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# Hemolytic activity of copper sulfate as influenced by epinephrine and chelating thiols

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**KEY WORDS** hemolysis; copper sulfate; epinephrine; succimer; unithiol; penicillamine; homocysteine; ceruloplasmin; albumins; chromium compounds

**AIM:** To study the effects of epinephrine, homocysteine, and other complexing agents on the cytotoxicity of copper sulfate. **METHODS:** *In vitro* suspensions of human red cells incubated with cupric sulfate were used, and hemolysis was determined by extracellular hemoglobin. **RESULTS:** The hemolytic activity of $CuSO_4$ (0.3 mmol·L$^{-1}$) was enhanced by the presence of epinephrine and to a lesser extent by homocysteine, whereas *D*-penicillamine, succimer, and mercaptodextran reduced the copper-induced hemolysis. The latter 3 chelating thiols also reduced the copper-epinephrine-induced hemolysis. The plasma protein ceruloplasmin reduced markedly the copper-epinephrine-induced hemolysis, even upon concentrations < 20 % of that of copper. Chromic chloride, as well, acted anti-hemolytically. **CONCLUSION:** The latter protectors may interact with the production or activity of toxic oxygen, while classical copper chelators sequester cupric ions from interaction with epinephrine or homocysteine.

Raised blood plasma levels of $Cu^{2+}$ may initiate an intravascular hemolysis[1]. In healthy humans, the concentration of $Cu^{2+}$ in blood is very low, considerably < 1 pmol·L$^{-1}$. This is partly due to the high affinity of $Cu^{2+}$ for reactions with thiol, hydroxyl, or amino groups on proteins[1,2]. When introduced in excess into the circulation, a considerable part of $Cu^{2+}$ is rapidly chelated to thiol groups on the erythrocyte membrane and/or intracellularly. This is a consequence of the high lg *K*-values (stability constants) of the complexes between $Cu^{2+}$ and thiols, that are usually above 15 or 16[3]. However, some thiols (eg, homocysteine) and some complexing agents (eg, epinephrine) possess strong reducing abilities, and might bring about a conversion of $Cu^{2+}$ to $Cu^{+}$, that may induce undesirable reactions. $Cu^{+}$ can interact with molecular $O_2$ in the solution and generate superoxide radicals (Fig 1), which are potential initiators of lipid peroxidation and hemolysis.

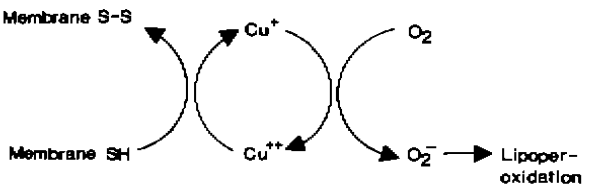


**Fig 1. Proposed mechanism of the interaction of $Cu^{2+}$ with cellular membrane constituents under aerobic conditions.**

The lg *K*-value of the $Cu^{2+}$-epinephrine-complex is as high as about 15, and the corresponding value for the homocysteine-complex is presumably about 16[3,4].

Hyperhomocystinemia and local epinephrine accumulations around receptor sites have been hypothesized to accelerate cell injury. Both agents are strong copper and iron chelators. Upon chelation and reduction of the metal ions to species that interact with oxygen, activated oxygen species may be generated in the vicinity of membrane surfaces *in vivo*. Thus, these agents may play a role in the pathogenesis of several diseases, including acute hemolytic crisis characterizing severe copper poisoning.

In the present paper the erythrocytes were used, when studying the potential membrane toxicity of the copper-epinephrine and copper-homocysteine interactions, and the possible protective effects of the therapeutic agents penicillamine, succimer, and other chelators, as well as the scavenging agents ceruloplasmin and albumin.

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## MATERIALS AND METHODS

**Chemicals** *D*-penicillamine (Pen), *L*-homocysteine (Hom), Succimer (Suc), and epinephrine were obtained from Sigma Chemical Co, USA. Unithiol (Uni) was obtained from Heyl & Co, Berlin, Germany. Mercaptodextran with average molecular weight 20 000 (SH-20) was synthesized as described previously[5]. Human serum albumin (Alb) and ceruloplasmin (Cer) was purchased from Kabi AB, Stockholm, Sweden.

**Experimental** Freshly drawn venous blood from a healthy adult, mixed with citrate, was centrifuged[2]. The red blood cells were washed thrice in saline containing glucose 1 mmol·$L^{-1}$, buffered to pH 7.4 with Tris 5 mmol·$L^{-1}$. The erythrocytes were resuspended in 9 volumes of the same buffer. Aliquots with a final volume of 5 mL of this cell suspension, mixed with $CuSO_4$ 0.3 mmol·$L^{-1}$ and the chelating agent to be tested, were incubated at 37 ℃ in a shaking water bath for 180 min. The hemolysis was quantified by measuring the hemoglobin concentration in the supernatant, using Drabkin's cyanmethemoglobin method. The hemolysis was expressed in % of total hemolysis as obtained by addition of one drop of Triton X-100.

