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Increased Serum Leptin Levels and Leptin mRNA Gene Expression by Zinc Depletion in Rats

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Zinc deficiency has been shown to result in poor appetite, causing anorexia. However, the role of zinc in the regulation of food intake is not well understood. In the present study, we hypothesized that zinc deficiency dysregulates circulating leptin level and leptin mRNA gene expression, and that whether these changes were occuring as a direct result of, or as a compensatory effect of zinc deficiency in rats. After an adaptation period of 4 weeks, Sprague Dawley rats were provided with three different level of zinc, as one week of a Zn-adequate (30 mg/kg) diet, then two weeks of a Zn-depletion (1 mg/kg), and finally by two weeks of a Zn-repletion (50 mg/kg) diet. At the end of each dietary experimental period, one third of the 26 rats were killed. Zinc levels of blood subfractions (plasma, red blood cells and mononuclear cells) and in the liver were substantially decreased, despite the fact that food intake was not substantially decreased during the Zn-depletion period. Serum leptin concentration was significantly increased during the zinc depletion period. Leptin mRNA in adipose tissue was also shown to be highly expressed during the Zn-depletion period. Presumably, increased leptin level and leptin mRNA induction during Zn-depletion conditions may be the cause of lowered appetite which is the common symptom of Zn-deficiency. In conclusion, These increases in circulating leptin levels and in leptin gene expression would be the direct result of, rather than the compensatory effect of, zinc deficiency.

Key word : zinc, leptin, food intake

INTRODUCTION

The main symptom of zinc deficiency in experimental animals, and to a lesser extent in humans, is anorexia which causes weight loss, growth retardation and poor appetite[1] ; however, the mechanism for zinc regulation in food intake has not yet been clarified. It can be assumed that zinc deficiency may alter brain neurotransmitter metabolism or other hormonal signal which affects appetite. Typically, zinc deficiency in experimental animals results in a cyclical 3-4 day pattern of decreased food consumption[2),3)] and in a high expression of hypothalamic neuropeptide Y (NPY), a potent orexigenic agent.[2)] Increased NPY levels are explained by a compensatory response for lowered food intake status due to zinc deficiency.

Food intake is controlled by regulation of appetite and hunger signals in the hypothalamus. Leptin, as the 16-kDa *ob* gene hormone which was first identified by positional cloning in mice, is secreted from white adipose tissue and acts as a satiety signal.[4)] Following the receipt of leptin signals from the bodys fat stores by the hypothalamic centers, these centers then control food intake and energy expenditure by regulating hypothalamic neuropeptide Y(NPY), a stimulator of food intake, and corticotropin-releasing hormone (CRH), an inhibitor of food intake.[5),6)] Mutant *ob/ob* mice that lack functional leptin have been found to be obese and to have reduced activity and metabolism.[4)]

Elevated serum leptin concentration can contribute to anorexia which causes weight loss. On the other hand, it has also been reported that obese subjects showed elevated leptin concentrations and hypozincemia.[7)-9)] In the study by Considine et al, the elevated leptin concentrations in obese subjects was explained as an impared response or resistance to leptin.[9)] In other studies, zinc deficient subjects were found to have hypoleptinemia which was ameliorated by zinc repletion.[10),11)] As leptin has only recently become a focus of research interest, the precise relation between zinc status and leptin signals has not yet been understood.

In the present study, we investigated the hypothesis that zinc deficiency contributes to reduced food intake by dysregulating circulating leptin concentrations and gene expression in rats. This study also set out to determine whether this change in circulating leptin levels and in leptin gene expression occurs as a direct result of,

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Table 1. Compositions of the mineral mix (g/kg diet)

Ingredient	Zn-adequate (Zn 30mg/kg)[1]	Zn-depleted(Zn-) (Zn 1mg/kg)[2]	Zn-repleted(Zn+) (Zn 50mg/kg)[3]
Calcium phosphate, dibasic	500	500	500
Sodium chloride	74	74	74
Potassium citrate, monohydrate	220	220	220
Potassium sulfate	52	52	52
Magnesium oxide	24	24	24
Manganous carbonate	3.5	3.5	3.5
Ferric citrate	6	6	6
Zinc carbonate	1.6	0.053	2.677
Cupric carbonate	0.3	0.3	0.3
Potassium iodate	0.01	0.01	0.01
Sodium selenite	0.01	0.01	0.01
Chromium potassium sulfate	0.55	0.55	0.55
Sucrose, finely powdered	118.03	119.577	116.953
Total amount (g)	1000	1000	1000

