

Mg and Ca deficiencies additively increase the Zn concentrations and metallothionein expression in the rat liver

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Running head: Hepatic metallothionein in Mg-deficient rats

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Abbreviations: CAD, calcium-deficiency; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; MCD, magnesium- and calcium-deficiency; MGD, magnesium-deficiency; MT, metallothionein; TBARS, thiobarbituric acid-reactive substances.

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Abstract

Mg deficiency increases the concentration of Zn in the liver. We investigated the effect of Mg deficiency on the expression of Zn-regulating factors such as Zn transporters and metallothionein (MT) in the rat liver. Because Ca deficiency alleviates some of the effects of Mg deficiency, we also investigated the interactions associated with Ca and Mg deficiencies. Growing male rats were given a control diet, an Mg-deficient diet, a Ca-deficient diet, and an Mg- and Ca-deficient diet for 3 weeks. Mg and Ca deficiencies additively increased the mRNA levels of *MT-1* and *MT-2*, the MT protein concentration, and the concentration of Zn in the liver. The hepatic level of *Zip14* mRNA increased with Mg deficiency but not with Ca deficiency. The dietary treatments did not affect the mRNA levels of other Zn transporters such as *Zip1*, *Zip5*, *ZnT1*, *ZnT5*, and *ZnT6* in the liver. Ca deficiency was found to decrease the amount of femoral Zn and increase the serum Zn concentration. This did not occur in the case of Mg deficiency. These results suggest that Mg deficiency enhances hepatic Zn uptake by up-regulation of *Zip14* expression and increases the hepatic Zn concentration, leading to the enhancement of the MT expression. Ca deficiency causes a transfer of Zn from the bone to the liver, which increases the hepatic Zn concentration and, in turn, up-regulates the expression of MT. Because Mg and Ca deficiencies increase the hepatic Zn concentration and increase MT expression by different mechanisms, their effects are additive.

Mg deficiency is known to disturb Zn metabolism. Mg deficiency increases the concentration of Zn in the liver⁽¹⁻³⁾ while the Zn concentration decreases in the testis⁽¹⁾ and the heart⁽²⁾. Although the liver plays a central role in the metabolism of Zn, it has not been clarified how Mg deficiency enhances Zn accumulation in the liver.

Metallothionein (MT) is a cysteine-rich, low molecular weight protein consisting of four isoforms. Isoforms MT-1 and MT-2 are expressed in the liver⁽⁴⁾ and are known to have diverse functions in Zn homeostasis and in protection against heavy metal toxicity and oxidative stress⁽⁵⁾. The expression of MT is up-regulated by metals, including Zn, and by oxidative stress in the liver⁽⁶⁾. Because Mg deficiency is known to induce oxidative stress in the liver^(3,7,8), it has been speculated that oxidative stress caused by Mg deficiency would increase the hepatic Zn concentration through up-regulation of MT expression⁽³⁾.

Solute carrier family 39 members such as Zip1⁽⁹⁾, Zip5⁽¹⁰⁾, and Zip14⁽¹¹⁾ and solute carrier family 30 members such as ZnT1⁽¹²⁾, ZnT5⁽¹³⁾, and ZnT6⁽¹⁴⁾ are expressed in the liver. Solute carrier family 39 members increase the cytosolic Zn concentration by importing Zn into the cytosol from the extracellular fluid or intracellular organelles, and solute carrier family 30 members decrease the cytosolic Zn concentration by promoting Zn efflux from the cytosol⁽¹⁵⁾. Thus, these transporters affect Zn metabolism in the liver. However, the effect of Mg deficiency on the expression of Zn transporters has not been reported.

Mg frequently acts as a natural Ca antagonist⁽¹⁶⁾, and Ca deficiency was reported to ameliorate some of the effects of Mg deficiency⁽¹⁷⁾. Therefore, simultaneous Ca and Mg deficiency possibly suppresses Zn accumulation observed in the liver of Mg-deficient animals.

We performed an investigation to clarify the effects of Mg and Ca deficiencies, and the effects of their interactions on the Zn concentration and the expression of MT and Zn transporters in rat liver.