In the 1st series of experiments the efficacy of various agents to influence the copper-induced hemolysis was assayed by simultaneous addition of $CuSO_4$ 0.3 mmol·$L^{-1}$ and one of the agents, final concentration 0.3 mmol·$L^{-1}$: Pen, Hom, Suc, Uni, SH-20, epinephrine, or $CrCl_3$. Alb 0.05 mmol·$L^{-1}$ or Cer 0.05 mmol·$L^{-1}$, Pen 0.05 mmol·$L^{-1}$ or epinephrine 0.05 mmol·$L^{-1}$ were also tested. Six parallels of each trial were carried out.

In another series of experiments the simultaneous addition of epinephrine, $CuSO_4$ 0.3 mmol·$L^{-1}$ was combined with Pen, Hom, Suc, $CrCl_3$, or Alb 0.05 mmol·$L^{-1}$.

In the 3rd series of experiments $CuSO_4$ and epinephrine were added simultaneously, while Pen, Hom, or $CrCl_3$ was added after 10 min.

## RESULTS

After 3 h of incubation of red cells with $CuSO_4$ 0.3 mmol·$L^{-1}$ the hemolysis was about 10 % (Tab 1).

**Tab 1. Hemolysis induced by $CuSO_4$ 0.3 mmol·$L^{-1}$ during 3 h of incubation with human red cells and agents to be tested. Extracellular hemoglobin ($\bar{x}$ and range of 6 parallels) in % of total hemoglobin.**

| $CuSO_4$ plus | Hemoglobin/% |
|---|---|
| – (Controls) | 9.8 (8.8 – 12.5) |
| Epinephrine | 54.5 (43.5 – 59.4) |
| Unithiol | 18.2 (15.8 – 22.5) |
| Homocysteine | 16.5 (14.1 – 19.2) |
| Succimer | 2.2 (1.6 – 2.5) |
| Penicillamine | 2.1 (1.8 – 2.4) |
| Mercaptodextran | 2.4 (1.5 – 2.9) |
| $CrCl_3$ 0.3 mmol·$L^{-1}$ | 3.0 (1.5 – 3.9) |
| Albumin 0.05 mmol·$L^{-1}$ | 2.1 (1.3 – 2.9) |
| Ceruloplasmin 0.05 mmol·$L^{-1}$ | 1.9 (1.1 – 2.8) |

The spontaneous hemolysis in the absence of copper was 2 % (range 1.0 % – 2.8 %). The presence of the chelating agents without $CuSO_4$ did not significantly change this spontaneous hemolysis.

Pen, Suc, or SH-20 0.3 mmol·$L^{-1}$ reduced the hemolysis to the same values as seen in the absence of $CuSO_4$, viz about 2 %. Non-stoechiometrically low concentrations of Alb or Cer 0.05 mmol·$L^{-1}$ had a comparable efficacy. In contrast, such low concentration of Pen 0.05 mmol·$L^{-1}$ had only a small protecting potential, reducing the hemolysis to 5.8 % (range 5.0 % – 6.8 %).

Uni 0.3 mmol·$L^{-1}$ increased the hemolytic activity of copper to about 18 %. Hom 0.3 mmol·$L^{-1}$ and a low epinephrine concentration 0.05 mmol·$L^{-1}$ brought about a comparable acceleration of the hemolysis, whereas epinephrine 0.3 mmol·$L^{-1}$ gave rise to a considerably increased copper-induced hemolysis, to about 55 % after 3 h of incubation.

$CrCl_3$ protected against the hemolysis induced by copper alone, as well as against the cytolysis induced by the combined action of copper and epinephrine (Tab 2).

Pen, Suc, and SH-20, protected against the combined cytotoxic action of copper and epinephrine, and again a relatively low Alb concentration 0.05 mmol·$L^{-1}$ was as efficient as Pen 0.3 mmol·$L^{-1}$. Hom did not offer any significant protection in this respect. Upon addition after a delayed period of 10 min Hom again was without any effect, while Pen and

$CrCl_3$ still had some protecting ability, and Alb also acted as a protector (Tab 3).

**Tab 2. Hemolysis during 3 h incubation of red cells with $CuSO_4$ 0.3 mmol·L$^{-1}$ and epinephrine 0.3 mmol·L$^{-1}$ in the presence of chelating agents 0.3 mmol·L$^{-1}$. Extracellular hemoglobin ($\bar{x}$ and range of 6 parallels) was given in % of total hemoglobin.**

| $CuSO_4$ + epinephrine plus | Hemoglobin/% |
|---|---|
| – (Controls) | 54.5 (43.5 – 59.4) |
| Succimer | 5.1 (3.9 – 6.9) |
| Penicillamine | 4.5 (3.1 – 5.9) |
| Mercaptodextran | 3.9 (2.1 – 5.6) |
| Homocysteine | 53.4 (45.4 – 59.0) |
| $CrCl_3$ 0.3 mmol·L$^{-1}$ | 3.9 (2.2 – 5.0) |
| Albumin 0.05 mmol·L$^{-1}$ | 4.5 (3.0 – 6.0) |

