Effects of Mild and Severe Vitamin B₁ Deficiencies on the Meiotic Maturation of Mice Oocytes

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ABSTRACT: We investigated the effects of vitamin B₁ deficiency on the meiosis maturation of oocytes. Female Crl:CD1 (ICR) mice were fed a 20% casein diet (control group) or a vitamin B₁–free diet (test group). The vitamin B₁ concentration in ovary was approximately 30% lower in the test group than in the control group. Oocyte meiosis was not affected by vitamin B₁ deficiency when the deficiency was not accompanied by body weight loss. On the contrary, frequency of abnormal oocyte was increased by vitamin B₁ deficiency when deficiency was accompanied by body weight loss (referred to as severe vitamin B₁ deficiency; frequency of abnormal oocyte, 13.8% vs 43.7%, P = .0071). The frequency of abnormal oocytes was decreased by refeeding of a vitamin B₁–containing diet (13.9% vs 22.9%, P = .503). These results suggest that severe vitamin B₁ deficiency inhibited meiotic maturation of oocytes but did not damage immature oocytes.

KEYWORDS: Vitamin B₁, deficiency, oocyte quality, mouse, oocyte meiosis

Introduction

A major factor in infertility and fetal loss (miscarriage) is poor-quality oocytes with chromosome and spindle abnormalities.¹ Recent studies have shown that macronutrients affect oocyte maturation,²,³ and that high-fat diets induce abnormal oocyte formation in mice with chromosomal misalignment and spindle defects.² Another study showed that caloric restriction without malnutrition reduced the increases of age-related oocyte aneuploidy and chromosome misalignment.⁴ In addition, we previously reported that biotin deficiency increased the proportion of abnormal oocytes with spindle defects and chromosomal misalignments in mice, and that readministration of biotin could not induce recovery in abnormal oocytes.⁵ It is no clear relationship between other vitamins and oocyte quality.

Vitamin B₁ is a water-soluble vitamin. Vitamin B₁, associated with energy metabolism, pentose phosphate cycle as a coenzyme, thiamin diphosphate. Oocytes grow in the ovaries, where they develop large nuclei known as germinal vesicles (GVs) but are arrested in the prophase of meiosis I. In response to hormonal stimulation or removal from the ovaries, fully grown oocytes resume meiosis by undergoing GV break down and the subsequent emission of the first polar body, and the resulting oocytes then arrest at the meiosis II (MII). During maturation, the energy source of oocytes is pyruvic acid, not glucose or lactic acid.⁶–⁸ Oxidative metabolism of pyruvic acid is a vital energy source during meiotic maturation and is essential for the proper completion of oogenesis. Pyruvic acid is supplied to oocytes from surrounding cumulus cells via a gap junction between them. Pyruvic acid is then catabolized to acetyl-CoA by a pyruvate dehydrogenase (PDH) complex, and the acetyl-CoA then enters the Krebs cycle. As PDH is a vitamin B₁–dependent enzyme, its activity in vitamin B₁–deficient mice is reduced.⁹ PDH E1 alpha 1 (PDHα1) deficient female mice are sterile as their oocytes cannot complete meiotic maturation and/or have gross abnormalities of the meiotic spindle and chromatin. PDHα1 deficient oocytes are also reported to have reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H).¹⁰

Vitamin B₁ is associated with PDH activity and also plays roles in other metabolic pathways such as the synthesis of neurotransmitters,¹¹,¹² nucleic acids, lipids,¹³ and steroids.¹⁴ To our knowledge, however, there are no reports regarding the role of vitamin B₁ in the maturation of oocytes. Here, we investigated the effect of a vitamin B₁ deficiency on the maturation of oocytes in female mice.

Materials and Methods

Diet

Vitamin-free milk casein and gelatinized cornstarch, mineral mixture (AIN-93-G-MX),¹⁵ vitamin mixture (AIN-93-MX),² vitamin B₁–free vitamin mixture, dextrin, and cellulose were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan) l-Methionine was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) Corn oil was obtained from Ajinomoto Co., Inc. (Tokyo, Japan) The composition of the 2 diets, a nutritionally complete diet (referred to as control diet) and a vitamin B₁–free diet, is shown in Table 1.
Table 1. Diet compositions.

