Prenatal Zinc Supplementation of Zinc-Adequate Rats Adversely Affects Immunity in Offspring1–3

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Abstract

We previously showed that zinc (Zn) supplementation of Zn-adequate dams induced immunosuppressive effects that persist in the offspring after weaning. We investigated whether the immunosuppressive effects were due to in utero exposure and/or mediated via milk using a cross-fostering design. Pregnant rats with adequate Zn nutrition were supplemented with either Zn (1.5 mg Zn in 10% sucrose) or placebo (10% sucrose) during pregnancy (3 times/wk). At postnatal d 3, 4 pups of Zn-supplemented dams (Zn-P) were exchanged with 4 of placebo-supplemented dams (P-Zn). The remaining pups continued with their biological mothers (Zn-Zn and P-P). Pups were orally immunized with dinitrophenol ovalbumin-BSA and/or cholera toxin B subunit (CTB), and serum Zn concentrations and cellular and humoral responses were assessed. Pups of Zn-supplemented dams had higher serum Zn when fostered either by placebo- or Zn-supplemented dams compared to pups of placebo-supplemented dams (P < 0.01). Postnatal Zn exposure reduced the number of Peyer’s patches in both the Zn-Zn and P-Zn groups (P < 0.01). Prenatal Zn exposure suppressed CTB-(P = 0.05) and BSA-specific proliferation response of Peyer’s Patch lymphocytes (P = 0.07). Prenatal Zn exposure effects on the splenocyte cytokine response were differently influenced by fostering mothers’ Zn status. Antigen presenting cell (APC) activity of splenocytes was lower in the Zn-Zn group than in the P-P group (P < 0.08). In conclusion, prenatal Zn exposure increases serum Zn levels in pups and suppresses antigen-specific proliferation and antibody responses and APC function, whereas postnatal exposure may suppress the mucosal immune reservoir. J. Nutr. 141: 1559–1564, 2011.

Introduction

Zinc (Zn) is essential for animal and human health, where it plays a crucial role in the normal functioning of the immune system (1). Zn modulates the activity of virtually all immune cells and suboptimal Zn status affects multiple aspects of both innate and adaptive immunity (2–4). However, Zn supplementation can reverse some of these adverse effects of Zn deficiency (5–9).

Marginal Zn deficiency in mice during gestation was shown to have both short- and long-term detrimental effects in their offspring, with reduced lymphoid organ size and Ig concentrations (10). Studies in Zn-deficient mice showed that several immunodeficiencies observed at birth persisted through adulthood, even when offspring were fed a Zn-adequate diet after weaning of the dams (11). Intrauterine growth retardation in humans linked to moderate maternal Zn deficiency induced persistent cellular immune deficiency in the offspring (12).

Zn is generally considered nontoxic, even in dosages that exceed the recommended daily intake (13). However, there are some reports of high-Zn intake adversely affecting immune function. Earlier studies have shown that excess Zn in adults and children results in copper deficiency, anemia, growth retardation, and immunodepression (13–15). High-dose Zn supplementation of healthy adults and excess Zn in vitro studies showed suppressive effects on functions of T-cells and granulocytes (16–19) and complement activation and complement-mediated phagocytosis (20). High-dose Zn supplements in mouse pups during the perinatal period reduced humoral responses (21), whereas in minks, it led to lymphopenia, suppressed lymphocyte proliferation response, and reduced growth rate in offspring (22). These studies suggest that excessive Zn supplementation during pregnancy may have adverse carryover effects in the offspring. Studies of the impact of maternal Zn supplementation during pregnancy on infant immune function are scarce. Osendarp et al. (23) showed that prenatal Zn supplementation had no effect on Haemophilus Influenzae Type b conjugate vaccine-specific responses in infants, although some improvement in delayed hypersensitivity immune responses to Bacilli Calmette-Guerin

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3 Supplemental Figures 1 and 2 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
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vaccine was noted in low-birth weight children only. In another study, infants born to mothers receiving Zn during pregnancy had fewer episodes of diarrhea but more episodes of cough and increased IL-6 production than infants born to mothers not receiving Zn (24). More research is required on the potential beneficial or adverse effects of prenatal Zn supplementation, especially concerning the possible modifying effect of pre-existing maternal Zn status.

