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Interrelationships between Dietary Iron and Tissue Zinc and Copper Levels and Serum Lipids in Rats[1] (39944)

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Introduction. Studies on the relationship between mineral and lipid metabolism have indicated that both deficiencies and excesses of minerals such as calcium (1), vanadium (2), and fluoride (3) in the diet are associated with elevated serum lipids. Hyperlipidemia has been demonstrated in rats (4, 5) and chicks (6) on diets low in iron and in rats fed diets with increased zinc/copper ratios (7). Thus it appears that multiple interactions among minerals may be involved in the regulation of serum lipid levels. In addition there is considerable evidence of interactions between minerals. Sourkes *et al.* (8) reported increased levels of copper in livers of iron-deficient rats. High intakes of zinc result in lower levels of iron in liver (9–12), blood (13), kidney (11), heart (11), and milk (14), and in lower hemoglobin levels (13). The effects of copper deficiency on decreased ceruloplasmin activity and increased liver iron concentration have been well established by Frieden (15).

This study was one of a series of experiments dealing with iron nutriture and lipid metabolism. Based on the literature cited above it was postulated that there were multiple interactions among minerals involved in the regulation of serum lipid levels. In this paper the hypothesis that maternal iron deficiency produces changes in offspring tissue zinc and copper concentrations was tested. A second hypothesis that changes in tissue zinc and copper levels are related to serum lipid levels was also tested.

Materials and methods. Animals. Nulliparous Sprague-Dawley rats were bred at a weight of 180–220 g. On the day pregnancy was suspected by the presence of sperm in a vaginal smear the dams were randomly assigned to one of three experimental groups (5, 29, or 307 ppm iron) and fed the appropriate diet and glass-distilled water *ad libitum*. The composition of the diets is shown in Table I. To avoid iron contamination, all materials which were used in contact with the rats or diets were stainless steel, glass, or plastic.

On the day following parturition the litter size was reduced to two males and four females. The pups were suckled by their mothers until the 18th day of lactation. Food cups were placed out of the reach of pups to ensure that milk was the only source of nourishment for the young.

Following a 4-hr fasting period on the 18th day, blood was collected by cardiac puncture for determination of triglycerides, cholesterol, and phospholipids in serum. Tail blood samples from pups and dams were taken for hemoglobin and hematocrit determinations (18). Livers of pups and dams were perfused *in situ* with 20 ml of cold 0.9% NaCl to remove circulating blood. Livers and spleens were removed, rinsed with glass-distilled water, blotted dry, and frozen for mineral analysis.

Milk was collected from dams under sodium pentobarbital anesthesia 6 hr after the removal of young on Day 18. The milk, collected from each nipple by gentle palpation, was frozen and later analyzed for iron, copper, and zinc.

Determination of lipids. Lipids were extracted from serum samples pooled from two females and one male of each dam's litter by the method of Folch *et al.* (19) and separated on thin-layer chromatography plates developed in a solvent system of petroleum ether:ethyl ether:acetic acid (70/30/1, v/v/v). Following iodine vapor exposure, lipid fractions were identified and recovered, and colorimetric assays were used to quantitate the lipid fractions. Triglyceride concentration was determined by the

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TABLE I. COMPOSITION OF DIETS.

	Iron		
	307 ppm	29 ppm	5 ppm
Casein[a] (%)	22.00	22.00	22.00
Sucrose (%)	29.70	29.75	29.76
Cornstarch (%)	29.70	29.76	29.76
Iron-free salt mix[b] (%)	5.48	5.48	5.48
Vitamin mix[c] (%)	1.00	1.00	1.00
Corn oil[d] (%)	10.00	10.00	10.00
Cellulose[e] (%)	2.00	2.00	2.00

[a] Vitamin-free casein, Teklad, Chagrin Falls, Oh.

[b] The level of minerals used is at least 12.5% more than NRC recommendations for lactation (16). Composition of salt mixture (mg/100 g of diet): $CaCo_3$, 1680.0; $CoCl_2 \cdot H_2O$, 0.1; $CuSO_4 \cdot 5\ H_2O$, 9.8; $MgSO_4 \cdot 7\ H_2O$, 278.5; $MnSO_4 \cdot H_2O$, 19.1; KI, 0.02; NaCl, 1000.0; K_2HPO_4, 2483.6; $ZnCl_2$, 3.0. $FeSO_4 \cdot 7\ H_2O$ was added to the supplemented diets in place of sucrose and cornstarch.