1 AIN-76 Mineral mix, Harlan, Teklad, USA
2,3 Zn content was modified using AIN-76 Mineral mix.

Table 2. Compositions of the experimental diets(g/kg mineral mix)

Ingredient	Zn-adequate (Zn 30mg/kg)	Zn-depleted(Zn-) (Zn 1mg/kg)[1]	Zn-repleted(Zn+) (Zn 50mg/kg)[2]
Casein	200	200	200
Sucrose	500	500	500
Corn Starch	150	150	150
Fiber (Cellulose)	50	50	50
Corn oil	50	50	50
Mineral mix	35 (AIN-76 mineral mix)	35 (Zn-depleted mineral mix)	35 (Zn-repleted mineral mix)
Vitamin mix	10	10	10
Choline bitartrate	2	2	2
DL-methionine	3	3	3
Total amount (g)	1000	1000	1000
Energy (kcal)	3850	3850	3850

1,2 Modified mineral mix, using AIN-76 Mineral mix formula (Harland, Teklad, USA) (Table 1), was used for each diet preparation.

or as a compensatory effect of, zinc deficiency.

MATERIALS AND METHODS

Animals and diets

Twenty-six Sprague Dawley male rats (5 wks old, with an average weight of 135.6±5.2 g upon arrival) consumed a nonpurified pellet diet (Rodent Diet, PMI Feeds Inc, Japan) *ad libitum* for a 4 week adaptation and growth period until the beginning of the experiment. Rats were housed in individual hanging wire-mesh cages in rooms with a 12-hours light-dark cycle (lights off 19:00) maintained at 22-23℃.

The compositions of experimental diets and mineral mixes are given in Tables 1 and 2. The Zn-depletion and Zn-repletion diets were designed to contain 1 and 50 mg Zn/kg diet, respectively; these diets were modified from the rodent AIN-76 mineral mix (30 mg Zn/kg diet), and final zinc concentration was confirmed *via* atomic absorption spectrophotometry (using Boschstra*Be* 10 Spectro Analytical Instruments, Germany).

Experimental design and sample collection

The experimental design is shown in Fig 1. After 4 wks of adaptation in the laboratory, all rats were weighed (averaging 307.4±14.6 g), and were provided with the Zn-adequate diet (30 mg Zn/kg diet) for one week. After one week with the Zn-adequate diet, one third of the rats (n=8) were killed. The remaining 18 rats were given

the Zn-depletion diet (1 mg Zn/kg diet). After two weeks with the Zn-depletion period, half of the rats (n=9) were killed. The remaining 9 rats were then provided with the Zn-repletion diet (50 mg Zn/kg) diet for another two weeks, and were killed. Body weights were measured weekly and food intake was recorded daily.

On the last day of each dietary period, the assigned rats were anesthetized with Na pentobarbitol (10mg/kg body weight). Whole blood was collected by abdominal aorta insertion and blood was subfractuated as plasma, mononuclear cells (MNCs) and red blood cells (RBCs); using a Histopaque- 1077 (Sigma). Serum was also separated for the measurement of circulating leptin levels. Liver and various adipose tissues (epididydmal, liguinal subcutaneous, omental, and abdominal) were collected for leptin mRNA expression analysis by RT-PCR. Adipose tissues were immediately frozen with liquid nitrogen upon removal from the animals, and were stored at 80C until the RNA assays were undertaken.

