis by 53%. In the presence of 50 μM zinc chloride no inhibitory effect is observed (Table III).

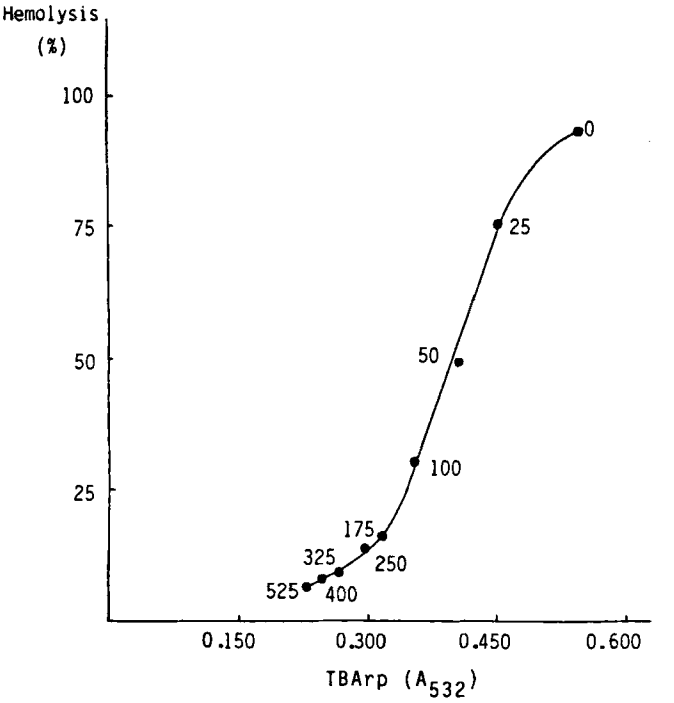

FIGURE 2 Relations between TBArp formation and hemolysis in the erythrocytes exposed to different concentrations of $MnCl_2$ (written on the curve as μM) and to 175 μM $CuCl_2$. Incubation time – 75 min.

4) Comparison of the Influence of Manganese and of Zinc on the Lipid Peroxidation induced by Copper in Plasma

As is shown in Table IV, the inhibitory effect of 125 μM manganese chloride on plasma lipid peroxidation induced by 125 μM copper chloride is approximately double that of the inhibition produced by 125 μM zinc chloride.

DISCUSSION

Several investigations have shown that the *in vitro* hemolytic effect of copper is a consequence of the peroxidation of the lipids of the red cell membrane. This may result from the generation of free radicals, related to: (a) the oxidation of copper (I) generated during the slow oxidation of oxyhemoglobin catalysed by copper;[11] (b) the reoxidation of copper near the membrane, after oxidation of thiol groups of membrane proteins by copper (II);[12] (c) a direct effect of copper on the initiation of lipid peroxidation[13] and (d) the activation of membrane enzymes which catalyse reactions which generate superoxide radicals.[14] In a previous investigation[1] we conclude that the first mechanism is predominant and that the hydroxyl radical participates in the initiation of lipid peroxidation. The fact that copper promotes the binding of hemoglobin to the red cell membrane facilitates these effects.[15] The interaction of hemo-

