Effects of Zinc Deficiency and Supplementation on Leptin and Leptin Receptor Expression in Pregnant Mice

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Leptin is an adipose-derived hormone that primarily regulates energy balance in response to nutrition. Human placental cells produce leptin, whereas murine placental cells produce soluble leptin receptors (Ob-R). However, the roles of these proteins during pregnancy have not been elucidated completely. As an essential metal, zinc (Zn) is central to insulin biosynthesis and energy metabolism. In the present study, the effects of Zn deficiency and supplementation on maternal plasma leptin and soluble Ob-R regulation in pregnant mice placentas were examined using enzyme-linked immunosorbent assay, reverse transcription-polymerase chain reaction, and Western blotting. Nutritional Zn deficiency significantly reduced plasma insulin concentrations and fetal and placental weights in pregnant mice. Plasma leptin concentrations in pregnant mice also increased 20- to 40-fold compared with those in non-pregnant mice. Although dietary Zn deficiency and supplementation did not affect plasma leptin concentrations in non-pregnant mice, Zn-deficient pregnant mice had significantly reduced plasma leptin concentrations and adipose leptin mRNA expression. In contrast, Zn-supplemented pregnant mice had increased plasma leptin concentrations without increased adipose leptin mRNA expression. Placental soluble Ob-R mRNA expression also decreased in Zn-deficient mice and tended to increase in Zn-supplemented mice. These results indicate that Zn influences plasma leptin concentrations by modulating mRNA expression of soluble Ob-R in the placenta, and leptin in visceral fat during pregnancy. These data suggest that both adipose and placenta-derived leptin system are involved in the regulation of energy metabolism during fetal growth.

Key words leptin; leptin receptor; dietary zinc; pregnancy; placenta


dramatic changes in energy homeostasis are well recognized in pregnant women, with major features including increased food intake, decreased insulin sensitivity, and hyperlipidemia. Placental leptin may regulate conceptus growth and placental function by regulating glucose metabolism and insulin sensitivity. However, the roles of leptin in these tissues during pregnancy have not been fully elucidated.

Zinc (Zn) is an essential metal and present in various mammalian organs, particularly as a cofactor of approximately 300 known enzyme active sites that are critical for normal growth and development in humans and animals. Indeed, Zn nutrition during pregnancy is critical, and Zn deficiency has been shown to exert various critical influences on embryo survival. In particular, Zn deficiency during pregnancy and lactation has adverse effects in laboratory animals, causing congenital malformation and embryonic and fetal death. Zn also plays an important role in the synthesis, storage, and secretion of insulin, acting as an insulin mimic or insulin stimulator, and reducing diabetic hyperglycemia. Potentially, the anti-hyperglycemic effects of Zn are associated with the influence of leptin. The effects of Zn on the leptin system have been demonstrated, although in non-pregnant subjects.

Given the role of maternal leptin system during pregnancy, we investigated the effects of nutritional Zn deficiency and supplementation on the expression of leptin and leptin receptor in pregnant mice.

MATERIALS AND METHODS

Animals Pregnant ICR female mice (10 weeks old) were purchased at day 1 (DI) of gestation from Japan SLC Inc. (Shizuoka, Japan). Groups of five mice were housed in plastic cages and were maintained with a 12-h light/dark cycle, at 20°C and 50% humidity. AIN-93M (50 mg Zn/kg: control diet, Oriental Yeast Co., Osaka, Japan), Zn-deficient (7 mg Zn/kg: Oriental Yeast Co., Osaka, Japan).
ZnDF) or Zn-supplemented diets (170 mg Zn/kg: ZnSP) and water were provided ad libitum. Zn concentrations of ZnDF and ZnSP diets were selected based on previous studies.22–25 Non-pregnant mice were maintained under the same conditions as pregnant mice. All animal experiments were approved by the Animal Research Committee of Osaka Ohtani University in accordance with the Guidelines on Animal Experiments at Osaka Ohtani University and Japanese Government Animal Protection and Management Laws.

Measurements of Plasma Leptin and Insulin Concentrations, and Blood Glucose On D14 or D17 of gestation, pregnant mice were placed under general anesthesia and blood samples were collected from abdominal aortas. After euthanasia, placentas and fetuses were removed from uteruses, and maternal parametrial visceral fat was collected. Plasma was obtained by centrifugation of blood samples, and leptin and insulin concentrations were measured using IBL Mouse Leptin Assay Kit (Immuno-Biological Laboratories, Gunma, Japan) and Ultra Sensitive Mouse Insulin ELISA kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan), respectively. Plasma glucose was measured using a Glucose C-II test wako kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). All animal procedures were repeated identically in non-pregnant mice.