We previously reported that Zn supplementation to Zn-adequate rat dams during pregnancy suppressed cellular immune function in offspring (7). We aimed to further evaluate whether immunosuppressive effect in the offspring was mediated by in utero Zn exposure through prenatal Zn supplementation or breast milk consumption after birth. We studied the effects of Zn supplementation in moderate excess of the recommended intake for rats during pregnancy on immune function of Zn-adequate offspring in a cross-fostering design.

Materials and Methods

Rats. The study complied with the Guide for the Use and Care of Laboratory Rats and was conducted in the Animal Resource Services of the University of California, Davis. Virgin female Sprague-Dawley rats (n = 12, 6–7 wk old) were obtained from Charles River. The rats were maintained in plastic cages under constant conditions (22°C, 65% humidity) with a 12-h-dark-light cycle and consumed food ad libitum. After consumption of a standard rat diet (LabDiet 5001) (25) for a 3-d acclimatization period, rats were allowed to consume Zn-adequate diet (25 mg Zn/kg) and deionized water ad libitum throughout the study. Following pregnancy confirmation, rats were divided into 2 groups to receive 3 times/wk either Zn (1.5 mg Zn in 10% sucrose) or placebo (10% sucrose) mixed with jelly on a steel container throughout pregnancy only (Supplemental Fig. 1). The jelly intake was checked for any leftovers. No supplementation was given after the pups were born.

The rat diet contained 25 mg/kg Zn, slightly lower than the AIN-93 recommendation of 30 mg/kg for pregnancy (26). Rats consumed ~150 g of diet (3.75 mg) + 4.5 mg/wk of supplemental Zn (1.5 mg/rat 3 times/wk). Thus, Zn intake was in excess of the recommended rodent intake by ~2.2-fold (8.25/3.67). The Estimated Average Requirement (EAR) for Zn during pregnancy in humans is ~9.5 mg/d and supplementation trials in pregnant women have used Zn levels of 20 (27) or 25 (28) to 50 mg/d (29). Therefore, a pregnant woman received ~210–420 mg Zn/wk from combined dietary (66.5 mg) and supplemental (140–350 mg) sources. Assuming women are already taking in 9.5 mg Zn in food, the supplemental doses exceed the EAR by 2.1- to 5.2-fold. Thus, the rat Zn intakes were within the range used in human studies.

Cross-fostering. After birth, pups were randomly culled to 8 pups/dam at d 3. Thereafter, one-half of the pups (n = 4) of each of Zn-supplemented dams were exchanged with one-half (n = 4) of those of placebo-supplemented dams according to the study trial (Supplemental Figs. 1 and 2). The groups of pups were designated as follows: Zn-Zn, pups of Zn-supplemented dams that continued with their biological mothers (n = 4); Zn-P, pups from Zn-supplemented dams cross-fostered to placebo-supplemented dams (n = 4); P-P, pups of placebo-supplemented dams that continued with their biological mothers (n = 4); and P-Zn, pups from placebo-supplemented dams cross-fostered to Zn-supplemented dams (n = 4). After weaning, pups received the same diet as their mothers.

Immunoassays. The weight of thymus and total number of Peyer’s patches in the small intestine were recorded. Mononuclear cells (MNC) were harvested from spleen, Peyer’s patches, and thymus. Single cell suspensions of spleen (referred to as splenocytes hereafter) and thymus were made by passing through nylon mesh (100-µm pore size, BD Biosciences) with a sterile rubber spatula. All Peyer’s patches were teased out from the small intestine, applied to a grinder, and passed through nylon mesh to obtain a single cell suspension (31). These cells were used for cell proliferation assay and enzyme-linked immunospot (ELISPOT). Blood obtained by cardiac puncture was collected into trace element-free vials and serum was separated and stored at ~80°C for further analyses.