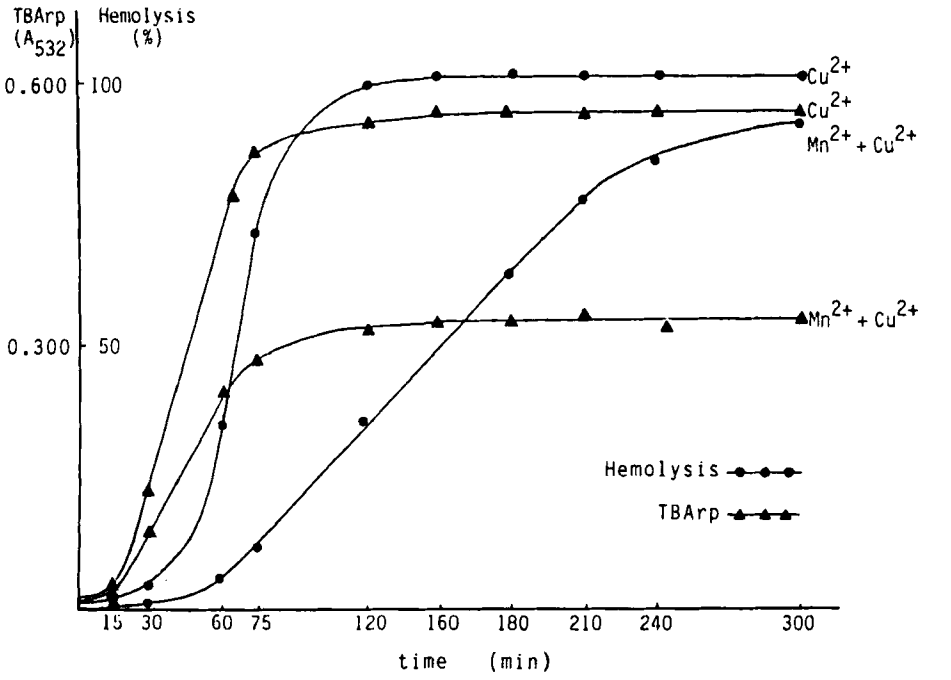

FIGURE 3 Evolution of TBArp formation and of hemolysis induced by 175 μM $CuCl_2$ during incubations with and without 175 μM $MnCl_2$.

TABLE II

Influence of manganese on lipid peroxidatin induced by copper in the plasma. Incubations with 125 μM $CuCl_2$ and 125 μM $MnCl_2$ during 3 h. The number of experiments is shown in parenthesis

Incubation	TBArp in the supernatant (8) A_{532}	TBArp in the precipitate (7) A_{532}
Plasma	0.011 ± 0.005	0.027 ± 0.006
Plasma + $MnCl_2$	0.011 ± 0.003	0.029 ± 0.007
Plasma + $CuCl_2$	0.218 ± 0.059	0.111 ± 0.023
Plasma + $MnCl_2$ + $CuCl_2$	0.069 ± 0.018	0.024 ± 0.009

The protective effect of manganese is significant ($p < 0.001$)

globin to the red cell membrane facilitates these effects.[15] The interaction of hemoglobin degradation products with the membrane may also be related to the hemolysis.[16]

The present investigation indicates that manganese counteracts the effect of copper on erythrocytes. It decreases both the oxidation of hemoglobin and lipid peroxidation and it also delays hemolysis. Manganese may compete with copper for entrance into the red cell, or for binding to the hemoglobin thiol groups. If it binds to these groups it does not cause the oxidation of the protein in contrast to copper.[17]

The decrease in methemoglobin and superoxide radical formation may be the main mechanism for the inhibition of lipid peroxidation. Other mechanisms may be: (a) the catalysis of superoxide dismutation which would prevent the formation of other