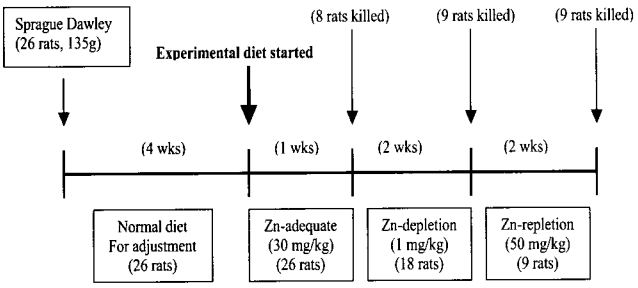

Fig 1. Experimental design

Zn, Cu and protein assay

RBCs and MNCs were homogenized in trace element free 0.125 M HCl before the trace mineral assay. The liver was dried and ashed at 500C in the furnace overnight. The liver and homogenized RBCs and MNCs were wet-digested using concentrated nitric acid (Sigma). Liver, plasma, and digested RBCs and MNCs were diluted with trace element free 0.125 M HCl (Fluka, Switzerland) at 1:10, 1:2, 1:30 and 1:15, respectively. The diluted samples were filtered using a 0.2 mm syringer filter (Corning) and measured using an inductively-coupled plasma spectrophotometer for Zn and Cu.

The analytical accuracy of the method for mineral anlysis was tested using standard reference material (SRM) obtained from the National Institute of Standards and Technology (NIST SRM 1577b, bovine liver, USA). The certified Zn and Cu values of NIST bovine Liver 1577b were 127±16 g/g and 160±8 g/g, respectively, and the measured Zn and Cu values obtained in our laboratory were 120±7 g/g and 154±7 g/g, respectively; this demonstrated the analytical accuracy of our method, as the measured values were 94.5 % of the certified value for Zn and 96.0 % for Cu(n=3).

Serum leptin assay

The quantitative sandwich enzyme immunoassay technique was used for the serum leptin assays using anti-mouse leptin conjugate (Quantikine M, mouse leptin immunoassay, R&D Systems, Inc. USA) by commercial instruction. For the leptin assays, blood samples were clotted for 2 hours at room temperature before centrifuging for 10 minutes at 2000 x g. The serum was removed and assayed immediately or aliquoted and stored at 20C.

RT-PCR for leptin mRNA gene expression

Adipose tissue RNA was extracted by the guanidinium-thiocyanate method[12] using Trizol Reagent (Gibco BRL, USA). To remove fat, the method was modified for adipose tissue by including an additional low speed spin and by increasing the volume of Trizol Reagent by 1.5 fold.

For first-strand cDNA synthesis, 100 ng of RNA from each sample were reverse transcribed using 20 U of AMV reverse transcriptase and Oligo-p(dT)15 1X random examers (Roche Diagnostics, USA). The resulting cDNAs were PCR-amplified by using a mixture of the corresponing forward and reverse primers. Primers for the leptin gene were designed on the basis of the sequences reported by the Pub-Med database for rats: forward primer 5 AAG AAG ATC CCA GGG AGG AA and reverse primer 5 TCA TTG GCT ATC TGC AGC AC (size 320 bp), and forward primer 5 ATC GTG GGG CGC CCC AGG CAC and reverse primer 5 CTC CTT AAT GTC ACG CAC GAT TTC for -actin (size 543 bp), as the internal standard. After each cDNA template had been amplified with β-actin, target leptin cDNA was amplified with the corresponding primers, with the same amount of cDNA template as was used for β-actin amplification. The PCR reactions were carried out on aliquots of the cDNA preparation, in 20 L volumes containing 2 l 10X PCR buffer, 1 l 2.5 mM dNTPs, 0.4 l of each target gene primer, 2.4 l cDNA template, 0.2 l *Taq* DNA polymerase (Advanced Biotechnologies, USA), and 0.2 l 5X Qual-up solution; autoclaved distilled water was added to make up to 20 l. The PCR reactions were implemented at 95C for 10 min, then 35 cycles at 95 C for 1 min, 60C for 1 min and 72C for 1 min, with a final extension at 72C for 5 min. The PCR products were separated on 1.2 % agarose gel and visualized by ethidium bromide staining. The amplified products were photographed with a digital camera for comparisons of band intensities.

