Effects of zinc depletion and repletion on natural killer cell activity in aged mice

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The effects of zinc depletion and repletion on spleen natural killer (NK) cell activity in aged mice were studied. Sixty 24-month-old male Balb/c mice were divided equally into three groups according to their weight: group I were fed the zinc-deficient diet (1.3 μg/g Zn). Group II were fed the zinc-supplemented diet (58 μg/g Zn), but their intake was restricted to the average daily amount consumed by the zinc-deficient group and group III were fed the zinc-supplemented diet (58 μg/g Zn) ad libitum. After 4 weeks, 10 mice were taken out from each group. The determinations of NK cell activity and plasma zinc level were performed in aged mice. Then, the other 10 mice in each group were all fed the zinc-supplemented diet. After another 4 weeks, they were also killed and used for the measurements of NK cell activity and plasma zinc level. The results showed that the level of plasma zinc and spleen NK cell activity were both significantly lower in zinc-deficient mice than in the restricted mice and in the ad libitum controls ($P<0.05$). There was no statistical difference in plasma zinc level and NK cell activity between the restricted mice and the ad libitum controls ($P>0.05$). Supplementation of zinc for 4 weeks enabled a satisfactory recovery of all the indices in the zinc-deficient mice. The data suggest that zinc deficiency significantly impairs the spleen NK cell activity in aged mice, which can be satisfactorily recovered by an adequate zinc supplementation.

Introduction

Zinc, an essential element in man and animals, is important for stabilization or function of numerous metalloenzymes involved in protein synthesis, protein catabolism, energy metabolism, and both DNA and RNA synthesis. Zinc deficiency in man and animals has been associated with hypogonadism, growth retardation, anorexia, gastrointestinal malfunction, and dermatitis characterized by hyperkeratosis and parakeratosis[1].

Recent studies indicate that zinc is important for normal immune function and may have a key role in regulating some lymphocyte functions including responses to mitogens and antibody synthesis. Zinc also appears to be involed in T-cell activation and natural killer cell functions[2].

Studies [1–7] of both animal models and human patients have revealed that, as a single nutritional variable, zinc deficiency resulted in profound diminution in thymus size and function, deficiency of T-cell numbers, reduced proliferative response of T-lymphocytes, impaired T-cell helper function, detective development of T killer lymphocytes, decreased T-cell-dependent antibody production, detective development of both direct and indirect plaque-forming cells after immunization in vivo

with sheep erythrocytes (SRBC) and decreased natural killer cell activity. The immunodeficiencies associated with each of these states can be corrected by sufficient dietary supplementation with zinc.

Natural killer (NK) cells, a subpopulation of lymphocytes that differ from mature T or B cells or macrophages, are large granular lymphocytes derived from bone marrow and found in spleen, lymph nodes, peripheral blood and peritoneum. NK cells are thought to play a significant role in immunosurveillance against microoganisms and tumour and in resistance against metastases. They kill directly without prior sensitization and have a selectivity for malignant cells. NK cells are unique in that do not require previous antigenic stimulation, are antigenic non-specific and are capable of recognizing and destroying xenogenic target cells. They may be part of the first line of defense against cancer by destroying tumour cells before T cells and macrophages can be mobilized[8,9].

Recent studies show an extensive discussion concerning the natural killer cell function in aging. One group reports age-related decrease. Others find no change. Some groups find an increased number of NK cells[10]. Little is known, however, about the effects of zinc depletion and repletion on natural killer cell activity in aged mice.

The purpose of this study was to determine if zinc depletion can decrease NK cell activity and if the decreased NK cell activity might be satisfactory recovered by zinc repletion in aged mice.

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Materials and methods

Animals

24-month-old Balb/c male mice were obtained from the Academy of Sciences of China and caged individually in stainless steel cages and maintained at 22–24°C and 45–55% relative humidity. Acid-washed glass food jars and polyethylene bottles with polyethylene stoppers were used. Diets and deionized water were provided fresh twice weekly ad libitum unless otherwise specified. All the utensils used in providing the diets were either stainless stell or acid washed. All diets were analysed for zinc by atomic absorption spectrophotometry and the samples were prepared for analysis by using a dry ashing method[11]. Diet consumption was measured daily and the mice were weighed once a week.

