Anemia induced by ingestion of excess zinc in chicks: importance of red blood cell turnover?

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Three studies were conducted to determine whether ingestion of excess zinc induces anemia, at least partially, by increasing red blood cell fragility directly or through changes in copper-zinc-dependent superoxide dismutase (Cu-Zn-SOD). Chicks from the cross New Hampshire × Single Comb White Leghorn were fed adequate (0.72–0.76 μmol Zn/g diet) or high (22.60–25.17 μmol Zn/g diet) levels of zinc and adequate (0.11–0.14 μmol Cu/g diet) or high (3.13–3.45 μmol Cu/g diet) levels of copper. To assess whether excess zinc could induce a hemolytic anemia, we monitored red blood cell (RBC) Cu-Zn-SOD, RBC fragility in vitro, and erythrocyte $t_{1/2}$ in vivo. Cu-Zn-SOD activity was depressed among chicks fed excess zinc and the ingestion of extra copper restored Cu-Zn-SOD activities to the levels of the control chicks. However, lysis of erythrocytes in diluted saline (0.35% NaCl) was lower when chicks were fed high levels of zinc and the ingestion of extra copper further decreased lysis. With these counteracting influences, the lifespan of erythrocytes was not affected by any of the treatments in one study and was greater in chicks fed both high zinc and copper in another study. These data indicate that the anemia induced by excess zinc is not a hemolytic anemia.

Keywords: anemia; zinc; copper; chick; superoxide dismutase

Introduction

We previously reported that feeding excess zinc (31 μmol/g diet) to chicks resulted in anemia.[1,2] We hypothesized, as had Settlemire and Matrone,[3] that this effect might not be solely due to the observed decreased absorption of Fe-59 by chicks fed excess zinc.

Several mechanisms may contribute to the anemia caused by excess dietary zinc. Ingestion of excess zinc could indirectly reduce stability of red blood cell (RBC) membranes by reducing copper absorption and retention[4] and thus decreasing activity of copper dependent enzymes such as superoxide dismutase.[5,6] Ingestion of excess zinc or copper might also have more direct effects on RBC stability. The addition of copper ions to in vitro solutions has been observed to increase hemolysis of erythrocytes.[7] However, erythrocytes from zinc- or copper-deficient rats have been found to be more susceptible to lysis in hypotonic saline or to sodium dodecyl sulfate than erythrocytes of normal rats.[8-13]

Thus, the objectives of these experiments were the following: 1) to investigate whether ingestion of excess zinc could depress tissue copper concentrations and the activity of copper-zinc-dependent superoxide dismutase (Cu-Zn-SOD) sufficiently to alter RBC fragility and $t_{1/2}$ life; and 2) to assess if this could be a mechanism by which excess zinc induced anemia.

Materials and methods

Experimental design

In three studies, chicks were randomly assigned to a 2 × 2 factorial arrangement of treatments. Chicks were fed adequate (0.76, 0.75, 0.72 μmol Zn/g diet in studies 1–3, respectively) or high zinc levels (23.33, 25.17, 22.60 μmol Zn/g diet in studies 1–3) and adequate (0.14, 0.14, 0.11 μmol Cu/g diet in studies 1–3) or high copper levels (3.41, 3.45, 3.13 μmol Cu/g diet in studies 1–3). The diets in studies 1–3 contained 2.24, 1.97, and

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1.70 μmol Fe/g diet, respectively. Diets were fed from hatching to 3 weeks of age to 32, 48, and 28 chicks in studies 1, 2, and 3, respectively.

Animals and diets

Chicks from the cross of New Hampshire × Single Comb White Leghorn were housed in individual stainless steel cages with wire floors in rooms maintained at 30° C with 24-hr lighting. The facilities met the requirements and the protocol was approved by the Institutional Animal Care and Use Committee.

Chicks were fed a semi-purified diet described previously.[1] Feed consumption and body weights were recorded weekly in all studies. Deionized water was supplied ad libitum throughout the studies.

Sample collection and analyses

On day 21, chicks were bled via cardiac puncture using syringes rinsed with heparin solution and the resulting blood was used to determine packed cell volume and hemoglobin levels (studies 1–3) as well as in vitro osmotic fragility (studies 1 and 2), Cu-Zn-SOD activity (studies 1 and 2) and in vivo erythrocyte $t_{1/2}$ (studies 2 and 3).