Materials and methods

Animals and diets

Twenty-four male specific pathogen-free Sprague-Dawley rats aged 4 weeks were purchased from SLC Japan (Shizuoka, Japan) and cared for according to the Guide for the Care and Use of Laboratory Animals (Animal Care Committee, Kyoto University). The rats were individually housed in stainless steel cages in a temperature-, humidity- and light-controlled room (24°C, 60%, and 12-h light/dark cycle, respectively). All rats were fed the AIN-93G diet⁽¹⁸⁾ for a 5-d adaptation period. Then the animals were divided into 4 groups of 6 rats as a 2 × 2 factorial arrangement (2 dietary Mg levels and 2 dietary Ca levels): one group was given the control diet, another group was fed an Mg-deficient (MGD) diet, another group was fed a Ca-deficient (CAD) diet, and the remaining group was fed an Mg- and Ca-deficient (MCD) diet. These diets were prepared on the basis of the AIN-93G diet⁽¹⁸⁾ with Mg- and Ca-free AIN-93G mineral premix. MgO was added at 26.1 mmol/kg for the control and CAD diets at the expense of cornstarch. CaCO₃ was added at 130 mmol/kg for the control and the MGD diets and at 13 mmol/kg for the CAD and the MCD diets. The dietary concentrations of Mg, Ca, and Zn are shown in Table 1. Animals were pair-fed with their respective experimental diets adjusted to match the feed intake of the MGD group. The rats were allowed free access to demineralized water for 3 weeks.

Data and sample collection

We measured the feed intake and body weight during the experiment. Feed efficiency was calculated from the ratio of weight gain to feed intake. Inflammatory skin lesions were evaluated by the inflammatory score⁽¹⁹⁾ with a slight modification based on the following criteria: 0 points, no skin lesion; 1 point, hyperemia of one ear accompanied by swelling; 2 points, hyperemia of both ears accompanied by swelling; 3 points, inflammation of one ear, including slough; 4 points, inflammation of both ears, including slough; 5 points, inflammation of both ears and tail.

Under isoflurane anesthesia, a blood sample was obtained from the abdominal aorta with a plastic syringe at the end of the experiment. Serum was separated by centrifugation at 3,000 × g for

30 min at 4°C. The left femur was removed, cleaned of adhering tissues, and weighed. The serum and femur samples were stored at -20°C until analyses. The liver was promptly excised, weighed, washed with ice-cold isotonic saline, placed in liquid nitrogen, and stored at -80°C until analyses.

Determination of minerals, thiobarbituric acid-reactive substances, and MT protein

The liver, femur, serum, and diet samples were wet-digested with trace-element-grade nitric acid and hydrogen peroxide (Wako Chemicals, Osaka, Japan). Then we determined the Mg, Ca, and Zn concentrations in the serum and tissue samples with an atomic absorption spectrophotometer (AA-6600F; Shimadzu, Kyoto, Japan).

The liver samples were homogenized in chilled saline and the homogenate was centrifuged at $105,000 \times g$ for 30 min at 4°C (CS100X, Hitachi Koki, Tokyo, Japan). The concentration of thiobarbituric acid-reactive substances (TBARS) in the supernatant was determined using a commercial kit (OXI-TEK TBARS Assay Kit; ZeptoMetrix, Buffalo, NY, USA) according to the instruction manual.

The total hepatic MT protein concentration was determined according to the Cd-Chelex method^(20,21). Briefly, the liver samples were homogenized in a 250-mM sucrose solution on ice and centrifuged at $105,000 \times g$ for 60 min at 4°C. The supernatant was mixed with 0.55-mM CdCl₂ in 20-mM Tris-HCl buffer (pH 8), incubated for 10 min at room temperature, and then heated at 100°C for 3 min. After cooling, the mixture was centrifuged at $8,000 \times g$ for 10 min at 4°C. Then, 16 mg/mL of the ion-exchange resin (Chelex 100; Bio-Rad Japan, Tokyo, Japan) was added to the supernatant and shaken for 30 min. The resin and precipitate were removed by centrifugation at $8,000 \times g$ for 10 min at 4°C. The Cd concentration in the supernatant was determined as the form binding to MT by an inductively-coupled plasma mass spectrometer (Elan 6000; Perkin Elmer, Waltham, MA, USA). The protein concentration of MT was calculated using a molecular weight of 6,600 for MT and the assumption that 7 g of Cd atoms corresponds to 1 mol of MT⁽²⁰⁾.

RNA preparation and quantitative RT-PCR

Total RNA was extracted from liver samples with TRIzol reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Absorbance at 260 nm was measured to quantify the RNA concentration, and simultaneously the ratio of absorbance at 260 nm to that at 280 nm was monitored to assess the purity of the RNA. Quantitative RT-PCR was carried out as described previously⁽²²⁾. The gene transcripts of *MT-1*, *MT-2*, *Zip1*, *Zip5*, *Zip14*, *ZnT1*, *ZnT5*, *ZnT6*, and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were amplified by cDNA-specific primers (Table 2). The relative levels of gene expression were normalized against the mRNA level of *Gapdh*.

