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The Interrelation of Fluoride and Iron in Anemia[1] (39559)

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A reduced fluoride intake by mice has been reported to increase the severity of the anemia in pregnancy and infancy (1). The anemia has been characterized in 15-day-old mouse pups as a microcytic hypochromic anemia, highly suggestive of an iron or copper deficiency (2). The diet employed in these studies (3) has a high content of whole wheat flour (58%) which contains phytic acid, a chelator of cations capable of interfering with the intestinal absorption of iron (4) and copper (5). The diet has a low fluoride content (0.5 ppm or less), is marginal in iron (29 ppm), and submarginal in copper (2.7 ppm). The requirements for iron and copper for growth of rodents are reported to be 25 and 5 ppm, respectively (6).

Since there are indications that the fluoride effect on the anemia that has been previously reported may be related to iron metabolism, a series of studies with mice on high (50 ppm) and low (deionized water) fluoride aqueous intake, fed the experimental diet supplemented with iron as $FeCl_3 \cdot 6H_2O$ (195 ppm) and the unsupplemented diet, was carried out. In addition to the experimental groups of animals, a control group was fed commercial rat chow (Purina Laboratory) containing 200 ppm of iron, 8 ppm of copper, and 20–60 ppm of fluoride. This group was provided tap water containing 1.0 ppm of fluoride. The effect of the various regimens on the hematocrit value of the newborn and 15-day-old pup was determined. Other regimens with copper supplementation indicated that the copper in the diet did not play an important role in reducing the severity of the anemia. For this reason, the emphasis of this publication is limited to the interrelation of iron and fluoride.

The effect of the maternal fluoride intake on the total body content of iron was determined in newborn and 15-day-old mouse pups. The dam's milk is the sole source of iron during the first 15 days of life and the measurement of the concentration of iron in the milk of the lactating mouse was considered important in determining the role of this source of iron. The absorption and retention of dietary iron was investigated in animals between the age of 15 and 23 days by providing an oral tracer dose of radioactive iron (^{59}Fe) to each animal in the series.

Experimental procedures. Experimental Animals. The mice employed were Swiss-Webster random bred mice placed on the experimental or control regimens as weanlings. All animals were weaned at 20 days of age and mated at 8 weeks of age. Food and water were provided *ad libitum*. At birth, each litter was reduced to six pups to provide a more uniform growth rate for the pups and uniform stress for the nurturing animal. Animals used in the nutritional studies were housed in polypropylene cages with wood shavings provided as litter and nesting materials. Mice involved in the iron absorption studies with radioactive iron (^{59}Fe) were housed in hanging mouse cages to limit coprophagy.

Bleeding and hematocrit determinations. Samples of tail blood were collected in Pre-Cal preheparinized microhematocrit tubes (Clay Adams, Division of Becton, Dickinson, and Company). The tubes were centrifuged for 4 1/2 min in a microhematocrit centrifuge and the hematocrit was determined.

Iron determinations. Five to six grams of diet or the entire bodies of newborn and 15-day-old mice were ashed overnight at 500°. The ash was dissolved in 5 ml of *N* HCl, evaporated to dryness, and reashed at 500° for 4–5 hr. The ash was again dissolved in 1 ml of *N* HCl and made to 50 ml before being analyzed with a Jarrell-Ash atomic absorption spectrometer (7).

The micromethod of Caraway (8) was

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used for serum iron determination but was modified for the assay of milk since the higher levels of lipid and protein in milk were found to interfere with the assay. A protein-free supernate was obtained by using 100 μl of milk, 50 μl of 1% ascorbic acid in 0.2 *N* HCl, 50 μl of 20% trichloroacetic acid, and 0.05 ml of chloroform. One-hundred-fifty microliters of supernatant was combined with 0.1% 2,4,6-tripyridyl-*S*-triazine (TPTZ) solution and the pH was adjusted to between 4 and 5 with 4% ammonium acetate buffer. The TPTZ–iron complex was then extracted with 50 μl of nitrobenzene, the aqueous layer was discarded, and 200 μl of absolute ethanol was added to the nitrobenzene to reduce surface tension so that microcuvettes used with Beckman Model 25 spectrophotometer could be easily filled without interfering air bubbles.