Table 1. Composition of basal diet.

Ingredients	Concentration (%)
Casein	20.0
DL-methionine	0.3
Sucrose	50.0
Cornstarch	15.0
Fiber-celufil	5.0
Corn oil	5.0
Mineral mixture*	3.5
Vitamin mixture†	1.0
Choline bitartrate	0.2

*The mineral mixture had the following composition (g/kg of mixture) : calcium phosphate, dibasic, 500.0; sodium chloride, 74.0; potassium citrate, monohydrate, 220.0; potassium sulphate, 52.0; magnesium oxide, 24.0; manganous carbonate, 3.5; ferric citrate, 6.0; zinc carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; chromium potassium sulphate, 0.55; sucrose, 118.0.

†The vitamin mixture had the following composition (g/kg of mixture) : thiamine HC1, 0.6; riboflavin, 0.6; pyridoxine-HC1, 0.7; nicotinic acid, 0.2; D-biotin, 0.02; cyanocobalamin (vit.B_{12}), 0.001; retinyl palmitate (vit A), 0.8; D-L-α-tocopheryl acetate (vit E), 20.0; cholecalciferol (vit D_3), 0.00025; manaquionine (vit K), 0.005; sucrose, 972.9.

The mice were randomly assigned to one of the following experimental groups of 20 mice each. (1) Control group was fed the control diet ad libitum (58 μg/g Zn). The composition of the control and zinc-deficient diet has been described previously (Table 1)[12]. (2) Zinc-deficient group was fed a zinc-deficient diet ad libitum (1.3 μg/g Zn), which was the control diet except that no zinc was added to the salt mixture. Because animals on the zinc-deficient diet were known to consume less food than average for mice[6], an equal number of pair-fed mice on the control diet were also included for comparison. A companion group of mice was fed control diet but was restricted to the average amount of diet consumed each day by the zinc-deficient mice.

After 4 weeks, one half of the mice from each group were anaesthetized with ether and blood samples were collected by cutting the armpit vein for zinc analysis. Spleens were excised aseptically and used for natural killer cell activity assay.

The experiment was continued for 4 weeks after which the remaining mice were killed for examination. The zinc-deficient group were returned and fed the control diet (58 μg/g Zn) ad libitum. The restricted group received the control diet but was limited to the average amount of diet consumed the previous day by the mice of the deficient group.

A diagram of the study design is given in Fig. 1.

Target cells

The YAC-1 subline of YAC lymphoma cells, induced in mice by moloney leukaemia virus, was grown in suspension culture with RPMI-1640 containing 10% heat-inactivated (56°C, 30 min) fetal calf serum, 100 U/ml penicillin, 100μg/ml streptomycin and 25 mM HEPES (complete medium, CM) and served as a source of target cells for mouse effector cells in ^{125}I-labelled iodo-deoxyuridine microcytotoxicity assays.

