

Maternal Zinc Deficiency Raises Plasma Prolactin Levels in Lactating Rats¹

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ABSTRACT There is an inverse relation between zinc (Zn) intake and plasma prolactin in men and nonpregnant women. Whether a relation exists in lactating women is unknown, despite the potential consequences of perturbations in prolactin regulation on lactation performance. We examined the effects of low Zn intake on prolactin concentration, the prolactin regulatory pathway in the pituitary gland, and lactation performance in lactating rats. Female rats were fed diets containing 7 (zinc deficient; ZD), 10 (marginally zinc deficient; MZD) or 25 mg Zn/kg (control) from 70 d pre-conception to lactation d 11. Rats were killed, pituitary glands dissected, and tissues and plasma collected and analyzed for prolactin concentration. Pituitary gland pituitary factor 1 (Pit-1), dopamine 2 receptor (D2R), and prolactin receptor mRNA expression were measured in the pituitary gland. Liver, mammary gland, plasma, and milk Zn were measured. Milk intake of the pups was also recorded. Plasma prolactin concentration was higher in rats fed the ZD (125.9 μ g/L) diet compared with control rats (21.7 μ g/L). Pituitary gland prolactin concentration was higher in rats fed the ZD diet (69.8 mg/g total protein) compared with controls (29.0 mg/g). Plasma Zn concentration was lower in rats fed the MZD and ZD diets, and mammary gland and milk Zn concentrations were lower in rats fed the ZD diet compared with control rats. Rats fed the ZD diet had lower D2R, prolactin receptor, and Pit-1 mRNA levels, whereas rats fed the MZD diet had lower prolactin receptor and Pit-1 mRNA levels compared with control rats. Milk intake was lower in pups of rats fed the MZD and ZD diets. Our results suggest that marginal Zn nutriture may compromise milk production despite increased prolactin levels. In addition, increased circulating prolactin concentration is not due to altered nursing behavior, but may be due to alterations in the prolactin regulatory pathway in the pituitary gland. *J. Nutr.* 134: 1314–1319, 2004.

KEY WORDS: • lactation • pituitary gland • milk • zinc • prolactin

Although severe zinc (Zn) deficiency is considered to be rare, mild or moderate Zn deficiency is believed to be widespread throughout the world. Pregnant and lactating women require ~1–2 mg more Zn/d than do nonpregnant, nonlactating women as a consequence of fetal growth and milk production, respectively; thus, pregnant and lactating women are at increased risk for Zn deficiency if dietary Zn intake is inadequate or of low bioavailability. In fact, it has been estimated that 82% of pregnant women worldwide consume an inadequate amount of Zn compared with the U.S. recommended dietary allowance, and the prevalence of inadequate Zn intake may approach 100% in developing countries (1). Lactating women were also shown to consume less than the recommended amount of Zn (2,3).

Breast-fed infants are dependent on the mother's ability to produce an adequate volume of milk that contains optimal levels of nutrients during this period of rapid growth; both lactogenesis (the initiation of milk production) and galactopoiesis (the maintenance of established milk production) are regulated through complex hormonal interactions (4). Prolactin is the primary hormone responsible for regulating milk protein synthesis and maintaining lactation; some physiologic conditions such as dieting (5) and malnutrition (6) were shown to affect serum prolactin

levels in lactating women. McCrory et al. (5) showed that plasma prolactin concentration is higher in lactating women who are dieting. Furthermore, Lunn et al. (6) showed that plasma prolactin concentration is higher in lactating women with poor nutrition, and decreases when they consume dietary supplements to increase their energy intake. Zn deficiency has been associated with hyperprolactinemia in men (7–9); however, the effects of Zn deficiency on prolactin in women during lactation are currently unknown.