[c] Teklad, Chagrin Falls, Ohio, Cat. No. 40060. Supplies (mg/kg of diet) when added at 1% of diet: *P*-aminobenzoic acid, 110.1; ascorbic acid, 991.2; biotin 0.4; vitamin B_{12}, 0.03; calcium pantothenate, 66.1; choline, 1433.7; folic acid, 2.0; inositol, 110.1; menadione, 49.6; niacin, 99.1; pyridoxine HCl, 22.0; riboflavin, 22.0; thiamine HCl, 22.0. Supplies (units/kg of diet) when added at 1% of diet: vitamin A palmitate, 19824; vitamin D_2, 2203; vitamin E acetate, 121. Cornstarch dilutent QS.

[d] Mazola corn oil, Best Foods, Englewood Cliffs, N.J.

[e] Alphacel non-nutritive cellulose, Teklad, Chagrin Falls, Ohio, with iron extracted by the method of Houk *et al.* (17).

method of Stern and Shapiro (20), nonesterified cholesterol and cholesterol ester by the method of Searcy and Bergquist (21), and phospholipid concentration by the method of Chen *et al.* (22).

Determination of minerals in liver, spleen, and milk. Two livers and four spleens pooled from each litter and maternal organs were used for mineral analyses. The samples were dried at 100° for 48 hr. The weights of dry samples were recorded and dry samples digested on a hot plate with a 4/1 (v/v) ratio of concentrated nitric and 70% perchloric acid. Additional nitric acid was added until the digests were clear. Digests were brought to an appropriate volume with glass-distilled water. Iron, copper, and zinc concentrations were determined with the Perkin–Elmer 403 atomic absorption spectrophotometer. One-milliliter samples of milk were treated similarly and assayed for iron, copper, and zinc.

Statistical methods. Analysis of variance was used to determine differences among group means. Duncan's multiple range test was used to find the means which differed significantly. Pearson product moment correlations were determined between appropriate variables (23).

Results. Effects of iron restriction on iron status and tissue iron levels. Dams in the 5 ppm iron group had hemoglobin and hematocrit levels which were significantly lower ($P < 0.01$) than those of dams in the 29 and 307 ppm iron groups (Table II). Although the 29 ppm iron diet contains considerably less than the 240 ppm iron which is recommended by the National Research Council for reproduction in the rat (16), hemoglobin and hematocrit levels in this group were not significantly lower than in dams receiving 307 ppm iron.

In the 18-day-old pups three different hematological responses were produced by feeding 5, 29, and 307 ppm iron to maternal animals (Table II). Pups in the 5 ppm iron group had the lowest hemoglobin and hematocrit levels, followed by those in the 29 ppm group, which were lower than those in the 307 ppm iron group ($P < 0.001$).

Iron stores in both liver and spleen were significantly lower in dams on 5 and 29 ppm iron diets ($P < 0.001$) than in dams receiving more dietary iron (Table II). Although iron stores in the liver and spleen of dams in the 29 ppm iron group were higher than those of dams in the 5 ppm iron group, the differences were not statistically significant.

Similarly, pups in the 5 and 29 ppm iron groups had significantly less iron in their livers and spleens than pups in the 307 ppm iron group (Table II). Thus, although pups in both the 5 and 29 ppm iron groups had similar iron stores in liver and spleen, they had two different degrees of anemia, defined by hematocrit and hemoglobin levels, which were significantly different.

Effects of iron on tissue levels of zinc and copper. Copper and zinc concentrations of livers and spleens of dams did not differ significantly among groups (Table III). In the spleen, there is a trend toward higher copper in the 307 ppm iron group and higher zinc in the 5 ppm iron group. How-

TABLE II. BODY WEIGHTS, HEMOGLOBIN, HEMATOCRIT, AND TISSUE IRON LEVELS OF DAMS AND PUPS ON DAY 18 OF LACTATION (MEAN ± SE, n = 8).