Effector cells

Mice were killed and the spleens were collected aseptically. Spleen cells were obtained by teasing in CM with two cured needles. Large cellular debris was
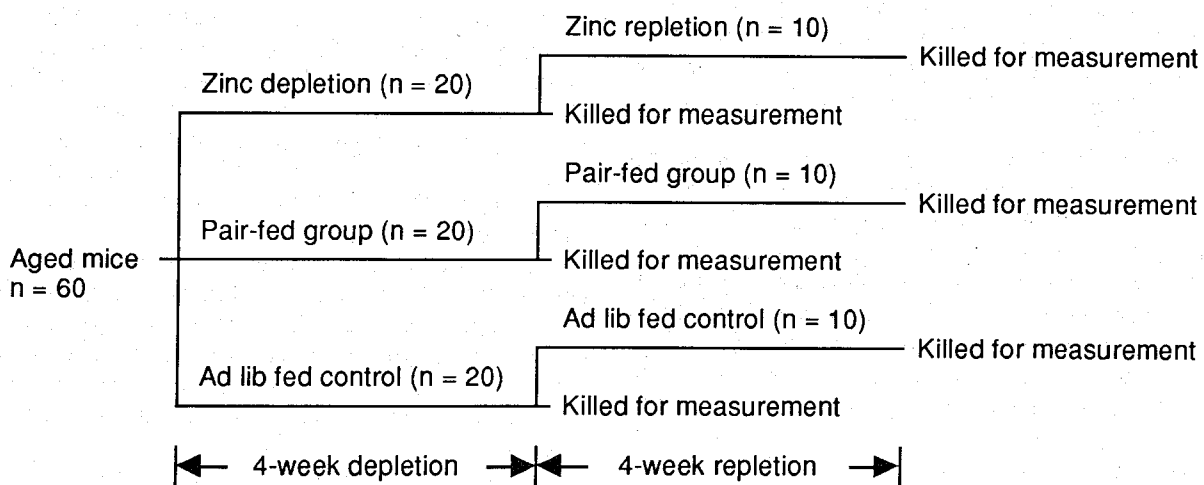
Figure 1. The study design. The study consisted of 2 stages, ie 4-week depletion stage and 4-week repletion stage. Each stage had randomly assigned, 10 mice fed ad libitum, control group (58 μg/g Zn), 10 mice restricted fed control group (58 μg/g Zn), and 10 mice zinc depletion or repletion group (1.3 or 58 μg/g Zn). Diet consumption was measured daily, body weight was measured once a week, plasma zinc level and NK cell activity were measured at the end of each stage.

removed by sedimentation, and a single-cell suspension was obtained by passing the material through graded needles. The lymphocytes were counted with a standard haemocytometer. For separating the lymphocytes, the suspension was layered over Ficoll-Hypaque with a specific gravity of 1.09 and centrifuged at $400 \times g$ for 30 min at room temperature. The lymphocytes which formed a visible interface were collected, washed three times in RPMI-1640 at $200 \times g$ for 10 min each, counted and adjusted to a concentration of 2.0×10^7 cells/ml in CM. This stock was used as effector cells in the natural killer cell activity assay.

Assay for natural killer cell activity

Natural killer cell activity was determined by means of the ^{125}I-labelled iododeoxyuridine ([125]IUdR) micro-cytotoxicity assay[13] with the highly sensitive YAC-1 as a target. For labelled, 10 μCi^{125} IUdR (specific activity 547 μCi/ml; China Nuclear Co, Beijing) was added to 5 $\times 10^5$ target cells/ml in 1.0 ml of CM. Fluorodeoxyuridine (FUdR) was included in the labelling medium to give a final concentration of $10^{-6}M$ in the medium with the cells to be labelled. The target cells in the labelling medium were incubated at $37°C$ in a 5% CO_2 incubator for 2 to 4 h to allow [125]IUdR uptake by the cells. After incubation, the target cells were washed three times in CM at $200 \times g$ for 10 min each at room temperature. Viable cells were counted and resuspended to the final concentration of 1.0×10^5 cells/ml.

