Studies of marginal zinc deprivation in rhesus monkeys. III
Use of liver biopsy in the assessment of zinc status1–3

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ABSTRACT Studies of marginal zinc deficiency in rhesus monkeys have demonstrated that plasma Zn levels are often a poor indication of Zn status. To better assess the Zn status of these animals, we examined their liver concentration of Zn as well as of other minerals, metallothionein (MT), and superoxide dismutase (SOD). Liver-wedge biopsies were obtained from adult rhesus monkeys fed for 15 mo, either a control (100 μg Zn/g) or a marginally Zn deficient diet (4 μg/g; ZD). Liver Zn and MT concentrations were lower in ZD monkeys than in controls whereas iron concentration was higher in ZD monkeys than in controls. Liver copper, manganese, and magnesium concentrations and activities of CuZnSOD and MnSOD were similar in the two groups. Data from the groups were pooled for regression analysis. Measurement of liver Zn and MT concentrations are useful in the assessment of the effects of long-term Zn deprivation in primates. Am J Clin Nutr 1988;47:1041–5.

KEY WORDS Zinc, trace minerals, rhesus monkeys, metallothionein, iron

Introduction

The essentiality of adequate dietary Zinc during pregnancy and early development was firmly established in laboratory rodents and domestic animals (1, 2). In the last few years there also were several studies indicating that Zn deprivation during pregnancy and early development can have detrimental effects on growth, behavior, and immune function in human populations (3–7). However, in contrast to studies with experimental animal models where the extent of the dietary Zn deficiency is often severe, human populations are more likely to consume diets that are only marginally Zn deficient. For this reason our group has been studying the effects of marginal Zn deficiency on pregnancy outcome and infant development in rhesus monkeys. To date we have shown that marginal Zn deficiency during gestation and the postnatal period can result in a syndrome characterized by growth retardation, delayed bone growth and mineralization, decreased taste sensitivity, behavioral lethargy, and immune dysfunction (8–12). These findings have significant implications with regard to human Zn nutriture and its physiological effects.

Although the studies cited above clearly demonstrated that a diet marginal in Zn can have a profound effect on pregnancy outcome and infant development in a non-human primate, an understanding of the biochemical lesions underlying these developmental defects has in part been limited by a lack of methods that accurately assess soft-tissue Zn status. Under conditions of high anabolism, such as late pregnancy and the rapid growth phases of infancy and adolescence, marginal Zn deprivation can be detected by group comparisons of plasma Zn concentration (8, 9, 13). However, it was shown in rodent models that plasma Zn concentration can change by as much as 50% over a 24-h period when animals previously fed a control diet are fed a Zn-deficient diet or vice versa (14). Thus plasma Zn concentration can reflect recent diet history rather than the tissue Zn status of the animal. We have therefore, in the current study, determined whether liver samples obtained by biopsy would accurately reflect Zn status. In addition to measuring liver Zn concentration, we determined the activity of the Zn-containing enzyme copper-Zn superoxide dismutase (CuZnSOD) and the concentration of the putative Zn storage protein metallothionein (MT) to establish

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lish whether changes in the concentration of Zn or Cu were functionally significant.

Materials and methods

Animals and diet

Female rhesus monkeys (Macaca mulatta) 4–10 y of age were selected from the breeding colony of the California Primate Research Center. All animals were in good health and adapted to individual caging. They were housed individually in stainless-steel cages in a temperature and light-controlled room (12-h light-dark cycle). Deionized distilled water was provided ad libitum in plastic bottles. Food was given in stainless-steel containers to minimize both spillage of the purified diet and Zn contamination. The precautions used to avoid Zn contamination were described in detail (13).

