Zinc Supplementation Increases Zinc Status and Thymopoiesis in Aged Mice

Carmen P. Wong, Yang Song, Valerie D. Elias, Kathy R. Magnusson, and Emily Ho

Abstract

The age-related decline in lymphocyte development and function coincides with impaired zinc status in the elderly. Thymic involution and reduced immune responsiveness are classic hallmarks of both aging and zinc deficiency, resulting in decreased host defense and an increased susceptibility to infections. Thus, compromised zinc status associated with aging may be an important contributing factor in reduced thymopoiesis and impaired immune functions. Our goal in this study was to understand how dietary zinc supplementation affects thymopoiesis in aged mice. We hypothesized that impaired zinc status associated with aging would mediate the decline in thymic function and output and that restoring plasma zinc concentrations via zinc supplementation would improve thymopoiesis and thymic functions. In this study, groups of young (8 wk) and aged (22 mo) mice were fed a zinc-adequate (30 mg/kg zinc) or zinc-supplemented diet (300 mg/kg) for 25 d. Aged mice had impaired zinc status, with zinc supplementation restoring plasma zinc to a concentration not different from those of young male C57Bl/6 mice. Zinc supplementation in aged mice improved thymopoiesis, as assessed by increased total thymocyte numbers. In addition, improved thymic output was mediated in part by reducing the age-related accumulation of immature CD4⁺CD8⁻CD44⁺CD25⁻ thymocytes, as well as by decreasing the expression of stem cell factor, a thymosuppressive cytokine. Taken together, our results showed that in mice, zinc supplementation can reverse some age-related thymic defects and may be of considerable benefit in improving immune function and overall health in elderly populations. J. Nutr. 139: 1–5, 2009.

Introduction

Age-related decline in immune function encompasses multiple defects (1). Among them, thymic involution and reduced T-cell production are one of the most recognized hallmarks of aging. Whereas the age-dependent changes in thymus function have been well described, factors important in controlling the process remain to be fully elucidated. The progressive age-related decline in thymic function and output coincides with reduced zinc status and suppressed immune responses in the elderly population. Thus, the reduced zinc status that occurs with aging may play an important role in mediating reduced thymopoiesis and contribute to a progressive decline in immune responsiveness. This culminates in a higher incidence of infection, cancer, and autoimmune diseases with increasing age (2–4).

Zinc is a key component for the functions of numerous proteins and is an essential micronutrient required for numerous cellular processes. In particular, zinc is necessary for the normal development and function of the immune system (5). Alterations in dietary zinc intake, zinc uptake, retention, or secretion can lead to zinc deficiency and affect zinc-dependent functions. Zinc deficiency can significantly depress immune response and impair host defense (6). Zinc homeostasis is critically involved in the signaling events in immune cells and changes in zinc status affect multiple immune cell types involved in both innate and adaptive immunity (7–9). Severe zinc deficiency dramatically affects the development of the immune system, resulting in immune dysfunction. Hallmarks of severe zinc deficiency include thymic involution, lymphopenia, and accelerated apoptosis in lymphocytes. One population particularly at risk for zinc deficiency is the elderly, who have impaired zinc absorption and reduced dietary intake (10,11). Elderly patients with reduced zinc status have higher frequencies of infections (12) and reduced immune responses to vaccinations (13–15). On the other hand, restoring normal zinc levels in individuals with low zinc status via zinc supplementation can improve T-cell–mediated functions and decrease the incidence of infections in the elderly (16–18).

Zinc supplementation has been shown to reverse thymus involution in aged mice (19,20). In human studies, zinc supplementation improved immune functions in the elderly. However, the mechanism of how this is accomplished is currently unclear. In particular, whether improving zinc status through zinc supplementation would improve thymopoiesis and thymic functions in aged individuals remains to be investigated. We studied the
effects of zinc supplementation on thymic development and function in aged mice. We hypothesized that the impaired zinc status of the elderly plays an important role in the age-related decline in thymopoiesis and that zinc supplementation of an aged population would improve zinc status and have direct effects on thymic health by reversing age-related defects in the thymus.