Statistical analysis

Data were expressed as means ± SD and were anal-

Table 3. Rat body weight and body weight gain (g)[1,2,3]

Zn dietary Period	Normal pellet diet					Zn-adequate (30mg/kg)	Zn-depletion (1mg/kg)		Zn-repletion (50mg/kg)	
Weeks	On arrival (n=26)	1st week (n=26)	2rd week (n=26)	3th week (n=26)	4th week (n=26)	5th week (n=26)	6th week (n=18)	7th week (n=18)	8th week (n=9)	9th week (n=9)
Body weight(g)	135.6±5.2^{a}	197.4±7.6^{b}	247.1±10.4^{c}	286.9±13.2^{d}	307.6±16.6^{e}	335.3±19.2^{f}	364.2±21.5^{g}	362.9±17.1^{g,h}	370.0±24.6^{g,h}	381.7±27.1^{h}
Body weight gain(g)	-	61.8±6.5^{a}	49.7±4.7^{b}	39.8±5.9^{c}	20.7±5.9^{d}	27.7±5.2^{d,e}	28.9±4.1^{e}	1.3±4.8^{f}	7.1±3.3^{f}	11.8±4.6^{f}

1 Rats consumed normal pellet diet ad libitum for 4 wks of adaptation and growth. Then, all rats were provided with the Zn-adequate diet for following one wk. After one wk of Zn-adequate diet, rats (n=8) were killed. The remaining 18 rats were then provided with Zn-depletion diet during another following two wks. After then, rats (n=9) were killed, and the remaining 9 rats were then provided with Zn-repletion diet during the last two wks, after then, the rats (n=9) were killed.
2 Rat body weight was measured weekly.
3 Statistical significance were analyzed by one-way ANOVA among the wks and each weekly mean body weight and body weight gain were compared by Turkey HSD test at $p<0.05$.

yzed with the SPSS program. Differences were considered significant at $p<0.05$. Statistical analyses of the data were performed by one-way ANOVA to test the effect of the three different Zn dietary period groups (Zn-adequate, Zn-depletion and Zn-repletion period). Weekly comparisons for body weight and body weight gain within groups, and for other variables within dietary periods, were performed using the Turkey HSD test.

RESULTS

Body weight and body weight gain

Body weight and body weight gain of rats during the experimental diet period are shown in Table 3. Weekly body weights during Zn-adequate and Zn-depletion period were different weekly ($p<0.05$), but not that much different between Zn-depletion and Zn-repletion period. Mean body weight at the end of the Zn-adequate period (335.3±19.2 g) was significantly lower than weights at the end of each of the two weeks of the Zn-depletion period (364.2±21.5g and 362.9±17.1g) or at the end of each of the two weeks of the Zn-repletion period (370.0±24.6g and 381.7±27.1g) at $p<0.05$. In this study, body weight change due to Zn-depletion diet was decreased at the end of Zn-depletion period at 7th wk, comparing to at the end of Zn-adequate period at 5th wk.

Daily food intake

Daily food intake and energy intake during the different dietary zinc periods are shown in Fig 2. Daily food intake decreased during the Zn-depletion period (17.9±1.4 g/d) compared with the Zn-adequate period (19.6±1.8 g/d), and then increased back during the Zn-repletion period (18.6±1.6 g/d). Correspondingly, energy intake follows the same pattern as daily food intake during the three different dietary zinc periods.

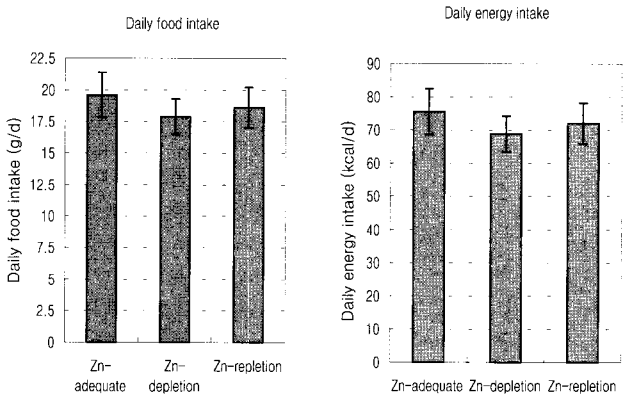

Fig 2. Daily food and energy intake in rats during three different dietary zinc period. No significance was shown among the dietary periods.