**Tab 3. Hemolysis during 3 h of incubation of red cells with $CuSO_4$ and epinephrine (equimolar concentrations, 0.3 mmol·L$^{-1}$). A chelating agent or $CrCl_3$ 0.3 mmol·L$^{-1}$ was added 10 min after the addition of copper and epinephrine.**

| Delayed addition of | Hemoglobin/% |
|---|---|
| – (Controls) | 54.5 (43.5 – 59.4) |
| Penicillamine | 25.4 (20.7 – 29.9) |
| Homocysteine | 52.5 (44.4 – 58.4) |
| $CrCl_3$ 0.3 mmol·L$^{-1}$ | 23.9 (19.8 – 27.9) |

## DISCUSSION

The present study shows that epinephrine and some reducing thiols (Uni and Hom) can accelerate copper-induced hemolysis. We have previously reported that $Cu^{2+}$ could interact with Uni or membrane thiol groups, and thereby give rise to superoxide anion formation[6]. Apparently, reducing thiol groups of some cellular membranes[2] as well as of Uni and Hom can promote the conversion of $Cu^{2+}$ to $Cu^{+}$ (Fig 1). Epinephrine has also been reported to bring about such reduction of $Cu^{2+}$[7].

Under aerobic conditions, the $Cu^{+}$ thus formed, undergoes spontaneous autoxidation to $Cu^{2+}$, accompanied by reduction of molecular $O_2$, to the superoxide form (Fig 1), that can be further transformed to more reactive and toxic oxygen radicals, which bring about the hemolytic action[8].

It appears that the $Cu^{2+}/Cu^{+}$-interchanges in the model system used here involve at least two important steps, first the reduction of $Cu^{2+}$ by a reducing agents, and subsequently the autoxidation requiring the presence of oxygen, that leads to formation of activated oxygen species.

The first of these steps can be markedly inhibited by addition of a chelating agent with high affinity for the $Cu^{2+}$, such as Pen[2], Suc[9], or SH-20[5], that renders the $Cu^{2+}$ unavailable for the reducing agents.

The second step, the autoxidation, can be inhibited by lowering of the $pO_2$ in the solution and is completely blocked under anaerobic conditions[10].

The presence of $Cr^{3+}$ appears to inhibit the reactivity of oxygen, and it is relevant that it inhibits peroxidations in membrane systems presumably by enzyme inhibition[11]. Cer can oxidize $Cu^{+}$ directly, without a simultaneous conversion of molecular oxygen to the superoxide anion, and may also act as a scavenger owing to its inherent superoxide dismutating ability[12]. The scavenging ability of sub-stoechiometric Alb concentrations (0.05 mmol·L$^{-1}$) can not be fully understood from its ability to chelate $Cu^{2+}$. Alb and Cer appear to possess oxygen radical scavenging potentialities.

In acute cases of clinical copper poisoning there is a marked excess of copper ions that rapidly invades blood plasma before any Cer induction can take place. A life-threatening hemolysis might be precipitated acutely in such cases[1].

The chronic copper retention characterizing cholestatic conditions is not considered to be hazardous in this respect, as it is associated with significantly increased synthesis of the protective agent Cer[13]. Patients suffering from the chronic copper retention of Wilson's disease, however, are at risk of developing acute hemolytic crisis, since their Cer synthesis is deranged[1], and their Alb synthesis may also be reduced. The latter patients need the protection offered by life-long treatment with Pen or other chelating agent[2].

In chronically copper-poisoned sheep an acute life-threatening hemolysis might be precipitated in a period with excessive physiologic stress[14], that most likely is accompanied by increased plasma levels of epinephrine. It should be noted however, that the prohemolytic

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epinephrine concentrations used in our *in vitro* model are several factors higher than average concentrations *in vivo*. But the possibility of localized accumulations of epinephrine on membrane surfaces can not be rejected.

Hom and epinephrine may initiate oxygen radical production and lipoperoxidation *in vivo*, in the presence of iron or copper ions. Such processes may involve not only cellular membranes, but also LDL lipoprotein particles. $Cu^{2+}$ or $Fe^{3+}$, in the presence of Hom and epinephrine may thereby accelerate the atherosclerotic process in humans[15].

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**受肾上腺素和巯基螯合剂影响的硫酸铜的溶血活性**



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**关键词** 溶血; 硫酸铜; 肾上腺素; 二巯丁二酸; 二巯丙磺钠; 青霉胺; 高半胱氨酸; 血浆铜蓝蛋白; 清蛋白; 铬化合物

## Solicit contributions

Acta Pharmacologica Sinica will be published monthly in 1999. Manuscripts in English of full length articles and mini-reviews from any part of the world are warmly welcome.