Measurements of Tissue Zn Concentrations Placentas and fetuses were weighed, wet-ashed using nitric acid at 80°C, and filtered using a Cosmocience Filter W (0.45µm, 13 mm, Nakalai Tesque, Kyoto, Japan). Plasma samples were wet-ashed in a similar manner. Zn concentrations were determined by flame atomic absorption spectrophotometry (Z-2300 Polarized Zeeman Atomic Absorption Spectrophotometer, HITACHI, Tokyo, Japan) according to the manufacturer’s instructions.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from placentas using TRIZol reagent (Life Technologies, CA, U.S.A.) according to the manufacturer’s instructions. Total RNA was extracted from visceral fat using an RNaseasy Lipid Tissue Mini kit (Qiagen, Hilden, Germany). RNA concentrations in samples were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, U.S.A.). Total RNA obtained from placenta and adipose tissues was reverse transcribed to cDNA using a ReverTra Ace qPCR RT kit or ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) in accordance with the manufacturer’s instructions. Real-time RT-PCR amplification and analysis was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad, CA, U.S.A.). Reactions were performed in final volumes of 20 µL containing 0.5 µM primers, 4 µL cDNA, and 10 µL SYBR Green Realtime PCR Master Mix Plus (TOYOBO, Osaka, Japan) in accordance with the manufacturer’s instructions. Real-time RT-PCR primer sequences were as follows: leptin,21 5'-AAG TCC AGG ATC ACA CCA AAA CC-3' (forward), 5'-GCT CCA TCT TGG ACA AAC TCA GAA-3' (reverse); soluble Ob-R,26 5'-TGT TAT ATC TGG TTA TGG ATG GG-3' (forward), 5'-CAT TAA ATG ATT TAT TAT CAG ATG TGC-3' (reverse); Zn concentrations of ZnDF pregnant mice significantly increased to 130% (p < 0.01) of that observed in control pregnant mice at D17. Zn concentrations in placentas and fetuses did not significantly differ between ZnSP and control pregnant mice, but were significantly lower in ZnDF pregnant mice than in control pregnant mice (Table 1). Zn concentrations did not significantly differ between ZnSP and control pregnant mice but were significantly lower in ZnDF pregnant mice than in control pregnant mice (Table 1). Zn concentrations did not significantly differ between ZnSP and control pregnant mice but were significantly lower in ZnDF pregnant mice than in control pregnant mice (Table 1). Zn concentrations did not significantly differ between ZnSP and control pregnant mice but were significantly lower in ZnDF pregnant mice than in control pregnant mice (Table 1). Zn concentrations did not significantly differ between ZnSP and control pregnant mice but were significantly lower in ZnDF pregnant mice than in control pregnant mice (Table 1). Zn concentrations did not significantly differ between ZnSP and control pregnant mice but were significantly lower in ZnDF pregnant mice than in control pregnant mice (Table 1). Zn concentrations did not significantly differ between ZnSP and control pregnant mice but were significantly lower in ZnDF pregnant mice than in control pregnant mice (Table 1).

RESULTS

Effects of Maternal Zn Deficiency and Supplementation on Growth and Fertility Numbers of fetuses (Table 1) and resorption sites (data not shown) were unaffected by dietary Zn deficiency and supplementation. The daily food intake did not significantly differ between the three groups of mice (data not shown). In addition, maternal weights did not significantly differ between the three groups of mice (Table 1). Fetal and placental weights and maternal parametrial visceral fat masses did not significantly differ between ZnSP and control pregnant mice, but were significantly lower in ZnDF pregnant mice than in control pregnant mice (Table 1). Zn concentrations in placentas and fetuses did not significantly differ between ZnSP and control pregnant mice but were significantly lower in ZnDF pregnant mice than in control pregnant mice (Fig. 1). The plasma Zn concentration in ZnDF pregnant mice significantly decreased to 40% (p < 0.01), whereas that observed in ZnSP pregnant mice significantly increased to 130% (p < 0.01) of that observed in control pregnant mice at D17.