Statistical analyses

Statistical analyses (except for the inflammation score) were performed using SAS statistical software (version 9.1; SAS Institute, Cary, NC, USA). The results were expressed as means with SEM. Data reflecting mRNA levels were log-transformed to provide an approximation of a normal distribution before analysis. The data were analysed as a 2×2 factorial using the GLM procedure. The statistical model included the main effects of dietary Mg, dietary Ca and their interaction. When an interaction was significant, the significant differences between means were evaluated using a multiple comparison procedure (Student-Newman-Keuls) or the significant difference between 2 means was evaluated using the two-sided *t*-test. The inflammation score data were expressed as means with SEM, and a significant difference between groups was determined each day by the Steel-Dwass test⁽²³⁾. When all rats in more than two groups had no inflammation, the data of one group were adopted as representative data in the statistical analysis because of methodological limitations. Statistical significance was considered to be $P < 0.05$.

Results

No skin lesions were observed in the control group and in the CAD group throughout the experiment. The inflammation score in the MGD group was significantly higher than that in the MCD group on day 4 ($P < 0.05$); this score peaked on day 5 and had essentially disappeared by day 13 (Fig. 1). Although two rats in the MCD group had a relatively high score on the last 2 d, the inflammation score did not differ significantly among the control, CAD, and MCD groups throughout the experiment.

The dietary treatments did not significantly affect the final body weight (Table 3). Ca deficiency and Mg deficiency did not affect body weight gain. However, interaction between Ca deficiency and Mg deficiency was significant ($P = 0.029$). Body weight gain did not differ among the control, CAD, and MCD groups but body weight gain was significantly lower in the MGD group than in the other groups ($P < 0.05$). The dietary treatments did not affect the feed intake because the animals were pair-fed. Therefore, feed efficiency was similarly affected as body weight gain by the dietary treatments.

The serum Mg concentration was significantly decreased by Mg deficiency ($P < 0.001$) but was significantly increased by Ca deficiency ($P < 0.001$) (Table 4). Interaction between Ca deficiency and Mg deficiency was also significant ($P = 0.008$). The serum Mg concentration was significantly lower in the Mg-deficient groups than in the Mg-sufficient groups ($P < 0.05$). The CAD group had a significantly higher serum Mg concentration than the control group ($P < 0.05$). Although the multiple comparison analysis showed that the serum Mg concentration did not differ significantly between the MCD group and the MGD group, the Mg concentration was 40% higher ($P = 0.01$ by *t*-test) in the MCD group than in the MGD group. The dietary treatments did not affect the serum Ca concentration. The dietary Ca concentration had a significant effect on the serum Zn concentration ($P < 0.001$); Ca deficiency significantly increased the serum Zn concentration. On the other hand, Mg deficiency did not affect the serum Zn concentration and did not affect the increase in serum Zn concentration caused by Ca deficiency.

Ca deficiency significantly decreased the femoral weight ($P = 0.009$); however, Mg deficiency did not affect either the femoral weight or the reduction of femoral weight caused by Ca deficiency. The amount of femoral Mg was significantly decreased by either Mg or Ca deficiency ($P < 0.001$). An interaction between Ca deficiency and Mg deficiency was also significant ($P < 0.001$). The Mg-deficient groups had significantly smaller amounts of femoral Mg than the Mg-sufficient groups ($P < 0.05$). The femoral Mg was significantly lower in the CAD group than in the control group ($P < 0.05$). Additionally, the amount of Mg was significantly lower in the MCD group than in the MGD group ($P < 0.05$). Ca deficiency significantly affected the amount of femoral Ca ($P < 0.001$); Ca deficiency decreased the amount of Ca. Mg deficiency did not affect the amount of femoral Ca and the extent of decreased femoral Ca by Ca deficiency. The amount of femoral Zn was significantly decreased by Ca deficiency ($P = 0.026$) but was slightly increased by Mg deficiency ($P = 0.007$). Interaction between Ca deficiency and Mg deficiency was not observed.

The dietary treatments did not affect the liver weight (Table 5). Either Mg or Ca deficiency significantly increased the Zn concentration in the liver (Mg deficiency, $P = 0.009$; Ca deficiency, $P = 0.004$). An interaction was not observed, indicating that Mg and Ca deficiencies additively increase the hepatic Zn concentrations. Because the dietary treatments did not affect liver weight, the amount of hepatic Zn was similarly affected as its concentration by the dietary treatments. Either Mg or Ca deficiency increased the concentration of MT protein in the liver (Mg deficiency, $P = 0.001$; Ca deficiency, $P = 0.003$). An interaction was not observed, indicating that Mg and Ca deficiencies additively increase the MT protein concentrations. The hepatic TBARS concentration was increased by Mg deficiency ($P = 0.034$), but Ca deficiency affected neither the hepatic TBARS concentration nor the extent of increased TBARS concentration by Mg deficiency.

