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Zinc deficiency, erythrocyte production, and chromosomal damage in pregnant rats and their fetuses

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During periods of rapid growth and development, chromosomal and DNA damage has been observed in hepatic tissue samples of zinc-deprived animals. In this study, the erythrocyte micronucleus assay was used to estimate cytogenetic damage due to zinc deprivation in pregnant rat dams and their fetuses. Rat dams were assigned to one of three dietary groups on day 0 of gestation: zinc deficient (<0.5 μg of zinc/g of diet), zinc adequate (50 μg of zinc/g of diet), and pair-fed (50 μg of zinc/g of diet in an amount matched to daily food intake of the zinc-deficient group). By day 19 of gestation, the zinc-deprived dams had gained no weight during pregnancy and had resorbed nearly half their implantation sites. Of their remaining fetuses, one-third were malformed, and all were growth-retarded. Fetuses from the zinc-deficient group had only one-half of the liver zinc concentration of fetuses in the zinc-adequate and pair-fed groups. In contrast, zinc deprivation of the dams caused no change in liver zinc concentrations but reduced plasma and bone zinc concentrations and elevated liver iron concentrations nearly 2 fold. In spite of their excessively high liver iron stores, erythrocyte production, estimated from the ratio of newly formed to older cells, was suppressed in the zinc-deprived dams. There was no observable increase in the frequency of micronuclei, a measure of chromosomal damage, in recently formed or mature erythrocytes in zinc-deficient rat dams or their fetuses. (J. Nutr. Biochem. 6:263–268, 1995.)

Keywords: zinc deficiency; micronucleated erythrocytes; chromosomal damage; gestation; iron; erythropoiesis

Introduction

Severe zinc deficiency in rodents is characterized by the rapid onset of metabolic abnormalities and impaired function.[1–3] The metabolic insult is proportional to the duration and intensity of the zinc deprivation, especially during periods of rapid growth and development, e.g., weanling[4] and gestation.[5,6]

The metabolic defects of zinc deficiency have been hypothesized to include several effects related to DNA and RNA synthesis. Impaired nucleic acid synthesis, reduced thymidine kinase activity, mitotic dysfunction, excessive chromatin condensation, and oxidative stress have been observed in zinc-deficient animals and humans.[7–10] In addition, zinc is a component of many proteins that alter gene activation and repression.[11,12]

Data from three studies suggest that impaired nucleic acid synthesis due to zinc deprivation is associated with chromosomal damage.[13–15] Bell et al.,[13] using metaphase analysis, observed chromosomal damage in liver tissue samples from magnesium- and zinc-deficient Sprague–Dawley rat dams and their fetuses. These investigators identified chromosomal gaps, deletions, and fragmentation with zinc deficiency, suggesting chromosomal damage due to disrupted synthesis of nucleic acids. More recently, two groups[14,15] using an alkaline precipitation assay have reported DNA single-strand breakage in liver tissue of zinc-deficient, weanling Sprague–Dawley rats and infant Rhesus monkeys.

The purpose of this study was to use the erythrocyte micronucleus assay,[16–18] a screening test for in vivo cytogenetic damage, to determine if zinc deficiency during pregnancy causes chromosomal damage in erythrocyte progenitors. Micronuclei arise in cells when chromosomes or

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their fragments lag at anaphase and are not incorporated into the daughter cell nucleus. Acentric chromosomal fragments, bridged chromosomes, and chromosomes lost due to spindle abnormalities all result in micronucleus formation. MacGregor et al.[19,20] reported increased frequency of micronuclei (Howell–Jolly bodies) in circulating red blood cells with dietary deprivation of folate or magnesium. Because RBC progenitors are a rapidly dividing cell population and because micronuclei are known to arise from the types of genetic damage reported in liver during zinc deficiency, we expected this endpoint to be a sensitive indicator of genetic damage resulting from zinc deficiency.

Materials and methods

Virgin Sprague–Dawley rats weighing 200 to 220 g (Simonsen, Gilroy, CA) were housed individually in stainless steel hanging cages in temperature (22 to 23°C) and photoperiod (12 hr/day) controlled rooms. The rats were allowed to acclimate for 10 days and were provided commercial chow diets (Purina Rat Chow, St. Louis, MO) and tap water ad libitum. The rats were mated, and day 0 of gestation was determined by the presence of sperm plugs.