Materials and Methods

**Mice, diets, and study design.** Young (8 wk) C57Bl/6 male mice were purchased from Jackson Laboratory. Aged (22 mo) C57Bl/6 male mice were purchased from the National Institute on Aging. Mice were housed in stainless steel suspended cages in a temperature- and humidity-controlled environment and randomly assigned to either a zinc-adequate (ZA)2 diet containing 30 mg/kg zinc or a zinc-supplemented (ZS) diet containing 10 times the zinc concentration (300 mg/kg zinc) of the ZA diet that was previously shown to be well tolerated (21). Purified diets were purchased from Research Diets and were custom prepared using an egg white-based AIN-93G diet with zinc provided as zinc carbonate (Table 1) (22). Mice were fed the assigned diets for 25 d and consumed food and water ad libitum. The dietary intakes and body weights of all mice were monitored throughout the study. At the termination of the experiments, mice were killed by CO2 asphyxiation. Blood was collected for plasma isolation. Thymus from each mouse was collected and made into single cell suspensions. Cell suspensions were passed through a 70-μm cell strainer to remove cell debris and prepared for cell counting and flow cytometry analysis. The animal protocol was approved by the Oregon State University Institutional Laboratory Animal Care and Use Committee.

**Plasma zinc concentrations.** Plasma zinc concentrations were measured using inductively coupled plasma-optical emission spectroscopy as previously described, with minor modification (23). Briefly, plasma samples (100 μL) were added to 1 mL 70% ultrapure nitric acid and incubated overnight. Incubated samples were diluted with chelex-treated nanopure water to a final concentration of 7% nitric acid, centrifuged at 3000 × g; 1 min at 25°C, and analyzed using the Prodigy High Dispersion inductively coupled plasma-optical emission spectroscopy instrument (Teledyne Leeman Labs) against known standards.

**Cell counts and flow cytometry analysis.** Thymocyte numbers were determined using the Z1 Coulter Particle counter (Beckman Coulter). After counting, one-half of the thymocytes were used for RNA isolation and the remaining thymocytes were used in flow cytometry. For flow cytometry analysis, thymocytes were resuspended in flow cytometry buffer (PBS, 2% fetal bovine serum, 1 mmol/L EDTA). Cells were incubated with CD4-FITC, CD25-PE, CD44-PerCP-Cy5.5, and CD8-APC for 30 min on ice in the dark. All antibodies were purchased from eBioscience. After extensive washing, cells were resuspended in buffer for flow cytometry acquisition and analysis. A minimum of 300,000 events in the lymphocyte gate were collected. Data were acquired using FACSCalibur (BD Biosciences). Data analyses were performed using Summit software (DakoCytonamation).

**RNA isolation, cDNA synthesis, and real-time quantitative PCR.** Total RNA from thymocytes was isolated using Trizol reagent (Invitrogen). One microgram of total RNA was reverse transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix for quantitative real-time PCR (Invitrogen). Real-time PCR was performed using primers specific for mouse stem cell factor (SCF) (forward: 5'-CAACCTCTCAGATGAACAC-3', reverse: 5'-CGAACCTCAGATGAACAC-3'), or 18S ribosomal RNA (forward: 5'-CCGAGCTTGAATACATGAT-3', reverse: 5'-CGAACCTCAGATGAACAC-3'). Real-time PCR were performed using DyNAmo HS SYBR Green qPCR kit (New England Biolabs). Gene copies were determined using the standard curve method. A standard curve was generated from serial dilutions of purified plasmid DNA that encoded for each gene of interest. Data represent the copy number of the gene of interest normalized to the copy number of the 18S ribosomal RNA housekeeping gene in individual mice.

**Statistical analysis.** Data are reported as means ± SEM. The main effects of age and dietary zinc and their interaction were analyzed using 2-way ANOVA, followed by the Bonferroni post hoc test when the interaction was significant. Where necessary, data were log transformed to correct for unequal variances prior to statistical analyses. Non-transformed data are shown in tables and figures. All analyses were performed using GraphPad Prism version 4.01. Significance was defined as P < 0.05.

## Results

**Plasma zinc concentration.** Both dietary zinc and age affected zinc status as assessed by the plasma zinc concentration (Fig. 1A). It was lower in aged mice than in young mice and was greater in ZS mice than ZA mice. The greater plasma zinc in the ZS mice was not due to differences in food intake or body weight gain; these variables did not differ between ZS and ZA mice of either age (data not shown).