The mechanisms through which prolactin exerts its actions are complex. During lactation, prolactin is secreted primarily by the pituitary gland (10) and binds to the prolactin receptor in the mammary gland initiating both the JAK/STAT5 (11) and mitogen-activated protein kinase (12) signaling cascades. The prolactin receptor is also expressed in other tissues, including the pituitary gland itself where it may serve as the receiving end of a negative feedback loop (13). Additionally, dopamine (14), which is produced by hypothalamic neurons that synapse upon the pituitary gland, binds to the dopamine 2 receptor to inhibit prolactin secretion from pituitary cells (15). Prolactin gene expression is mediated in part by the binding of the pituitary-specific transcription factor 1 (Pit-1)³ to the Pit-Oct-Unc domain of the prolactin promoter (16). These all represent potential

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³ Abbreviations used: D2R, dopamine 2 receptor; MZD, marginally zinc deficient; Pit-1, pituitary specific transcription factor 1; ZD, zinc deficient.

regulators of prolactin expression, synthesis, and release and, as such, may be affected by Zn deficiency.

In this study, we hypothesized that similar to the observations of Zn deficiency-induced hyperprolactinemia in men, marginal Zn intake during lactation would result in increased prolactin concentration, thereby affecting milk production. Furthermore, we address several regulatory aspects of the prolactin pathway in the pituitary gland that may be affected by a diet marginally or moderately deficient in Zn.

MATERIALS AND METHODS

Diets. Rats were fed an egg white-based semipurified experimental diet based on the AIN-93 recommendations (17). The experimental diets differed only in Zn content, containing 25 mg Zn/kg (control), 10 mg Zn/kg (marginally Zn deficient, MZD), or 7 mg Zn/kg (moderately Zn deficient, ZD), which was confirmed by atomic absorption spectrophotometry (18).

Rats. This study complied with the NIH guidelines and was administered under the auspices of Animal Resource Services of the University of California, Davis, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. Virgin Sprague-Dawley rats ($n = 42$, 14 rats \times 3 diets; \sim 250 g) were obtained from a commercial source (Charles River). The rats were maintained in stainless steel hanging cages in a temperature-controlled facility with a 12-h dark:light cycle and allowed to consume purified, deionized water ad libitum. After consumption of a standard nonpurified diet (Ralston Purina) for a 7-d acclimation period, rats ($n = 14$ /diet) were randomly assigned to 1 of the 3 experimental diets. Rats were fed the diets from 70 d pre-conception through d 10 of lactation. Throughout the experiment, food intake was recorded every other day and animal weight was recorded weekly. Following birth, the litter size and pup weights were recorded. On d 2 of lactation, litters were culled to 8 pups/dam.

For determination of milk intake on d 10 of lactation ($n = 6$ dams/diet), pups were removed from dams for 4 h and manually stimulated to urinate. Litter weight was recorded, and the litter was returned to each dam. Pups were allowed to suckle for 60 min and reweighed after manual stimulation of urination. Maternal nursing behavior (gathering of pups to nest, time until initiation of nursing, and duration of suckling) was observed. Estimation of milk volume was calculated as the difference between pre- and postsuckle litter weight.

For tissue and plasma collection on d 11 of lactation, dams ($n = 10$ dams/diet) were separated from the pups. After 4 h, rats were anesthetized by CO₂ inhalation and blood was collected into heparinized vials by heart puncture. Plasma was separated by centrifugation at $1500 \times g$ for 15 min at 4°C and stored at -20°C until analysis. Dams were quickly decapitated; pituitary glands were dissected, placed in 1 mL of RNase inhibitor solution (RNAlater, Ambion), and stored at -20°C until RNA extraction or snap frozen in liquid nitrogen and stored at -70°C until determination of prolactin concentration. Liver and mammary gland were dissected and frozen at -20°C until measurement of tissue Zn concentration. Milk was collected on d 10 of lactation (5 rats/diet) as previously described

(19). Milk volume was recorded and milk Zn concentration was measured.