On day 0 of gestation, the rat dams were assigned randomly to one of three diet groups. The control group (ZnAd, $n = 12$) was fed a nutritionally complete, semipurified 25% egg white albumin diet containing 50 μg of Zn/g of diet. The experimental group (ZnDef, $n = 13$) was fed the same diet as the ZnAd group, except that the zinc content was less than 0.5 μg of Zn/g of diet. The pair-fed control group (Prfed, $n = 8$) was fed the 50 μg of Zn/g of diet in an amount equal to the mean daily food intake of the ZnDef group. The ZnAd and ZnDef groups were fed ad libitum.

All diets contained 50 μg of Fe/g. The dietary formulation and preparation have been described previously[21]; only the protein source was changed. Triply deionized water was provided ad libitum from plastic water bottles with stainless-steel sipper tubes (Nalgene, Rochester, NY). Food intake and body weight were recorded daily.

On day 19 of gestation, dams were anesthetized by CO_2 asphyxiation. Laparotomies were performed, and blood was collected from each dam by cardiac puncture into polypropylene syringes containing zinc-free heparin (LyphoMed, Rosemont, IL). Blood was centrifuged for 15 min at 2500*g*. Plasma was removed with plastic transfer pipettes and stored frozen in acid-washed polypropylene tubes until analysis.

At necropsy, the uterus was examined intact, and the number of fetuses and resorption sites were recorded. The uterus and conceptus were dissected from the attached tissues and weighed. Individual fetuses were removed, weighed, and examined for gross structural anomalies, e.g., limb morphology and cleft lip. Fetal tissues were pooled for mineral determinations, except for the second fetus from the dam's right uterine horn, which was reserved for micronucleus evaluation. This fetus was killed by severing the cervical column with a razor blade; blood was collected in a microcapillary tube, 5 to 10 μL was smeared onto a slide, then air-dried, and fixed as described below.

In the rat dams, bone marrow rather than blood was used for the micronucleus evaluation because the mature spleen removes micronucleated erythrocytes from the peripheral circulation.[22] Therefore, bone marrow was sampled from the dams for slide preparation. The left femur was immediately removed from the dam at necropsy and flushed with 0.5 mL of fetal calf serum as described by Schmid.[23] Smears were prepared, air-dried, fixed in absolute methanol for 2 min, and then air-dried again.

Smears were stained with acridine orange, a fluorescent dye that differentiates DNA from RNA or RNA-protein complexes, as described previously by Hayashi et al.[24] Microscope conditions utilized a fluoroscein (FITC) filter set (excitation is 450 to 490 nm, with a 510 nm dichromatic beam splitter and a 520 nm barrier filter). Under these conditions, RNA fluoresces red–orange, and DNA fluoresces bright yellow. Normochromatic erythrocytes are unstained or a very dull greenish color, yet visible.

Slides were randomized and scored at 630× under oil immersion by a skilled observer who was unaware of the identity of the sample on the slides (i.e., slides were coded and scored "blind"). In the dams' slides, micronuclei were scored in two populations of at least 500 cells each, and the ratio of polychromatic erythrocytes (PCEs, newly formed, RNA-positive erythrocytes) to normochromatic erythrocytes (NCEs, older, RNA-negative erythrocytes) was determined by a count that included at least 500 of the prevalent cell type. In the fetal smears, micronuclei were scored in three populations[18]: two distinct populations of RNA-positive erythrocytes (newly formed, uniformly stained erythrocytes [UEs]; or older, stippled erythrocytes [SEs]), as well as more mature RNA-negative erythrocytes (NCEs). The percentage of each subpopulation, i.e., newly formed and older RBCs, was calculated from the total number of cells scored, which included at least 500 of each RNA-positive cell type.

Maternal plasma, liver, and the right femur were removed and stored at −20°C for subsequent analysis of zinc and iron concentrations by atomic absorption spectrophotometry (AAS, Thermo-Jarrell Ash 22, Franklin, MA). The spleen and kidney were also removed and weighed. Soft tissue was ashed in a low temperature asher[25] (LTA, Branson IPC, Hayward, CA), and the ash was diluted in 0.1 N HNO_3. Bone was wet ashed in concentrated nitric acid according to the method of Clegg et al.[26] Metal standards and reference bovine liver (SRM 1577 (a), National Bureau of Standard [NBS], Gaithersburg, MD) and an internal plasma standard were prepared in the same matrix as samples and with each analysis. The bovine liver standard was analyzed to contain 1.78 ± 0.76 μmol Zn/g. Published values are 1.88 ± 0.12 μmol Zn/g. Within- and between-run coefficients of variation were 1.8 and 2.8% for bovine liver and the internal standard, respectively.

