The Effects of Cobalt on Superoxide Dismutase Activity, Methemoglobin Formation and Lipid Peroxide in Rabbit Erythrocytes

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In a previous study\(^1\), it was demonstrated that the degree of lipid peroxidation increased in the erythrocytes and plasma of rabbits treated with cobalt chloride when measured by malonyldialdehyde (MDA) formation. Tudhope\(^2\) demonstrated that the formation of MDA was accompanied by the bluing of the erythrocyte membrane when it was stained supravitally with crystal violet. Shen and Levy\(^3\) have reported that the addition of cobalt accelerates the formation of methemoglobin (metHb) in human blood \(\text{in vitro}\), but Bucciero and Orten\(^4\) found that administering cobalt to normal rats results in polycythemia without any evidence of metHb formation. The superoxide radical ion \(\text{O}_2^-\) is produced by the autoxidation of oxyhemoglobin (oxyHb) in erythrocytes\(^5\). The \(\text{O}_2^-\) produced can oxidize the heme groups in oxyHb and reduce those in metHb. In normal erythrocytes, superoxide dismutase (SOD) catalyses the removal of \(\text{O}_2^-\) and suppresses the reaction with hemoglobin\(^6\).

In the present investigation, the action of cobalt to form blue cells was examined in terms of its relation to lipid peroxidation in erythrocytes. The accelerated production of metHb from oxyHb in the blood of cobalt-treated rabbits and the ability of SOD to inhibit \(\text{O}_2^-\) generation, which promotes the formation of metHb from oxyHb, were investigated.

**MATERIALS AND METHODS**

Ten male white rabbits weighing 3–4 kg were injected subcutaneously with 25 mg/kg cobalt chloride (CoCl\(_2\)) once daily for three days and were deprived of food after the first injection. Ten control rabbits were given injections of 1 ml saline solution daily and also deprived of food. Blood was drawn from the cobalt-treated animals 24 hr after the last injection and from the control animals, 72 hr after food deprivation began.

Erythrocyte lipid peroxidation was determined by measuring MDA concentration according to the method described by Sotcks and Dormandy\(^9\) and plasma lipid peroxidation was measured following the method of Ohishi\(^10\). Supravital staining of peroxidized blue cells by crystal violet was performed according to the method of Tudhope and Hopkins\(^2\). Blood methemoglobin and hematocrits were estimated by the micromethod of Hegesh et al.\(^11\). Erythrocyte superoxide dismutase activity was as in Scudder et al.\(^12\). Reticulocytes were counted as by Simons\(^13\). Plasma triglyceride determination followed that of Kawade\(^14\).

**RESULTS**

The administration of cobalt chloride to rabbits for three days caused a remarkable rise in plasma triglycerides. Hematocrit values were the same for both cobalt-treated and control groups (Table 1), while a slight but significant increase in reticulocyte count was observed for the cobalt-treated animals. MDA concentration, a measure of the degree of lipid peroxidation, increased not only in erythrocytes but also in the plasma of the experimental group.

No significant difference was found in the percentage of blue cells between the cobalt-treated and control groups, which suggests that there was no relation between the percentage of blue cells and the degree of lipid peroxidation. Methemoglobin content increased in the erythrocytes of cobalt-treated rabbits, while superoxide dismutase activity was inhibited when compared to that of the control group.
DISCUSSION

Giovannini et al.\textsuperscript{15} reported that rabbits exhibited a significant and rapid increase in hematocrit level within a short time after even a single intramuscular injection of 250 $\mu$ mole/kg CoCl$_2$. Yastrebov\textsuperscript{16} found that the period of increase in erythrocyte count after the administration of cobalt nitrate was preceded by a period (7-8 days) of some decrease. He ascribed this initial decrease to an increase in the destruction of erythrocytes. Furthermore, the number of reticulocytes increased throughout the investigation (50-60 days) with the greatest rise between the 7th and 11th day after the beginning of cobalt administration. Thorling and Erslev\textsuperscript{17} found increased PO$_2$ levels in subcutaneous air pockets in cobalt-treated rats. The sharp rise in oxygen tension suggests that the cobalt chloride inhibited cellular oxygen uptake and utilization. It was hypothesized that the cobalt-induced release of endogenous erythropoietin is caused by generalized cellular hypoxia. Under the influence of increased erythropoietin stimulation, erythrocyte maturation is modified and marrow reticulocytes are prematurely delivered into the blood\textsuperscript{18,19}.

In the present experiment, cobalt did not cause an increase in hematocrit values, but increased reticulocytes. It would seem that the three injections were insufficient to induce erythropoiesis but were sufficient to release reticulocytes from bone marrow into the blood stream.