In vitro osmotic fragility was determined by adding 50 μL of whole blood to 4 mL of 0%, 0.35%, or 0.9% NaCl solutions.[14] Absorbances (Abs) were read at 540 nm. Percent lysis was determined as follows: % lysis = (Abs in 0.35% NaCl − Abs in 0.9% NaCl) × 100 ÷ (Abs in 0% NaCl − Abs in 0.9% NaCl).

Erythrocyte Cu-Zn-SOD (E.C.1.15.1.1) activity was based on the inhibition of auto-oxidation of pyrogallol at pH 8.2. The reaction was conducted at room temperature and monitored by recording absorbance at 420 nm.[15] One unit of SOD activity was defined as the amount of enzyme that inhibits the reaction by 50%. Specific activity was expressed on the basis of mg of protein in the enzyme preparation. Protein was determined by the Bradford method using human gammaglobulin as a protein standard.[16]

To determine in vivo erythrocyte $t_{1/2}$, chicks at day 7 of age were injected in the wing vein with 370 kBq Cr-51 (17.04GBq/mg Cr, Na_2CrO_4 in saline, Dupont Company, Wilmington, DE, USA). Blood samples were collected at 2, 7, 10, and 14 days after injection of the isotope. They were centrifuged and washed three times with phosphate buffer saline (PBS) to remove any free label in plasma. Radioactivity in erythrocyte pellets was determined and the results were expressed as the percent of dose injected. Lifespan was determined from the inverse of the slope resulting from plotting the percent of dose injected (as log) over time.[17-19]

The radioactivity values were corrected for background and decay. Radioactivity of erythrocytes pellets and of tissues were measured in an automatic gamma counter (Gamma Trac 1191 TM Analytic Inc., Elk Grove Village, IL, USA) set at the photopeaks of Cr-51.

Diets and tissues were analyzed for zinc and copper by atomic absorption spectrophotometry (Perkin-Elmer Corp. Model 372, Norwalk, CT, USA). Bovine standards (SRM #1577a) obtained from the National Institute of Standards and Technology (NIST) were analyzed with most of the sets of samples. The analyzed standards (n = 8) were within 97% ± 2% for copper and 98% ± 2% for zinc of the certified NIST values.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using the general linear model.[20] Effects of zinc, copper, and their interactions were then determined. Mean treatment differences were determined by least square differences (LSD) with a level of statistical significance of 5%. Percentage data were transformed to arc sin angles prior to statistical analysis.

Results

Feeding high levels of zinc significantly decreased packed cell volume and hemoglobin levels of chicks (*Table 1*). These effects were partially reversed in study 2 when chicks consumed more copper.

Analysis of variance indicated that zinc intake significantly affected body weight in all three studies (*Table 2*). Feed intake was affected by zinc intake only in study 3. Feed efficiency (kg body weight/kg feed consumed) was lower when chicks were fed excess rather than adequate zinc in all three studies. Copper intake did not affect weight gain, feed intake, or feed use in any study.

Liver copper concentrations were elevated when high levels of copper were fed (*Table 3*). Ingestion of excess zinc in study 3 significantly decreased copper concentrations in livers of chicks fed the high level of copper. Tibia copper concentrations were insensitive to dietary changes.

Copper intake did not affect tissue zinc levels in these studies (*Table 4*). Liver and tibia, but not spleen, zinc concentrations were sensitive to variation in zinc intake.

Table 1 Hematological status of chicks fed various levels of zinc and/or copper

Diet zinc*	A	A	H	H	Statistical		
Diet copper	A	H	A	H	significance of:‡		
Packed cell volume			(l)		Cu	Zn	Cu × Zn
Study 2	0.28 ± 0.01†[a]	0.30 ± 0.01[a]	0.25 ± 0.01[b]	0.27 ± 0.01[ab]	0.05	0.01	ns§
Study 3	0.27 ± 0.01[a]	0.26 ± 0.01[a]	0.23 ± 0.01[b]	0.20 ± 0.01[b]	ns	0.0001	ns
Hemoglobin			(g/l)				
Study 2	88 ± 8[a]	95 ± 3[a]	75 ± 6[b]	84 ± 4[ab]	0.05	0.01	ns
Study 3	70 ± 4[a]	76 ± 6[a]	54 ± 8[b]	39 ± 5[c]	ns	0.001	ns