The mRNA level of *MT-1* was significantly increased by either Mg or Ca deficiency in the liver (Mg deficiency, $P < 0.001$; Ca deficiency, $P = 0.001$) and an interaction was not observed (Table 6). The mRNA level of *MT-2* was significantly increased by either Mg or Ca deficiency in the liver ($P < 0.001$) and an interaction was not observed. These results indicate that Mg and Ca

deficiencies additively increase the mRNA levels of *MT-1* and *MT-2*. Mg deficiency was found to significantly increase the hepatic level of *Zip14* mRNA ($P = 0.014$) but Ca deficiency affected neither the mRNA level of *Zip14* nor the increase in mRNA level caused by Mg deficiency. The dietary treatments did not affect the mRNA levels of *Zip1*, *Zip5*, *ZnT1*, *ZnT5*, and *ZnT6*, indicating that the increased hepatic Zn concentration caused by Ca or Mg deficiency is not related to the mRNA levels of these transporters.

Discussion

Although Mg deficiency did not affect the serum Zn concentration, Mg deficiency increased the hepatic Zn concentration and the *Zip14* mRNA level without affecting the mRNA levels of the other Zn transporters. *Zip14* imports Zn into the cytosol through the plasma membrane in the liver⁽¹⁵⁾. The suppression of *Zip14* expression by a specific small interfering RNA decreases the extent of Zn uptake by the hepatocytes⁽²⁴⁾, and the up-regulation of *Zip14* expression is considered to increase the extent of hepatic Zn accumulation⁽¹¹⁾. These results suggest that Mg deficiency increases Zn uptake by the liver through up-regulation of *Zip14* expression at the mRNA level. Hepatic expression of *Zip14* is transcriptionally enhanced by interleukin-1 β ⁽²⁵⁾ and interleukin-6⁽¹¹⁾. These cytokine concentrations in the plasma were reported to increase in rats fed a low-Mg diet for 3 weeks⁽²⁶⁾. It is possible that the elevation of these cytokine levels up-regulates mRNA expression of *Zip14* in the livers of Mg-deficient rats.

Mg deficiency increases the concentration of MT protein with increasing mRNA levels of *MT-1* and *MT-2* in the liver. *MT-1* transcription is enhanced by increased cytosolic Zn concentrations and oxidative stress⁽⁶⁾. Thus, we consider that the expressions of *MT* mRNA and MT protein are enhanced by an increased Zn uptake through up-regulation of *Zip14* expression in the liver of Mg-deficient animals. This is supported by reports showing that over-expression of *Zip14* increased the mRNA expression of *MT-1* via an increased Zn uptake in HEK 293T cells⁽¹¹⁾ and that its down-regulation decreased the expression of *MT-1* mRNA through suppression of Zn uptake in cultured hepatocytes⁽²⁴⁾. Mg deficiency increased hepatic Zn concentration by 20% and increased hepatic MT expression by 40%. An experiment using subcutaneous injection with Zn indicated that MT concentration highly and linearly correlated with Zn concentration in the liver of rats and that MT concentration largely responded to changes in Zn concentration⁽²⁷⁾. Therefore, we consider that the increase in Zn concentration is enough to explain the enhancement of MT expression in the liver of Mg-deficient rats. Additionally, Mg deficiency induced oxidative stress, which is reflected by increased TBARS concentrations measured in this study. This is supported by previous reports^(3,7,8). Therefore, it is also likely that oxidative stress partly contributes to the up-regulation of expression of MT mRNA in the liver of Mg-deficient animals.