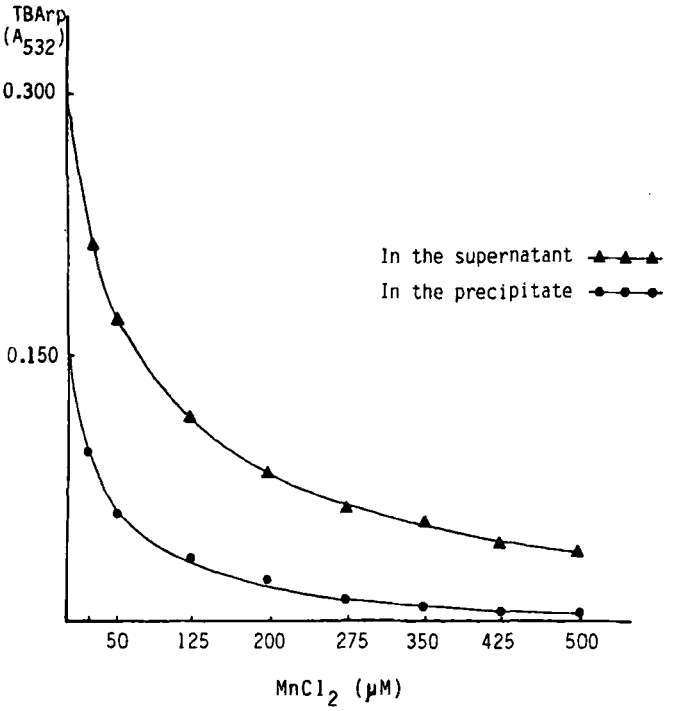

FIGURE 4 Protection by different concentrations of $MnCl_2$ against plasma lipid peroxidation induced by 125 μM $CuCl_2$. TBArp were determined in the supernatant and in the precipitate obtained at the deproteinization step of the TBA method.

TABLE III
Comparison of the influences of manganese and of zinc on the effects of copper in the red blood cells. $CuCl_2$-175 μM, $ZnCl_2$-50 μM, $MnCl_2$-50 μM. Incubation time – 75 min. Number of experiments – 7

Incubation	TBArp A_{532}	Hemolysis (%)
rbc	0.019 ± 0.004	1.0 ± 0.1
rbc + $CuCl_2$	0.544 ± 0.047	70.7 ± 7.0
rbc + $ZnCl_2$	0.020 ± 0.006	1.2 ± 0.4
rbc + $MnCl_2$	0.018 ± 0.004	0.9 ± 0.4
rbc + $ZnCl_2$ + $CuCl_2$	0.529 ± 0.068	73.3 ± 10.8
rbc + $MnCl_2$ + $CuCl_2$	0.408 ± 0.046	33.3 ± 1.4

In the presence of manganese the effects of copper are decreased significantly ($p < 0.01$)

active forms of oxygen; (b) interference with the copper stimulated hemoglobin-membrane binding; (c) the competition with copper for the binding to membrane thiol groups and (d) the binding to other membrane constituents thereby making them less susceptible to initiation or propagation of lipid peroxidation. Hypotheses based on a competitive mechanism are strengthened by the observation that the inhibitions become weaker when the concentration of manganese is higher than that of copper.

Hemolysis is not inhibited by manganese but it is simply delayed. The small amount

TABLE IV
Comparison of the influences of manganese and of zinc on the copper induced plasma lipid peroxidation. Each salt was used in a final concentration of 0.125 mM. Incubation time – 3 h. Number of experiments – 3

Incubation	TBArp in the supernatant A_{532}	TBArp in the precipitate A_{532}
Plasma	0.010 ± 0.008	0.026 ± 0.010
Plasma + $CuCl_2$	0.206 ± 0.047	0.106 ± 0.028
Plasma + $ZnCl_2$	0.010 ± 0.002	0.029 ± 0.010
Plasma + $MnCl_2$	0.011 ± 0.001	0.025 ± 0.009
Plasma + $ZnCl_2$ + $CuCl_2$	0.134 ± 0.049	0.072 ± 0.014
Plasma + $MnCl_2$ + $CuCl_2$	0.061 ± 0.011	0.026 ± 0.014

of lipid peroxidation not inhibited by manganese seems sufficient to induce irreversible damage to the red cell.

Manganese also reduces lipid peroxidation induced in plasma by copper. The mechanisms may be similar to those occuring in red cells. The protective effect of manganese was demonstrated to be of similar magnitude both when determination of TBArp is studied in the supernatant or in the precipitate obtained after deproteinization of the incubated plasma mixture. This fact favours of the validity of the utilization of either of these fractions for the study of lipid peroxidation.

Several investigations have suggested that zinc is part of the antioxidant defence of tissues.[18,19] In the present investigation we found that zinc does not inhibit either lipid peroxidation or hemolysis caused by copper in red cells. However, it decreases lipid peroxidation in plasma but to a smaller degree than manganese. The reason for this lower effect is as yet unclear. Our results confirm that manganese may have an important function in the antioxidant defense of the tissues.[20]

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