# Maternal Dietary Zinc Influences DNA Strand Break and 8-Hydroxy-2′-Deoxyguanosine Levels in Infant Rhesus Monkey Liver (43623)

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*Abstract.* **Severe zinc deficiency in rodent models has been shown to influence the frequency of single-strand breaks in DNA isolated from liver. In the current study, we investigated whether DNA isolated from infant monkeys born to mothers fed zinc-restricted diets would be characterized by higher than normal levels of DNA damage. DNA was isolated from 30-day-old infants born to dams fed low zinc (2 or 4 μg Zn/g) or control zinc (50 μg Zn/g) diets. The amount of single-strand breaks in liver DNA was significantly higher in the low zinc group than in controls; consistent with the above, there was a trend for higher steady state levels of liver 8-hydroxy-2′-deoxyguanosine in the low zinc group. While evidence for DNA damage in the low zinc group was obtained, the activities of several antioxidant enzymes were similar between the low zinc and control groups. In summary, infants born to monkeys fed low zinc diets are characterized by evidence of DNA damage shortly after birth; this damage may be due to an increased rate of oxidative damage and/or a reduction in the rate of DNA repair.** [P.S.E.B.M. 1993, Vol 203]

During the past decade, there has been increasing interest in the idea that maternal nutritional status may be a critical predictor for embryonic and fetal development, as well as the growth and development of the neonate. One nutrient that has received considerable attention is zinc, with a number of studies supporting the thesis that maternal zinc status can be a predictor of pregnancy outcome in humans (1–4). The idea that maternal zinc status would be a predictor of pregnancy outcome in primates is consistent with the well-documented teratogenicity of maternal zinc deficiency in several other species, such as rodents and sheep (5, 6). However, in contrast to studies with experimental animal models in which the extent of the induced zinc deficiency is often severe, human populations are more likely to develop conditions of marginal zinc deficiency. For this reason, our research group has been studying the effects of marginal zinc deficiency on pregnancy outcome and infant development in rhesus monkeys. We have demonstrated that marginal maternal zinc deficiency, induced by feeding diets containing 2–4 μg Zn/g during pregnancy and lactation, can result in a syndrome characterized in part by growth retardation, delayed bone growth and mineralization, and immune dysfunction (7–12). While the induction of zinc deficiency clearly represents a developmental insult to the animal, the biochemical lesions underlying zinc deficiency-associated abnormal development have not been agreed upon. Recently, there has been interest in the idea that one consequence of zinc deficiency may be an alteration in DNA integrity. Severe zinc deficiency in rat models has been reported to result in hypomethylation of liver DNA (13), an increased resistance of liver chromatin to micrococcal nuclease (14), and an increase in the frequency of single-strand breaks in DNA isolated from liver (15). Given the above observations, in the current study we investigated whether DNA isolated from liver of infant monkeys born to mothers fed zinc-restricted diets would be characterized by higher than normal levels of damage.

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## Materials and Methods

**Animals and Tissue Collection.** Female rhesus monkeys (*Macaca mulatta*) 4–10 years of age were obtained from a breeding colony of healthy, multiparous monkeys maintained at the California Regional Primate Research Center. Animals were housed individually in stainless steel cages in a temperature (20–29°C) and light-controlled room (14:10 hr light:dark cycle). Deionized distilled water was provided *ad libitum* via an automatic water system. Food was given in stainless steel containers to minimize zinc contamination and spillage of the purified diet.

Experimental diets were initiated 2 weeks before mating and were fed throughout gestation and lactation. Dams in the control group (C; $n = 7$) were fed purified diets that contained 50 μg Zn/g. Dams in marginal zinc (M; $n = 5$) and moderate zinc-deficient (MZD; $n = 8$) groups were fed the same diet, but with 4 μg Zn/g or 2 μg Zn/g, respectively. The detailed composition of the diet has been published (12). Mating was accomplished by transferring a female monkey to the cage of a proven breeder for a 2-hr period on 2 alternate days around the estimated time of ovulation as determined from menstrual cycles. A total of 38 dams were mated successfully. General health of the dams was monitored daily throughout gestation. Infants remained caged with dam after birth and obtained nutrition mainly via nursing.

