# Effect of the Administration of Toxic Amounts of Copper with Other Chelating Compounds such as EDTA, Thiomolybdate, Ascorbate and Deferoxamine on Growth, Mortality and Hepatic Minerals of Chick Embryos

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## ABSTRACT

We administered copper and other chelating or reducing compounds directly into the air sacs of 14-day-old fertile eggs and after 5 days of incubation we examined embryonic growth, mortality and hepatic minerals of chick embryos. Adminstration of EDTA-2Na with copper (II) stimulated copper absorption and hepatic copper accumulation while that of tetrathiomolybdate with copper completely suppressed them. Although other agents such as deferoxamine mesylate, bathophenanthroline sulfonic acid, L-ascorbic acid and D-penicillamine conld alleviate the copper toxicity to some extent, they were not so effective as EDTA or thiomolybdate in either stimulatory or suppressive ways for the hepatic copper accumulation. Examination of the hepatic minerals (Cu, Zn, Fe) revealed that the treatment group which had a higher death rate from excess copper absorption showed significantly higer tissue iron deposition probably due to the occurrence of hemolysis. These results suggest that excess copper absorption increases tissue iron deposition and that both free copper and free iron contribute to produce harmful radical species for membrane lipid peroxidation.

## INTRODUCTION

According to our previous reports[1-3] copper becomes a growth promoter for young pigs when 200 ppm of copper as $CuSO_4$ is supplemented to diets, and if other antibiotic or bacteriostatic agents such as tylosin and olaquindox are supplemented, the growth-promoting effect of copper will disappear. By the above copper supplementation the copper contents in the liver and kidney cortex are significantly elevated. Hepatic copper accumulation is a useful bioassay criterion for determining bioavailability of inorganic copper[4-6]. To know the interaction of trace metal with other chelate compounds in its absorption

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and tissue distribution processes we have used an experimental system using fertilized hen eggs[7,8]. We administered such compounds directly into the air sacs of 14-day-old-eggs, and after 5 days incubation, examined the hepatic mineral contents of 19-day-old chick embryos. The liver and legs (toes) are selected for copper or selenium retention and vanadium retention, respectively. The object of the present work was to know the effects of chelaing or reducing agents on copper (Ⅱ) absorption, retention and toxicity by using the experimental system of developing chick embryos.

## MATERIALS AND METHODS

Copper disodium ethylenediaminetetraacetate, disodium ethylenediamineteraacetate, and bathophenanthroline sulfonic acid disodium salt were from Dojindo Lab. ; deferoxamine mesylate was from Sigma Chem. Co. ; D-penicillamine was from Aldrich Chem. Co. ; and ammonium tetrathiomolybdate was a gift from Rowett Research Institute, UK. Other reagents were from Wako Pure Chem. Ind.

The method was the same as that previously shown[8]. Fertile White Leghorn eggs were incubated in an incubator. Two small holes were made in the eggshells just above the air sacs of 14-day-old fertile eggs and 100 μl of aqueous mineral or chelate solutions were separately introduced into the air sacs through the holes. The holes were coated with vaseline and the eggs were incubated for a further 5 days. Then wet embryonic weights and the liver weights were measured. In some embryos the weigths of legs plus toes were also measured. Dry tissue samples were digested by using the nitric acid digestion method and the mineral contents were determined by atomic absorption.

## RESULTS

Embryonic death rate increased with increased administration levels of $CuCl_2$. Administration of 1 μ mole of $CuI_2$ per egg caused about 90% death rate. Compared with the $CuCl_2$ administration the $CuSO_4$ administration appeared to be less toxic. Following exeriments were done with an administration of 1 μ mole $CuCl_2$.

Administration of copper alone (positive control) significantly decreased embryonic weights and significantly increased death rate ($p < 0.05$) in comparison to the no-treatment group (negative control) as shown in Table 1. Administration of tetrathiomolybdate (1 μ mole) or EDTA-2Na (1 μ mole) significantly contributed to alleviate the copper toxicity of the positive control group.

The treatment groups administered EDTA with copper or Cu-EDTA complex showed the highest hepatic copper content ; that administered copper alone (positive control) second highest copper ; and that administered thiomolybdate with copper or negative control group the lowest copper. Although the data are not shown in Table, the administration of 2 μ mole of Cu-EDTA complex caused 80% embryonic death with the copper accumulation of 242 ppm in the liver dry matter. Administration of EDTA contributed to an increase in copper absorption and hepatic copper retention.