Measurement of prolactin concentration in plasma and pituitary gland. Pituitary glands ($n = 5$ dams/diet) were homogenized in 2 mL of 0.1 mol/L PBS, pH 7.4, with protease inhibitors [0.2 mmol/L 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.1 mmol/L EDTA, 13 μ mol/L bestatin, 1.4 μ mol/L M E-64, 0.1 μ mol/L leupeptin, 0.03 μ mol/L aprotinin; Sigma], with 6 strokes of a hand-driven Dounce glass-Teflon homogenizer. Total protein concentration was determined by the Bradford assay. Pituitary gland homogenate and plasma ($n = 5$ dams/diet) were analyzed for prolactin concentration using a commercially available ELISA kit (Amersham Pharmacia Biotech). Prolactin concentration was expressed relative to total protein.

Mineral analysis. Liver, mammary gland, and milk ($n = 5$ dams/diet) were digested in ultrapure concentrated nitric acid for 1 wk and wet-ashed using a modification of Clegg et al. (18). Plasma was digested with 0.1 mol/L ultrapure nitric acid at room temperature for 5 d. Zn concentration was analyzed by flame atomic absorption spectrophotometry as previously described (19).

Measurement of mRNA expression by semiquantitative RT-PCR. After the removal of RNase inhibitor solution, pituitary gland total RNA ($n = 4-6$ dams/diet) was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction using the method of Chomczynski and Sacchi (20) (TriZol, Invitrogen) and stored at -80°C until mRNA isolation. Poly T+ columns (Invitrogen) were used to extract mRNA from 20 μ g of total RNA according to the manufacturer's instructions. mRNA (200 ng) was used for first strand cDNA synthesis using the cDNA Cycle Kit (Invitrogen) according to the manufacturer's instructions. PCR coamplification of dopamine 2 receptor (D2R), prolactin receptor, or Pit-1 with β -actin was conducted with Taq polymerase (Platinum Taq, Invitrogen) using gene-specific primers (Table 1). The reaction consisted of an initial denaturation of 60 s at 94°C, followed by the gene-specific conditions listed in Table 1. Duplicate PCR reactions were performed for each mRNA sample. Pituitary gland gene primers were designed using the Primer3 program (21), and β -actin primers were purchased (Classic QuantumRNA β -actin Internal Standards, Ambion). Prolactin receptor primers were designed to detect both long and short isoforms, although the long form predominates in the pituitary gland (22). Modified β -actin primers (competimers), which are not extendable by the polymerase, were also included in the PCR reaction to reduce the quantity of the β -actin transcript and allow accurate normalization (Ambion). PCR cycles were limited to within an experimentally determined linear range, and the appropriate ratio of β -actin primers to competimers was determined empirically according to the manufacturer's instructions. PCR products were separated on a 2% agarose gel by electrophoresis, stained by Syber-Gold nucleic acid stain (Molecular Probes), and visualized under UV light. Digital images were captured (Chemidoc, BioRad), band intensity was quantified using Chemidoc software (BioRad Quantity One, Version 4.2.2), and gene expression was expressed as band intensity relative to β -actin band intensity.

Statistical analysis. Results are presented as means \pm SD; statistical comparisons were made using one-way ANOVA and post-tested using the Tukey-Kramer test (Prism Graph Pad). Differences were considered significant at $P < 0.05$.