Serum Zn analysis. Serum Zn was measured by atomic absorption spectrophotometry (Smith-Heiﬁger 4000, Thermo Jarrell Ash) as previously described (32). Briefly, 0.1 mL of serum was digested in acid-washed vials with 0.9 mL of 1 mol/L HNO3 for 48 h. Samples were centrifuged and the supernatant was collected and measured by atomic absorption spectrophotometry.

Lymphocyte proliferation response. Thymocytes, splenocytes, and Peyer’s patch lymphocytes were stimulated with 1 g/L of BSA (Sigma) as previously described (33). Splenocytes and Peyer’s patches were stimulated with 10 mg/L of CTB. To identify the optimum dose, different concentrations (0.5, 1, and 2.5 mg/L) of the monogat concanavalin A (ConA) (Sigma) were used as positive control. The optimum concentration of ConA was found to be 2.5 mg/L. The proliferation response was assessed by bromodeoxyuridine incorporation using commercial kits (R&D Systems). The cell proliferation or stimulation index (SI) was defined as absorbance of antigen (CTB or BSA) stimulated cell supernatant by absorbance of unstimulated control cell supernatant, where absorbance was measured at 450 nm (reference wavelength 690 nm).

Flow-cytometric analysis. Phenotypic analysis of subpopulation of mature T lymphocytes from thymus was conducted by using a fluorescent cell sorter (FACSCalibur E3393; Becton Dickinson) using phycoerythrin (PE)-labeled mouse anti-rat CD4 (Clone OX-35, BD Biosciences) and PerCP-labeled mouse anti-rat CD8a (peridinin chlorophyll protein) (Clone OX-8, BD Biosciences). Before data acquisition, instrument settings were checked and optimized by using Cali-BRITE beads (Becton Dickinson). Data acquisition and analysis were done with CellQuest software (version 4.2, Becton Dickinson). All samples were analyzed by setting appropriate forward and side-scatter gates around the lymphocytes and the percentage of positive cells was estimated.

Detection of total and specific Ig-secreting cells. Splenocytes and Peyer’s patch lymphocytes were assayed for numbers of total and specific

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**Abbreviations used:** APC, antigen presenting cell; ConA, concanavalin A; CTB, cholera toxin B subunit; DNP, dinitrophenol ovalbumin; EAR, Estimated Average Requirement; ELISPOT, enzyme-linked immunospot; ES, effect size; MNC, mononuclear cell; P-P, pups of placebo-supplemented dams which continued with their biological mothers; P-Zn, pups of placebo-supplemented dams transferred to Zn-supplemented dams; Zn-P, pups of Zn-supplemented dams transferred to placebo-supplemented dams; Zn-Zn, pups of Zn-supplemented dams which continued with their biological mothers.
antibody secreting cells by ELISPOT assay (34,35). For CTB-specific ELISPOT, a nitrocellulose-bottomed, 96-well MultiScreen-HA Filter Plate (Millipore) was first coated with monosialoganglioside (0.5 mg/L) from bovine brain (Sigma) in PBS (Sigma) overnight at 4°C, followed by washes and coating with purified CTB (2.5 mg/L) for 3 h at ambient temperature. Wells coated with BSA (1 g/L) only were used as control. For each determination, duplicates of 2 different cell concentrations (1 × 10^6/well, 1 × 10^5/well) were applied. Biotinylated mouse anti-rat conjugates were used followed by streptavidin-conjugated HRP. For Peyer’s patch cells, biotinylated mouse anti-rat IgA or IgM (BD Biosciences) were added in separate wells. For splenocytes, biotinylated mouse anti-rat IgA (BD Biosciences) or IgG1 or IgG2a (Southern Biotech) was added in individual wells. Plates were developed with AEC Substrate Reagent Set (BD Biosciences). The number of red spots corresponding to each Ig-secreting cells per well was counted using an inverted phase-contrast microscope (Leica WILD M3Z). Results were expressed as Ig-secreting cells/10^5 cells. For DNP-specific ELISPOT, the procedure was similar except that wells were coated with DNP-Ovalbumin (1 mg/L) (Biosearch Technologies).

To count total IgA- and IgM-secreting cells from Peyer’s patches and total IgA-, IgG1-, and IgG2a-secreting cells from spleen, individual wells were coated with mouse anti-rat IgA, mouse anti-rat IgM (BD Pharmingen), mouse anti-rat IgG1, or IgG2a (Southern Biotech).