Zn and Cu concentrations in blood subfractions and liver

The zinc concentrations in blood subfractions, as in plasma, red blood cells (RBCs) and mononuclear cells (MNCs) are shown in Fig 3 depending on different dietary zinc periods. Zn concentration in plasma, RBCs and MNCs showed a consistent pattern depencling each of the different dietary zinc periods. Zinc concentration decreased during the Zn-depletion dietary period and increased back during the Zn-repletion period, in plasma, RBCs ($p<0.01$) and MNCs ($p<0.01$). The zinc concentration in MNCs was the most sensitive index of dietary zinc level in this study, as it was much more affected by the Zn-depletion diet, being decreased by about one fifth compared with the Zn-adequate diet.

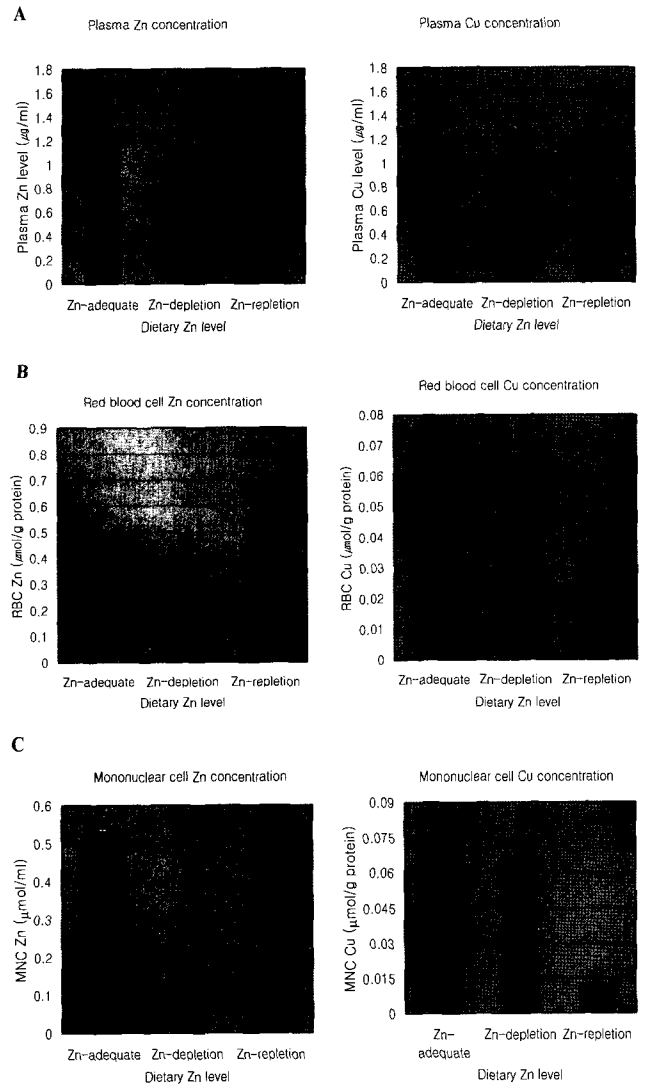

Fig 3. Plasma (A), red blood cell (B) and mononuclear cell (C) Zn and Cu concentration in rats consuming the three different dietary zinc level. Different superscripts indicate statistical significance at $p<0.05$.

Since copper competes with zinc absorption, blood copper was also measured during the different dietary zinc periods. During the Zn-depletion period, copper levels did not change in plasma, or slightly decreased in RBCs, compared to during the Zn-adequate period. However, the copper level was substantially increased in those MNCs where the zinc level was the most decreased during the Zn-depletion period.

Zinc concentrations in the liver during the different zinc periods are shown in Fig 4; these followed the same pattern of change as the concentrations of zinc in the blood, according to the different dietary zinc periods. During the Zn-depletion period, liver zinc was decreased to only 22% of its level of the Zn-adequate period, but increased back during the Zn-repletion period. Liver copper levels did not significantly differ between the various dietary zinc levels; this implies that copper concentrations during the Zn-repletion period didn't affect liver copper levels.