Milking. Milk was collected from lactating dams anesthetized with diethyl ether on the ninth or tenth day of lactation after ip injection of 0.1 ml of oxytocin (2 units). The pups were removed from the dam's cage approximately 6 hr before milking. The mammary glands were massaged, forcing the milk toward the nipple so that the droplets could be collected in a fine-tipped micropipet.

Fluoride. The humeri of adult females were removed, cleansed of adhering muscle and tendon, and the bones were split, the marrow was removed, dried at 100°C, and ashed at 500° for 8 hr. The bone ash was analyzed by the method of Singer and Armstrong (9).

Radioiron. A tracer dose of radioactive iron (^{59}Fe) was given by gastric intubation (4 μCi and 0.0188 μg of iron as $FeCl_3$ in 0.1 ml) to 15-day-old animals housed with their dam. Food and water, in accordance with the dietary group assignment, were provided *ad libitum*. On the twenty-third day of age, the pups were sacrificed under ether anesthesia after blood had been collected in small capillary tubes. After the animal was sacrificed, the gastrointestinal tract was removed. The gastrointestinal tract and the eviscerated body were placed in separate crucibles, charred under infrared lamp, and ashed at 500°. The ash was dissolved in 6 ml of HCl and was diluted to 5 and 10 ml, respectively. Sufficient volumes (20–100 μl) were taken for radioactive counting to provide a reasonable rate of counting. Whole blood (1 ml) samples were ashed at 500°, dissolved by five serial treatments (1 ml) with 1 *N* HCl, and the final volume was adjusted to 10 ml. Duplicate 100-μl aliquots of the ashed solution were taken for radioactive counting. Plasma radioactivity was determined with 50-μl samples pipetted into the scintillation vial. All preparations were counted in scintillation vials containing 15 ml of fluid (0.3% diphenyloxazole in a 70:30 solution of toluene and absolute ethanol) with a Packard Tricarb scintillation counter.

Results and discussion. The fluoride concentrations found in the ashed humeri of dams of the groups (1 year of age) receiving high and low fluoride intake and the diet with and without iron supplementation are given in Table I. Although there is a large difference between the mean fluoride concentration in the bones of adult animals raised on low or high fluoride intakes, it is apparent that supplementation of the diet with iron did not influence the fluoride uptake by bone.

The effect of iron and fluoride intake provided the mothers on the mean hematocrits and weights of 1- and 15-day-old mouse pups is shown in Table II. Animals of all groups had relatively normal hematocrit levels at birth. Iron supplementation of the diet of the dam resulted, however, in an increase in the hematocrit of the newborn as compared to newborn pups of dams provided the unsupplemented diets. Birth weights were similar for all groups. The difference previously reported (1) between the hematocrit for the 15-day-old pups of low and high fluoride mothers raised on the unsup-

TABLE I. FLUORIDE CONCENTRATION IN THE ASHED HUMERI OF FEMALE MICE.

Fluoride intake	Supplement	No. of animals	Percentage F in ash ± SE
Low	None	10	0.0135 ± 0.0010
	Fe	17	0.0154 ± 0.0019
High	None	9	1.23 ± 0.109
	Fe	15	1.26 ± 0.045

plemented regimen was observed. Namely, the high fluoride animals (15 days old) had a less severe anemia than animals of the same age from the low fluoride group. There was an apparent beneficial effect on the hematocrit of the young animals resulting from additional iron in the supplemented diet. These later values are similar to those seen for the pups of the control group whose mothers were raised on a commercial laboratory diet (Purina Laboratory Diet).

The total body iron contents of the newborn pups of the control and iron-supplemented experimental groups (Table III) had high and similar levels of body iron stores, whereas the pups from animals maintained on the unsupplemented basal diet had much lower and similar iron contents. The mothers of the low and high fluoride-unsupplemented groups were apparently providing their pups with a smaller iron pool at a time when growth is being highly accelerated and expansion of body fluids is enormous. The total iron present in the 15-day-old pups of dams on a diet unsupplemented with iron and receiving a high fluoride intake was significantly higher ($P < 0.005$) than that of pups of similarly fed dams receiving the low fluoride intake. The high fluoride pups had gained about 198 μg of iron during the 15-day period as compared to 145 μg for the low fluoride pups. In addition, the serum iron concentration in control (10 animals), high fluoride (18 animals), and low fluoride groups (18 animals) was 381 ± 41, 276 ± 32, and 152 ± 8 μg of Fe/ml, respectively. There was a significant difference between the control and high fluoride groups ($P < 0.05$) and between the high and low fluoride groups ($P < 0.0005$). This suggests that (a) there is less dietary iron being provided the low fluoride pups in their early life or (b) there is a more efficient absorption and/or retention of iron from the gastrointestinal tract when the fluoride intake is high.