Enumeration of IFNγ- and IL-4-secreting cells. Cytokines of the T helper cell type 1, IFNγ, and T helper cell type 2, IL-4, were measured from splenocytes and Peyer’s patch lymphocytes stimulated with CTB and BSA using rat IFNγ ELISPOT (BD Biosciences) and rat IL-4 ELISPOT (BD Biosciences). Splenocytes and MNC of Peyer’s patches were stimulated (33) with CTB (10 mg/L) and BSA (1 g/L) for 72 h. Cells were collected at centrifugation and applied to a 96-well MultiScreen-HA Filter Plate precoated with cytokine capture antibodies and the assay was performed as described by the manufacturer. Triplicate cultures for CTB and BSA in RPMI containing 10% FBS with or without stimulation were incubated for 24 h at 37°C in 5% CO2. Immunospots were counted as described earlier. Results were expressed as number of cytokine spot-forming cells per million MNC.

Determination of APC function: cytokine response in CD4+ T-cell. In Trial 2, isolation of APC from spleen of unimmunized Zn-Zn (n = 2) and P-P (n = 2) pups was done by preparing a single cell suspension of splenocytes as described earlier (36). Isolated cells were allowed to adhere to a plastic tissue culture plate (Nunc) and nonadherent cells were removed from the top. Adherent cells containing macrophages and dendritic cells were stimulated with BSA (1 g/L) for 2 h and thereafter collected by a sterile rubber spatula. This mixture of stimulated cells was referred to as APC thereafter. Spleen cells were isolated from the P-P group (n = 4) as described above. MagCellect Rat CD4+ T Cell Isolation kit (R&D Systems) was used to isolate CD4+ T-cells via a negative selection principle from the spleen of P-P pups immunized with DNP-BSA. The resulting cell preparation was highly enriched with CD4+ T-cells that were confirmed with flow cytometry. Cell suspension of CD4+ T-cells and APC was prepared in a ratio of 10:1 and cultured with 1 g/L of BSA. Supernatant was removed after 72 h and cells were added to a 96-well MultiScreen-HA Filter Plate precoated with cytokine capture antibodies (purified anti rat IL-4, purified anti rat IFNγ, BD Biosciences) for analysis of IFNγ- or IL-4-secreting cells as described earlier above (33). Results were expressed as number of cytokine spot-forming cells per MNC (MNC consisting of APC and CD4 cells).

Statistical analysis. Statistical analyses were conducted using the statistical software packages SIGMASTAT (version 3.1; Jandel Scientific) and SPSS for WINDOWS (release 17; SPSS Institute). When a variable was not normally distributed, an appropriate transformation (e.g. log or square root) was used to better achieve approximate normality and analyses were performed on the transformed variables. When the data could not be normalized, nonparametric analysis (rank-sum test) was performed. Differences were significant if P < 0.05. Results were expressed as means ± SD. Repeated-measures ANOVA was used to test body weight data on various days as within-subject variable with Zn supplementation as the between-subject factor. Two-way ANOVA was performed to determine significant interactions between prenatal and postnatal Zn exposure, and when interaction was significant, the Holm-Sidak post hoc comparison procedure was used to compare the effects of prenatal (in utero) and postnatal (lactation) Zn exposure on immune measures. The overall significance level of these tests was set at P < 0.05. Effect sizes (ES) of prenatal and postnatal zinc exposures were estimated. ES can be interpreted in terms of the mean percentile standing of the mean experimental group relative to the mean control group or in terms of the percent of nonoverlap of the experimental group’s scores with those of the control group (37). An ES of 0.0–0.2 indicates that the mean of the experimental group is between the 50th and 58th percentile of the control group and score distribution for the experimental group overlaps almost completely with the score distribution for the control group with 0–14.7% of nonoverlap (small). An ES between 0.6 and 0.8 indicates that of nonoverlap, respectively. An ES between 0.3 and 0.5 indicates that the mean of the experimental group is between the 62nd and 69th percentile (medium) of the control group with a nonoverlap of 21–33% between the 2 distributions. An effect size ≥ 2 indicates that the mean of the experimental group is at the 97.7th percentile or more of the control group with >81.1% of nonoverlap. The description of the individual effect size is given in the footnote of the tables.