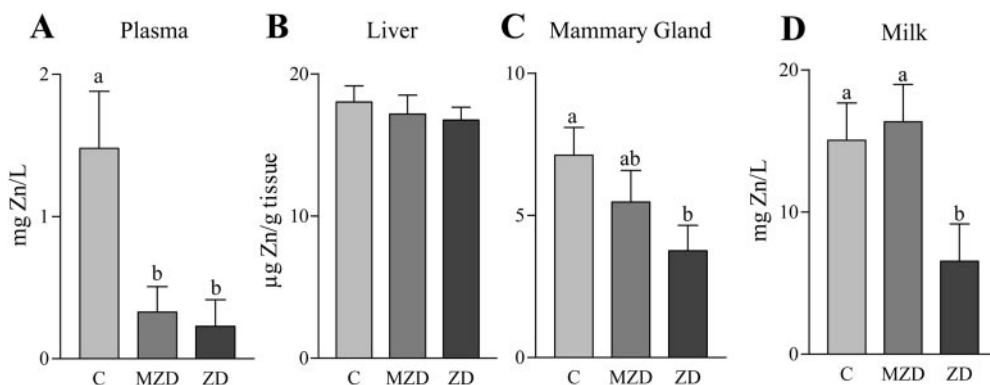
TABLE 1

Primers used for semiquantitative RT-PCR¹

Gene	Accession number	Primers (5' \rightarrow 3')	PCR cycle conditions
Prolactin receptor	NM_012630	F: 298gcccacgaaccaaagtggaagcagt321 B: 805gcccaggaaactggtggaagatgc782	34 cycles: 94°C, 30 s; 68°C, 3 min
D2R	NM_012547	F: 1254tgcccctgtcctactggtggtcc1273 B: 1760cgcccttgcaaacacagctccttg91733	34 cycle s: 94°C, 30 s; 68°C, 3 min
Pit-1	NM_013008	F: 296cggaggaccaacagcctctgaat319 B: 830tgcccttggtgcaaacacact807	32 cycles: 94°C, 30 s; 68°C, 3 min

¹ Primer F and B denote forward and backward primers, and numerical subscripts denote location of primer relative to the start site (+1).

FIGURE 1 Concentrations of Zn in plasma (A), liver (B), mammary gland (C), and milk (D) of lactating rats fed the control (C), MZD, or ZD diet analyzed by atomic absorption spectrophotometry. Values are means \pm SD, $n = 5/\text{diet}$. Means without a common letter differ, $P < 0.05$.



RESULTS

Maternal food intake, litter size, and dam weight were not significantly affected by maternal diet (data not shown), indicating that these diets did not cause severe Zn deficiency. Pup weight at birth was not affected by maternal diet. However, body weight at d 11 was higher ($P < 0.05$) for pups from control dams (25.9 ± 2.9 g) than from dams fed the MZD (20.1 ± 5.6 g) and ZD diets (22.7 ± 2.8 g).

Plasma Zn concentration was significantly lower in dams fed the MZD and ZD diets compared with control rats (Fig. 1A, $P < 0.05$). Diet did not affect the liver Zn concentration (Fig. 1B), whereas mammary gland Zn concentration was lower in rats fed the ZD diet than in those fed the control diet (Fig. 1C, $P < 0.05$). Milk Zn concentration was lower in rats fed the ZD diet than in rats fed the MZD and control diets (Fig. 1D, $P < 0.05$).

Rats fed the ZD diet had a significantly higher plasma prolactin concentration than rats fed the control diet (Fig. 2A, $P < 0.05$), and rats fed the MZD diet had a plasma prolactin concentration ($45.4 \mu\text{g/L}$) more than double that of control rats ($21.7 \mu\text{g/L}$), but the difference was not significant ($P = 0.10$). In addition, prolactin concentration in the pituitary gland from rats fed the ZD diet was higher (Fig. 2B, $P < 0.05$) than in rats fed the control diet. Although there was no effect on maternal nursing behavior, dams fed the MZD and ZD diets had a lower milk volume at lactation d 10 than dams fed the control diet (Fig. 3, $P < 0.05$), despite a higher plasma prolactin concentration in dams fed the ZD diet.

Rats fed the ZD diet had lower D2R ($P < 0.01$), prolactin receptor ($P < 0.05$), and Pit-1 ($P < 0.05$) mRNA levels than

rats fed the control diet (Fig. 4). Rats fed the MZD diet had lower prolactin receptor ($P < 0.05$) and Pit-1 ($P < 0.01$) mRNA levels; however, D2R mRNA levels were not different from rats fed the control diet.