Hepatic Zn concentration is 2-fold lower in *MT-1* and *MT-2* null mice than in wild-type mice when excess Zn is provided, and 72% of the total Zn in the liver is associated with MT in the wild-type mice⁽²⁸⁾. Therefore, the large difference of MT expression probably affects the Zn concentration in the liver. On the other hand, it was reported that the total Zn concentration was not changed in rat liver even when Zn-bound MT was increased by 5- and 7-fold by cold stress and treatment with CCl₄, respectively⁽²⁹⁾. The administration of lipopolysaccharides was reported to cause approximately a 12-fold increase in the mRNA level of *MT-1* in mouse liver but did not affect the total Zn concentration⁽³⁰⁾. Mg deficiency was found to increase the expression of MT protein and *MT-1* mRNA by 40% and 7-fold, respectively. These results suggest that the increase in the MT expression is not enough to increase the Zn concentration in the liver of Mg-deficient animals. The increase in the MT expression does not contribute significantly to increasing the Zn concentration. We consider that Mg deficiency increases Zn uptake by enhancing *Zip14* expression in the liver, and that MT expression is up-regulated by an increased Zn concentration and possibly by oxidative stress. Then, MT traps Zn loaded to the liver by Mg deficiency.

The levels of circulating Zn were reported to decrease immediately after inducing *Zip14* expression in the mouse liver⁽¹¹⁾ and MT expression in the rat liver⁽²⁹⁾. However, the up-regulation

of *Zip14* mRNA and MT protein expression did not affect serum Zn concentration in the Mg-deficient rats. These results suggest that the serum Zn concentration is not affected by chronic up-regulation of expression of *Zip14* or MT in Mg-deficient rats. An adaptive mechanism of Zn may prevent hypozincemia in Mg-deficient rats with chronically enhanced expression of *Zip14* or MT.

In contrast to the observations in cases of Mg deficiency, Ca deficiency was found to increase the serum Zn concentration along with reducing the amount of femoral Zn. Bone is a major reservoir of Zn in the body, and Ca deficiency accelerates the release of Zn from bones. This is accompanied by bone resorption⁽³¹⁾. A low Ca diet was reported to increase the plasma Zn concentration⁽³²⁾. This is supported by the present results. Therefore, we suggest that Ca deficiency increases the serum Zn concentration through stimulation of bone resorption in the present experiment. The present results indicated that Ca deficiency increased the concentrations of Zn and MT in the liver. Furthermore, Ca deficiency increased the mRNA levels of *MT-1* and *MT-2* in the liver without affecting the mRNA levels of Zn transporters. As mentioned above, MT concentration highly and linearly correlated with Zn concentration in the liver of rats and that MT concentration largely responded to changes in Zn concentration⁽²⁷⁾. These results suggest that the enhanced Zn supply to the liver increases the hepatic Zn concentration in Ca-deficient rats, leading to the up-regulation of MT expression through increases in the mRNA expressions of *MT-1* and *MT-2*. Then hepatic MT traps Zn supplied from bone in Ca-deficient animals.

Reductions in the dietary Zn beyond the capacity to maintain homeostasis lead to the utilization of Zn from pools located in the bone and the liver⁽³³⁾, and hepatic MT serves as a Zn reservoir that can be utilized when Zn is deficient⁽²⁸⁾. The amount of hepatic Zn was 0.68 μmol higher in the CAD group than in the control group. The amount of Zn in the left femur was 0.25 μmol lower in the CAD group than in the control group; femoral Zn was 17% lower in the CAD group than in the control group. According to the equation of House and Wastney⁽³⁴⁾, the amount of whole bone Zn was calculated to be 23 μmol in the rats weighing 177 g. This body weight corresponds to the final mean body weight for both groups. Therefore, Zn loss from the whole bone was estimated to be 3.9 μmol (23 μmol multiplied by 0.17) during Ca deficiency based on the assumption that each bone responds to Ca deficiency in a similar manner as the femur. These results show that the increase in hepatic Zn corresponds to 17.4% (0.68 μmol divided by 3.9 μmol) of Zn loss from the skeletal tissues. This suggests that the liver only partly saves the released Zn from the bone during periods of Ca deficiency.

Skin lesions are a clinical sign of inflammation in Mg-deficient rats⁽³⁵⁾. Mg deficiency-induced skin lesions were observed to appear shortly after the initiation of the experiment. This was resolved by simultaneous Ca deficiency. Additionally, Mg deficiency reduced weight gain and feed efficiency, which was also alleviated by simultaneous Ca deficiency. These results indicate that Ca deficiency rescues the effects of Mg deficiency on inflammation and growth retardation. Mg acts as a natural Ca antagonist⁽¹⁶⁾ and the reduction of the extracellular ratio of Mg to Ca was reported to lead to an inflammatory response⁽³⁶⁾. The dietary restriction of Ca with hypocalcemia was reported to suppress the incidence of skin lesions in Mg-deficient rats, suggesting that hypocalcemia could be beneficial by decreasing external Ca availability and Ca entry, and thus, decreasing the intracellular Ca concentration in Mg-deficient animals⁽¹⁷⁾. On the other hand, Ca deficiency did not affect the serum Ca concentration in the present experiment probably because Ca levels in Ca-deficient diets were 20-fold higher in the present experiment than in the above-cited report⁽¹⁷⁾. Therefore, it is unlikely that the amelioration of skin lesions and growth retardation are related to the reduction of the antagonistic effect of extracellular Mg on Ca entry in the present experiment. Bones hold the major pool of Mg in the body and release Mg in response to Mg deficiency⁽³⁷⁾. This is supported by the present results, which indicate that Mg deficiency causes a significant decrease in the femoral Mg level without affecting the femoral Ca level. Furthermore, the required dietary Mg decreases linearly with a reduction in dietary Ca in rats fed with low Mg diets (based on the recovery body weight gain)⁽³⁸⁾. The restriction of dietary Ca decreases the amount of femoral Mg