On postnatal Day 30, the infants were sacrificed by an overdose of pentobarbital. The liver was quickly removed, weighed, and frozen in liquid nitrogen. Livers were stored at −70°C until analyzed for steady state levels of 8-hydroxy-2′-deoxyguanosine (oxo$^8$dG) and single-strand breaks (SSB) in DNA as indices of DNA damage. In addition, copper, zinc, iron, and metallothionein (MT) concentrations, and copper-zinc superoxide dismutase (SOD), manganese SOD, glutathione peroxidase (GSHPx), and glutathione reductase (GSHRed) activities were measured in the infant livers. Additional information concerning maternal and infant data collected during this study can be found in Refs. 11, 12, and 16–18.

**Determination of oxo$^8$dG in DNA.** Levels of oxo$^8$dG were determined in liver DNA of monkey infants in the C and MZD groups. DNA was isolated using modifications of a procedure reported previously (19). Enzymes were purchased from Boehringer-Mannheim Corp. (Indianapolis, IN). Frozen liver (150 mg) was homogenized in 1 ml of 0.1 *M* NaCl/30 m*M* Tris/10 m*M* EDTA (pH 8.0) with 10 up and down strokes using a hand-held Potter-Elvehjem glass and polytetrafluoroethylene pestle tissue grinder. The 3000-rpm pellet was washed, centrifuged, and resuspended in 300 μl of extraction buffer (0.1 *M* NaCl/0.1 *M* Tris/20 m*M* EDTA [pH 8]). Samples were incubated with 20 μl of RNase A/T (50 μg/ml RNase A and 5 units/ml RNase T1) for 60 min at 50°C, followed by an incubation with 40 μl of proteinase K (5 mg/ml) and 40 μl of 10% *N*-lauroyl sarcosine for 60 min at 50°C. The samples were extracted successively with 1 vol each of extraction buffer-saturated ultrapure phenol (Clonetech Laboratories, Palo Alto, CA), 1:1 mixture of phenol/Sevag (chloroform:isoamyl alcohol, 24:1), and Sevag. The phases were separated by centrifugation at 10,000*g*, at 4°C for 10 min. After the addition of 0.1 vol of 3 *M* sodium acetate (pH 5.0), the DNA was precipitated by the addition of 2 vol of absolute EtOH, precooled to −20°C. The tubes were inverted several times and kept at −20°C for at least 1 hr. After centrifugation (10,000*g* at 4°C for 10 min), the supernatant was removed and the pelleted DNA washed with 1 ml of precooled 70% EtOH and recentrifuged. Traces of EtOH were removed from the DNA pellet by evaporation (Speed-Vac concentrator; Savant Instruments Inc., Hicksville, NY). The DNA was dissolved in 20 m*M* sodium acetate (pH 4.8), followed by the addition of nuclease P1 (final concentration in sample, 0.1 mg/ml), and incubated for 15 min at 70°C. The resulting nucleotide 5′-monophosphates were further digested by the addition of 20 μl of 1 *M* Tris-HCl (pH 8.0) and 4 μl of 1 unit/μl calf intestine alkaline phosphatase and incubated for 1 hr at 37°C. Samples were centrifuged in Millipore ultrafree filter units (30,000 NMWL; Millipore Corp., Bedford, MA) and the filtrate was used for the determination of oxo$^8$dG.

The amount of oxo$^8$dG in the DNA was measured by high-performance liquid chromatography with an electrochemical detector (20–22). The oxo$^8$dG standard was prepared as described by Kasai and Nishimura (23). Levels of oxo$^8$dG are expressed relative to the amount of DNA, quantitated by measuring the absorbance of a known amount of a given nucleoside at 300 nm and using its molar ratio to calculate the amount of DNA present. The peak area of a 2′-deoxyguanosine standard was compared with the peak area of 2′-deoxyguanosine in the samples, and the conversion factor of 0.648 μmol dG/mg DNA was used.