Initially there were four animals per group matched in pairs for age, weight, and parity. Each group was fed a purified diet containing either 4 mg/kg Zn (Zn-deprived, ZD) or 100 μg/g Zn (control) for 15 mo. The detailed composition of the diet was previously published (13). With severe Zn deficiency a reduction in food intake can occur (1, 2); thus the control diet was fed in a pair-fed protocol. However, over the course of the study, the ZD animals did not demonstrate signs of anorexia and the control animals often ate less food than the amount provided; thus the control group was not actually food restricted. One control monkey was found to be anemic and was very thin (ponderal index 20 cm/kg compared to a range of 17–18 in the remaining animals) at the time of biopsy. Data for this animal were not included in the group’s statistical evaluation.

Animal surgery

After laparotomy under general anesthesia (ketamine, halothane, and nitrous oxide), wedge biopsy samples were obtained under general anesthesia performed by the veterinary staff of the California Regional Primate Center. The biopsy samples from each monkey were collected from approximately the same site on the ventral lobe. Samples (1–1.5 g) were frozen immediately after removal with dry ice and stored at −70 °C in acid-washed plastic vials. All samples were coded and evaluated in a blinded fashion.

Assurance 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 (15). The California Primate Research Center is fully accredited by AALAC. Individual protocols were approved by the campus veterinarian under the auspices of the Animal Care and Use Committee of the University of California, Davis, CA.

Mineral analysis

Liver samples (0.3 g) were treated by the wet ashing procedure with 16 mol/L nitric acid (2 mL) Ultrex grade, JT Baker Co, San Francisco, CA), concentrated by evaporation, and diluted with distilled deionized water as described by Clegg et al (16). Zn, Cu, Fe, Mn, and Mg concentrations were determined in the diluted ashed samples by flame atomic absorption spectrophotometry (Instrument Laboratories IL 551, Wilmington, MA). Certified reference standards purchased from Fisher Scientific (Santa Clara, CA) were used to assure reproducibility. Recovery of these elements from liver average 98–102% by these methods. A sample of NBS bovine liver (SRM 1577) purchased from the US Department of Commerce, National Bureau of Standards, Washington, DC was included with the monkey liver samples to ensure accuracy of the elemental analysis.

Plasma samples collected throughout the 15-mo deprivation period were also analyzed for Zn concentration by the wet ashing procedure described above.

Metallothionein

Hepatic MT concentration was analyzed by the cadmium saturation method of Onosaka and Cherian (17). Liver samples (0.3 g) were homogenized in 0.25 mol/L sucrose and centrifuged at 10,000 × g. Supernatants were removed and exposed to a 10 μg/mL Cd solution allowing maximum binding of Cd to MT. Excess Cd was removed by addition of hemoglobin and subsequent heat precipitation. Cd in the supernatant was then measured by flame atomic absorption spectrophotometry. Concentrations of MT were determined by analyzing for Cd concentration and assuming a ratio of 6 mol Cd to 1 mol MT.

Superoxide dismutase activity

SOD activity was determined by inhibition of the autoxidation of pyrogallol as described by Marklund and Marklund (18). Samples of liver (0.8 g) were homogenized in cold 0.25 mol/L sucrose (20%) and sonicated for 2 min (Insonator Model 500, Savant Instruments, Inc, Hicksville, NY). The homogenates were centrifuged at 17,000 × g for 30 min at 4 °C and the supernatants were removed for assay. Total SOD activity was determined in 50 mmol/L tris-cacodylic acid and 1 mmol/L diethylentriamine pentaacetic acid, pH 8.2, at 25 °C. MnSOD activity was measured under the same conditions with the addition of 1 mmol/L potassium cyanide, which inhibits the CuZnSOD.

Statistics

Statistical differences between groups were evaluated with independent Student’s t test. Pooled data from the two groups were analyzed by Pearson product-moment correlations to determine the strength of relationships between variables (19).

Results

The plasma Zn concentration of the two groups during the 15-mo experimental period is shown in Table 1. There was no significant difference between the ZD and control monkeys until they had consumed the diets for 425 d.