and increases the serum Mg concentration, regardless of the dietary Mg level in the present experiment. This suggests that the supply of Mg from the bone to the soft tissues is enhanced by Ca deficiency even in Mg-deficient animals. We suggest that Ca deficiency ameliorates Mg deficiency by increasing the Mg supply from bone. This leads to alleviation of skin lesions and growth retardation. However, it must be noted that the restriction of dietary Ca did not allow complete recovery from the low concentration of serum Mg in Mg-deficient rats. The MCD rats remained Mg deficient.

The MGD and MCD groups suffered severe Mg deficiency and the CAD and MCD groups suffered severe Ca deficiency in the present experiment because the Mg or Ca concentration in the deficient diets was set as low as 10% of the requirement levels. Mg or Ca deficiency shown in the present experiment may not take place actually. On the other hand, the Ca deficiency did not affect serum Ca concentration and body weight gain. These results suggest that Ca homeostasis is maintained in the Ca-deficient animals and the extent of Ca deficiency does not reach to induce pathological conditions in the present experiment. As reviewed by Nielsen⁽³⁹⁾, severe Mg deficiency induces many pathological conditions in experimental animals but subclinical Mg deficiency *per se* does not. On the other hand, severe Mg deficiency (14% of the requirement) increased hepatic Zn concentration within 10 days in rats and the increase in hepatic Zn concentration was also observed but delayed when rats suffered moderate Mg deficiency (22% of the requirement)⁽³⁾. Therefore, we can postulate that MT and *Zip14* mRNA expression is also up-regulated when rats are given a moderate Mg-deficient diet for long time.

In conclusion, Mg and Ca deficiencies up-regulate MT expression in the liver through increasing Zn uptake by different mechanisms. Mg deficiency up-regulates *Zip14* mRNA expression, and Ca deficiency increases the Zn supply from the bone to the liver. As a result, Mg and Ca deficiencies additively increase the expression of MT and the Zn concentrations in the liver.

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Table 1. Mineral concentration of the experimental diets (mmol/kg diet)

	Diet			
	Control	MGD	CAD	MCD
Ca	136.3	131.7	13.1	13.2
Mg	20.37	1.69	20.29	1.65
Zn	0.61	0.61	0.61	0.61

MGD, Mg-deficient; CAD, Ca-deficient; MCD, Mg- and Ca-deficient.

Table 2. Sequence of the PCR primers for amplification

Gene	Forward primer	Reverse primer	GenBank accession number
<i>MT-1</i>	5'-CACCAGATCTCGGAATGGAC-3'	5'-GCAGCAGCTCTTCTTGCAG-3'	NM_138826
<i>MT-2</i>	5'-ACCTCCTGCAAGAAAAGCTG-3'	5'-ACTTGTCCGAAGCCTCTTTG-3'	NM_001137564
<i>Zip1</i>	5'-CCTGGCTACCTGTCTTCTGG-3'	5'-CTCTTGCAAGGGGAAGTAA-3'	NM_001134577
<i>Zip5</i>	5'-CCTCGGGCCTAGACCTCTT-3'	5'-AGCTGGGAACCATTCAGACA-3'	NM_001108728
<i>Zip14</i>	5'-TTCCTCAGTGTCTCACTGATTAA-3'	5'-GGAAAAGTGCGTTAGAGAGC-3'	NM_001107275
<i>ZnT1</i>	5'-AACACCAGCAATTCCAACG-3'	5'-CCACTGGATCATCACTTCTCAA-3'	NM_022853
<i>ZnT5</i>	5'-TGACACAAACATGCTGACACC-3'	5'-CATGACTGTGGGCGTGACT-3'	NM_001106404
<i>ZnT6</i>	5'-GTGCCATCAACGTGCTGT-3'	5'-GGTGAGGTAAGTATAAGCAGTTAAAG-3'	NM_001106708
<i>Gapdh</i>	5'-ACAACCTTTGGCATCGTGGA-3'	5'-CTTCTGAGTGGCAGTGATGG-3'	NM_017008