**Measurement of SSB in DNA.** The alkaline unwinding assay of Morris and Shertzer (24) was used to measure SSB in liver DNA of the monkey infants. This assay measures the rate of transition of double-stranded DNA to single-stranded DNA during alkaline denaturation, in which DNA strand breaks serve as points at which unwinding is initiated (25). Artifactual strand breakage was minimized by preparing liver samples in subdued light and suspending liver cells in the homogenizing buffer using the squashing technique of Cox *et al.* (26). DNA concentrations were measured using the fluorescent dye Hoechst 33258 (27). The fraction of DNA unwound was calculated as

$$\frac{(total\ DNA - DS\ DNA)}{(total\ DNA)}_t - \frac{(total\ DNA - DS\ DNA)}{(total\ DNA)}_0 \quad [1]$$

where the subscripts indicate times 0 and $t$ (20 min) of incubation in alkaline solution.

**Mineral Analysis.** Tissues were wet-ashed with 16 *N* nitric acid (Baker's Instra-analyzed; J. T. Baker Co., Philipsburg, NJ), evaporated, and diluted with 0.1 *N* nitric acid (Baker's Instra-analyzed), as described by Clegg *et al.* (28). Concentrations of copper, zinc, and iron were determined in the samples by flame atomic absorption spectrophotometry (model 551; Thermo-Jarrel Ash, Wilmington, MA). Certified reference solutions (1000 μg metal/ml; Fisher Scientific, Santa Clara, CA) were used to generate standard curves for each element. A sample of National Bureau of Standards bovine liver (SRM 1577; U.S. Department of Commerce, National Bureau of Standards, Washington, DC) was included with the samples to ensure accuracy and reproducibility.

**Enzymatic Analyses.** For assays of SOD, GSHPx, and GSHRed activities, liver was homogenized (10% w/v) in 0.25 *M* sucrose/10 m*M* Tris (pH 7.0) with a Potter-Elvehjem glass and polytetrafluoroethylene pestle tissue grinder and centrifuged at 10,000*g* for 30 min. Enzyme assays were conducted on the supernate. Manganese SOD and copper-zinc SOD activity was determined as described by Marklund and Marklund (29). One unit of SOD activity is defined as the amount of enzyme needed to obtain 50% inhibition of pyrogallol oxidation, expressed per mg protein. GSHPx activity was determined by the method of Lawrence and Burk (30). Selenium-dependent GSHPx activity was specifically measured by utilizing 5 m*M* hydrogen peroxide in the assay system. Activity is expressed as nmol NADPH oxidized/min/mg protein. GSHRed activity was measured as described by Rogers and Augestyn (31) and is expressed as nmol NADPH oxidized/min/mg protein. Protein concentration was determined using the method of Bradford (32), using bovine serum albumin as the standard. Liver MT concentrations were determined as described by Onosaka and Cherian (33).

**Data Analysis.** Sex was not found to be a significant factor for the infant variables examined and, therefore, data for males and females were combined. Data were analyzed using one-way analysis of variance. Fisher's least significance difference test was used to examine differences between group means. A $P$-value $\leq 0.05$ was considered statistically significant. Data are shown as mean ± SE.

**Assurances of Compliance with Animal Codes.** All procedures conformed to the guidelines of the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals of the National Research Council (34). The California Regional Primate Research Center is fully accredited by American Association for Accreditation of Laboratory Animal Care. Individual protocols were approved by the campus veterinarian under the auspices of the Animal Care and Resources Committee of the University of California, Davis, Davis, CA.