Effects of Maternal Zn Deficiency and Supplementation on Plasma Insulin and Glucose Concentrations Plasma insulin concentrations in ZnSP non-pregnant mice were significantly increased to 300%, whereas in ZnDF non-pregnant mice insulin concentrations tended to decrease to 50% of that observed in control non-pregnant mice (Fig. 2). During pregnancy, plasma insulin concentrations did not significantly differ between ZnSP and control mice, but in ZnDF dams at D17, insulin concentrations decreased to 15% of that observed
in control mice (Fig. 2).

Plasma glucose concentrations were unaffected by dietary Zn intake (Table 1).

**Effects of Maternal Zn Deficiency and Supplementation on Plasma Leptin Concentrations** Plasma leptin concentrations did not significantly differ between the three groups of non-pregnant mice (Fig. 3). However, plasma leptin concentrations in control pregnant mice remarkably increased to 20–40-fold compared with that observed in non-pregnant mice (Fig. 3). At D17 of gestation, plasma leptin concentrations significantly increased to 170% in ZnSP mice, whereas leptin concentrations in ZnDF mice remarkably decreased to 15% of that observed in control mice (Fig. 3).

**Effects of Maternal Zn Deficiency and Supplementation on the Expression of Leptin and Leptin Receptor** Visceral

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Table 1. Effects of Maternal Zn Deficiency and Supplementation on Growth and Fertility

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>ZnDF</th>
<th>ZnSP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>38.8±1.9</td>
<td>36.2±3.6</td>
<td>39.5±1.5</td>
</tr>
<tr>
<td>D14</td>
<td>50.8±2.9</td>
<td>49.6±2.1</td>
<td>55.2±3.4</td>
</tr>
<tr>
<td>D17</td>
<td>64.1±5.9</td>
<td>58.7±2.2</td>
<td>68.8±6.5</td>
</tr>
<tr>
<td><strong>Fetal weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D14</td>
<td>0.23±0.02</td>
<td>0.27±0.04</td>
<td>0.25±0.01</td>
</tr>
<tr>
<td>D17</td>
<td>1.13±0.05</td>
<td>0.96±0.04*</td>
<td>1.13±0.07</td>
</tr>
<tr>
<td><strong>Placental weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D14</td>
<td>0.085±0.010</td>
<td>0.078±0.006*</td>
<td>0.089±0.009</td>
</tr>
<tr>
<td>D17</td>
<td>0.115±0.014</td>
<td>0.094±0.008*</td>
<td>0.108±0.006</td>
</tr>
<tr>
<td><strong>Visceral fat mass (g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>0.590±0.107</td>
<td>0.466±0.149</td>
<td>0.494±0.166</td>
</tr>
<tr>
<td>D14</td>
<td>0.405±0.060</td>
<td>0.365±0.111</td>
<td>0.457±0.097</td>
</tr>
<tr>
<td>D17</td>
<td>0.455±0.083</td>
<td>0.232±0.098*</td>
<td>0.430±0.192</td>
</tr>
<tr>
<td><strong>Number of fetuses per dam</strong></td>
<td>14.2±2.8</td>
<td>15.6±0.9</td>
<td>15.2±1.5</td>
</tr>
<tr>
<td><strong>Plasma glucose (mg/dL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>156.6±28.1</td>
<td>140.7±21.2</td>
<td>153.8±24.7</td>
</tr>
<tr>
<td>D14</td>
<td>139.7±11.5</td>
<td>149.8±17.9</td>
<td>150.7±10.6</td>
</tr>
<tr>
<td>D17</td>
<td>147.0±12.4</td>
<td>151.6±25.9</td>
<td>142.2±12.1</td>
</tr>
</tbody>
</table>

Values are presented as the mean±S.D. (n=5). *p<0.05, **p<0.01 compared with the control value.
fat leptin mRNA expression significantly reduced on D14 of pregnancy, but by D17 was recovered to levels observed in non-pregnant mice, except in ZnDF mice. Leptin mRNA expression in ZnDF pregnant mice reduced to 50% of that observed in control pregnant mice. In contrast, the Zn-deficient diet had no effect on leptin mRNA expression in non-pregnant mice (Fig. 4). No leptin mRNA was detected in mice placentas (data not shown).

At D17 of gestation, the expression of soluble Ob-R mRNA in the placentas of ZnDF mice significantly reduced to 75%, whereas that observed in ZnSP mice insignificantly increased to 120% of control levels (Fig. 5). The expression of Ob-R protein in the placenta of ZnDF mice significantly decreased to 40%, and insignificantly increased to 140% of the control levels in ZnSP mice (Fig. 6).