MT, metallothionein; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

Table 3. Effects of Mg and Ca deficiencies on growth parameters in rats(Mean values with their standard errors, *n* 6)

Group	Control		MGD		CAD		MCD		Effect (<i>P</i> =)		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mg	Ca	Mg × Ca
Dietary Mg level	Sufficient		Deficient		Sufficient		Deficient				
Dietary Ca level	Sufficient		Sufficient		Deficient		Deficient				
Initial body weight (g)	105.4	3.9	105.5	3.3	106.1	1.6	105.8	3.9	0.875	0.982	0.950
Final body weight (g)	177.7	5.6	170.0	2.5	177.4	2.6	179.2	3.7	0.252	0.450	0.229
Weight gain (g/d)	3.62 ^a	0.15	3.23 ^b	0.08	3.57 ^a	0.11	3.67 ^a	0.07	0.071	0.188	0.029
Feed intake (g/d)	11.7	0.1	11.5	0.1	11.6	0.1	11.6	0.1	0.808	0.157	0.325
Feed efficiency (g/g)	0.31 ^a	0.01	0.28 ^b	0.01	0.31 ^a	0.01	0.32 ^a	0.01	0.365	0.381	0.023

MGD, Mg deficient; CAD, Ca deficient; MCD, Mg and Ca deficient.

When the interaction was significant, the significant differences among means were evaluated using Student-Newman-Keuls test; ^{a,b}mean values within a row with unlike superscript letters were significantly different (*P* < 0.05) among the 4 groups.

Table 4. Effects of Mg and Ca deficiencies on mineral concentrations in serum and the amount of mineral in rat femur(Mean values with their standard errors, *n* 6)

Group	Control		MGD		CAD		MCD		Effect (<i>P</i> =)		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM			
Dietary Mg level	Sufficient		Deficient		Sufficient		Deficient				
Dietary Ca level	Sufficient		Sufficient		Deficient		Deficient				
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mg	Ca	Mg × Ca
Serum											
Mg (μmol/L)	840 ^b	33	226 ^{c*}	12	1202 ^a	80	313 ^{c*}	25	<0.001	<0.001	0.008
Ca (mmol/L)	2.70	0.14	2.85	0.09	2.69	0.12	2.35	0.25	0.577	0.129	0.156
Zn ((μmol/L)	26.6	1.2	24.0	0.6	36.2	1.4	33.6	3.1	0.177	<0.001	0.962
Femur											
Wet weight (g/femur)	0.71	0.03	0.84	0.04	0.66	0.04	0.69	0.04	0.056	0.009	0.264
Mg (μmol/femur)	44.4 ^a	1.2	15.2 ^c	0.8	29.6 ^b	1.2	10.3 ^d	0.8	<0.001	<0.001	<0.001
Ca (mmol/femur)	1.66	0.05	1.73	0.02	0.90	0.03	1.00	0.03	0.203	<0.001	0.716
Zn (μmol/femur)	1.39	0.06	1.42	0.02	1.14	0.04	1.36	0.07	0.007	0.026	0.086

MGD, Mg deficient; CAD, Ca deficient; MCD, Mg and Ca deficient.

When an interaction was significant, the significant differences among means were evaluated using Student-Newman-Keuls test or the significant difference between 2 means was evaluated using the two-sided *t*-test; ^{a,b,c,d} mean values within a row with unlike superscript letters were significantly different (*P* < 0.05) among the 4 groups; *mean values were significantly different (*P* = 0.01) between the 2 groups.

Table 5. Effects of Mg and Ca deficiencies on Zn, metallothionein and thiobarbituric acid-reactive substances concentrations in rat liver
(Mean values with their standard errors, *n* 6)

Group	Control		MGD		CAD		MCD		Effect (<i>P</i> =)		
Dietary Mg level	Sufficient		Deficient		Sufficient		Deficient		Mg	Ca	Mg × Ca
Dietary Ca level	Sufficient		Sufficient		Deficient		Deficient				
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM			
Wet weight (g/liver)	6.38	0.40	6.22	0.15	6.95	0.29	7.01	0.21	0.865	0.204	0.690
Zn (μmol/g)	0.41	0.02	0.49	0.03	0.48	0.02	0.60	0.05	0.009	0.004	0.471
Zn (μmol/liver)	2.63	0.13	3.04	0.12	3.31	0.15	4.18	0.29	0.003	<0.001	0.240
MT protein (μg/g)	83.4	9.0	120.6	12.3	128.1	12.7	212.2	28.2	0.001	0.003	0.194
TBARS (nmol/g)	45.4	3.2	52.8	1.7	42.7	5.7	53.8	4.5	0.034	0.834	0.658

MGD, Mg deficient; CAD, Ca deficient; MCD, Mg and Ca deficient; MT, metallothionein; TBARS, thiobarbituric acid-reactive substances.