All data are reported as mean ± SEM. One-way analysis of variance was used to compare growth, food intake, mineral concentrations, and fetal number and teratology with dietary zinc intake and caloric restriction. The Scheffe post hoc test was used to compare mean values of the ZnAd, Prfed, and ZnDef groups. Stepwise regression was used to evaluate relationships between variables (StatView SE Abacus, Inc., Berkeley, CA). The Kastenbaum–Bowman test[27] was used to compare frequencies of micronuclei in red blood cell population types of each treatment group with the control.

Results

Gestational weight gain

From day 0 to day 19 of gestation, ZnAd dams gained an average of 90 ± 4 g, ZnDef dams gained no weight ($P < 0.001$), and Prfed dams gained 67 ± 2 g (*Figure 1*), significantly less than the ZnAd and significantly more than the ZnDef ($P < 0.05$, *Table 1*). Thus, by day 19, ZnAd dams increased their body weight by 40% and Prfed dams by 30%. Weights of the liver, kidney, and spleen were reduced in proportion with body weight in the ZnDef and Prfed groups.

Food intake averaged 19 g/day for the ZnAd and 13.5 g/day for the ZnDef and Prfed dams. The ZnAd and Prfed dams had the same gain in body weight for each gram of

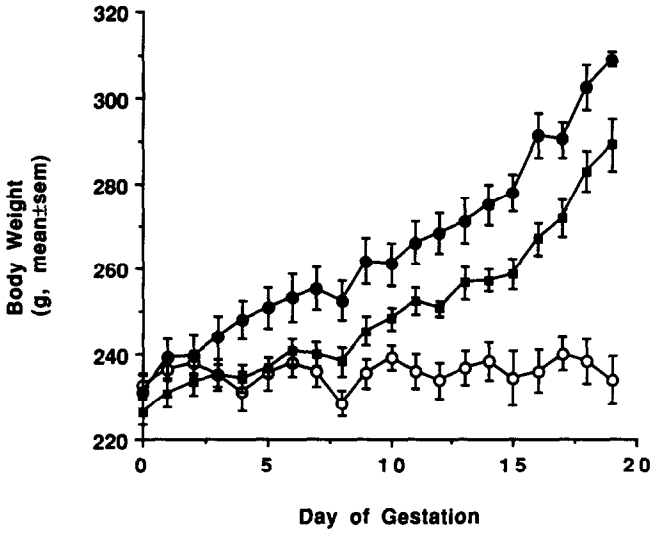

Figure 1 Body weight (g) from day 0 through day 19 of gestation in dams fed diets containing 50 μg of Zn/g ad libitum (ZnAd,-●-), or calorically matched to the group fed the zinc deficient diet (Prfed, -⊟-), or fed a zinc-deficient diet containing 0.5 μg of Zn/g (Zndef,-⊖-).

food ingested (i.e., the feed efficiency was similar), but the ZnDef dams had no net weight gain ($P < 0.05$) (*Table 1*).

Fetal growth and development

At day 19 of gestation, the average weight of pups from the ZnDef dams was less than 50% of those of the ZnAd and Prfed dams ($P < 0.05$) (*Table 1*). Litter size was reduced more than 40% in the ZnDef compared with the ZnAd and Prfed groups (*Table 1*). On day 0, 23 rats in the ZnDef group were positive for sperm plugs, but more than 40% showed no evidence of uterine implantation on day 19. In 85% of the ZnAd and Prfed dams, pregnancy outcome was successful. ZnDef dams further resorbed 44% of their observable implantation sites, and 34% of their fetuses had gross teratology (*Table 1*). For example, the most common malformation was short or curly tail ($n = 13$), followed by cleft palate ($n = 8$), fused or missing digits ($n = 5$), and open gut ($n = 4$); types of malformations tended to be similar among litter mates. In contrast, there were only two resorption sites in the ZnAd group and none in the Prfed group, and no deformities in either (*Table 1*).