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Diet composition1</th>
<th>ZA diet</th>
<th>ZS diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg white, spray dried</td>
<td>203</td>
<td>203</td>
<td></td>
</tr>
<tr>
<td>l-Histidine</td>
<td>0</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Corn starch</td>
<td>494.5</td>
<td>494.5</td>
<td></td>
</tr>
<tr>
<td>Maltodextrin 10</td>
<td>35</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
<td>0.014</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>Mineral mix S19409 (no added Ca, P, K, or Zn)2</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Potassium phosphate, monobasic</td>
<td>6.86</td>
<td>6.86</td>
<td></td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>8</td>
<td>8</td>
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<tr>
<td>Potassium citrate</td>
<td>2.48</td>
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</tr>
<tr>
<td>Calcium phosphate</td>
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<td></td>
</tr>
<tr>
<td>Vitamin mix V100372</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Biotin, 1%</td>
<td>0.4</td>
<td>0.4</td>
<td></td>
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<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Zinc carbonate (52.1% zinc)</td>
<td>0.058</td>
<td>0.58</td>
<td></td>
</tr>
</tbody>
</table>

1 Final zinc concentration is 30 mg/kg zinc in the ZA diet and 300 mg/kg zinc in the ZS diet.
2 Mineral and vitamin mix as previously described (22).

**Total and double negative thymocyte numbers.** Dietary zinc, age, and their interaction affected the number of thymocytes (Fig. 1B). As expected, aged mice had fewer thymocytes than young mice. Zinc supplementation did not alter thymocyte numbers in young mice but resulted in a significant 52% increase in thymocytes in aged mice.

We evaluated whether zinc status affected thymocyte development in young and aged mice. Thymocyte maturation is divided into 4 main differentiation stages based primarily on the...
expression of CD4 and CD8. The earliest immature thymocytes are double negative (DN) thymocytes (CD4\(^{-}\)CD8\(^{-}\)), which transition to immature double positive (DP) (CD4\(^{+}\)CD8\(^{+}\)) thymocytes, and finally differentiate into mature single positive (SP) CD4\(^{+}\)CD8\(^{-}\) and CD8\(^{+}\)CD4\(^{-}\) thymocytes that exit the thymus and enter the bloodstream (Fig. 2A). Dietary zinc and age, but not their interaction, specifically affected DN thymocytes; there were more in aged mice than in young mice (Table 2). Zinc supplementation resulted in a lower frequency of DN thymocytes compared with ZA mice. DP and SP thymocytes were not affected by dietary zinc in aged or young mice.

**DN1 thymocyte subsets.** The development of DN thymocytes can be further divided into 4 distinct maturation steps based on the expression of CD44 and CD25, namely DN1 (CD44\(^{+}\)CD25\(^{-}\)), DN2 (CD44\(^{+}\)CD25\(^{+}\)), DN3 (CD44\(^{+}\)CD25\(^{+}\)), and DN4 (CD44\(^{+}\)CD25\(^{-}\)) (24). During aging, there is a specific blockade in thymocyte development that prevents DN1-DN2 transition, resulting in the accumulation of DN1 thymocytes (Fig. 2A) (25,26). Dietary zinc and age, but not their interaction, specifically affected DN1 thymocytes (Fig. 2B). As expected, aged mice had a higher frequency of the DN1 subset than young mice. Zinc supplementation reduced the proportion of DN1 thymocytes compared with ZA mice.

**Thymic cytokines.** Dysregulation of various thymic cytokines, including SCF and IL-7, have been associated with thymic atrophy and the suppression of thymopoiesis during aging (27). Dietary zinc, age, and their interaction affected thymic SCF expression (Fig. 1C). Specifically, SCF expression was greater in the thymus of aged mice than in young mice. Zinc supplementation in young mice did not alter SFC expression but significantly reduced SCF expression in aged mice. Neither age nor zinc supplementation affected thymic IL-7 expression (data not shown).

**Discussion**

There is a growing body of evidence that suggests that the impaired zinc status associated with aging may directly be involved in age-related immunological decline. Thus, it has been postulated that zinc supplementation to restore zinc levels in aged individuals may have beneficial effects on immune health. This study

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**TABLE 2** CD4/CD8 thymocyte distribution in young and aged male C57Bl/6 mice fed ZA or ZS diets for 25 d\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Aged</th>
<th>ANOVA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZA</td>
<td>ZS</td>
<td>ZA</td>
<td>ZS</td>
</tr>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DN</td>
<td>2.8 ± 0.7</td>
<td>2.1 ± 0.2</td>
<td>8.2 ± 2.5</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>DP</td>
<td>88.9 ± 0.5</td>
<td>88.0 ± 0.5</td>
<td>81.2 ± 3.6</td>
<td>88.2 ± 2.5</td>
</tr>
<tr>
<td>CD4 SP</td>
<td>6.6 ± 0.7</td>
<td>5.0 ± 0.3</td>
<td>8.2 ± 0.8</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>CD8 SP</td>
<td>1.6 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>2.3 ± 0.3</td>
<td>1.9 ± 0.1</td>
</tr>
</tbody>
</table>

\(^{1}\)Values are means ± SEM, n = 8.