Mouse pups up until about 15 days of age do not eat the food provided the mother but are nurtured only on milk. It seemed reasonable that, although milk has a low iron content, perhaps the milk from dams of the two experimental fluoride groups might be sufficiently different to account for the difference in iron accumulation during early life as suggested above. The milk obtained from the low and high fluoride dams contained 2.24 ± 0.34 (eight animals) and 3.39 ± 0.22 ppm (nine animals) of iron (mean ± SE), respectively. It is evident that the fluoride intake of the dam apparently influences the iron content of the milk produced by

TABLE II. EFFECT OF MATERNAL INTAKE ON HEMATOCRITS VALUES AND BODY WEIGHT OF MOUSE PUPS.[a]

Maternal diet		Newborn			15 Days of age		
Mineral added	Fluoride intake	Number	Hematocrit (%)	Body wt (g)	Number	Hematocrit (%)	Body wt (g)
None	Low	51	42.6 ± 0.78	1.55 ± 0.04[b]	36	28.3 ± 0.76	10.7 ± 0.28
None	High	75	41.0 ± 0.65	1.65 ± 0.03	44	33.5 ± 0.96	10.0 ± 0.37
Fe	Low	26	49.3 ± 0.88	1.60 ± 0.03	16	41.1 ± 0.72	10.2 ± 0.31
Fe	High	26	48.9 ± 0.19	1.67 ± 0.05	17	40.1 ± 0.49	9.5 ± 0.36

[a] Mean ± SE.
[b] Twenty animals of each group.

TABLE III. IRON CONTENT OF MOUSE PUPS.[a]

Maternal dietary regimen	Newborn		15 Days of age	
	Iron (μg/pup)	Body wt (g)	Iron (μg/pup)	Body wt (g)
Control	81 ± 5.5 (10)	1.60 ± 0.03	338 ± 35.3 (8)	8.3 ± 0.48
Low fluoride	51 ± 2.4 (18)	1.58 ± 0.07	196 ± 11.1 (27)	9.2 ± 0.33
High fluoride	52 ± 2.6 (17)	1.54 ± 0.06	249 ± 12.3 (20)	8.2 ± 0.40
Low fluoride iron-supplemented	83 ± 2.3 (7)	1.46 ± 0.07	335 ± 13.6 (10)	8.0 ± 0.58
High fluoride iron-supplemented	87 ± 2.4 (6)	1.63 ± 0.05	352 ± 41.6 (9)	9.9 ± 0.86

[a] Mean ± SE (number).

TABLE IV. RADIOACTIVE ^{59}Fe CONCENTRATIONS IN MICE.[a]

Measurement	Maternal dietary regimen		
	Low fluoride	High fluoride	Control
% Dose retained in eviscerated animal	63.5 ± 1.61[b c] (43)	70.22 ± 1.33[b c] (34)	79.8 ± 1.54[b] (18)
% Dose retained in GI tract	3.52 ± 0.22[b] (41)	2.98 ± 1.25[b] (33)	1.9 ± 0.18[b] (16)
% dose per 0.01 ml of serum	0.058 ± 0.0045 (38)	0.057 ± 0.0058 (31)	0.061 ± .0072 (17)
% Dose per 0.1 ml of blood	7.06 ± 0.800 (30)	8.47 ± 0.563 (32)	8.7 ± 0.70 (18)
Hematocrit at sacrifice	35.0 ± 0.59[b] (43)	37.1 ± 0.74[b] (33)	42.4 ± 0.49[b] (18)

[a] Twenty-three days of age. Values are mean ± SE.
[b] Control significantly higher or smaller ($P < 0.005$) than low or high F group.
[c] Comparison between low and high F groups, $P < 0.005$. All other comparisons are not significant.