**Table 1.** Effects of administration of EDTA and thiomolybdate with copper on growth, mortality and hepatic minerals of chick embryos.

| Treatment | Embryonic wt(g) | Death rate (%) | Liver Cu ppm/DM | Liver Zn ppm/DM | Liver Fe ppm/DM |
|---|---|---|---|---|---|
| None (21) | $26.9^{a}$ | $5^{a}$ | $62^{a}$ | 63 | $131^{a}$ |
| +Cu (30) | $16.1^{d}$ | $90^{b}$ | $94^{b}$ | $70^{a}$ | $397^{b}$ |
| +Cu+EDTA (10) | $21.4^{bc}$ | $50^{c}$ | $161^{c}$ | 65 | $263^{a}$ |
| +Cu-EDTA Complex (9) | $26.2^{bc}$ | $22^{a}$ | $154^{c}$ | $55^{b}$ | $150^{a}$ |
| +Cu+$MoS_4$ (10) | $27.8^{a}$ | $0^{a}$ | $42^{a}$ | $74^{a}$ | $228^{a}$ |

Numbers in parentheses in Treatment show numbers of eggs used.
There are significant differences ($P<0.05$) among the values denoted by different superscripts.

There were some significant differences in hepatic zinc content but the difference in zinc content among treatment groups seemed to be much smaller than those of other minerals. Hepatic iron content of the group administered only copper (positive control) which showed the higest death rate became significantly higher than those of the other treatment groups. Although the data are not shown in Table, iron content of the legs plus toes in the positive control group was also significantly elevated, suggesting that extensive iron deposition occurred not only in the liver but also in other tissues.

Adding effects of deferoxamine, bathophenathroline sulfonic acid, L-ascorbic acid and D-penicillamine to the positive control group on embryonic weight, death rate and hepatic mineral contents are shown in Table 2. Addition of deferoxamine (1 μmole), bathophenanthroline (1 μmole), ascorbic acid (2 μmole) and penicillamine (2 μmole) contributed to the decrease of copper toxicity to some extent. Although hepatic copper content did not change significantly, the addition of deferoxamine, bathophenanthroline or ascorbic acid (2 μmole) significantly decreased hepatic iron content. Although data are not shown in Table, copper content in the liver dry matter was 73.1 ppm when 3 μmole of ascorbic acid was adminstered. The increase in the administration rate of ascorbic acid tended to decrease hepatic copper content.

Correlation coefficients between death rate and embryonic weight, hepatic copper content, hepatic zinc content or hepatic iron content in a total of 14 administration experiments are shown in Table 3. High correlation coefficients were shown between the death rate and embryonic weight and between the death rate and hepatic iron content. The latter is shown in Fig. 1. These relationships suggested that excess

copper absorption caused the increase in death rate and the higher death-rate group showed higher iron deposition. This higher iron deposition may be caused by the occurrence of oxidative hemolysis due to the accumulation of excess free copper ions in the tissues.

**Table 2.** Effects of administration of deferoxamine (Def), bathophenanthroline (Batho), ascorbate (Asc) and penicillamine (Pen) with copper on growth, mortality and hepatic minerals of chick embryos

| Treatment | Embryonic wt(g) | Death rate (%) | Liver Cu ppm/DM | Liver Zn ppm/DM | Liver Fe ppm/DM |
|---|---|---|---|---|---|
| +Cu (30) | 16.1 | 90 | 94 | 70 | 397 |
| +Cu+Def (9) | 22.2* | 33* | 77 | 61 | 161* |
| +Cu+Batho (8) | 22.3* | 38* | 108 | 46* | 178* |
| +Cu+Asc-1 μmole(9) | 18.2 | 67* | 113 | 73 | 379 |
| +Cu+Asc-2 μmole(9) | 21.5* | 11* | 98 | 69 | 211* |
| +Cu+Pen-1 μmole(9) | 16.1 | 56* | 101 | 75 | 320 |
| +Cu+Pen-2 μmole(9) | 20.9* | 56* | 96 | 49* | 360 |

Numbers in parentheses in Treatment show numbers of eggs used.
There are significant differences ($P < 0.05$) between the value of +Cu treatment and other values denoted by an asterisk character(*).

**Table 3.** Correlation coefficients between death rate and embryonic weight, hepatic copper content, hepatic zinc content or hepatic iron content.

| | Correlation coefficient (N=14) |
|---|---|
| Death rate versus embryonic wt | −0.77 |
| Death rate versus liver Cu | 0.21 |
| Death rate versus liver Zn | −0.14 |
| Death rate versus liver Fe | 0.71 |