	Dietary treatment							
	Dams				Pups			
	Iron (ppm)				Iron (ppm)			
	5	29	307	Significance	5	29	307	Significance
Body weight (g)	315 ±9	294 ±19	267 ±11	NS	32.6 ±2.6	35.7 ±2.4	33.0 ±3.3	NS
Hemoglobin (g/dl)	14.9[a,*] ±0.5	18.3[b] ±0.9	18.8[b] ±1.3	$P < 0.01$	4.5[a] ±0.3	9.1[b] ±0.4	11.8[c] ±0.3	$P < 0.001$
Hematocrit (%)	49.0[a] ±1.2	56.0[b] ±2.4	57.1[b] ±1.7	$P < 0.01$	20.7[a] ±1.9	39.8[b] ±1.3	45.8[c] ±1.0	$P < 0.005$
Spleen dry weight (g)	0.112 ±0.006	0.115 ±0.007	0.095 ±0.008	NS	0.019[a] ±0.002	0.029[b] ±0.002	0.032[b] ±0.003	$P < 0.005$
Spleen iron (μg/g dry wt)	647.6[a] ±79.3	1081.1[a] ±122.0	4243.9[b] ±640.4	$P < 0.001$	151.1[a] ±35.0	225.9[a] ±35.0	347.7[b] ±38.6	$P < 0.005$
Liver dry weight (g)	3.037 ±0.232	2.873 ±0.203	2.549 ±0.177	NS	0.322 ±0.039	0.262 ±0.024	0.265 ±0.024	NS
Liver iron (μg/g dry wt)	114.5[a] ±7.6	215.8[a] ±27.2	871.0[b] ±111.3	$P < 0.001$	40.2[a] ±1.4	41.7[a] ±8.5	69.8[b] ±10.4	$P < 0.05$
Milk iron (μg/ml)	3.10[a] ±0.97	7.69[b] ±0.45	9.37[b] ±1.10	$P < 0.005$				

* Means in a row followed by different letters (a, b, c) are significantly different.

TABLE III. ZINC AND COPPER CONCENTRATIONS AND ZINC/COPPER RATIO IN SPLEEN, LIVER, AND MILK OF DAMS AND PUPS ON DAY 18 OF LACTATION (MEAN ± SE, n = 8).

	Dietary treatment							
	Dams				Pups			
	Iron (ppm)				Iron (ppm)			
	5	29	307	Significance	5	29	307	Significance
Spleen								
Zinc (μg/g dry wt)	84.00 ±1.39	80.79 ±2.20	80.45 ±3.53	NS	87.53 ±10.03	93.18 ±6.68	99.61 ±7.75	NS
Copper (μg/g dry wt)	21.20 ±0.78	21.78 ±1.86	26.31 ±2.21	NS	12.74 ±2.01	10.43 ±1.07	9.91 ±0.93	NS
Zinc/copper	4.00[a,*] ±0.12	3.83[a] ±0.19	3.21[b] ±0.21	$P < 0.01$	7.24[a] ±0.23	9.42[b] ±0.70	10.25[b] ±0.31	$P < 0.005$
Liver								
Zinc (μg/dry wt)	84.15 ±2.70	85.50 ±4.53	86.35 ±3.49	NS	48.20 ±11.86	62.03 ±15.81	111.28 ±26.77	NS
Copper (μg/g dry wt)	14.15 ±0.69	15.23 ±1.27	14.61 ±0.89	NS	16.61 ±4.54	11.55 ±3.52	9.37 ±2.08	NS
Zinc/copper	6.01 ±0.32	5.76 ±0.31	6.09 ±0.38	NS	3.22[a] ±0.46	6.60[a] ±1.43	11.33[b] ±1.68	$P < 0.002$
Milk								
Zinc (μg/g dry wt)	13.1 ±1.5	9.7 ±1.3	11.8 ±1.4	NS				
Copper (μg/g dry wt)	3.1 ±0.8	2.1 ±0.2	2.5 ±0.5	NS				
Zinc/copper	4.72 ±0.65	4.59 ±0.52	5.18 ±0.55	NS				

* Means in the same row followed by different letters (a, b) are significantly different.

ever, the ratio of zinc/copper, (μg of zinc/g of tissue)/(μg of copper/g of tissue), in dams in the 307 ppm iron group was significantly lower ($P < 0.001$) than that in dams in the 5 and 29 ppm iron-deficient groups.