* A = adequate (0.72–0.75 μmol Zn/g diet and 0.11–0.14 μmol Cu/g diet); H = high (22.60–25.17 μmol Zn/g diet and 3.13–3.45 μmol Cu/g diet).
† Values are mean ± SEM; n = 12 in study 2 and n = 7 in study 3. Means in a row not sharing a common superscript letter differ significantly ($P < 0.05$).
‡ Data analyzed by 2 × 2 ANOVA and LSD tests applied to differentiate among means that were significantly different.
§ ns, not significant.

Table 2 Body weight and feed intake of chicks fed various levels of zinc and/or copper

Diet zinc* / Diet Copper	A / A	A / H	H / A	H / H	Statistical significance of:‡		
Body weight at 3 weeks		(g)			Cu	Zn	Cu × Zn
Study 1	125 ± 4†[a]	129 ± 8[a]	112 ± 8[ab]	103 ± 8[b]	ns§	0.001	ns
Study 2	148 ± 7[a]	135 ± 7[ab]	115 ± 10[b]	115 ± 8[b]	ns	0.001	ns
Study 3	176 ± 12[a]	184 ± 13[a]	138 ± 12[b]	136 ± 14[b]	ns	0.01	ns
Feed intake during 3 weeks		(g)					
Study 1	213 ± 7	218 ± 10	204 ± 10	202 ± 16	ns	ns	ns
Study 2	234 ± 10	216 ± 9	210 ± 9	208 ± 8	ns	ns	ns
Study 3	255 ± 17[a]	255 ± 19[a]	211 ± 10[b]	208 ± 19[b]	ns	0.01	ns

* A = adequate (0.72–0.76 μmol Zn/g diet and 0.11–0.14 μmol Cu/g diet); H = high (22.60–25.17 μmol Zn/g diet and 3.13–3.45 μmol Cu/g diet).
† Values are mean ± SEM; n = 8 in study 1, n = 12 in study 2, and n = 7 in study 3. Means in a row not sharing a common superscript letter differ significantly ($P < 0.05$).
‡ Data analyzed by 2 × 2 ANOVA and LSD tests applied to differentiate among means that were significantly different.
§ ns, not significant.

Table 3 Tissue copper concentration of chicks fed various levels of zinc and/or copper

Diet zinc* / Diet copper	A / A	A / H	H / A	H / H	Statistical significance of:‡		
Liver copper		(μmol/g wet tissue)			Cu	Zn	Cu × Zn
Study 1	0.14 ± 0.02†[b]	0.35 ± 0.06[a]	0.11 ± 0.02[b]	0.39 ± 0.06[a]	0.001	ns§	ns
Study 2	0.14 ± 0.02[ab]	0.24 ± 0.05[a]	0.06 ± 0.02[b]	0.31 ± 0.11[a]	0.05	ns	ns
Study 3	0.22 ± 0.03[b]	0.34 ± 0.33[a]	0.13 ± 0.02[b]	0.20 ± 0.02[b]	0.001	0.001	0.001
Tibia copper							
Study 1	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.03 ± 0.03	ns	ns	ns
Study 2	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.05 ± 0.02	ns	ns	ns
Spleen copper							
Study 1	0.06 ± 0.02[b]	0.06 ± 0.02[b]	0.06 ± 0.02[b]	0.16 ± 0.02[a]	0.05	0.05	0.005
Study 2	0.06 ± 0.02[b]	0.36 ± 0.02[b]	0.05 ± 0.02[b]	0.09 ± 0.02[b]	0.001	0.05	0.01

* A = adequate (0.72–0.76 μmol Zn/g diet and 0.11–0.14 μmol Cu/g diet); H = high (22.60–25.17 μmol Zn/g diet and 3.13–3.45 μmol Cu/g diet).
† Values are mean ± SEM; n = 8 in study 1, n = 12 in study 2, and n = 7 in study 3. Means in a row not sharing a common superscript letter differ significantly ($P < 0.05$).
‡ Data analyzed by 2 × 2 ANOVA and LSD tests applied to differentiate among means that were significantly different.
§ ns, not significant.