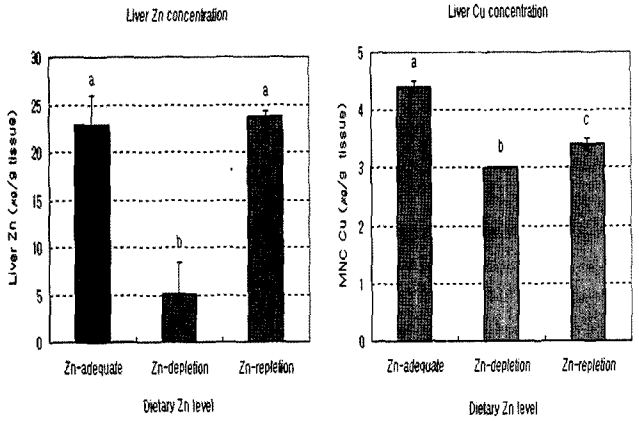

Fig 4. Liver Zn and Cu concentration during three different zinc dietary period in rats. Different superscripts indicate statistical significance at $p<0.05$.

Serum leptin concentration

Results for circulating serum leptin levels are shown in Fig 5. Serum leptin levels increased significantly during the Zn-depletion period (1236.0 ± 123.9 pg/ml), relative to the Zn-adequate dietary period (880.9 ± 112.5 pg/ml) at $p<0.05$. During the Zn-repletion period, serum leptin levels decreased almost to levels of the Zn-adequate period (998.3 ± 171.4 pg/ml) ($p<0.05$). Serum leptin concentration during Zn-depletion period was increased to approximately 140% of the Zn-adequate period, and then, during Zn-repletion perio, was decreased back to almost the same level (113%) of the Zn-adequate period.

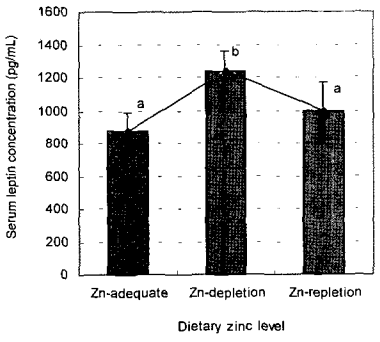

Fig 5. Serum leptin concentration in rats during three different dietary zinc period. Different superscripts indicate statistical signficance at $p<0.05$ by Turkey HSD, one-way ANOVA.

Leptin gene expression in adipose tissues

Leptin mRNA expressions in liguinal adipose tissue during the three different dietary Zn periods are shown

in Fig 6. Leptin mRNA expression during the Zn- depletion period is higher compared to the Zn-adequate period, but is not much different from that during the Zn-repletion period. Higher leptin mRNA expression levels during the Zn-depletion period, compared to the Zn-adequate period, are consistent with the results for circulating serum leptin levels which were also shown as higher during the Zn-depletion period, compared to either of the Zn-adequate or Zn-repletion periods.

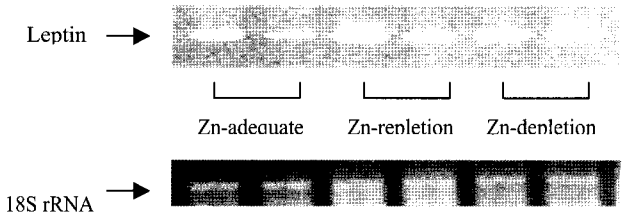

Fig 6. Leptin mRNA expression in liguinal adipose tissue in rats consuming three different level of dietary zinc. Total RNA isolated from individual rats was subjected to electrophoresis and RT-PCR.

Leptin mRNA expression in the various adipose tissues in rats during the 2 week Zn-depletion period is shown in Fig 7; the object is to compare tissue specificity in leptin gene expression among the different adipose tissues. Leptin mRNA was expressed as its highest level in liguinal subcutaneous adipose tissue, followed by abdominal and omental adipose tissue. The expression of Leptin mRNA during Zn-depletion period was the least in epididymal adipose tissue.