Results

Body and thymus weight, serum Zn, and number of Peyer’s patches. Birth weight, body weight (data not shown), and ratio of thymus and body weight at d 52 (Table 1) did not differ among the groups. There were significant effects of prenatal and postnatal Zn exposure on the serum Zn levels. Zn-Zn and Zn-P pups had higher serum Zn compared with P-P and P-Zn pups (P = 0.01). The effects of fostering mothers were also detected; Zn-Zn and Zn-P pups had higher serum Zn concentrations than Zn-P and P-P pups (P = 0.03) (Table 1). Zn-Zn and Zn-P pups had fewer Peyer’s patches in the small intestine compared with P-P and P-Zn pups (P < 0.01); thus, increased milk Zn may reduce the number of Peyer’s patches. A marginally significant interaction of prenatal and postnatal (P < 0.09) Zn exposure on the number of cells per Peyer’s patch was noted. The number of cells per Peyer’s patch tended to be lower in the P-P pups compared with Zn-P pups (P = 0.11) (Table 1). For serum Zn levels, ES was 3.2 (large) for Zn-Zn, 3.09 (large) for Zn-P, and 2.23 (large) for P-Zn compared with the P-P group. For numbers of Peyer’s patches, ES for Zn-Zn was 1.75 (large) compared with the P-P group.

Prenatal Zn exposure suppresses proliferation response of Peyer’s patches lymphocytes. Prenatal Zn exposure decreased the antigen-specific proliferation response of Peyer’s patch lymphocytes in Zn-Zn and Zn-P pups compared with P-P and P-Zn pups receiving prenatal placebo supplementation (P = 0.05). Proliferation responses to CTB (P = 0.05) and BSA (P = 0.07) tended to be lower in Zn-Zn and Zn-P pups compared with P-P and P-Zn pups (Table 2). For CTB-specific proliferation response of Peyer’s patch lymphocytes, the ES was 0.62 (large) for the Zn-Zn group and 0.56 (medium) for the Zn-P group compared with the P-P group. No significant difference was observed in the ConA- or antigen-specific proliferation response of thymocytes and splenocytes (data not shown).

APC function and subsequent T-cell response is suppressed in the Zn-Zn group. Zn-Zn rats tended to have lower frequencies of IFNγ-secreting cells (418 ± 10.6/million MNC) compared with P-P rats (493 ± 10.6) (P = 0.07). Similarly, the Zn-Zn group tended to have lower frequencies of IL-4-secreting
cells (52.5 ± 3.54/million MNC) compared with P-P pups (114 ± 14.2) (P = 0.08).


Prenatal Zn exposure suppresses antigen-specific humoral immunity in splenocytes. Zn-Zn and P-P pups tended to have lower percentage of DNP-specific IgG1 in total IgG1-secreting splenocytes compared with P-P and Zn-P pups (P = 0.1) (Table 2). There were no differences in antigen- (DNP- and CTB-) specific IgA, IgG2a, and IgM-secreting Peyer’s patch lymphocytes (data not shown).

Antigen-specific cytokine response is differently affected by pre- and postnatal Zn status. There was an interaction of prenatal and postnatal Zn exposure on CTB-specific IL-4 responses of splenocytes (P = 0.03) (Table 2). The Zn-P group tended to have greater CTB-specific IL-4 responses of splenocytes than the Zn-Zn group (P = 0.07) and the P-P group (P = 0.09) (Table 2). The P-Zn group did not differ from the Zn-Zn group (P = 0.14) or the P-P group (P = 0.17).

The frequency of BSA-specific IL-4–secreting cells and antigen-specific IFNγ-secreting cells did not differ among the groups. Cytokine production by Peyer’s patch lymphocytes against specific antigens did not show any significant differences between groups (data not shown).