DISCUSSION

Previous studies in men showed an inverse relation between Zn intake and plasma prolactin concentration. For example, an association between Zn deficiency and hyperprolactinemia in uremic men was observed (7,8). Furthermore, Mahajan et al. (8) reported that plasma Zn concentration and serum prolactin concentration were inversely correlated, and Zn treatment increased plasma Zn level and reduced serum prolactin concentration. Thus, both clinical and biochemical data support a link between Zn deficiency and the regulation of prolactin levels. To our knowledge, there is currently no information concerning the existence of this relation in lactating women; however, the association between higher plasma prolactin levels in women in developing countries (6) in combination with observations of inadequate Zn nutrition (1) in these same populations suggest that there may be a relation. Furthermore, the consequences of perturbations in prolactin regulation may have a greater effect in lactating women due to the role of this hormone in the establishment and maintenance of lactation.

Similar to reports on Zn-deficient men, we observed higher plasma prolactin concentration in lactating female rats fed a moderately Zn-deficient diet. There are several factors that affect the plasma prolactin concentration, and they are driven by maternal or neonatal stimuli. One possibility is that the higher prolactin level is the result of changes in suckling

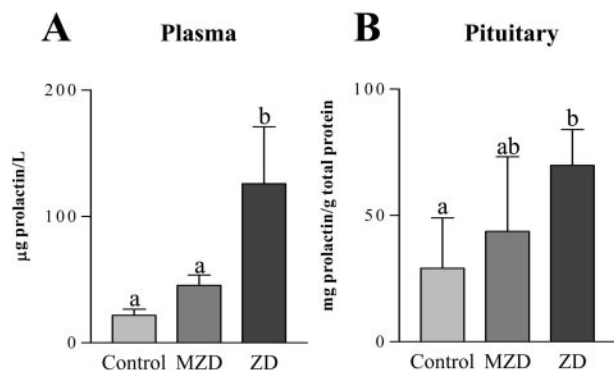


FIGURE 2 Concentrations of plasma (A) and pituitary (B) prolactin of lactating rats fed the control (C), MZD, or ZD diet analyzed by ELISA. Values are means \pm SD, $n = 5/\text{diet}$. Means without a common letter differ, $P < 0.05$.

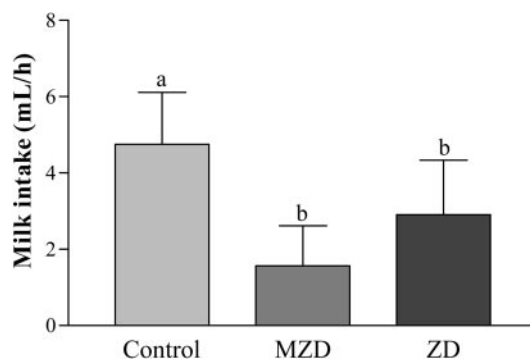


FIGURE 3 Milk intake of pups from lactating rats fed control (C), MZD, or ZD diet. Values are means \pm SD, $n = 6/\text{diet}$. Means without a common letter differ, $P < 0.05$.