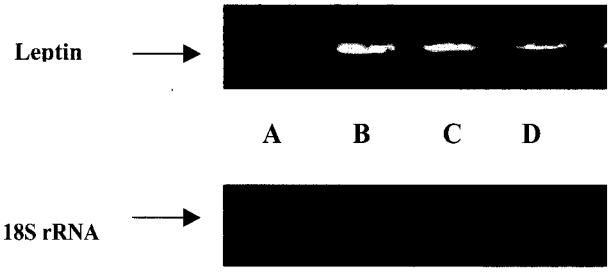

Fig 7. Leptin mRNA expression in the various adipose tissues in rats consuming Zn-depletion diet. Total RNA isolated from individual rats was subjected to electrophoresis and RT-PCR. (Lane A, epididymal adipose tissue; lane B, liguinal subcutaneous tissue; lane C, abdominal tissue; lane D, omental adipose tissue).

DISCUSSION

Leptin, a hormone that is secreted by adipose tissue in proportion to fat stores, regulates energy balance and appetite. Leptin is increased in adipose tissue in response to feeding, and results in decreased food intake.[13] Circulating serum leptin levels have been shown to be regulated in response to a variety of stimuli including food intake,[14-16] insulin,[17] glucocorticoids[18] and reproductive events.[19] However, the relationship between dietary zinc level and serum leptin concentration has not been extensively studied.

There are a few studies that reported that zinc depletion lowered leptin levels in normal rodents[10] and humans.[11] In another study, plasma leptin concentration and body weight were lower in Zn-deficient rats compared with pair-fed controls.[10] Also, in a human study, zinc depletion reduced leptin concentrations, which were then restored to normal values by Zn repletion.[11] As indicated in the above findings, low leptin concentrations are associated with weight loss and reduced body mass. On the other hand, high leptin concentrations are linked to obese states. Also, a zinc and leptin association was proven in metallothionein In their study, (MT)-null mice, since zinc is the dominant metal binding to metallothioneine[20] ; compared to the control, metallothionein-null mice became obese and showed higher plasma leptin levels and leptin mRNA gene expression. In their studys MT-null mice which showed higher liver zinc levels compared to the control, still showed obesity and higher leptin gene expression levels.

In contrast with the findings above, decreased food intake which would be the status of Zn-deficiency, induced high leptin mRNA in fasted adipose tissue in hamsters.[13),21] In the same study, tumor necrosis factor (TNF) and interleukin-1 (IL-1), which induced anorexia, increased leptin mRNA expression in adipose tissue; also, Zn-repletion conditions increased these endotoxins and cytokines. This finding supports our results, under which anorexia was evident and leptin mRNA was sisgnificantly induced during Zn-depletion conditions. It may be tentatively concluded from our study that decreased food intake during Zn-depletion results from increased leptin induction and secretion, thus sustaining an anorexic status during zinc deficiency.

The relationship between zinc deficiency and leptin level is considered to be based on neuropeptide Y (NPY) which acts as an appetite stimulant under anorexia. Generally, leptin as an appetite reducer, and NPY as an appetite stimulant, act reciprocally in the body as food intake regulators. Zn depletion upregulates NPY gene expression, whereas Zn repletion downregulates NPY expression.[2),22] It is assumed that NPY gene expression, as an appetite stimulant, is acting as a negative-feedback system under the decreased appetite conditions of Zn-deficiency. These studies suggested that NPY is functioning normally during Zn deficiency to restore food intake, and that other physiological changes may be considered to explain the decreased food intake during Zn-depletion. Increased serum leptin levels, as shown in the present study, would be another possible physiological

change under Zn-depletion conditions. From the results of the present study, increased serum leptin levels in Zn-depletion rats may act as a positive-feedback system, where Zn-deficiency induces decreased appetite and, in turn, leptin is increased as an appetite reducer.

In conclusion, increased leptin levels and leptin mRNA induction during Zn-depletion conditions may lower appetite, and this is the main symptom of Zn-deficiency in rats. This change in levels of circulating leptin and in leptin gene expression would be a direct result of, rather than a compensatory effect of, zinc deficiency. The reported study results, about the effects of interactions between zinc status and leptin level on food intake, are contradictory; however, this may be partly due to differences between animal or human subjects in Zn-depletion. Further studies on interactions among Zn, leptin, and neuropeptides are needed to further understand the complicated process of food intake regulation.

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