animals fed the unsupplemented experimental diet. Even though the supply of iron in the milk is low, it is the only source available in early life and the difference in iron content between the milk supplies appears to be of major importance. The fluoride content of rodent's milk is not known; however, based on the low levels (0.1–0.2 ppm) reported in milk of other species (10) and the apparent regulation of the fluoride content of milk in normal and fluorosed cattle (11), it can be assumed that the fluoride content of rodent's milk under the conditions of this study is extremely low. If any difference in fluoride intake occurs between the young animals of the two groups during the first 10 days of life, it is insignificant.

The radioactivity (^{59}Fe) remaining in 23-day-old animals 8 days after intubation of the radiotracer dose when the animals were 15 days of age (Table IV) indicates that the control animals absorbed and/or retained a significantly higher amount of the dose in the 8 days following the intubation than did the two experimental unsupplemented groups of animals ($P < 0.005$). Animals on the experimental diet begin to recover from their severe anemic state between 15 and 20 days of age (1). The experimental group of animals receiving 50 ppm of fluoride in the water had significantly ($P < 0.005$) more of the dose in the eviscerated carcass than did the low fluoride animals. The amount of the dose in the gastrointestinal tract was higher for the experimental groups than for the control group ($P < 0.005$), although the total difference in radioiron present was small. The radioiron is taken up by the mucosa, but it would appear that the iron is not as well utilized by the experimental animals as by the control animals receiving a commercial diet containing 20–60 ppm of fluoride or that the iron stores are being turned over by the experimental animals at a faster rate than is observed for the control animals.

Messer *et al.* in an earlier publication (12) have shown that the fluoride content of the calvaria of the newborn mouse pup of the group given 50 ppm decreased with age up to 10 days of age and was only three times that of the group given the low fluoride diet. By 20 days of age, it was also reported that the fluoride content of the calvaria had increased sharply as a result of the pups being partially weaned and consuming water with a high fluoride content (50 ppm). We have examined the calvaria of 15-day-old pups of the high fluoride group and find that the fluoride content was increased from 0.026 to 0.039% of the ashed bone between 10 and 15 days of age. This circumstance could only occur if the pups had consumed some of the water with a high fluoride content, since the experimental diet had a low fluoride content. The low fluoride group maintained a constancy of fluoride in the bone ash at approximately 0.008%. The young mouse pup although not consuming the food of the dam until approximately 15 days of age, apparently drinks water at an earlier age, and therefore, the 15-day-old animal with dams provided water with 50 ppm of fluoride will consume much more fluoride

than those in the low fluoride group having access to deionized water.

Comparisons of the radioactive results indicate that the control group had a greater absorption and/or retention of the orally administered iron than the experimental groups and that an elevated fluoride intake increased the absorption and/or retention of iron when the iron and fluoride in the prepared experimental diet was minimal. This observation is in agreement with the earlier speculations of Ruliffson *et al.* (13), based on inadequate information, that fluoride intake enhanced the absorption of iron.

The high fluoride animals had a significantly higher concentration of radioactive iron in the blood than the low fluoride animals ($P < 0.025$). This is consistent with the increased production of reticulocytes reported in the high fluoride experimental group over that of the animals receiving the low fluoride regimen or the control animals fed a commercial diet (2).

Summary. It has been demonstrated that iron supplementation can completely prevent the anemia observed in 15-day-old mice raised on a low or high fluoride experimental diet. A higher fluoride intake was again shown to reduce the severity of the anemia seen in low fluoride animals. An increased fluoride intake has been shown to improve iron metabolism when animals were fed an experimental diet marginally adequate in its iron content. The young animals of the high fluoride group were found to have a higher total body iron content at 15 days of age than similar animals of the low fluoride group, although there was no appreciable difference in the iron content of the newborn. The higher fluoride intake of the dam also was shown to increase the iron content of the milk produced by the lactating dams, and the difference in concentration in the milk from animals in the two groups may account for the observation that there is a higher total body iron burden at 15 days of age in the high fluoride group. In older animals (15–23 days old), the higher fluoride intake was demonstrated to increase the absorption and/or retention of iron by the young mouse provided the experimental diet.

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