**Discussion**

In this study, we found that prenatal Zn supplementation in Zn-adequate rats increased serum Zn levels in the pups compared with prenatal placebo supplementation. Additionally, a postnatal effect of lactation was also seen in serum Zn levels of pups when fostered by Zn-supplemented dams. Pregnant women in Zn supplementation trials received Zn supplements by 2.2- to 5.2-fold in excess of the EAR (27–29). In our study, pregnant rats also received about 2.1-fold more Zn in supplements than the EAR for rodents (26); thus, rat Zn intakes were within the range used in human studies. However, it is important to mention that a direct comparison of excess dosages cannot be made between humans and animals, because most of the human trials were conducted in populations with suspected zinc deficiency and prior screening for Zn status was not done.

In earlier studies, epigenetic effects of Zn deficiency resulting in immunodeficiency in mice for 3 generations have been shown (10). Additionally, we have shown that the negative effects of prenatal and postnatal Zn exposure on serum Zn, number of Peyer’s patches, and ratio of thymus and body weight in rats

<table>
<thead>
<tr>
<th>Variable</th>
<th>Zn-Zn</th>
<th>Zn-P</th>
<th>P-P</th>
<th>P-Zn</th>
<th>Prenatal Zn</th>
<th>Postnatal Zn</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TABLE 1</strong> Effect of prenatal and postnatal zinc (Zn) exposure on serum Zn, number of Peyer’s patches, and ratio of thymus and body weight in rats¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn concentration, µmol/L</td>
<td>20.2 ± 2.5</td>
<td>15.2 ± 1.4</td>
<td>9.68 ± 0.4</td>
<td>13.4 ± 1.3</td>
<td>0.01</td>
<td>0.03</td>
<td>0.74</td>
</tr>
<tr>
<td>Peyer’s patches, n</td>
<td>12.3 ± 0.33</td>
<td>15.3 ± 0.33</td>
<td>16.3 ± 1.32</td>
<td>13.3 ± 0.33</td>
<td>0.21</td>
<td>0.00</td>
<td>0.61</td>
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<tr>
<td>Cells/Peyer’s patch, n x 10⁶</td>
<td>0.08 ± 0.14</td>
<td>0.77 ± 0.11</td>
<td>0.53 ± 0.14</td>
<td>1.08 ± 0.25</td>
<td>0.36</td>
<td>0.91</td>
<td>0.09</td>
</tr>
<tr>
<td>Thymus weight/body weight, mg/g</td>
<td>2.65 ± 0.24</td>
<td>2.42 ± 0.20</td>
<td>2.40 ± 0.40</td>
<td>2.74 ± 0.11</td>
<td>0.90</td>
<td>0.30</td>
<td>0.83</td>
</tr>
</tbody>
</table>

¹ Values are mean ± SD. Data were ln transformed as it was not normally distributed.

² Zn-Zn, pups of Zn-supplemented dams that continued with their biological mothers (n = 4); Zn-P, pups of Zn-supplemented dams transferred to placebo-supplemented dams (n = 4); P-P, pups of placebo-supplemented dams which continued with their own dams (n = 4); P-Zn, pups of placebo-supplemented dams transferred to Zn-supplemented dams (n = 4).

³ Effect size was 3.2 (large) for the Zn-Zn group, 3.0 (large) for the Zn-P group, and 2.2 (large) for the P-Zn group compared with the P-P group. The effect size between 2 and 3 indicates that the mean of the supplemented group is at the 97.7th percentile (81.1% of nonoverlap) of the P-P group.

⁴ Effect size was 1.75 (large) for P-Zn compared with the P-P group. The effect size of 1.75 suggests that the mean of the P-Zn group is at the 95.5th percentile (75.4% of nonoverlap) of the P-P group.