|                     | CONTROL DIET, G/KG DIET | VITAMIN B1–FREE DIET, G/KG DIET |
|---------------------|--------------------------|---------------------------------|
| Vitamin-free milk casein | 200                      | 200                             |
| L-Methionine        | 2.0                      | 2.0                             |
| Gelatinized cornstarch | 376                      | 376                             |
| Sucrose             | 188                      | 188                             |
| Corn oil            | 80                       | 80                              |
| Dextrin             | 50                       | 50                              |
| Cellulose           | 50                       | 50                              |
| Mineral mixture (AIN-93-G-MX)a | 42                        | 42                              |
| Vitamin mixture (AIN-93)a | 12                    | —                               |
| Vitamin B1–free vitamin mixture (AIN-93)a | —                        | 12                              |

*aComposition of mineral and vitamin mixtures formulated to meet AIN-76A.15

Mice

Female 5-week-old ICR mice (n = 58) were purchased from Charles River Laboratories (Tokyo, Japan). Thirty-eight mice were immediately placed in individual metabolic cages (CL-0335; CLEA Japan, Tokyo, Japan) to collect 24-hour urine samples and analyze the frequency of abnormal oocyte. The remaining 20 mice were placed in plastic cages to measure the vitamin B1 concentrations in the liver, uterus, and ovary, and the estradiol concentration in plasma. To acclimatize to their conditions, all mice were ad libitum fed a control diet for 1 week (Table 1).

At 09:00 of day 0 of the experiment, 6-week-old mice were divided into 2 groups: a control group in metabolic cages (n = 16) and in plastic cages (n = 10), and a test group in metabolic cages (n = 22) and in plastic cages (n = 10). The control group was fed a 20% casein diet containing vitamin B1 (Table 1) for 62 days. The test group was initially fed a vitamin B1–free diet (Table 1) until day 20 and then the control diet until day 62 of the experiment.

Animals were allowed food and water ad libitum, and body weight was measured daily at 09:00. Food intakes of mice in metabolic cages were measured daily. Temperature was maintained at approximately 20°C with 60% humidity and a 12-hour light/dark cycle (lights on at 06:00 and off at 18:00). The care and treatment of the experimental animals conformed to the guidelines for the ethical treatment of laboratory animals set by the University of Shiga Prefecture (Shiga, Japan).

Superovulation and collection of oocytes

To obtain oocytes, the control mice were randomly selected from metabolic cages on days 13, 20, and 62 of the experiment and the test group mice from metabolic cages on days 13, 20, 40, and 62 (n = 4–7 per group per day). Mice underwent superovulation with an intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG; product no. E164A; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) followed by 5 IU human chorionic gonadotropin (hCG; product no. E801A; ASKA Pharmaceutical Co., Ltd.) after 46 to 48 hours. Oocytes were collected 18 hours after hCG injection in EmbryoMax 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid sodium salt (HEPES) buffered medium (FHM; Millipore Corp., Tokyo, Japan). Retrieved oocytes were denuded of cumulus cells using 5% hyaluronidase (Sigma-Aldrich, Inc., Tokyo, Japan) for 5 minutes at room temperature. From the control group mice, the following numbers of oocytes were collected: 161 (day 13), 88 (day 20), and 87 (day 62). From the test group mice, the following numbers of oocytes were collected: 235 (day 13), 130 (day 20), 137 (day 40), and 118 (day 62).

Immunofluorescence

A proportion of oocytes were collected by inducing superovulation, puncturing the oviducts with forceps, washing with FHM, and adding 5% protease (Sigma-Aldrich, Inc.) to soften and remove the zona pellucida. Oocytes were then extensively washed with FHM and fixed in phosphate-buffered saline (PBS) containing 4% parafomaldehyde for 15 minutes at room temperature. The oocytes were washed with PBST (0.05% Tween 20 in PBS) and permeabilized with 0.2% Triton X-100 in PBS for 15 minutes at room temperature, washed in PBST again, then blocked for 1 hour in 5% goat serum (Sigma-Aldrich, Inc.) in PBST at room temperature. Oocytes were then washed with PBST and incubated for 1 hour in a 1:4000 dilution of mouse anti-α-tubulin antibody (Cell Signaling Technology, Inc., Tokyo, Japan) in PBST containing 5% goat serum at room temperature. Oocytes were washed again and incubated for 1 hour in a 1:500 dilution of mouse anti-α-tubulin antibody (Cell Signaling Technology, Inc., Tokyo, Japan) followed by 5 IU pregnant mare serum gonadotropin (PMSG; product no. E164A; ASKA Pharmaceutical Co., Ltd.) after 46 to 48 hours. Oocytes were then washed with PBST and incubated for 1 hour in a 1:500 dilution of mouse anti-α-tubulin antibody (Cell Signaling Technology, Inc., Tokyo, Japan) and analyzed by confocal fluorescence microscopy (FV10i; Olympus, Inc., Tokyo, Japan). Oocytes were then mounted using PermaFluor Aqueous Mounting Medium (Thermo Fisher Scientific, Inc., Kanagawa, Japan) and analyzed by confocal fluorescence microscopy (FV10i; Olympus, Inc., Tokyo, Japan). Oocytes with barrel-shaped bipolar spindles and distinct and well-organized microtubule fibers, along with tightly aligned chromosomes on the metaphase plate, were classified as normal (Figure 1A).