Milk levels of zinc and copper and zinc/copper ratios (Table III) reveal no significant differences among the three dietary groups.

Zinc and copper concentrations in livers and spleens of pups are shown in Table III. While there was a trend toward higher copper and lower zinc in spleens of pups in the 5 ppm iron group, the difference was not

significant. However, the zinc/copper ratio in the spleens of pups in the 5 ppm iron group was lower ($P < 0.005$) than that found in the spleens of either of the groups receiving more dietary iron.

Thus the effect of maternal iron restriction is to increase the zinc/copper ratio in spleens of dams, but to decrease it in the spleen of the pups.

The changes in zinc and copper in livers of pups were similar to the changes found in the spleens. Although the decrease in zinc concentration and the increase in copper concentration with decreased dietary iron were not significant, the zinc/copper ratios in liver were significantly lower in groups whose dams received less dietary iron (5 and 29 ppm) than in the group receiving 307 ppm iron ($P < 0.002$).

Serum lipid levels of pups. Serum from pups of dams fed the diet containing 5 ppm iron had a visible hyperlipidemia. As shown in Table IV, serum triglycerides were up to 20 times higher in the 5 ppm iron group than in the other two dietary groups ($P < 0.005$). Nonesterified cholesterol was from two to three times higher in serum from the 5 ppm iron group than from the 29 and 307 ppm iron groups ($P < 0.01$). In contrast, cholesterol esters were found in similar concentrations in the serum from all three experimental groups of pups. The 5 ppm iron group had serum phospholipid levels 50 to 100% greater than those of the other groups ($P < 0.005$).

The hyperlipidemia in the pups of the dams in the iron-deficient 5 ppm group was characterized by significantly elevated triglycerides, nonesterified cholesterol, and phospholipids. This elevation in serum lipid levels appeared in pups with the most severe iron-deficiency anemia. Although feeding 29 ppm iron to maternal animals produces anemia in pups, this degree of iron restriction was not associated with a significant elevation of serum lipids.

Relationships among serum lipid levels in pups and tissue mineral concentrations. Table V shows the correlations between tissue levels of iron and zinc/copper ratios and serum lipids in the pups. Tissue stores of iron in both the spleen and liver of the dam and milk iron were negatively correlated with serum triglycerides in the pups. A similar relationship was seen between spleen iron, but not liver iron, in pups and their serum triglycerides. But a greater negative correlation between zinc/copper in the pups' spleens and their serum triglycerides was observed (-0.66, $P < 0.005$).

Serum cholesterol levels varied inversely with the zinc/copper ratio in the spleens of pups ($P < 0.001$) but were not related to iron in tissues of dams or pups. Phospholipids varied with zinc/copper ratios in the livers of pups ($P < 0.05$) in addition to the level of iron in milk ($P < 0.05$) and pups' spleens ($P < 0.05$). Thus it appears that in the iron-deficient pups, the increased levels of serum triglycerides, cholesterol, and phospholipids were associated with both a decreased ratio of zinc/copper and lower iron in the tissues.

Discussion. Data from this study indicate

TABLE IV. SERUM TRIGLYCERIDES, NONESTERIFIED CHOLESTEROL, CHOLESTEROL ESTER, AND PHOSPHOLIPIDS IN 18-DAY-OLD PUPS (MEAN ± SE, $n = 8$).

	Treatment			
	Iron (ppm)			
	5	29	307	Significance
Triglycerides (mg/dl)	$405.0^{a,*} \pm 107.0$	$47.8^{b} \pm 15.2$	$20.6^{b} \pm 8.4$	$P < 0.005$
Nonesterified cholesterol (mg/dl)	$94.9^{a} \pm 23.4$	$31.9^{b} \pm 5.9$	$40.8^{b} \pm 4.9$	$P < 0.01$
Cholesterol ester (mg/dl)	107.8 ± 8.3	108.3 ± 15.7	109.5 ± 16.6	NS
Phospholipids (mg/dl)	$160.0^{a} \pm 17.9$	$87.5^{b} \pm 10.5$	$101.2^{b} \pm 8.8$	$P < 0.005$

* Means followed by different letters (a, b) are significantly different.