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**FIGURE 1** Plasma zinc concentration (A), thymocyte number (B), and thymic SCF expression (C) in young and aged male C57Bl/6 mice fed ZA or ZS diets for 25 d. Values are means ± SEM, n = 8. *Different from corresponding ZA, P < 0.001. NS, Nonsignificant, P > 0.05.

**FIGURE 2** Flow cytometry analysis of DN (CD4\(^{+}\)CD8\(^{-}\)), DP (CD4\(^{+}\)CD8\(^{+}\)), and SP (CD4\(^{+}\)CD8\(^{-}\) and CD8\(^{+}\)CD4\(^{-}\)) thymocyte cell populations from young and aged male C57Bl/6 mice fed ZA or ZS diets for 25 d. DN cell population was further analyzed to determine the frequency of DN1 (CD44\(^{+}\)CD25\(^{-}\)), DN2 (CD44\(^{+}\)CD25\(^{+}\)), DN3 (CD44\(^{+}\)CD25\(^{+}\)), and DN4 (CD44\(^{+}\)CD25\(^{-}\)). Representative flow cytometry data (A) and mean DN1 frequency (B) are shown. Values are means ± SEM, n = 8. NS, Nonsignificant, P > 0.05.
demonstrated that zinc status is impaired in aged mice and could be restored to levels comparable to young mice by dietary zinc supplementation. Moreover, our results indicated that zinc supplementation partially reversed the thymic defects associated with aging. In particular, zinc supplementation increased total thymocyte numbers in aged mice, in part by reversing the age-related accumulation of DN1 thymocytes and reducing the age-related elevated expression of the thymosuppressive cytokine, SCF. These data were consistent with our hypothesis that zinc status is an important variable in determining overall thymic health during the aging process. To our knowledge, this is the first report to directly examine the effects of zinc supplementation on thymocyte development and function during aging. Overall, our data suggest that impaired zinc status associated with the aging process may play an important role in the age-related decline in thymopoiesis.

Immuno-senescence, the age-related progressive decline in lymphocyte development and function, coincides with impaired zinc status in the elderly. Involution of the thymus and reduced immune responsiveness are classic hallmarks shared by aging and zinc deficiency. In particular, severe zinc deficiency results in a decline of CD4+CD8+ pre-T-cells in the thymus (5). Disruption of the hypothalamus–pituitary–adrenal axis leading to enhanced circulating corticosterone concentrations has been postulated to play an important role in zinc-deficiency related dysfunction (28). Elevated corticosterone levels are also apparent in aging and may also contribute to immuno-senescence (29). Whether alteration in corticosterone levels with age is directly related to alterations in zinc status is currently unknown. Decreases in zinc uptake and absorption increase the risk of zinc deficiency in the elderly (10,11). Murine models have been used to study the various effects of aging, including age-associated decline in zinc status (19,30). In agreement with others, our data demonstrated that aged C57Bl/6 mice, despite being fed a ZA diet, had a lower plasma zinc concentration than young mice (Fig. 1A). This suggested that aged mice, similar to humans, had impaired zinc uptake/absorption. In addition, the elderly are more prone to consuming inadequate levels of zinc in their diets. From the NHANES III data, it is estimated that 12% of the US population does not consume the current Average Estimated Requirement for zinc, but this number escalates to 35–45% in people over the age of 60 y (31). Because a high proportion of the elderly population (>60 y) consumes a low-zinc diet, there is likely a synergy between low dietary zinc intake and the age-related decline in zinc status that leads to immune dysfunction. Our studies demonstrate that zinc supplementation of aged mice restored plasma zinc to levels comparable to ZA young mice and partially restored age-related alterations in thymopoiesis thus, it is likely that compromised zinc status only partially accounts for the age-related decline in immune function. Understanding these interactions is an important area of future research with important public health implications and highlights the potential need for higher zinc requirements in an elderly population.