**Effects of Maternal Zn Deficiency and Supplementation on the Expression of GLUT1** At D17 of gestation, the expression of placental GLUT1 mRNA did not significantly differ between ZnSP and control mice, but that observed in ZnDF mice significantly decreased to 70% of that observed in control mice (Fig. 7).

**DISCUSSION**

In the present study, we investigate the effects of dietary Zn deficiency and supplementation on the leptin system during pregnancy. In these experiments, ZnDF pregnant mice had significantly reduced plasma leptin concentrations and adipose leptin mRNA expression, whereas ZnSP pregnant mice had increased plasma leptin concentrations without increased adipose leptin mRNA expression. In contrast, neither Zn deficiency nor supplementation affected plasma leptin concentrations in non-pregnant mice. Moreover, placental soluble Ob-R mRNA expression decreased in ZnDF pregnant mice but tended to increase with Zn supplementation. These data indicate that Zn nutrition influences plasma leptin concentrations by modulating soluble Ob-R mRNA expression in placenta and leptin mRNA expression in visceral fat during pregnancy. Hence, it appears that both adipose and placenta contribute to leptin-mediated energy metabolism during fetal growth.

The daily food intake did not significantly differ between the three groups of pregnant mice, and ZnSP pregnant mice did not show the symptom of acute zinc toxicity (data not shown). Schulz et al. demonstrated that the total areas of placenta and junctional zone were smaller in the food-restricted pregnant mice than in control pregnant mice, but not the labyrinth zone. It suggests that placentas from mothers exposed to food restriction must preserve the placental labyrinth zone at the expense of the junctional zone. When analyzing placental histopathology, we did not identify the difference in the average of blood space area within three randomly selected images of the junctional zone between the three groups (control: 100.0±6.4%; ZnDF: 102.5±5.2%; ZnSP: 105.0±4.8%).
ZnSP: 102.9±5.3%). Further study will be needed to establish precisely how deficiency and supplementation of Zn and placenta-derived leptin system alter the labyrinth zone. In contrast, plasma, placenta, and fetus Zn concentrations significantly decreased in ZnDF pregnant mice. Moreover, fetal and placental weights and maternal parametrial visceral fat masses were significantly lower in ZnDF than in control pregnant mice. The expression of placental GLUT1 mRNA in ZnDF pregnant mice was significantly decreased than that observed in control pregnant mice, whereas that observed in ZnSP mice was unchanged. Placental GLUT1 plays an important role in transporting glucose from mother to fetus.29,30 Thus, supplying glucose by use as a placental and fetal fuel may be decreased in ZnDF pregnant mice. Moreover, plasma insulin and leptin concentrations in ZnDF pregnant mice significantly decreased compared with control pregnant mice. These results suggest that the expression of placental GLUT1 mRNA may decrease to avoid the elevation of fetal blood glucose levels. Maternal GLUT1-mediated glucose transport is upregulated by AMP-activated protein kinase, the activity of which is regulated by nutrient availability and circulating hormones such as insulin and leptin.31–33 However the regulation of placental GLUT1 expression remains unclear.34 In accordance with the importance of Zn for fetal development, Zn deficiency was previously associated with congenital malformations, and embryonic and fetal deaths in laboratory animals.14,15

Plasma leptin concentration remarkably increased from D14 through D17 of pregnancy compared with that observed in non-pregnant mice. These observations may reflect the high growth rate of fetuses relative to that of placentas during the gestational period from D14 to D17. Tomimatsu et al. suggested that plasma leptin concentrations dramatically rise from D10.5, peak during the third trimester of pregnancy, and rapidly decrease to non-pregnant levels during lactation.9,35,36 These dramatic increases suggest that leptin may be particularly important during stages of fetal growth and development (late pregnancy) but less so during stages of organogenesis (early pregnancy). During pregnancy, leptin may act on the hypothalamus and regulate energy expenditure, neuroendocrine functions, glucose metabolism, and insulin sensitivity in dams. Furthermore, leptin is transferred to the fetus and may regulate fetal development and growth.3