Tudhope and Hopkins\textsuperscript{2} have shown that there is an overall blue coloration of erythrocytes, after they have been stained supravitally with crystal violet following exposure to hydrogen peroxide vapor. Such cells are designated “blue cells”, and there was a close relationship between the percentage of blue cells and the degree of lipid peroxidation. Here, no correlation was found between these blue cells and MDA formation, but the reason for this discrepancy is unclear.

In normal erythrocytes, both in the circulatory system and in vitro, hemoglobin is constantly being oxidized to metHb by autoxidation. Erythrocytes are susceptible to autoxidation not only because of polyunsaturated membrane phospholipids, high intracellular oxygen concentrations, but also because of an abundance of hemoglobin. Shen et al.\textsuperscript{3} observed metHb formation when normal human blood was incubated at 37°C for 24 hours in the presence of 0.2% cobalt chloride. They suggested that cobalt does inhibit the enzymatic reduction system which is concerned with the reconversion of metHb to hemoglobin. Bucciero and Orten\textsuperscript{4} have shown that the administration of cobalt to normal animals or man results in polycythemia without any evidence of methemoglobin formation. The increase of metHb formation in rabbit erythrocytes treated with CoCl$_2$ here may be explained by the fact that cobalt inhibits the

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
 & Unit of & Number of & Controls & CoCl$_2$-treated group \\
 & measurement & animals & & \\
\hline
Ht & % & 10 & 41.0±2.9 & 41.5±2.9 \\
Reticulocytes & % & 10 & 1.2±0.4 & 2.6±0.6$^*$ \\
Plasma & mg/dl & 10 & 52±32 & 266±108$^*$ \\
Triglyceride & & & & \\
Erythrocyte & n moles/g Hb & 10 & 272±100 & 328±60$^*$ \\
MDA & n moles/ml & 9 & 0.69±0.26 & 1.88±0.63** \\
Plasma & & & & \\
Blue cells & % & 10 & 21.5±6.0 & 18.4±5.2 \\
Erythrocyte SOD & units/ml & 10 & 681±61 & 600±63$^*$ \\
activity & erythrocytes & & & \\
metHb & % & 10 & 0.242±0.061 & 0.294±0.053$^*$ \\
\hline
\end{tabular}
\caption{Effects of cobalt on MDA concentration, blue cell formation, SOD activity and metHb formation in rabbit blood}
\end{table}

The values are the means ± SD

$^*$: significant at the level of $p<0.05$ or $^*$: $p<0.01$
enzymatic reduction system which is concerned with the reconversion of metHb to Hb. The present authors, however, did not investigate metHb reductase activity and the precise mechanism by which metHb reconverts to Hb remains unclear.

The autoxidation of oxyHb to metHb is believed to involve the displacement of the superoxide radical. This has been confirmed by Misra and Fridovich, and Wever et al. Lynch et al. have shown that the formation of metHb from oxyHb is inhibited by SOD and their data support the notion of the mediation of metHb formation by $O_2^-$. If SOD prevents metHb formation in erythrocytes, cells with a reduced SOD content should have an accelerated metHb rate when exposed to superoxides in vitro and the hemoglobin denaturation in cells from an animal deficient in SOD should increase in vivo. Efforts to test these predictions are being made on swine rendered deficient in SOD by means of dietary copper deprivation. In the present experiments, cobalt inhibited SOD activity. SOD inhibition may facilitate the conversion of oxyHb to metHb and as a consequence increase metHb formation. The problem of whether metHb formation is accelerated directly by a reduction in metHb reductase activity in cobalt-treated animals or indirectly by the inhibition of SOD activity, is a subject for future research.

**SUMMARY**

Daily administrations of 25 mg/kg cobalt chloride to rabbits for three days evoked a significant increase of lipid peroxides in erythrocytes and plasma accompanied by an increase in the reticulocyte count.

The proportion of blue cells that is an overall blue coloration after the supravital staining with crystal violet, did not correlate with the increase of lipid peroxides in erythrocytes of cobalt-treated rabbits. Cobalt induced a slight but nevertheless significant increase in the percentage of methemoglobin but caused a decrease in SOD activity in erythrocytes.

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**REFERENCES**

コバルトがウサギの赤血球のスーパーオキサイドディスムターゼの活性、メトヘモグロビンの生成と過酸化脂質におよぼす影響

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体重3〜4 kgのオスのウサギを2群にわけ1群には塩化コバルトを25 mg/kg/日の割合で、他の群には同量の生理的食塩水をそれぞれ3日間筋肉注射した。

4日目に採血した結果、コバルト注射群のヘマトクリット値は対照群に比べて有意の差を示さなかったが、網状赤血球の割合は増加した。赤血球ならびに血漿中の過酸化脂質（マロンニルジュアルデヒドであらわした）はコバルト群で有意に増加したが“blue cells”的割合との相関はみられなかった。

コバルト注射群のメトヘモグロビン含量は有意に増加したが、赤血球のスーパーオキサイドディスムターゼの活性は低下した。