**TABLE 2** Effect of prenatal and postnatal zinc (Zn) exposure on antigen-specific immune responses of lymphocytes in rats¹

<table>
<thead>
<tr>
<th>Variable</th>
<th>Zn-Zn²</th>
<th>Zn-P</th>
<th>P-P</th>
<th>P-Zn</th>
<th>Prenatal Zn</th>
<th>Postnatal Zn</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation response of Peyer’s patch lymphocytes, [SI]³</td>
<td>0.9 (0.56–1.3)</td>
<td>0.96 (0.7–1.3)</td>
<td>2.0 (0.67–2.4)</td>
<td>1.8 (1.4–2.7)</td>
<td>0.05</td>
<td>0.65</td>
<td>0.52</td>
</tr>
<tr>
<td>BSA, 1 g/L</td>
<td>0.6 (0.3–0.95)</td>
<td>0.6 (0.5–0.7)</td>
<td>1.5 (1.0–1.54)</td>
<td>1.0 (0.99–2.3)</td>
<td>0.07</td>
<td>0.74</td>
<td>0.72</td>
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<tr>
<td>Cytokine response of splenocytes</td>
<td>9.0 (8.0–11.5)</td>
<td>16.5 (11.5–28.0)</td>
<td>9.0 (2.5–14.0)</td>
<td>13.0 (13.0–18.0)</td>
<td>0.08</td>
<td>0.73</td>
<td>0.03</td>
</tr>
<tr>
<td>DNP-specific splenocytes</td>
<td>9.0 (5.2–14.0)</td>
<td>18.7 (9.7–29.6)</td>
<td>36.0 (6.7–62.7)</td>
<td>4.5 (2.0–12.9)</td>
<td>0.71</td>
<td>0.10</td>
<td>0.75</td>
</tr>
</tbody>
</table>

¹ Values are median (25–75 percentiles), n = 4, within brackets. Data were ln transformed as they were not normally distributed.

² Zn-Zn, pups of Zn-supplemented dams which continued with their biological mothers; Zn-P, pups of Zn-supplemented dams transferred to placebo-supplemented dams; P-P, pups of placebo-supplemented dams which continued with their own dams; P-Zn, pups of placebo-supplemented dams transferred to Zn-supplemented dams.

³ Stimulation index (SI) was defined as absorbance of antigen (CTA or BSA) stimulated cell supernatant by absorbance of unstimulated control cell supernatant, where absorbance was measured at 450 nm (reference wavelength 690 nm).

⁴ Effect size was 0.62 (large) for the Zn-Zn group and 0.56 (medium) for the Zn-P group compared with the P-P group. The effect size of 0.62 indicates that the mean of the Zn-Zn group is at the 73rd percentile (38.2% of nonoverlap) of the P-P group. The effect size of 0.56 suggests that the mean of the Zn-Zn group is at the 69th percentile (33% of nonoverlap) of the P-P group.
excess Zn during pregnancy are also carried over to the next filial generation, resulting in immunosuppressive effects in certain aspects of cellular immunity in the offspring (7). In the earlier study, we found that the suppressive effect on the antigen-specific proliferation response of Peyer’s patch lymphocytes occurred due to in utero Zn exposure (7). In the present study, we further explored whether persistence of an immunosuppressive effect in offspring was caused by in utero exposure to excess Zn or mediated via breast milk consumption after birth, or both. Pups of both Zn- and placebo-supplemented dams had fewer Peyer’s patches when they were fostered by Zn-supplemented dams, suggesting that milk Zn may reduce the number of Peyer’s patches in the small intestine and therefore result in a reduced mucosal immune reservoir of lymphocytes. Our earlier study showed that rat dams fed a regular diet containing 25 mg Zn/kg had a milk Zn concentration of 0.16 ± 0.02 mmol/L, which decreased progressively throughout the course of lactation to about one-half that level (38). Supplements of 40 mg Zn/d given to lactating mothers increased serum Zn levels and delayed the normal decline of milk Zn concentration up to 6 mo, whereas 20 mg Zn/d had no effects (39). Thus, Zn supplementation during lactation can affect the Zn content of breast milk. However, in the present study, Zn supplements were given during pregnancy only and were stopped at birth. Thus, suppressive effects of milk may not be mediated by Zn directly but by factors, e.g., hormones such as prolactin, that are regulated by Zn. Zn deficiency increases the prolactin concentration in milk (40). Prolactin has an immunomodulatory role and lymphocytes are an important target tissue for circulating prolactin. We do not know whether high Zn levels can reduce the prolactin concentration and lead to imbalance in immune responses.