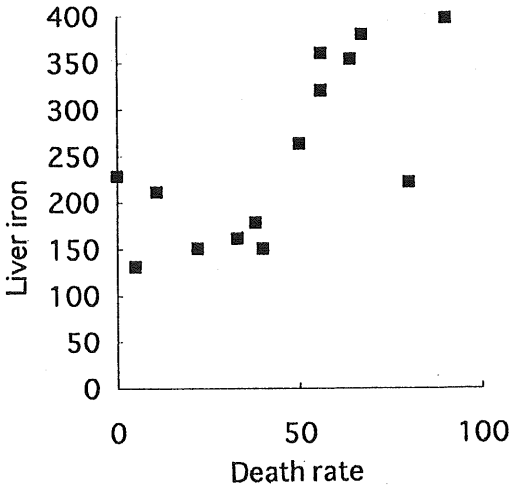


**Fig. 1.** Relationship between death rate (%) and hepatic iron content (ppm/DM).

## DISCUSSION

Most copper compounds introduced into the air sacs pass through the eggshell membrane and can be absorbed into the blood system through the allantoic membrane. When absorbed, copper is rapidly taken up into the liver by a mechanism that does not appear to be saturable[9]. In the liver most copper stays as metallothionein complexes. Copper is then excreted into bile or integrated into ceruloplasmin and secreted into plasma. If excess copper surpassing the complexing capacity of metallothionein in the liver is absorbed into the embryonic blood system, free ions will produce oxidative radical species to facilitate hemolysis. In our previous works[10,11] the *in vitro* hemolysis of erythocytes occurred at 10 μM copper (II) concentration irrespective of vitamin E status. Free copper ions induce the peroxidation of membrane lipid fraction through a free-radical mediated pathway.

The absorption mechanism of copper from the gastrointestinal tract is still a matter of debate. Many agents to increase or decrease the absorption are known[12]. EDTA complexing with copper increased the copper absorption rate from inorganic copper sources. The administration of 1 μmole of inorganic copper alone caused 90% embryonic death with significant growth depression whereas that of 1 μmole of EDTA-complexed copper alone caused only 22% embryonic death with no growth retardation. In the latter treatment more copper accumulated safely in the liver as copper-metallothionein complexes. Copper toxicity as shown by death rate did not correlate with hepatic copper content but did correlate well with hepatic iron content in administration of less than 1 μmole of copper.

Copper accumulates more readily in the livers of male rats and they are less resistant to the toxic

effects[13]. The signs of toxicity are a reduced growth rate and early liver damage. This occurs beyond the maximum capacity of retaining copper in the liver. The liver storage compartment provides an emergency means for removing ionic copper from the blood, thereby preventing its toxic buildup in tissues[14]. Compared with the present copper work, in our previous work[8] the administration of vanadyl-EDTA complex does not increase vanadium content in the legs, suggesting that (VO)-EDTA is unabsorbable or unusable in contrast to Cu-EDTA. According to Camargo et al.[15] the administration of copper-EDTA chelate to sheep results in a rapid accumulation of the copper in the liver.

In contrast to EDTA addition, thiomolybdate addition could completely inhibit copper absorption and its hepatic deposition. Copper-thiomolybdate complex must be unabsorbable or unusable. The intravenous administration of tetrathiomolybdate prevents the development of chronic copper poisoning in sheep, reduces the rate of accumulation of copper in the liver of copper-dosed sheep but increased the copper contents of kidneys[16,17].

Deferoxamine, bathophenanthroline, ascorbic acid and penicillamine were effective to ameliorate the copper toxicity to some extent without changing the copper absorption and deposition significantly. Deferoxamine is an iron chelator and it reduces hepatic iron concentration[18]. Effectiveness of deferoxamine to alleviate the excess copper toxicity may be due to its chelating action to iron because the peroxidation of membrane phospholipids is detected if micromolar concentrations of free iron ions are present[19]. Ascorbic acid may reduce cupric ions to cuprous ions in the air sacs and the absorption of cuprous ions may be less toxic to animals than that of cupric ions. Ascorbic acid shows positive and negative regulatory functions for copper absorption[20]. Ascorbic acid can augment ceruloplasmin synthesis and shows a sterospecific postabsorption role in the metabolism of copper[21]

According to Chareonpong et al.[22] the hemolysis occurring in long-term selenium deficiency in rats contributes to increase the tissue iron content and to decreae iron-binding capacity. According to our present experiment the copper overload showed the same phenomenon as shown by Chareonpong et al. In the experiment of Ledoux et al.[5] iron concentration in the liver becomes the greatest in chicks fed a diet of the highest copper supplementation. The development of hemolytic anemia as a complication of acute copper intoxication is well documented[23]. According to Gooneratne et al.[16] copper-loaded sheep undergoing hemolysis show the presence of increased iron levels in the liver and kidney, and the iron probably arises from hemolyzed blood. In contrast to our present experimental evidence, copper deficiency also increases the hepatic iron content because iron transport from the liver is inhibited due to the lack of celuroplasmin[24]. So both copper deficiency and copper excess states can increase hepatic iron deposition and both free copper and free iron contribute to produce harmful radical species for membrane lipid peroxidation. The chelating compounds to facilitate complex-formation with copper or iron can be used as protective agents.

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