For setting up the assay, 100 μl labelled target cell suspension (1.0×10^4 cells) and 100 μl effector cell suspension (2.0×10^6 cells) were delivered into each plastic tube. This corresponded to an effector target ratio of 200 : 1. Spontaneous [125]IUdR release from target cells was measured in the absence of effector cells, and the maximum release was determined by treating the target cell with 100 μl/tube of a 10% detergent solution. The volume in each tube was maintained at 1.0 ml by adding an appropriate amount of CM. The tubes were then incubated at $37°C$ in 5% CO_2 incubator for 16 to 18 h. The assay was set up in quadruplicate for each sample and incubation period. After incubation, the tubes were centrifuged at $200 \times g$ for 10 min. 0.5 ml of supernatant was collected from each tube and counted for radioactivity in a gamma counter. Cytotoxic activity results were expressed as percentage specific lysis and determined as follows. Percentage specific lysis = (mean
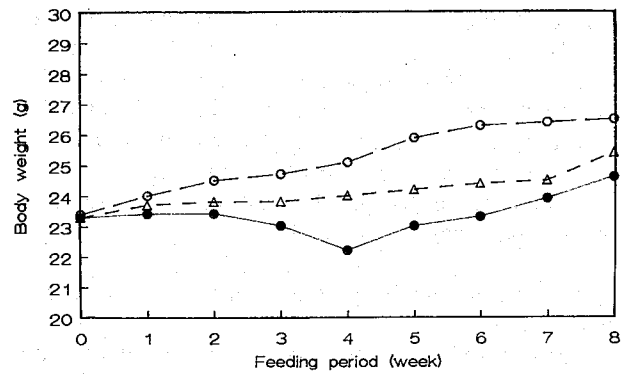

Figure 2.   Effects of zinc-deficient (●), pair-fed (△), and ad libitum fed (○) diets on body weight of Balb/c male mice for 4 weeks and effects of zinc repletion (●) on body weight of zinc-deficient mice for another 4 weeks.

cpm of experimental release – cpm of spontaneous release) / (mean cpm of maximum release – mean cpm of spontaneous release) × 100%.

Plasma zinc measurement

Plasma zinc was determined by direct aspiration of 1 : 8 dilution of plasma in deionized water into the atomic absorption spectrophotometer[14].

Statistical methods

All data were examined by analysis of variance with statistical significance of treatment differences being determined by Student's t-test[15].

Results

Effects of zinc deficiency on general appearance, body weight and diet consumption of Balb/c male mice

24-month-old male mice on the zinc-deficient diet did not lose weight during the first 2 weeks; from the third week onward, a gradual loss of body weight occurred (Fig. 2 and Table 2). Pair-fed mice on the zinc supplementation diet, although growing, gained less weight than did mice on the ad libitum zinc supplementation diet. At the end of 4 weeks, the zinc-deficient mice weighed 2.9 g less than the ad libitum controls (Table 2) and on the average they consumed 23.2 g less diet per day out of a total of 80 g consumed by the ad libitum controls during the same 4-week period. The

Table 2.   Body weight and diet consumption of Balb/c male mice after 4 weeks on zinc-depletion or zinc-supplementation diet.

Dietary group	Initial body wt(g)	Final body wt(g)	Food consumption (g/day)
Zinc-deficient (1.3μg/g Zn)	23.3±2.0	22.2±2.0	57.4±9.6
Pair-fed control (58 μg/g Zn)	23.3±2.0	24.0±1.6*	57.0±9.4
Ad lib-fed control (58 μg/g Zn)	23.4±2.0	25.1±2.1†	80.6±7.8†

Data are shown as mean ± SEM. n= 20 per treatment.
*Significantly different from zinc-deficient group at $P < 0.05$.
†Significantly different from zinc-deficient group at $P < 0.01$.

restricted mice also consumed 23.2 g less diet per day than the controls. In spite of this, the restricted mice weighed only 1.6 g less than the controls as compared to 2.9 g less for the zinc-deficient mice. Nearly 25% of the mice on the zinc-deficient diet were less active, had lost hair, had acrodermatitis, had diarrhoea, and had typical skin lesions on the tail and paws. No such lesions occurred in the pair-fed and ad libitum fed mice on the zinc supplementation diet.

Effects of zinc deficiency on plasma zinc levels and NK cell activity

Table 3 shows the plasma zinc levels in the three groups. Zinc levels ranged from 16 to 19 µmol/l in pair-fed and ad lib fed mice; in mice fed zinc-deficient diets they ranged from 6 to 8 µmol/l after the animals had been on the diet for 4 weeks. The plasma zinc levels were significantly lower in the zinc-deficient mice than in the ad lib controls and the pair-fed mice. Food restriction (pair-fed mice) did not decrease the plasma zinc levels decreased by zinc deficiency. However, zinc deficiency had a greater effect than food restriction alone on the plasma zinc levels ($P<0.05$).