Abnormal oocytes were identified according to the following criteria: (1) spindles exhibited serious malformations (Figure 1B), (2) chromosomes failed to align on otherwise normal meiotic spindles (Figure 1C), (3) GV stage oocyte (Figure 1D).

Reverse-transcriptase polymerase chain reaction (RT-qPCR) analysis of messenger RNA (mRNA) expression

Expression levels of PDHa1 mRNA in isolated oocytes were assessed by RT-qPCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal loading control for...
standardization. Glyceraldehyde-3-phosphate dehydrogenase mRNA expression has been used as a housekeeping gene in reports about oocyte quality.2,16 Pan et al17 reported that GAPDH mRNA expression in old mice MII oocytes analyzed by microarray did not differ from GAPDH mRNA expression levels in young mice MII oocytes. We therefore used GAPDH as a housekeeping gene.

Five or 10 MII oocytes were collected from each superovulated mouse. Total RNA from MII oocytes was isolated using the RNeasy Micro kit (Qagen, Tokyo, Japan), and we obtained 14 µL of total RNA solution per 1 sample. The 14 µL of total RNA solution was used to perform reverse transcription using a Superscript VILOTM cDNA Synthesis Kit (catalog number 11754050, Invitrogen Co., Waltham, Massachusetts, USA), which yielded 20 µL of complementary DNA (cDNA) solution per 1 sample. The conditions of reverse transcription were 42°C for 60 minutes followed by 85°C for 5 minutes. The cDNA solution was 2-fold diluted with Milli-Q water (Merck Millipore Co., Tokyo, Japan). The mixtures were used to analyze mRNA expression with the LightCycler 480 II (Roche Ltd., Tokyo, Japan), and 8.2 µL of H2O. Final volume was about 20 µL. The mixtures were used to analyze mRNA expression with the LightCycler 480 II (Roche Diagnostics K.K., Tokyo, Japan). The RT-qPCR conditions were 95°C for 10 minutes and 10 seconds, 60°C for 10 seconds, and 72°C for 10 seconds, for 45 cycles. The dates were analyzed by LightCycler 480 Software (Roche Diagnostics K.K.).

Measurement of vitamin B1
On days 9, 16, 36, and 58, 24-hour urine samples were collected from mice in metabolic cages using amber bottles containing 1 mL of 1 mol/L HCl. Samples were stored at −30°C until analysis. To avoid the effect of superovulation on the metabolism of the mice, urine samples were collected 24 hours prior to PMSG injection.

The frozen acidified urine samples (about 500 µL) were thawed and centrifuged at 10000 g for 10 minutes at 4°C. The supernatant was retained and used to measure thiamin, which is a major form of vitamin B1 in urine. To 250 µL of the supernatant, 50 µL of 1% cyanogen bromide was added. The mixture was then added to 50 µL of 5% NaOH. After being kept for 10 minutes at room temperature, 80 µL of 1.5 mol/L HCl was added. The mixture was then centrifuged at 10000 g for 10 minutes at 4°C. The resulting supernatant was passed through a 0.45-µm microfilter equipped with Hydrophilic Durapore (polyvinylidene difluoride [PVDF]; Millipore Corp.). The filtrate (20 µL) was directly injected into a high-performance liquid chromatography system to measure the level of thiochrome (vitamin B1–derived fluorescent compound). A Tosoh ODS-100S (15 mm × 3.2 mm, I.D., average particle size: 5 µm) column was used as a precolumn, and a Tosoh ODS-100S (250 mm × 4.6 mm, I.D., average particle size: 5 µm) column was used as an analytical column. Samples were separated using the following mobile phase: 0.1 mol/L KH2PO4-K2HPO4 buffer (pH 7.0) containing 3% acetonitrile. Flow speed was set at 1.0 mL/min. Fluorescence intensities were measured with an excitation wavelength of 375 nm and emission wavelength of 430 nm.18