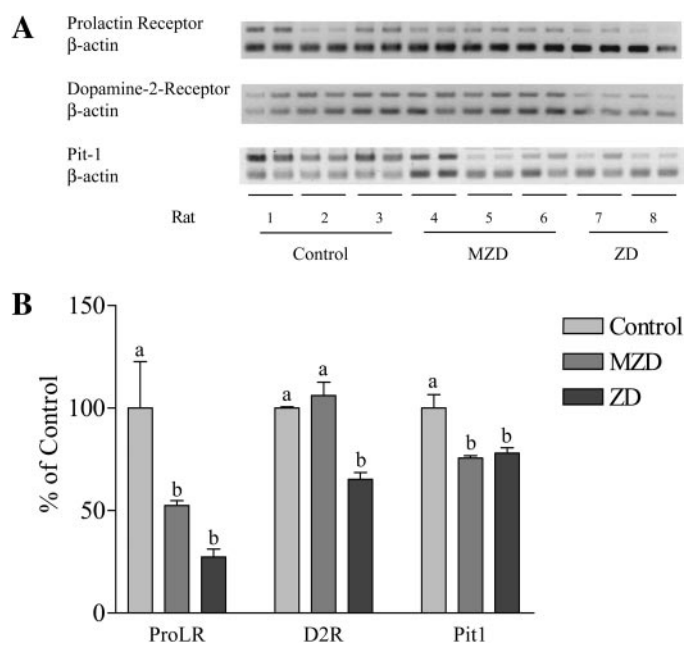


FIGURE 4 Effect of control ($n = 6$ /diet), MZD ($n = 6$), or ZD diet ($n = 4$) on the mRNA expression of the prolactin receptor (ProLR), D2R, and Pit-1 in the pituitary glands of lactating rats analyzed by semiquantitative RT-PCR. (A) Representative gels of RT-PCR products, run in duplicates. (B) Relative mRNA expression following densitometry analysis. mRNA expression of rats fed control diet was set at 100%. Values are means \pm SD. Means without a common letter differ, $P < 0.05$.

behavior, such as increased suckling frequency or duration by the neonate, because it was documented that prolactin secretion is correlated with nursing frequency (23,24). However, the litters were of equal size and the separation of the dams from the pups was carefully held constant to ensure that plasma prolactin reflected basal levels, and not those induced by suckling. Although no differences in maternal or neonate suckling behavior were noted in response to food restriction, as described by McGuire et al. (25), the possibility of increased suckling intensity was not addressed, and this may play a role in mediating prolactin levels.

We observed lower milk Zn concentration in the ZD group and decreased milk volume in both the MZD and ZD groups, suggesting that the higher prolactin levels were inadequate to maintain milk Zn concentration and milk volume. It was shown in humans that maternal plasma prolactin concentration generally increases under conditions of negative energy balance, and it was hypothesized that this may serve to protect lactation (26). In addition, several investigators documented increased β -casein expression in mouse mammary HC11 cells exposed to prolactin (27), indicating that prolactin can stimulate processes crucial for milk synthesis. Furthermore, prolactin exposure of prostate cells (28) and HC11 cells (Kelleher and Lönnerdal, unpublished observations) stimulates Zn uptake, which suggests that increased circulating prolactin levels could be a mechanism for increasing milk Zn concentration during periods of poor maternal Zn intake. Interestingly, our results suggest that increased plasma prolactin concentration neither maintains milk Zn concentration nor protects lactation during moderate maternal Zn deficiency. However, we observed that dams fed a marginally zinc-deficient (MZD) diet had decreased plasma Zn concentration and a trend toward increased plasma prolactin concentration, whereas mammary gland and milk Zn levels did not differ from control dams. This

suggests that the maintenance of milk Zn concentration may be facilitated by increasing circulating prolactin levels, possibly through changes in mammary gland zinc transporter expression during periods of marginal Zn intake (19). Dams fed a moderately Zn-deficient diet (ZD) had decreased plasma, mammary gland, and milk Zn concentrations despite increased pituitary and plasma prolactin concentrations, which suggests that pup Zn nutriture may be compromised despite the elevation in prolactin concentration during more severe maternal Zn deficiency.

Domellöf et al. (29) recently showed that milk Zn concentration is independent of maternal Zn status in humans. However, in that study, milk Zn concentration was measured during late lactation, whereas in the present study, milk Zn concentration was measured in mid-lactation. Milk Zn concentration decreases throughout lactation (30,31) and may be more sensitive to external factors early in lactation. In addition, milk Zn is considerably higher in rats than in humans (30,32), which reflects the high requirement of Zn for growth of the large number of pups in rats. It is likely that differences in species and lactation stage contributed to the discrepancies observed between the 2 studies. Interestingly, the results of Domellöf et al. (29) paralleled our findings for the MZD group, which also showed no correlation between plasma Zn concentration and milk Zn concentration. On the other hand, we observed a positive association in rats fed a more severely Zn-deficient diet. Thus, it is possible that the level of zinc deficiency in dams fed the MZD diet more closely resembles that of the human population studied by Domellöf et al. (29), whereas the ZD group may reflect a more severe Zn deficiency.