Table 1 Pregnancy outcomes of rat dams fed diets containing 50 μg of Zn/g and <0.5 μg/g from day 0 to day 19 of gestation*

	Zinc adequate	Pairfed	Zinc deficient
No. of dams	12	8	13
Dam wt gain (g/kg/d)	22.5 ± 3.2[a]	15.6 ± 2.2[b]	−0.1 ± 3.2[c]
Dam wt gain (g gain/g food intake)	0.25 ± 0.01[a]	0.26 ± 0.01[a]	0[b]
Fetal wt, d 19 (g)	2.3 ± 0.1[a]	2.1 ± 0.1[a]	1.0 ± 0.1[b]
Litter size	11.8 ± 0.3[a]	12.9 ± 0.7[a]	6.8 ± 1.4[b]
No. of implantation sites/dam	11.9 ± 0.6[a]	12.9 ± 0.7[a]	12.1 ± 0.3[a]
No. of resorption sites/dam	0.2[a]	0[a]	5.3 ± 1.0[b]
No. fetuses with gross malformations/ total no. fetuses	0/141[a]	0/103[a]	30/89[b]

*Values are shown as means ± SEM.
†Values not sharing a common superscript letter are significantly different at $P < 0.05$.

Tissue mineral concentrations

Compared with the ZnAd group, plasma zinc concentrations were reduced 25% in the Prfed and nearly 70% in the ZnDef groups ($P < 0.05$, *Table 2*). Femur zinc concentrations were also reduced significantly in Prfed and ZnDef dams ($P < 0.05$, *Table 2*).

In contrast, liver iron concentrations were elevated in the ZnDef ($P < 0.05$) dams compared with those fed adequate zinc diets (*Table 2*). Liver zinc concentrations were unchanged, however, and averaged 0.32 μmol Zn/g of wet tissue weight for all groups.

In the zinc-deficient rats that had no visible evidence of implantation at day 19 ($n = 8$), the pregnancy presumably failed at a very early stage. Because they have no direct comparison group, their tissue mineral concentrations are included for information only. Tissue mineral levels were slightly higher than the ZnDef pregnant dams, and measured: plasma zinc, 8.5 ± 0.1 μmol/dL; liver zinc and iron, 0.31 ± 0.04 and 4.12 ± 1.2, respectively; and femur zinc, 1.75 ± 0.08 μmol/g of wet tissue weight.

Fetal livers were pooled by litter for mineral determinations. In contrast to their dams, fetal liver iron concentrations were not elevated in the ZnDef group, and liver zinc concentrations were significantly reduced in ZnDef fetuses compared with groups fed adequate zinc diets ($P < 0.05$) (*Table 3*).

Erythrocyte micronuclei

Using the Kastenbaum–Bowman test, frequencies of micronucleated erythrocytes per 1000 erythrocytes showed no significant differences due to dietary treatment. The mean values were as follows: ZnAd dams ($n = 12$) had 1.3 ± 0.5, Prfed dams ($n = 8$) had 1.5 ± 0.5, and ZnDef dams ($n = 13$) had 1.4 ± 0.5 micronucleated PCEs/1000 PCEs;

Table 2 Tissue mineral concentrations of rat dams fed diets containing 50 or <0.5 μg Zn/g from d 0 to d 19 of gestation[1,2]

	Zinc adequate	Pairfed	Zinc deficient
No. of dams	12	7	11
Plasma zinc (μmol/dL)	18.2 ± 0.5[a]	13.5 ± 0.3[b]	6.3 ± 0.9[c]
Femur zinc (μmol/g)	2.11 ± 0.05[a]	1.94 ± 0.03[b]	1.59 ± 0.03[c]
Liver iron (μmol/g)	1.86 ± 0.18[a]	2.3 ± 0.18[ab]	3.31 ± 0.28[b]
Liver zinc (μmol/g)	0.30 ± 0.03[a]	0.33 ± 0.08[a]	0.33 ± 0.09[a]

*Values are shown as means ± SEM.
†Values not sharing a common superscript letter are significantly different at $P < 0.05$.

Table 3 Tissue mineral concentrations of fetuses of rat dams fed diets containing 50 or <0.5 μg of Zn/g from day 0 to day 19 of gestation*†‡

	Zinc adequate	Pairfed	Zinc deficient
No. of dams	12	7	8
Liver iron (μmol/g)§	4.24 ± 0.18[a]	4.11 ± 0.21[a]	3.99 ± 0.16[a]
Liver zinc (μmol/g)§	0.73 ± 0.03[a]	0.76 ± 0.03[a]	0.38 ± 0.3[b]

*Values are shown as means ± SEM.
†Values not sharing a common superscript letter are significantly different at $P < 0.05$.
‡Fetal tissues from same litter were pooled for mineral determinations.
§Wet weight.

and 0.16 ± 0.10, 0.0, and 0.44 ± 0.22 NCEs/1000 NCEs, respectively.