Measurement of vitamin B1 in liver, ovary, and uterus
To examine vitamin B1 nutrient status in the body, vitamin B1 concentrations were measured in the liver, ovary, and uterus. On days 13 and 62 of the experiment, 5 mice from each group were selected from plastic cages. The mice were decapitated, blood was collected, and the liver, ovary, and uterus were removed. The mice were

Figure 1. Normal oocyte and abnormal oocyte: MII oocyte α-tubulin and chromosomal misalignment were stained by immunofluorescence method: (A) Normal MII oocyte, (B) spindle defect oocyte, (C) chromosomal misalignment and spindle defect oocyte, and (D) GV stage oocyte. Representative examples of meiotic spindles in oocytes from indicated mice after labeling α-tubulin antibody (green) and counterstaining DNA with DAPI (aqua blue). DAPI indicates 4,6-diamidino-2-phenylindole dihydrochloride; GV, germinal vehicle; MII, metaphase II.
in the metabolic cages were also decapitated, and the liver removed on days 13, 20, 40, and 62. Control mice were not sacrificed on day 40, and their vitamin B₁ concentration data were unavailable.

Ten volumes of cold 5% trichloroacetic acid were added to the isolated liver, uterus, and ovary. The suspension of liver or ovary was homogenized in a Teflon-glass homogenizer (AS ONE Co., Osaka, Japan) and that of uterus was homogenized by POLYTRON PT 1200 CL (KINEMATICA AG, Tokyo, Japan). Each acidified homogenate was centrifuged at 10000 g for 10 minutes at 4°C. Retained supernatants were stored at –80°C until analysis.

The frozen acidified samples (about 600 µL) were thawed and centrifuged at 10000 g for 10 minutes at 4°C. The supernatant was retained and used to measure thiamin, thiamin monophosphate, and thiamin diphosphate. The supernatant (200 µL) was added to 40 µL of 1% cyanogen bromide and 80 µL of 5% NaOH. After being kept for 10 minutes at room temperature, 80 µL of 1.5 mol/L HCl was added. Measurements of thiamin were conducted as described in the “Measurement of urinary vitamin B₁” section. For measurement of tissue thiamin diphosphate, 0.1 mol/L KH₂PO₄-K₂HPO₄ buffer (pH 7.0) containing 1.5% acetonitrile was used as a mobile phase. Vitamin B₁ values in the tissues are affected by energy restriction as well as vitamin B₁ deficiency. At that stage, frequency of abnormal oocytes was markedly higher in the test group than in the control group (13.8% vs 43.5%, P < .01). In the test group, most oocytes had normal chromosome alignment with normal spindle fibers converging on highly localized poles. Most abnormal oocytes had spindle defects. Some of oocytes showed arrest at the GV stage on day 20. Its percentage occupied 12.2% of abnormal oocytes of the test group (Figure 3). Germinal vesicle stage oocyte was 4.3% of frequency of abnormal oocyte.

Measurement of plasma estradiol
Blood from the carotid artery was collected into EDTA-2Na tubes (Terumo Co. Ltd., Tokyo, Japan). Collected samples were centrifuged at 1700 g for 30 minutes at 4°C to obtain plasma samples, which were then stored at –80°C until analysis.

Estradiol concentration was determined in mice plasma on days 13 and 62 using an enzyme-linked immunosorbent assay kit (catalog number KB30-H1; Arbor Assays Inc., Ann Arbor, Michigan), as described in the manufacturer’s instructions.

Estrus cycle
Vaginal smears of mice were taken daily at 09:00. The mice were sacrificed on days 40 and 62 of the experiment. A bulb was gently depressed to expel 25 to 50 µL H₂O at the opening of the vaginal canal. This step was repeated 4 or 5 times. Fluid was placed on a glass slide, and the smear was allowed to dry completely at room temperature. Dry glass slides were stained with 1.6% Giemsa stain solution (Wako Pure Chemical Industries Ltd.) for 10 minutes and dried at room temperature. The estrus cycle consists of 4 sequential stages: proestrus, estrus, metestrus, and diestrus. Stages of the estrus cycle were determined by cytological evaluation of vaginal smears with a microscope (Carl Zeiss, Tokyo, Japan). Those with 4- or 5-day cycles were considered to have a regular cycle, whereas those that displayed a prolonged cycle (≥6 days) were considered to have an irregular cycle.