In non-pregnant mice, plasma leptin concentrations were unaffected by dietary Zn deficiency and supplementation. During pregnancy, plasma leptin concentrations in ZnDF mice significantly decreased from D14 (by 60%) through D17 (by 15%) compared with control mice, although leptin concentrations in ZnSP mice significantly increased to 170%. It was reported that secretions of soluble Ob-R, which is a leptin binding protein, into the maternal circulation may lower the rate of leptin clearance and increase plasma leptin concentrations.6–9 Thus, we measured maternal parametrial visceral fat leptin mRNA and placental soluble Ob-R mRNA expression. Interestingly, in non-pregnant mice, leptin mRNA expression in visceral fat was not influenced by maternal dietary Zn deficiency and supplementation. At D14 of gestation, leptin mRNA expression decreased to 30% of that observed in non-pregnant mice. However, in the absence of Zn deficiency, leptin expression recovered to non-pregnant levels by D17. Decreased leptin mRNA expression in visceral fat at D14 may limit placental soluble Ob-R mediated hyperleptinemia at the initiation of placental soluble Ob-R secretion in accordance with low fetal energy demands during early to middle gestation and the maternal body prepares for the elevated metabolic demands of late pregnancy and lactation.35,36 At D17, leptin mRNA expression in visceral fat from ZnDF pregnant mice significantly decreased to 50% and that of placental soluble Ob-R mRNA and Ob-R protein decreased to 75% and 40% compared with that observed in control mice, respectively. In contrast, the expression of placental soluble Ob-R mRNA and Ob-R protein in ZnSP pregnant mice tended to increase to 120% and 140%, respectively, compared with that observed in control mice. These data suggest that maternal Zn nutrition proportionally influences plasma leptin concentrations during pregnancy by modulating placental soluble Ob-R and visceral fat leptin mRNA expression.

Given the necessity of Zn for insulin biosynthesis,16,17 we investigated the effects of dietary Zn on maternal insulin concentrations in pregnant and non-pregnant mice. In ZnDF and ZnSP non-pregnant mice, plasma insulin concentrations decreased to 50%, and increased to 300%, respectively, compared with that observed in control mice. In contrast, insulin concentrations were unchanged in ZnSP pregnant mice but reduced from D14 through D17 in ZnDF pregnant mice. Indeed, despite Zn supplementation, plasma insulin concentrations did not increase during pregnancy. In ZnSP pregnant mice, the expression of placental soluble Ob-R mRNA tended to increase and plasma leptin concentration increased more than that observed in control pregnant mice. One of the functions of leptin is the regulation of insulin sensitivity. Thus, it can be suggested that hyperleptinemia in ZnSP pregnant mice at D17 may suppress maternal insulin concentration.

In ZnDF pregnant mice, plasma insulin and leptin concentrations were significantly lower than that observed in control pregnant mice. Although leptin circulates freely between the mother and fetus, maternal plasma insulin does not cross the placenta.5,37 Thus, abnormally low concentrations of plasma insulin and leptin during pregnancy and Zn deficiency may have serious implications in maternal and fetal glucose metabolism. Although plasma leptin concentrations of ZnDF non-pregnant mice were unchanged, those in ZnDF pregnant mice decreased with the duration of pregnancy, indicating an increasing influence of Zn and leptin deficiencies during fetal development.

In ZnSP pregnant mice, plasma leptin concentrations were significantly greater than those in control pregnant mice. Moreover, maternal and fetal weights and visceral fat masses did not decrease, despite the canonical relationship between leptin expression and reduced body weight and fat mass. Jaquet et al. suggested that continued fetal exposure to high concentrations of uterine leptin results in hyperleptinemia, which increases the risk of leptin resistance in offspring.38

Indeed, even transient imbalances in maternal nutrition during fetal development can trigger heritable epigenetic disturbances, such as abnormal placental DNA methylation and histone modifications, and subsequent risks of adult obesity and diabetes.39–41 The “developmental origin of health and disease (DOHaD)” hypothesis proposed by Barker et al. recognizes that maternal eating disorders during pregnancy can lead to adverse consequences in offspring.42–44 Based on reports indicating that intraterine leptin conditions contribute to DOHaD,45,46 it can be said that the control of the mater-
nal Zn nutrition may be critical.

In summary, the present data suggest that dietary Zn directly affects fetal development and placenta function and indirectly regulates fetal energy metabolism via the leptin system. Thus, investigations into the effects of other essential dietary metals on maternal leptin expression, trans-generation al influences, and heritable placental epigenetic consequences are required.

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REFERENCES