NK cell activity was assessed by ^{125}IUdR release assay from mouse YAC-1 cells. As shown in Table 3, there was a significant difference in NK activity of splenocytes obtained from mice fed zinc-deficient diets compared to controls ($P<0.05$). There was no statistical difference in NK activity between the pair-fed mice and ad libitum controls ($P>0.05$).

Table 3. Plasma zinc levels and natural killer cell activity of Balb/c male mice after 4 weeks on zinc depletion or zinc supplementation diet.

Dietary group	Plasma zinc levels (µmol/l)	Natural killer cell activity(%)
Zinc-deficient (1.3 µg/g Zn)	6.73 ± 0.45	17.37 ± 1.09
Pair-fed control (58 µg/g Zn)	17.21 ± 1.07*	22.13 ± 4.28†
Ad lib fed control (58 µg/g Zn)	17.71 ± 1.12*#	23.72 ± 4.35†#

Data are shown as mean ± SEM. n = 10 per treatment.
*Significantly different from zinc-deficient group at $P < 0.01$.
†Significantly different from zinc-deficient group at $P < 0.05$.
#Not significantly different from pair-fed control group at P > 0.05.

In addition, there were positive correlations between the plasma zinc levels and NK activity in zinc-deficient, pair-fed and ad lib fed groups, respectively ($P<0.05$).

Effects of zinc repletion on deficient sympotoms and signs in zinc deficient mice

After 4 weeks of re-feeding of a zinc supplementation diet, the formerly deficient mice rapidly increased body weight (Table 4 and Fig. 2). And the typical skin lesions on tail and paws disappeared. In addition, the diet consumption of zinc-deficient mice was significantly increased after the return to zinc supplementation diets for 4 weeks.

Effects of zinc repletion on plasma zinc levels and NK cell activity in deficient mice

As shown in Table 4, after the deficient mice had been on a zinc supplementation diet for 4 weeks, their NK cell activity and plasma zinc levels recovered. The plasma zinc levels and NK cell activity did not differ among the zinc-deficient and pair-fed mice ($P>0.05$).

Discussion

Since there is evidence in the literature that reduced energy intake may cause thymic atrophy, it was important to determine what fraction of the observed loss in immunity in the zinc-deficient mice could be attributed directly to the deficiency of zinc in the diet and what fraction was due to the reduced energy intake that developed as a secondary consequence of the zinc deficiency. For this reason, two pair-fed studies were initiated. In each case, aged Balb/c male mice were fed zinc-deficient diets (1.3µg/g Zn) ad libitum[6]. Thus, in all of our experiments, the immunological responses of zinc-deficient mice were compared to those of pair-fed mice on the zinc supplementation diet, thus controlling for the adverse effects of inadequate food intake on immune functions. In addition, comparisons were made with the responses of mice fed zinc supplementation diets ad libitum. Results show that there was no significant difference in NK cell activity between the pair-fed mice and ad libitum controls, although the pair-fed mice consumed 23.2 g less diet per day than the ad lib fed mice at the end of 4 weeks. Suffice to say that for this period of time and under these experimental conditions, inanition had no quantitative effect on the NK cell activity of the pair-fed mice. However, the absence of zinc in the diet for the same time period had a profound effect on the zinc-deficient mice and greatly impaired their cell activity. This clearly indicates that adequate dietary zinc is essential to NK cell activity.

Table 4. Body weight, diet consumption, plasma zinc levels and natural killer cell activity of Balb/c male mice after the zinc depletion mice had been fed a zinc supplementation diet for 4 weeks.