Statistical analyses
Data were analyzed by Student t test (unpaired, nonparametric test). Frequency of abnormal oocytes was analyzed by the chi-square test. Statistical analyses were conducted using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA), with P < .05 considered significant.

Results
Food intake and body weight
Food intake in the test group gradually decreased from day 10 and reached its lowest value from days 14 to 20 (Figure 2A). Anorexia appeared in the test group mice (Figure 2B). Upon refeeding of the vitamin B₁–containing diet (control diet) at 09:00 on day 21, the food intake immediately increased, and it was higher than that of the control group from days 21 to 24 (Figure 2A).

Effects of mild vitamin B₁ deficiency and severe vitamin B₁ deficiency on frequency of abnormal oocyte
Ovulated oocyte numbers did not differ between the control and test groups (Supplementary Table 1).

On day 13, the frequency of abnormal oocytes in the test mice did not differ from the control group (17.1% vs 14.6%). This stage was defined as mild vitamin B₁ deficiency status; the vitamin B₁ concentrations were lower in the test group than in the control group, but the body weights were similar.

On day 20, the test group was in a condition defined as severe vitamin B₁ deficiency status; the vitamin B₁ concentrations were lower in the test group than in the control group, and the body weights were also lower. Thus, the mice in the test group were affected by energy restriction as well as vitamin B₁ deficiency. At that stage, frequency of abnormal oocytes was markedly higher in the test mice than in the control group (13.8% vs 43.5%, P < .01). In the test group, most oocytes had normal chromosome alignment with normal spindle fibers converging on highly localized poles. Most abnormal oocytes had spindle defects. Some of oocytes showed arrest at the GV stage on day 20. Its percentage occupied 12.2% of abnormal oocytes of the test group (Figure 3).

Effect of refeeding vitamin B₁–containing diet on oocyte quality
Superovulated oocyte numbers were not different between the test group on day 40 (after the mice had been refed the vitamin B₁–containing diet for 20 days) and day 62 (after the mice had been refed the vitamin B₁–containing diet for 42 days), nor was there a difference compared with the control group on day 62 (Supplementary Table 1). In the test group, the frequency of abnormal oocytes on day 40 was 20.7% (Figure 3). Germinal vesicle stage oocyte was 4.3% of frequency of abnormal oocyte in the test group.
On day 62, the frequency of abnormal oocytes in the control group was 13.9% whereas that in the test group was 22.9% \((P = .503; \text{Figure 3})\). The frequency of abnormal oocyte in the test group was lower on day 62 than on day 20 \((P = .0503)\). The frequency of abnormal oocyte in the test group on day 40 did not differ significantly compared with that on day 62 \((P = .694)\).

**Pdhα1 mRNA expression in oocytes**

PDHα1 mRNA expressions in oocytes in the control and test groups on day 13 were \(1.00 \pm 0.39\) and \(0.82 \pm 0.22\), respectively (Figure 4A). PDHα1 mRNA expressions in the control and test groups on day 20 were \(1.01 \pm 0.17\) and \(0.92 \pm 0.40\), respectively (Figure 4B). Therefore, PDHα1 mRNA expression levels in the test group did not differ significantly from those of the control group on days 13 and 20.

**Effects of feeding of the vitamin B1–free diet and refeeding of the vitamin B1–containing diet on the vitamin B1 concentrations in urine, liver, uterus, and ovary**

The vitamin B1 concentrations of urine, liver, uterus, and ovary on days 13 and 20 in the test group were markedly decreased compared with those in the control group (Table 2).
After the vitamin B₁-deficient mice had been refed on a vitamin B₁–containing diet for 20 days, vitamin B₁ concentrations in the urine and the liver were restored (Table 2). On day 62, the vitamin B₁ concentrations in the liver, ovary, and uterus were similar in both groups (Table 2).