Table 6. Effects of Mg and Ca deficiencies on mRNA levels of *MT* and Zn transporters in rat liver(Mean values with their standard errors, *n* 6)

Group	Control		MGD		CAD		MCD		Effect (<i>P</i> =)		
	Sufficient		Deficient		Sufficient		Deficient				
Dietary Mg level	Sufficient		Deficient		Sufficient		Deficient				
Dietary Ca level	Sufficient		Sufficient		Deficient		Deficient				
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mg	Ca	Mg × Ca
MT-1	1.00	0.31	6.87	2.53	5.30	1.23	12.24	3.23	<0.001	0.001	0.222
MT-2	1.00	0.32	11.38	4.92	5.87	1.11	15.23	3.10	<0.001	<0.001	0.226
Zip1	1.00	0.10	1.02	0.15	0.94	0.13	1.02	0.24	0.292	0.868	.0533
Zip5	1.00	0.55	0.62	0.10	1.53	0.54	1.00	0.13	0.068	0.907	0.668
Zip14	1.00	0.14	1.42	0.22	1.07	0.18	1.75	0.18	0.014	0.383	0.659
Znt1	1.00	0.18	0.90	0.09	0.92	0.12	1.11	0.23	0.759	0.494	0.481
Znt5	1.00	0.15	1.10	0.09	1.05	0.19	1.15	0.12	0.690	0.553	0.940
Znt6	1.00	0.10	0.85	0.11	0.75	0.20	0.71	0.08	0.537	0.111	0.463

MGD, Mg deficient; CAD, Ca deficient; MCD, Mg and Ca deficient; MT, metallothionein.

Data are expressed as relative values with respect to the control group.

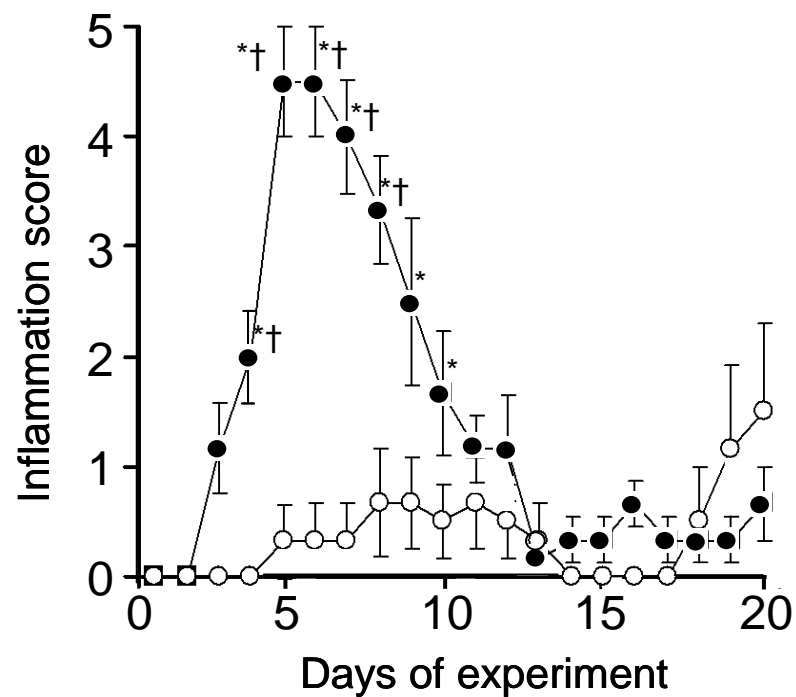


Fig. 1. Effects of Mg and Ca deficiencies on the inflammation score in the skin of rats

Values are expressed as means with their SE ($n\ 6$).

*Mean values were significantly different from the control and the Ca-deficient groups ($P < 0.05$).

†Mean values were significantly different from the Mg and Ca- deficient group ($P < 0.05$).

Lesions were not observed in the control group and the Ca-deficient group throughout the experimental period.

Mg-deficient group (-●-); Mg- and Ca-deficient group (-○-).