Mechanisms responsible for the effect of low Zn intake on plasma prolactin concentration may be attributed to prolactin production or secretion from the pituitary gland. Biochemically, Zn affects many factors involved in prolactin secretion. For example, the addition of Zn to cell media was shown to suppress prolactin release in both bovine and rat pituitary gland cultures (33,34). In addition, human prolactin was shown to bind Zn in secretory granules, leading to aggregation and stabilization of the hormone (35), and a mutant form of human prolactin unable to bind Zn was not efficiently secreted and quickly degraded (36). We were not able to show an increase in prolactin secretion when GH3 cells were made Zn deficient by exposure to DTPA or Chelex-treated media (Chowanadisai et al., unpublished observations), which suggests that there may be other mechanisms involved in the increased prolactin secretion observed in this study, or that dopamine receptors are required for the interaction with Zn. We observed lower Pit-1 expression in the pituitary glands of dams fed a MZD or ZD diet. Because Pit-1 upregulates prolactin expression, a decrease in Pit-1 expression would be expected to decrease prolactin expression, which was not observed in this study. The interaction of Pit-1 with the prolactin promoter also involves other factors such as Pitx1 and Pitx2 (37), and it is possible that there are other factors that affect the prolactin pathway, such as binding affinity to the prolactin promoter. Dopamine was shown to inhibit the transcriptional activation of the Pit-1 promoter (38). However, we also observed a decrease in D2R expression along with the decrease in Pit-1 expression, which suggests that dopamine may not be responsible for the downregulation of Pit-1. There are also other hormones that were shown to regulate Pit-1 expression, such as GHRH, GHRP-6, and ghrelin (39,40). Thus, it is likely that there are other factors that can affect the transcription, translation, or secretion of prolactin during Zn deficiency, and further investigation into these mechanisms is warranted.

The regulation of prolactin production and secretion by the pituitary gland is the result of complex positive and negative feedback mechanisms acting on the pituitary gland itself. Thus, it is possible that alterations in these secondary negative feedback responses result in an increase in circulating prolactin. The decrease in prolactin receptor expression observed in this study could possibly be explained by the high circulating concentrations of prolactin. Our observations that D2R expression was reduced by a ZD diet and prolactin receptor expression was reduced by MZD and ZD diets support this possibility. In the rat pituitary GC cell line, the addition of prolactin to the medium downregulated the expression of prolactin, as measured by a reporter construct containing the prolactin promoter, but only with cotransfection with prolactin receptor (41). *Ex vivo*, prolactin treatment inhibited the proliferation of lactotrophs from wild-type females, but not from D2R null female mice, which exhibit chronic hyperprolactinemia (42). Schuff et al. (43) suggested that this is due to downregulation or desensitization of the prolactin receptor due to the hyperprolactinemia. Furthermore, hyperprolactinemia induced by chronic treatment with the dopamine antagonist, domperidone, was shown to alter pituitary dopamine receptor sites (44). Thus, the downregulation of D2R expression observed in the Zn-deficient dams could be responsible for the increased prolactin concentrations. Further studies will be required to uncover the mechanisms behind these observations.

One interesting question is whether hyperprolactinemia during lactation has any negative consequences for the mother. Hyperprolactinemia due to pituitary tumors, amenorrhea, or antipsychotic medication can decrease bone mineral density and may increase the risk for osteopenia (45–48). In addition, lactation is associated with bone loss (49–51). Thus, it is conceivable that hyperprolactinemia due to maternal Zn deficiency could induce bone loss or exacerbate the decrease in bone density associated with lactation and result in increased risk for osteoporosis.

This study shows that feeding a Zn-deficient diet to lactating rats can increase plasma prolactin and alter the expression of genes involved in regulatory pathways in the pituitary gland. Although it remains to be determined whether hyperprolactinemia has a negative effect on offspring development, it was shown that maternal Zn deficiency is associated with poor infant growth and development. The effect of maternal Zn deficiency on prolactin regulation in humans remains to be determined and merits further study.

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