**Figure 4.** Pdhα mRNA expression in oocytes: Pdhα mRNA expression in oocytes taken from test group mice fed the V.B₁-free diet for (A) 13 days or (B) 20 days. Control mice were fed V.B₁-containing diet (control diet) during the experimental period. mRNA expression in test group mice during the V.B₁-free diet is shown as the fold change relative to control group (n=3-6). Data are shown as mean ± SEM. Data were analyzed by Student t test. P values are shown in the figure. N.S. indicates not significant; Pdhα, pyruvate dehydrogenase E1 alpha 1; V.B₁, vitamin B₁.

**Table 2.** V.B₁ concentrations in urine, liver, ovary, and uterus.

|                      | CONTROL GROUP (FEEDING CONTROL DIET FOR 63D) | TEST GROUP (FEEDING V.B₁–FREE DIET ON DAY 0-20 → CONTROL DIET ON DAYS 21-63) |
|----------------------|---------------------------------------------|--------------------------------------------------------------------------------|
|                      | MEAN | SE  | MEAN | SE  |
| **V.B₁–deficient experiment** |
| Day 13               |      |     |      |     |
| Urine, nmol/d<sup>a</sup> | 51.6 | 7.6 | 1.39 | 0.24*** |
| Liver, nmol/g        | 22.8 | 1.1 | 2.0  | 0.2***  |
| Uterus, nmol/g       | 4.82 | 1.09| 0.39 | 0.12**  |
| Ovary, nmol/g        | 0.68 | 0.09| 0.21 | 0.12*   |
| Day 20               |      |     |      |     |
| Urine, nmol/d<sup>a</sup> | 32.6 | 3.6 | 1.31 | 0.09*** |
| Liver, nmol/g        | 38.0 | 2.5 | 0.9  | 0.2***  |
| **Recovery experiment** |
| Day 40 (refeeding V.B₁ for 20d) |      |     |      |     |
| Urine, nmol/d<sup>a</sup> | —    | —   | 38.0 | 4.0   |
| Liver, nmol/g        | —    | —   | 35.8 | 4.8   |
| Day 62 (refeeding V.B₁ for 42d) |      |     |      |     |
| Urine, nmol/d<sup>a</sup> | 44.0 | 4.9 | 40.0 | 14.9  |
| Liver, nmol/g        | 34.0 | 3.3 | 33.1 | 3.2   |
| Uterus, nmol/g       | 3.65 | 0.67| 3.01 | 0.58  |
| Ovary, nmol/g        | 0.63 | 0.06| 0.70 | 0.10  |

Abbreviations: PMSG, pregnant mare serum gonadotropin; SE, standard error; V.B₁, vitamin B₁. Urine, n=4-7; liver, n=4-12; uterus and ovary, n=5. Mean values were significantly different from those of the control group (Student t test [unpaired, nonparametric test]): *P < .05, **P < .01, ***P < .001. 'We considered the effect of superovulation on urinary excretion of V.B₁, so 24-h urine was collected before injection of PMSG. Twenty-four hour urine on days 13, 20, 40, and 63 was collected on days 9, 16, 36, and 58, respectively.

Effects of feeding the vitamin B₁–free diet and refeeding the vitamin B₁–containing diet on plasma estradiol levels

Plasma estradiol concentration did not change even when mice were fed the vitamin B₁–free diet for 13 days.
(Supplementary Table 2). Refeeding of the vitamin B₁-containing diet to the vitamin B₁-deficient mice for another 42 days (until day 62 of the experiment) did not affect the concentrations (Supplementary Table 2).

**Effects of feeding of the vitamin B₁-free diet and refeeding of the vitamin B₁-containing diet on estrus cycle**

Estrus cycles in the control group were regular throughout the experimental period (5.1 ± 0.3, n = 5). In the test group, estrus cycles were also regular (5.4 ± 0.2 days, n = 8) until the arrest of estrus. When the test group mice were fed the vitamin B₁-free diet, estrus was arrested from around day 14 (days 11-17). However, when the vitamin B₁-deficient mice were refed the vitamin B₁-containing diet, the estrus cycles of the mice restarted on around day 25 (days 22-26). Estrus cycles were then gradually restored to regular cycles. At the end of the experimental period (days 40-62), the estrus cycles of mice in the test group were completely recovered (4.6 ± 0.4 days, n = 3).