TABLE V. PEARSON PRODUCT MOMENT CORRELATIONS AMONG MINERALS AND SERUM LIPIDS IN 18-DAY-OLD PUPS.

	Correlation coefficients[a]		
	Serum triglycerides	Serum cholesterol	Serum phospholipids
Iron, dam spleen	−0.51*	−0.18	−0.26
Iron, dam liver	−0.59**	−0.20	−0.18
Iron, milk	−0.61**	−0.49*	−0.55*
Iron, pup spleen	−0.46*	−0.19	−0.43*
Iron, pup liver	0.20	0.02	−0.32
Zinc/copper, dam spleen	−0.11	0.04	0.23
Zinc/copper, dam liver	0.30	0.24	0.25
Zinc/copper, pup spleen	−0.66***	−0.60***	−0.35
Zinc/copper, pup liver	−0.38	−0.36	−0.40*

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.005$.
[a] The number of values for each correlation was 20–24.

that the relationship between maternal dietary iron deficiency and hyperlipidemia in offspring involves not only iron status of the animals, but also the resulting changes in tissue zinc and copper levels in the pups. Dietary iron restriction in maternal animals produces a decrease in the tissue levels of zinc and an increase in copper, and the resulting zinc/copper ratio decreases in the tissues of iron-deficient pups. The cause and effect relationships have not been determined at this time. The correlation data suggest that serum triglycerides, nonesterified cholesterol, and phospholipids are related to both tissue iron in both pups and dams and zinc/copper levels in pups. It is now known whether the changes in serum lipids are due primarily to the iron deficiency or to the decreased ratio of zinc/copper.

Sourkes *et al.* (8) have reported an inverse relationship between dietary iron and liver copper in adult rats not found in this study. Our data show a similar trend of increased liver copper in iron-deficient pups, but not in the dam's liver and spleen. In addition the data reported here suggest that the alterations in the zinc as well as the copper content of iron-deficient rat tissues and the resulting changes in the zinc/copper ratio in tissues are involved.

Klevay (7) has also studied the relationship between zinc/copper ratio and cholesterol. He reported hypercholesterolemia produced by feeding weanling rats water containing a high ratio of zinc/copper. There are important differences in the design of Klevay's experiments and those reported here. He altered the intake of zinc/copper in drinking water of growing animals, and here the dietary iron of maternal rats was decreased and hyperlipidemia was observed only in the offspring. Klevay's experiments associated a high ratio of dietary zinc/copper with serum lipids, while our experiments showed a lowered ratio of tissue zinc/copper associated with increased serum lipids. Klevay reported lowered hematocrits in the rats fed a high ratio of zinc/copper (7), but did not report tissue levels of iron. It is possible that in feeding rats water with a high ratio of zinc/copper, the iron status and tissue iron levels were changed. The elevated serum cholesterol and phospholipids which are attributed to a high ratio of zinc/copper may also be related to changes in iron at the tissue level.

Dietary iron deficiency during pregnancy and lactation in rats not only results in iron deficiency at the tissue and blood levels in 18-day-old offspring, but also produces tissue level changes in zinc and copper. The livers and spleens of iron-deficient pups contain more copper and less zinc than do those of control pups. These interrelationships require more study to separate the effects of iron deficiency on serum lipid metabolism from the effects of changes in copper and zinc. The implications that simple iron deficiency results in more complex changes in tissue levels of other trace minerals require more study.

Summary. The effects of maternal iron restriction during pregnancy and lactation on serum lipids and tissue zinc and copper levels in rat pups were studied. Feeding diets containing 5 ppm iron to maternal rats produced hyperlipidemia and a decreased ratio of zinc/copper in livers and spleens of 18-day-old pups. Significant correlations were found between zinc/copper ratio in pups and serum triglycerides, cholesterol, and phospholipids.

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