Liver Zn concentration at day 450 was significantly lower in the ZD group than in controls (Table 2) whereas liver Fe was significantly higher in the ZD group. Liver Cu, Mn, and Mg concentrations were similar in the two groups. Concentrations of all the elements analyzed were similar to values previously reported for Macaca radiata (20).

Liver MT concentration was significantly lower in the ZD group than in controls (Table 3). CuZnSOD and MnSOD activities were similar in the two groups (Table 3), and values were similar to those previously reported for Macaca radiata (20).
LIVER BIOPSY IN ASSESSMENT OF ZN STATUS

Regression analysis showed significant correlations between liver Zn and MT concentrations (r = 0.91, p < 0.01), liver Cu and MT concentrations (r = 0.93, p < 0.01), and liver Cu and CuZnSOD activity (r = 0.91; p < 0.01). Strong correlations between liver Zn and CuZnSOD activity (r = 0.82) and liver Zn and Fe concentrations (r = -0.64) were also found; however, they were not statistically significant due to the small number of samples.

Discussion

As hypothesized, mean liver Zn and MT concentrations were lower in the ZD group than in the controls. Thus, the results support our hypothesis that liver biopsy can be useful in the evaluation of the soft tissue Zn status of nonhuman primates. As has been reported for Zn-deficient rats (21), the concentration of MT in the liver was low in the ZD monkeys compared with the controls. This observation suggests that the lower liver Zn concentrations of the ZD monkeys were physiologically significant because it is thought that MT serves as a labile pool of Zn in transfer of the element to apometalloproteins (22, 23). The observation of a strong correlation (r = 0.91, p < 0.01) between liver Zn and liver MT in the two groups adds further support to the idea that the liver Zn-MT pool is sensitive to dietary Zn intake. The inadequacy of current diagnostic methods for Zn status has been well recognized. Recently it was suggested that the measurement of plasma and soft-tissue MT levels may be more useful in the evaluation of Zn status than the determination of plasma Zn alone (21, 22). The basis for this suggestion is that it is now recognized that the synthesis of this protein is in part regulated by changes in cellular Zn concentration (21–24). The results here strongly support the idea that measurement of MT levels can be a useful indicator of Zn status in primates. The time course of the effects of marginal Zn deficiency on plasma and liver MT levels in primates needs to be established in future studies.

A surprising observation is the finding of a similarly strong correlation (r = 0.93, p < 0.01) between liver Cu and MT concentrations. This observation could suggest that in the monkey liver MT levels may also be sensitive to the Cu status of the animal. Alternatively it is possible that the correlation between Cu and MT concentration actually reflects the binding of Cu to Zn-MT (22, 23, 25). Further studies are needed to clarify this issue.

Liver concentrations of several elements other than Zn were measured to determine if any observed effects were specific for Zn or if they represented a generalized disturbance in mineral metabolism. With the exception of Fe, the concentrations of the other minerals analyzed were not affected by dietary treatment. The marked increase in liver Fe concentrations in the ZD monkeys is consistent with previous reports on liver Fe changes in Zn-deficient rats (26, 27). Because high levels of tissue Fe can result in oxidative stress and tissue damage (28, 29),

**TABLE 1**

Plasma zinc concentration in control and zinc-deprived monkeys*

<table>
<thead>
<tr>
<th>Diet</th>
<th>Days after initiation of diet</th>
<th>μg/mL (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>Control (n = 3)</td>
<td>1.48 ± 0.65</td>
<td>1.78 ± 0.60</td>
</tr>
<tr>
<td>(22.6 ± 9.95)</td>
<td>(27.2 ± 9.18)</td>
<td>(12.1 ± 2.60)</td>
</tr>
<tr>
<td>Zn deprived (n = 4)</td>
<td>0.94 ± 0.32</td>
<td>0.97 ± 0.11</td>
</tr>
<tr>
<td>(14.4 ± 4.89)</td>
<td>(14.8 ± 1.68)</td>
<td>(24.5 ± 8.26)</td>
</tr>
</tbody>
</table>