Table 4 Tissue zinc concentrations of chicks fed various levels of zinc and/or copper

Diet zinc* / Diet copper	A / A	A / H	H / A	H / H	Statistical significance of:‡		
Liver zinc		(μmol/g wet tissue)			Cu	Zn	Cu × Zn
Study 1	0.29 ± 0.02‡[b]	0.28 ± 0.02[b]	3.17 ± 0.49[a]	4.00 ± 0.60[a]	ns§	0.001	ns
Study 2	0.39 ± 0.02[b]	0.26 ± 0.02[b]	2.94 ± 0.44[a]	2.65 ± 0.21[a]	ns	0.0001	ns
Study 3	0.26 ± 0.03[b]	0.29 ± 0.05[b]	2.92 ± 1.31[a]	2.78 ± 0.96[a]	ns	0.001	ns
Tibia zinc							
Study 1	1.00 ± 0.07[b]	0.91 ± 0.09[b]	13.16 ± 1.30[a]	12.10 ± 1.37[a]	ns	0.001	ns
Study 2	0.84 ± 0.06[b]	0.89 ± 0.05[b]	12.95 ± 0.99[a]	12.68 ± 1.48[a]	ns	0.0001	ns
Spleen zinc							
Study 1	0.17 ± 0.03	0.20 ± 0.03	0.34 ± 0.11	0.20 ± 0.02	ns	ns	ns
Study 2	0.44 ± 0.08	0.44 ± 0.11	0.46 ± 0.03	0.63 ± 0.05	ns	ns	ns

* A = adequate (0.72–0.76 μmol Zn/g diet and 0.11–0.14 μmol Cu/g diet); H = high (22.60–25.17 μmol Zn/g diet and 3.13–3.45 μmol Cu/g diet).
† Values are mean ± SEM; n = 8 in study 1, n = 12 in study 2, and n = 7 in study 3. Means in a row not sharing a common superscript letter differ significantly ($P < 0.05$).
‡ Data analyzed by 2 × 2 ANOVA and LSD tests applied to differentiate among means that were significantly different.
§ ns, not significant.

Table 5 Activity of cytosolic copper-zinc superoxide dismutase (SOD) in erythrocytes, osmotic fragility at 0.35% saline and $t_{1/2}$ of erythrocytes from chicks fed various levels of zinc and/or copper

Diet zinc*	A	A	H	H	Statistical		
Diet copper	A	H	A	H	significance of§		
SOD activity		(units/mg of protein)†			Cu	Zn	Cu × Zn
Study 1	56 ± 4‡[a]	60 ± 3[a]	40 ± 6[b]	67 ± 7[a]	0.005	ns‖	0.05
Study 2	54 ± 4[a]	52 ± 2[ab]	44 ± 4[b]	57 ± 2[a]	ns	ns	0.01
Osmotic fragility		(lysis in vitro)					
Study 1	0.87 ± 0.04[a]	0.91 ± 0.03[a]	0.77 ± 0.11[ab]	0.60 ± 0.07[b]	ns	0.001	ns
Study 2	0.91 ± 0.06[ab]	0.93 ± 0.03[a]	0.73 ± 0.07[bc]	0.61 ± 0.08[c]	ns	0.005	ns
RBC $t_{1/2}$ in vivo		(days)					
Study 2	23.0 ± 0.8	24.9 ± 0.9	23.3 ± 1.1	24.2 ± 1.3	ns	ns	ns
Study 3	16.4 ± 0.7[ab]	16.2 ± 0.8[ab]	15.5 ± 0.6[b]	18.2 ± 0.9[a]	ns	ns	0.05

* A = adequate (0.72–0.76 μmol Zn/g diet and 0.11–0.14 μmol Cu/g diet); H = high (22.60–25.17 μmol Zn/g diet and 3.13–3.45 μmol Cu/g diet).
† One unit defined as the amount of enzyme that inhibits the rate of reaction by 50%.
‡ Values are mean ± SEM; n = 8 in study 1, n = 12 in study 2, and n = 7 in study 3. Means in a row not sharing a common superscript letter differ significantly ($P < 0.05$).
§ Data analyzed by 2 × 2 ANOVA and LSD tests applied to differentiate among means that were significantly different.
‖ ns, not significant.