In the fetuses, micronucleated UEs/1000 numbered 5.2 ± 0.8 in the ZnAd ($n = 12$), 5.2 ± 1.2 in the Prfed ($n = 8$), and 5.5 ± 0.9 in the ZnDef ($n = 10$) group; SEs/1000 SEs were 2.2 ± 0.7, 4.4 ± 1.7, and 2.6 ± 0.9, respectively. Micronuclei were not associated with degree or type of malformation.

Erythrocyte production

Effects on erythrocyte formation were monitored by comparing the proportion of the newly formed red blood cells (a parameter analogous to reticulocyte count) to older red blood cells. In ZnAd and Prfed dams the newly formed red blood cells, the PCEs, comprised nearly one-half of total bone marrow RBCs. In the ZnDef dams, PCEs accounted for less than one-third ($P < 0.05$) of total RBCs (*Table 4*). The percentage of newly formed RBCs was markedly reduced ($P < 0.05$) in the ZnDef dams, indicating suppression of bone marrow erythropoiesis in the dams at day 19 of gestation.

Table 4 Percent of erythrocyte subpopulations in rat dams fed diets containing 50 or <0.5 μg of Zn/g from day 0 to day 19 of gestation and their fetuses*†

	Zinc adequate	Pairfed	Zinc deficient
No. of dams‡	12	8	13
% PCE§	44 ± 3[a]	45 ± 3[a]	31 ± 2[b]
% NCE‖	56 ± 4[a]	55 ± 3[a]	69 ± 2[b]
No. of fetuses¶	12	8	10
% UE**	83 ± 7[a]	77 ± 7[a]	81 ± 7[a]
% SE††	15 ± 2[a]	22 ± 2[a]	15 ± 2[a]
% RNA negative‖	2.9 ± 0.7[a]	2.6 ± 0.3[a]	2.7 ± 0.5[a]

*Values are shown as means ± SEM.
†Values not sharing a common superscript letter are significantly different at $P < 0.05$.
‡Erythrocyte smears were made from bone marrow samples.
§Newly formed, RNA positive (polychromatic) erythrocytes.
‖RNA negative (normochromatic) erythrocytes.
¶Erythrocyte smears were made from peripheral blood.
**Newly formed, uniformly staining RNA positive erythrocytes.
††Older, stippled RNA positive erythrocytes.

The plasma zinc concentration was significantly correlated with erythrocyte formation ($r = 0.62$, $P < 0.001$). Using stepwise regression analysis, the addition of fetal liver zinc and dam femur zinc further strengthened the relationship of zinc status with erythrocyte formation in dams ($r = 0.77$, $P < 0.001$).

In the fetuses, there was no effect of zinc deprivation on the erythrocyte populations counted. In fetal blood, the RNA-positive erythrocyte populations of newly formed uniformly staining (UEs), older stippled (SEs) erythrocytes, and the RNA negative mature erythrocytes, were similar for all groups (*Table 4*). Due to the relative absence of mature RBCs, they were not included in the populations scored for micronuclei.

Discussion

Zinc status and suppression of erythrocyte formation

Compared with the rat dams fed adequate zinc diets, dams fed severely zinc-deficient diets had a significantly smaller proportion of newly formed erythrocytes despite elevated liver iron concentrations. In the adult rat, erythropoiesis takes place predominantly in the bone marrow, and the newly formed cells retain RNA for about 48 to 72 hr before it disappears from the cells. This residual RNA is an easily scored visible marker of newly formed cells (also known as reticulocytes in humans, because the vital staining generally employed causes the RNA to form a reticular pattern). In normal rat bone marrow the populations of RNA positive and RNA negative erythrocytes are approximately equal. The percentage of newly formed cells will decrease if erythropoiesis is blocked or retarded.

A relative iron deficiency exists if the liver is unable to mobilize ferritin or tissue iron into circulation and the blood forming tissues. Zinc deficiency may affect this process by impairing the synthesis or the function of transport proteins. Transferrin, a major iron transport protein,[28] was significantly decreased in adult men who had consumed a zinc-deficient diet for 4 to 9 weeks.[29] Rogers et al.[30] found that severe zinc deficiency (<0.5 μg of Zn/g of diet) impaired uptake of radioactive iron into the erythrocytes of pregnant rats. The same animals accumulated more iron in plasma, visceral, and intestinal tissues and less in erythrocytes than did control animals. Rats given marginally zinc-deficient diets (4.5 μg of Zn/g of diet) did not retain excessive amounts of iron in their tissue, nor did they have malformed fetuses.[30,31] Wehr and MacGregor (unpublished data) also fed marginally zinc-deficient diets to rapidly growing, weanling rats and to rat dams throughout gestation. Compared with controls, they observed no differences in the proportion of erythrocyte subpopulations or fetal teratology in the dams, but found reduced erythrocyte production in the weanlings. Severe zinc deficiency appears to limit erythrocyte production. Reduced erythrocyte production may decrease oxygen delivery to developing tissues and embryos, and this decrease could contribute to the poor outcome of severely zinc-deprived pregnancies.