## Results

Signs of pronounced zinc deficiency were not observed in any of the mothers from the M group throughout the study period; several mothers in the MZD group developed a dermatitis characteristic of zinc deficiency in the third trimester. Maternal plasma zinc concentrations were lower ($P \leq 0.05$) in the MZD group than in the M and C groups (gestation Day 90: 7.1 ± 0.8, 14.1 ± 1.3, and 14.2 ± 1.2 μ*M* zinc; term: 6.0 ± 0.8, 12.1 ± 1.3, and 10.8 ± 1.24 μ*M* zinc; Day-30 lactation: 8.3 ± 1.5, 11.0 ± 0.9, and 13.0 ± 1.3 μ*M* zinc, respectively). Additional information concerning these animals, including blood chemistry panels, immunologic status, and zinc status, is presented in Ref. 12.

Pregnancy outcome was similar in all groups and no gross malformations were noted in the neonates; body weights at birth and at Day 30 were similar among the groups. Plasma zinc concentrations were lower in the MZD infants compared with M and C infants at birth (8.2 ± 1.2, 10.3 ± 1.2, and 12.8 ± 1.3 μ*M* zinc; $P = 0.075$) and 14 days postpartum (7.0 ± 0.9, 11.9 ± 1.1, and 13.9 ± 1.5 μ*M* zinc; $P = 0.002$). Day 30 plasma zinc concentrations were similar among the infants (10.3 ± 1.0, 11.3 ± 1.0, and 12.2 ± 0.9 μ*M* zinc for MZD, M, and C infants, respectively).

Values for the parameters measured in the liver were similar between the M and MZD groups, and, thus, these data have been pooled for ease of presentation and interpretation. Liver zinc and MT concentrations were lower in the zinc-deprived groups than in controls (Table I).

The amount of SSB in liver DNA was higher in the zinc-deprived group than in controls (Table II). Consistent with the above, the concentration of liver

**Table I.** Influence of Maternal Dietary Zinc Intake on Liver Zinc, Copper, Iron, and Metallothionein Concentrations in Day-30 Infants[a]

| | Control ($n = 7$) | Low zinc ($n = 13$) |
|---|---|---|
| Zinc (nmol/g) | 400 ± 43 | 374 ± 33 |
| Copper (nmol/g) | 294 ± 67 | 311 ± 48 |
| Iron (nmol/g) | 5015 ± 716 | 6006 ± 888 |
| Metallothionein (nmol/g) | 244 ± 119 | 107 ± 38 |

[a] Values represent mean ± SE.

**Table II.** Influence of Maternal Dietary Zinc Intake on Liver Antioxidant Enzyme Activities and DNA Damage in Day-30 Infants[a]

| | Control ($n = 7$) | Low zinc ($n = 13$) |
|---|---|---|
| CuZnSOD activity (units/mg protein) | 18.0 ± 0.8 | 17.9 ± 0.9 |
| MnSOD activity (units/mg protein) | 3.65 ± 0.22 | 3.33 ± 0.24 |
| GSHPx activity (nmol NADPH oxid. $min^{-1}$ mg $protein^{-1}$) | 33.1 ± 4.8 | 29.3 ± 4.2 |
| GSHRed activity (nmol NADPH oxid. $min^{-1}$ mg $protein^{-1}$) | 3.24 ± 0.64 | 3.69 ± 0.28 |
| DNA SSB (% unwound DNA) | 30 ± 3.4 | 40 ± 1.5[b] |
| $Oxo^8dG$ (fmol dG/μg DNA) | 6.0 ± 0.6 | 8.1 ± 1.2[c] |

[a] Values represent mean ± SE. Enzyme abbreviations: CuZnSOD, copper-zinc SOD; MnSOD, manganese SOD.
[b] Significant difference from control group ($P < 0.01$).
[c] Value represents data from eight MZD infants.

$oxo^8dG$ tended to be higher in the zinc-deprived groups compared with controls ($P = 0.17$) (Table II).

Liver copper and iron concentrations were similar among the groups (Table I). There were no significant differences among the groups in liver copper-zinc SOD, manganese SOD, GSHPx, or GSHRed activities (Table II).