* p < 0.05

**TABLE 2**

Liver trace element concentrations in control and zinc-deprived monkeys*

<table>
<thead>
<tr>
<th>Diet</th>
<th>Zinc</th>
<th>Copper</th>
<th>Manganese</th>
<th>Iron</th>
<th>Magnesium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg/g wet wt (nmol/g wet wt)</td>
<td>μg/g wet wt (μmol/g wet wt)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>45</td>
<td>90</td>
<td>135</td>
<td>425</td>
</tr>
<tr>
<td>Control (n = 3)</td>
<td>45.5 ± 4.9</td>
<td>3.0 ± 0.5</td>
<td>1.6 ± 0.2</td>
<td>322 ± 73</td>
<td>158 ± 17</td>
</tr>
<tr>
<td>(696 ± 75)</td>
<td>(47 ± 7.9)</td>
<td>(29 ± 3.6)</td>
<td>(5.76 ± 1.3)</td>
<td>(6.49 ± 0.70)</td>
<td></td>
</tr>
<tr>
<td>Zn deprived (n = 4)</td>
<td>32.6 ± 1.2</td>
<td>2.2 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>704 ± 30</td>
<td>161 ± 4</td>
</tr>
<tr>
<td>(499 ± 18)</td>
<td>(35 ± 1.6)</td>
<td>(27 ± 5.5)</td>
<td>(12.6 ± 0.53)</td>
<td>(6.62 ± 0.16)</td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05

NS = not significant.
TABLE 3
Liver metallothionein and superoxide dismutase activity in control and zinc-deprived monkeys*  
<table>
<thead>
<tr>
<th>Diet</th>
<th>MT</th>
<th>CuZnSOD</th>
<th>MnSOD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/g wet wt</td>
<td>U/g wet wt</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14.5 ± 3.2</td>
<td>3311 ± 1174</td>
<td>367 ± 85</td>
</tr>
<tr>
<td>(n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn deprived</td>
<td>5.6 ± 1.1</td>
<td>2160 ± 396</td>
<td>407 ± 57</td>
</tr>
<tr>
<td>(n = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* x ± SEM at day 450.

the high concentrations of Fe in livers of ZD monkeys may result in liver pathology. The mechanism underlying the increase in tissue Fe observed with Zn deficiency has not been delineated. On the basis of the similar physicochemical properties of these two elements, it has been suggested that Zn and Fe may compete directly for a transport process into or out of cells (30). An additional possible explanation for the high liver Fe concentrations found in the ZD monkeys is that liver retroendocytosis of transferrin is affected by Zn deficiency. It is currently thought that once the transferrin-transferrin receptor complex has been internalized into a cell, retroendocytosis is facilitated by microtubules (31). Because microtubule assembly and structure are deranged in Zn deficiency (32, 33), Fe and transferrin efflux from the liver may be reduced. Data here show that the rhesus monkey may be an excellent model to study the mechanisms underlying Zn-Fe interactions and the physiological effects of this interaction.

The SOD enzymes were measured for two reasons; first, one of these enzymes has an absolute requirement for Zn and, second, there have been suggestions that increased tissue lipid peroxidation may be one consequence of Zn deficiency (34, 35). One mechanism by which increased tissue lipid peroxidation could occur is via a reduction in the activities of the antioxidant enzymes, CuZnSOD and/or MnSOD (36–38). Although there was a strong correlation (r = 0.82) between Zn concentrations and CuZnSOD activity, the extent of the Zn deprivation was not severe enough to result in a reduction in the activity of liver CuZnSOD. Thus this enzyme does not appear to be a sensitive marker for Zn status in the rhesus monkey. Unfortunately, because of the small amount of liver collected by the biopsy procedure used, we were unable to measure liver lipid peroxidation rates. The implications of these results with regard to the possible tissue accumulation of excess Fe in individuals who consume marginally Zn-deficient diets for prolonged periods of time need to be determined.

References