Fetal erythropoiesis was unaffected by the dietary treatments of the dams in this study; approximately 80% of circulating fetal erythrocytes were newly formed. Because

fetal erythropoiesis occurs primarily in the liver, it is unlikely that a reduction in circulating transport proteins impairs the formation of red blood cells. In contrast to the marked increase in maternal hepatic iron stores with dietary zinc deficiency, fetal liver iron concentrations were unaffected. These data support the hypothesis that maternal zinc deficiency modulates erythropoiesis in the dam but not in the fetuses.

It may be that changes in bone zinc concentration have functional significance for erythropoiesis. Bone zinc concentrations are known to be correlated with dietary zinc over a wide range of intakes.[32] In addition, marginal dietary zinc intakes resulted in reduced zinc content and tracer uptake in bone marrow as well as calcified bone.[33] This same dietary condition decreased the concentration of bone marrow red blood cell metallothionein (MT-1), a zinc storage protein.[34] However, when erythropoiesis was stimulated in anemic rats, zinc uptake and MT-1 concentration increased proportionately, but only in the marginally zinc deprived group.[35] These studies suggest that bone marrow zinc is utilized in erythrocyte production, possibly mediated by MT-1. Zinc status can modulate at least two aspects of erythrocyte synthesis, the activity of delta amino levulinic acid dehydratase (δALAD),[36] an enzyme involved in heme synthesis,[37] and cellular proliferation.[38,39] When zinc supplies are limiting, the available zinc appears to be directed into the liver, which has higher priority metabolic functions and more labile zinc pools, and away from blood-forming bone marrow tissue.[34]

Micronucleus studies

Although we observed severe fetal teratological defects in the offspring of the zinc-deficient dams in this study, we detected no evidence of chromosomal damage in the dams or their fetuses using micronucleus incidence as an index of cytogenetic damage. This finding contrasts with three previous zinc-deficiency studies, which reported chromosomal damage in liver tissue, using either metaphase smears to examine chromosomal structure[13] or alkaline precipitation to detect DNA single-strand breaks.[14,15] The effect of zinc deficiency on chromosomal damage may vary with the tissue source. The extent that the tissue utilizes thymidine kinase or thymidylate synthetase for deoxythymidine monophosphate synthesis in DNA synthesis may be one explanation for these tissue-specific effects. The pathway that uses thymidine kinase is considered a salvage pathway and requires preformed nucleotides; the folate-dependent pathway utilizing thymidylate synthetase is a de novo synthetic pathway. Zinc deficiency causes a reduction in thymidine kinase activity in several tissues,[40] including cells originating from the bone marrow, e.g., lymphocytes.[41] Some tissues are more sensitive to zinc deficiency than others, e.g., thymidine kinase activity is reduced to a greater extent in brain than in the liver.[42] Thus, the contribution of the de novo and salvage pathways to DNA synthesis was maintained at a relatively constant rate in the liver compared with other tissues, making the liver a sensitive tissue for measuring chromosomal damage due to zinc deficiency. Possibly, the de novo and salvage pathways in DNA synthesis, as well as the degree of thymidine kinase inhibition, in various tissues may influence the sensitivity of specific tissues to chromosomal damage during zinc deficiency.

In summary, rat dams fed severely zinc-deficient diets during gestation exhibited classic signs of zinc deficiency, e.g., decreased food intake, cyclical feeding, weight loss, poor zinc status, poor pregnancy outcome, and development of fetal terata. Zinc deprivation was also associated with reduced liver zinc concentrations in the fetuses. The dams fed zinc-deficient diets had excessively high liver iron stores and a reduced proportion of newly formed red blood cells. Suppressed erythrocyte production leading to reduced nutrient supply to the developing tissues and embryo may contribute to the pathology exhibited by zinc-deprived dams and their zinc-deficient fetuses.

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