The differentiation, selection, and output of mature naive T-cells take place in the thymus. The earliest immature thymocytes are bone marrow-derived precursors that are negative for both CD4 and CD8 (DN thymocytes). DN thymocytes are further divided in 4 distinct subsets (DN1–4) based on the expression of CD44 and CD25 (24). The frequency of immature DN1 thymocytes has been shown to be significantly increased in aged mice, suggesting an age-dependent defect or blockade in DN1-DN2 transition during thymocyte maturation (25,26). As a consequence, a reduced number of thymocytes are available for maturation and the output of naive T-cells that exit the thymus is reduced. Although zinc supplementation did not fully restore thymocyte numbers to those of young mice, it nevertheless indicated that improving zinc status in aged mice could enhance thymopoiesis by partially reversing involution of the thymus (Fig. 1B). Our data further suggested that the increase in thymocyte numbers was mediated in part by reducing the accumulation of DN1 thymocytes and relieving the age-specific DN1-DN2 block during thymopoiesis (Table 1; Fig. 2).

To date, the precise mechanism of age-induced thymic atrophy remains unclear. However, it likely involves changes in the thymic microenvironment, including the dysregulation of thymic cytokines, the loss of thymic epithelium, and/or changes in T-cell progenitors (1). Dysregulation of thymotrophic as well as thymosuppressive cytokines have been proposed to contribute to age-associated thymic involution (27). In particular, elevated expression of thymosuppressive cytokines such as SCF has been observed in aged thymus and administration of SCF into young mice induced acute thymic atrophy in vivo. Although the precise mechanism by which SCF affects thymopoiesis remains to be elucidated, we showed that zinc supplementation in aged mice was associated with a significantly reduced expression of SCF in the thymus (Fig. 1C). Interestingly, dietary supplementation of zinc similarly decreased expression of SCF in the small intestine in vivo (32). In addition to the restoration of normal expression of SCF in aged mice, zinc supplementation likely affects thymopoiesis via additional mechanisms. For example, dietary zinc may modulate the expression of genes involved in zinc homeostasis. Our preliminary survey of zinc transporters in the thymus revealed age-specific differences in zinc transporter expression (data not shown). In addition, Moore et al. (33) reported that the expressions of a number of genes were modulated in the thymus of zinc-deficient and zinc-supplemented young mice. Interestingly, genes differentially expressed with age, including various thymic cytokines (27) and zinc transporters (C. P. Wong and E. Ho, unpublished data), were not among the list of differentially expressed genes identified in this murine model of severe zinc deficiency. However, in this model, p56ck, a lymphocyte-specific tyrosine kinase important in the selection and maturation of thymocytes, was upregulated with zinc deficiency. This could be an important target for examination in future studies. Other studies have also found increased p56ck with zinc deficiency but without effects on T-cell maturation (34). It is possible that alterations found with age-related decline in zinc status are not fully comparable to young growing mice fed a severely zinc-deficient diet (<1 mg/kg zinc). Both the severity of zinc deficiency and the physiological response to zinc status may differ. At the same time, the effects of zinc supplementation likely have differential effects in young and aged mice. This is evidenced in our findings that the effect of zinc supplementation on SCF expression was exclusively observed in aged mice. Further study is needed to characterize the response of the immune system to nutritional status in the growing young, adult, and aged animals. The interactions among nutrition, age, maturity/decline of the immune system are important to address to be able to make appropriate nutrient intake recommendations across the lifespan. Future studies will focus on providing an in-depth characterization of the differential expression of genes in the thymus with age and examine how zinc status influences the expression of other thymic cytokines as well as genes involved in zinc homeostasis, such as metallothionein and zinc transporters. We will further explore how zinc status and age influence thymopoiesis. Specifically, it will be of great interest to study how diet and age interact and affect cell proliferation and/or apoptosis during thymocyte development.
In summary, we showed that zinc supplementation improved impaired zinc status and thymopoiesis in aged mice. Reversing age-related thymic involution and enhancing thymic development and function would be of considerable benefit to improve overall immune function in aged individuals. In particular, the elderly are at increased risk of complications and death from infections such as influenza. Improving thymic development and function in the elderly should lead to an enhancement in immune responsiveness and translate to a reduction in complications and mortality from infections. Our study showed that zinc supplementation can serve as a therapeutic agent in improving thymic health and provided the foundation for future studies to further investigate the mechanisms as well as therapeutic effects of zinc supplementation in reversing thymic involution and in improving immune response in the elderly.

**Literature Cited**