Although zinc intake per se did not affect Cu-Zn-SOD activity, there was a significant interaction of dietary copper and zinc on Cu-Zn-SOD activity (*Table 5*). Cu-Zn-COD activity was lower in chicks fed excess zinc and adequate copper than in other chicks.

Analysis of variance indicated that diet zinc, but not diet copper, significantly affected in vitro erythrocyte osmotic fragility (*Table 5*). However, LSD tests suggested that the ingestion of high levels of copper did exacerbate the effects of high levels of zinc. Erythrocytes from chicks fed excess zinc and copper hemolyzed less in a dilute saline solution (0.35% NaCl) than erythrocytes from chicks fed adequate zinc with either level of copper. The greater stability of these erythrocytes could reflect shorter $t_{1/2}$ of RBC in these chicks because younger RBCs are more resistant to hemolysis.[21] Erythrocytes of chicks fed excess zinc and copper in study 2 had longer $t_{1/2}$ than those of chicks fed excess zinc with only adequate copper. However, in vivo erythrocyte $t_{1/2}$ was not affected by zinc or copper intake per se.

Discussion

Settlemire and Matrone[3] stated that erythrocytes from rats fed 0.75% zinc lysed in 0.32% saline solution while erythrocytes from control rats lysed in a 0.4% saline solution. They did not report actual data on in vitro lysis of cells from their one study. However, our results also indicated that excess zinc intake must have stabilized erythrocyte membranes because cells from chicks fed excess zinc lysed less in hypotonic saline than those of chicks fed adequate diets.

Chavpil et al.[22] noted that high zinc intakes decreased the peroxidation of fatty acids in the livers of rats. The increased stability of membranes may be due to zinc binding to thiol and carboxyl groups of proteins or phosphate moiety of phospholipids.[13] Copper may augment this function of zinc because when both excess copper and zinc were fed, erythrocytes lysis in vitro decreased even further.

Settlemire and Matrone[3] injected a small group of rats with C-14 labeled glycine and measured the disappearance of label from hemin isolated from blood over a 52-day period. Using this methodology, they concluded that ingestion of excess zinc resulted in a shorter life span of RBCs in vivo. In contrast, we used Cr-51 to label RBCs and found that the addition of excess zinc and copper had no effect on erythrocyte $t_{1/2}$ in one study. In a second study chicks fed both high levels of zinc and copper had longer erythrocyte $t_{1/2}$ than chicks fed excess zinc with adequate copper. These discrepancies in both Settlemire and Matrone's results and our results could be due to species differences because mammalian erythrocytes are not nucleated as avian erythrocytes are. Our results on erythrocyte $t_{1/2}$ in chicks are consistent with our observation that Fe-59 turnover in erythrocytes was not affected by ingestion of excess zinc.[1] However, one limitation of the standard Cr-51 methodology is that some recycling of the label is possible.

Zinc and copper could also affect erythrocyte stability by mechanisms other than a direct effect on membrane stability. Rats fed high levels of zinc have been shown to have decreased Cu-Zn-SOD activity and decreased tissues copper concentrations.[4-6] Copper deficiency, which also induces these two symptoms, has been demonstrated to decrease $t_{1/2}$ of erythrocytes in pigs and rats.[10,11,22,23] We found that chicks fed excess zinc had depressed Cu-Zn-SOD activity and that the effect was overcome by feeding additional copper.

The balance between direct (as indicated by in vitro hemolysis tests) and indirect (i.e., through Cu-Zn-SOD) effects of zinc and copper on erythrocytes probably accounts for the observed lack of effect of excess zinc on erythrocyte $t_{1/2}$ in study 2. In study 3, the effect of copper on SOD activity was perhaps more important, although the dietary treatments were the same.

In theory, ingestion of excess zinc could depress tissue copper concentrations and decrease Cu-Zn-SOD activity sufficiently to increase RBC fragility and

hence decrease the in vivo $t_{1/2}$ of red blood cells. However, we were unable to demonstrate this sequence of events in chicks. The ingestion of excess zinc without added copper tended to depress Cu-Zn-SOD activity but did not adversely affect RBC fragility in vitro. In fact, exposure to excess zinc tended to decrease RBC fragility in vitro. Thus, it is doubtful that the anemia induced in chicks by feeding excess zinc is hemolytic in nature.

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