In the present study, we found that Zn-Zn rats that were exposed to Zn prenatally as well as via lactation tended to have suppressed antigen-presenting function of the APC compared with P-P rats that were not exposed to additional Zn. The study by Shi et al. (41) demonstrated that Zn deficiency impaired immune responses by limiting the capacity of the APC to induce T-cell responses such as proliferation and production of IL-4, IL-5, and IFNγ. Research in a Zn-deficient mouse model showed that Zn deficiency plays a major role in impairing the gut mucosal immune system, which also leads to changes in systemically disseminated immune responses (42). Zn directly interacts with monocytes and affects monokine release, whereas lymphocyte stimulation is the consequence of an indirect effect mediated by Zn-induced monokine secretion (19,43). Another study showed that the decreased T-cell proliferation during Zn deficiency was caused by a primary defect in the macrophage population and it could be reversed by increasing the APC numbers (44). Thus, our results suggest that the antigen-presenting functions of APC from the Zn-Zn group for stimulating CD4 T-cells may be suboptimal, resulting in fewer IFNγ- and IL-4 secreting T-cells.

Our data indicated that prenatal Zn exposure tended to suppress the percentage of DNP-specific IgG1 in total IgG1-secreting splenocytes compared with placebo supplementation. T-cell–dependent antigens (e.g., DNP, cholera toxin) require interaction with helper T-lymphocytes to stimulate B-lymphocytes to produce antibodies. Thus, lower antigen-specific T-cell proliferation and a lack of adequate response from T-helper cells to antigen-presenting B cells may result in lower specific antibody production. Immunologic memory is influenced by Zn (45,46). Earlier studies showed that Zn-deficient mice had reduced antibody recall responses to antigens for which they were immunized, and antibody production in response to T-cell–dependent antigens was more sensitive to Zn deficiency than antibody production in response to T-cell–independent antigens (47). Again, Zn levels higher and lower than normal are known to induce apoptosis in B cells (48). Notably, we have shown that numbers of Peyer’s patches (harboring both B and T lymphocytes) were reduced in Zn-Zn rats.

Additionally, we found that the antigen-specific cytokine response was differently affected by pre- and postnatal Zn status. There was a significant interaction effect between in utero Zn exposure and fostering mothers’ Zn status on CTB-specific IL-4 responses by splenocytes. Pups from Zn-supplemented dams cross-fostered to placebo dams tended to have higher CTB-specific IL-4 responses by splenocytes compared with pups remaining with their biological mothers. Th1 responses (IFNγ) are characterized by cellular immunity and production of IgG2a antibodies, whereas Th2 responses (IL-4) are characterized by humoral immunity, specifically the production of IgG1 and IgE antibodies. We did not find any relation between IL-4 responses and antigen-specific IgG1 responses.

One of the limitations of the present study was that Zn concentration in the breast milk was not measured, although it is well known that Zn supplementation during lactation does not increase the Zn concentration in milk. Another caveat was that functional aspects of antibodies (activation of complement and opsonization) were not evaluated. It is important to consider that the ELISPOT technique identifies antibody-secreting cells only; it does not discriminate between high- and low-avidity antibodies produced. Thus, the quantitative differences obtained may not reflect the functional capacity of the antibodies. The immune systems of mice or rats are frequently used as experimental tools for studying functions of the immune system in health and disease, providing insight into the functions of the human immune system. However, it is worth noting that there are differences in the innate and adaptive immune responses between humans and rats and any given response in a rat may not occur in the same way in humans (49). Thus, caution in drawing inferences based on animal studies is well warranted.

In conclusion, our study with a cross-fostering design showed that exposure to Zn during pregnancy at amounts well in excess of the EAR altered the immune function of the offspring, with profound suppressive effects on some aspects of cellular functions and cellular immune reservoir. Humoral immunity was marginally affected. These effects were caused by in utero exposure to excess Zn and via breast milk consumption. Just as Zn supplementation can reverse the impaired immunity of Zn deficiency, it can also induce immunosuppressive effects in moderate dosages. Further studies are needed to elucidate the mechanisms responsible for these altered responses.

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