## Discussion

The research described in this report concerns one aspect of a program that centers on defining the influence of maternal zinc status on infant growth and development. We have reported previously that infant rhesus monkeys born to mothers fed marginal zinc diets are characterized by growth, immune, and behavioral abnormalities (9–12). The results from the current study suggest that nucleic acid metabolism is also affected in these animals. Castro *et al.* (15) have reported recently that one effect of zinc deficiency in a rat model is an increase in the frequency of single-strand DNA breaks in liver. The magnitude of difference in the rate of strand breakage between the control and zinc-deficient groups was greater than that observed in the present study. However, in the Castro *et al.* study, the diets were severely zinc deficient (0.63 μg zinc/g) and they were directly fed to male weanling rats for 4 weeks before tissue collection. The rats fed the zinc-deficient diets were characterized by gross signs of zinc deficiency. Data from the present study show that for nonhuman primates, an increase in strand breaks can also occur even in conditions of marginal zinc deficiency when gross signs of zinc deficiency are not evident. Consistent with the higher frequency of strand breaks, liver DNA isolated from infant monkeys in the zinc-deprived groups was characterized by higher than normal concentrations of $oxo^8dG$.

There are at least two basic mechanisms that may explain the observed increase in the frequency of strand breaks and concentrations of $oxo^8dG$ in the zinc-restricted animals. First, zinc deficiency can be associated with an increase in oxidative damage due to enhanced free radical generation and/or impaired free radical scavenging. For example, dietary zinc deficiency has been reported to stimulate the production of hydrogen peroxide (35), which may result in an increased rate of free radical generation due to zinc deficiency-associated increases in membrane iron concentrations (36). The concentration of MT in liver obtained from the zinc-compromised monkeys was lower than in controls; this observation is significant, since zinc induction of MT has been reported to reduce cadmium-induced DNA single-strand breaks in cultured liver cells (37). Given that cadmium is thought to induce DNA damage via free radical generation (38), Coogan *et al.* (37) have suggested that zinc-MT is protective due to its high content of thiolate groups (39).

Although the idea that the higher frequency of SSB and higher level of $oxo^8dG$ in liver DNA from the zinc-restricted group are due to a compromised antioxidant defense system is attractive, the observation that several components of the antioxidant defense system (SOD, GSHPx, GSHRed) are similar in the control and low zinc monkeys suggests that a generalized defect in antioxidant defense has not occurred.

An increase in free radical-induced DNA damage in the zinc-deprived groups would also be predicted to occur if the zinc deficiency resulted in a reduction in the amount of zinc directly associated with DNA; sites that typically bind zinc (a redox-inactive element) can become occupied with redox-active metals such as copper and iron. Har-el and Chevion (40) have shown that the addition of zinc to isolated DNA will reduce redox metal-mediated single- and double-strand breaks. A similar protective role for zinc has also been shown in erythrocyte membranes, where its addition reduces the rate of iron-induced lipid peroxidation (41).

An additional mechanism by which zinc deficiency may affect DNA integrity could be through a reduction in DNA repair. Poly (ADP-ribose) polymerase, which catalyzes the covalent attachment of ADP-ribose units to nuclear acceptor proteins participating in the recognition of single-strand and double-strand DNA breaks, is a zinc-dependent enzyme (42). Seres *et al.* (43) have reported that the activity of this enzyme is lower than normal in liver obtained from zinc-deficient rats. Unfortunately, insufficient material was available to analyze for poly (ADP-ribose) polymerase activity in the current study.

The results from this study show that the offspring of monkeys fed low zinc diets are characterized by evidence of DNA damage shortly after birth. Although we cannot define mechanistically how the low zinc environment resulted in the DNA damage, two possi-

bilities that will be pursued in the future are an increased rate of oxidative damage in zinc-compromised animals and a reduction in the activity of key DNA repair enzymes.

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