Dietary group	Body weight (g)	Food consumption (g/day)	Plasma zinc levels (µmol/l)	Natural killer cell activity (%)
Zinc-repletion	24.6 ± 2.0	32.6 ± 4.7	15.94 ± 0.80	20.59 ± 2.81
Pair-fed control	25.4 ± 1.6	32.6 ± 4.7	17.51 ± 0.67	22.93 ± 3.05
Ad lib fed control	26.5 ± 2.1	43.4 ± 1.1	18.11 ± 0.75	24.55 ± 3.26

All mice were fed the zinc supplementation diet (58 µg/g Zn). Data are shown as mean ± SEM. n = 10 per treatment. Not significantly different from pair-fed control group to zinc repletion group at P > 0.05.

At the initial period of the experiment, there was no significant difference in the food consumption per day and body weight between zinc-deficient and ad libitum fed controls. But from the first or third week onward, a gradual loss of food consumption or body weight occurred respectively in zinc-deficient mice. At the end of 4 weeks, the food consumption and body weight differed among the zinc-deficient mice and ad lib fed controls. The data indicate that the deficiency of dietary zinc in mice produced gastrointestinal malfunction and loss of appetite and therefore caused a lower food intake and growth retardation.

It has been demonstrated by numerous published studies[3] that the poor appetite and growth of the zinc-deficient animals can be rapidly alleviated by zinc supplementation. We have observed that the body weight of zinc-deficient mice could rapidly reach the same level as the restricted-fed mice as a result of feeding a zinc supplementation diet for 4 weeks. And the experiments reported here clearly indicate that plasma zinc levels and NK cell activity of zinc-deficient mice significantly increased at the end of the 4-week repletion stage. However, the increment did not reach the same level as either the restricted-fed mice or ad libitum fed mice. There are two possible explanations for this. First, 4 weeks of dietary zinc repletion may not have improved zinc status to a level necessary for full recovery of NK cell activity. Second, it is clear that most of the measures of cellular immunity were strongly related to cellular zinc concentration. And plasma zinc did not correlate significantly with any of the cellular zinc concentrations measured[16-17]. This observation suggests that cellular zinc concentrations can provide additional information that may be helpful in evaluating the effects of zinc supplementation on immune function and that they are not simply a surrogate for plasma zinc. It is possible that the decreased NK cell activity in zinc-deficient mice was not or could not be fully recovered by short-term dietary zinc replation. An extension of the experimental time period could have resulted in a greater increment of NK cell activity in the zinc-repletion mice.

We conclude from these studies that in the case of the aged Balb/c male mouse inanition does not contribute significantly to the loss in NK cell activity in the zinc-deficient mouse if the studies are conducted within a 4-week period and the zinc deficiency significantly impairs the spleen NK cell activity in aged mice, which can be satisfactory recovered by an adequate zinc supplementation.

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摘　要

　　作者以老年小鼠爲試驗對象，研究了鋅耗竭與盈餘對脾臟自然殺傷細胞(NK)活性的影響。他們根據體重把60隻24個月大的雄小鼠(Balb/C)分成三組；第一組喂缺鋅飼料(每克飼料含鋅1.3微克)，第二組喂補充鋅的飼料(每克飼料含鋅58微克)，但限制它們的平均進食量與鋅缺乏組相同，第三組同樣喂補充鋅的飼料，但隨意進食。喂養10週以後，每組取出10隻小鼠，分折它們的自然殺傷細胞活性和血漿鋅水平。餘下每組的10隻小鼠均喂以鋅補充飼料，另十週以後，把所有小鼠殺掉並測定它們的自然殺傷細胞活性和血漿鋅水平。結果顯示，鋅缺乏組小鼠的血漿鋅水平和脾臟自然殺傷細胞活性二者均較鋅補充的兩組（限制進食組和隨意進食組）爲低（P<0.05）。在鋅補充的兩組中（限制進食與隨意進食組），其血漿鋅水平和自然殺傷細胞活性無統計學上的差異（P>0.05）。補充鋅飼料4週後可以使缺鋅小鼠的所有指標回復正常。作者從上述實驗數據提出，由於鋅缺乏而明顯損害脾臟殺傷細胞活性的老年小鼠，可以補充足夠的鋅而得到完滿地恢復。