**Discussion**

In the present study, we investigated the effects of mild and severe vitamin B₁ deficiencies on oocyte maturation. Mild vitamin B₁ deficiency was induced by feeding mice a vitamin B₁-free diet for 13 days. Severe vitamin B₁ deficiency was induced by feeding mice a vitamin B₁-free diet for 20 days. Mild vitamin B₁ deficiency did not affect the quality of oocytes. On the contrary, severe vitamin B₁ deficiency caused an increase in the frequency of abnormal oocytes. However, this frequency was lower than that in the Km value of pig heart PDH for thiamin diphosphate is 0.2 µmol/L. Adenosine monophosphate (AMP)/ATP ratio in the liver was reported to increase in the liver of mice fed a vitamin B₁-free diet. Oocyte meiosis needs more energy than the usual metabolic state. Adenosine triphosphate (ATP)/ADP ratio in the liver was reported to increase in mice fed a vitamin B₁-free diet. Oocytes with chromosome misalignment. Selesniemi et al reported that caloric restriction without malnutrition reduced the increases of age-related oocyte aneuploidy and chromosome misalignment. The report looks like conflicts with our study. In fact, mice treated with caloric restriction were fed a normal caloric diet with ad libitum for 1 month before oocyte collection. Therefore, experimental condition is different between the study of Selesniemi et al and our study.

There are 2 pattern mechanisms of abnormal oocytes (Figure 5). Immature oocytes at the GV stage are present in the ovary as primary follicles, which grow to Graafian follicles. Following a rapid increase in luteinizing hormone, immature GV break down and MII oocytes undergo ovulation. When oocytes undergoing meiotic maturation are damaged, other normal oocytes in the primary follicle are able to mature normally.
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Figure 5. Characteristic effects of vitamin B<sub>1</sub> deficiency and biotin deficiency on oocyte maturation: (A) Severe vitamin B<sub>1</sub> deficiency (top oocyte) inhibited normal oocyte meiosis maturation. Mild and severe vitamin B<sub>1</sub> deficiencies did not damage immature oocytes in primary follicle (second oocyte from the left), and the oocyte quality was recovered by refeeding vitamin B<sub>1</sub>-containing diet (second oocyte from the top). (B) Biotin deficiency inhibited oocyte meiosis maturation (second oocyte from the bottom). Biotin deficiency also damaged immature oocytes in the primary follicle (second oocyte from the left), and the oocyte quality was not recovered by refeeding a biotin-containing diet (bottom oocyte). GV indicates germinal vehicle.

Considering the different effects of vitamin B<sub>1</sub> and biotin, each nutrient has an individual role in oogenesis and meiotic maturation. To clarify in more detail the relationships between vitamin nutrition and oocyte quality, the effects of other B-group vitamins such as niacin, pantothenic acid, and folacin on oocyte quality should be investigated.

Author Contributions
AT and KS designed the study and prepared the first draft and wrote this article. AT performed the experiments. TN assisted with the data analysis.

Disclosures and Ethics
As required by the publication, the authors have provided signed confirmation of their compliance with ethical and legal obligations, including, but not limited to, compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

REFERENCES
1. Hassold T, Chiu D. Maternal age-specific rates of numerical chromosome abnormalities with special reference to trisomy. Hum Genet. 1985;70:11-17.
2. Luzzo KM, Wang Q, Purcell SH, et al. High fat diet induced developmental defects in the mouse: oocyte meiotic aneuploidy and fetal growth retardation/brain defects. PLoS ONE. 2012;7:e49217.
3. Muhlhauser A, Susiaja M, Rahbo C, et al. Biophenol A affects on the growing mouse oocyte are influenced by diet. *Biol Reprod.* 2009;80:1066–1071.

4. Seleniemi K, Lee HJ, Muhlhauser A, Tilly JL. Prevention of maternal aging-associated oocyte aneuploidy and meiotic spindle defects in mice by dietary and genetic strategies. *Proc Natl Acad Sci U S A.* 2011;108:12319–12324.

5. Tsuji et al. Acetyl-CoA deficit in brain mitochondria in experimental thiamine deficiency. *Biosci Biotechnol Biochem.* 2015;9:292–299.

6. Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oocyte and zygote. *Proc Natl Acad Sci U S A.* 1967;54:560–567.

7. Brinster RL. Oxidation of pyruvate and glucose by oocytes of the mouse and rhesus monkey. *J Reprod Fertil.* 1971;4:187–191.

8. Eppig JJ. Analysis of mouse oogenesis in vitro. Oocyte isolation and the utilization of exogenous energy sources by growing oocytes. *J Exp Zool.* 1976;198:375–382.

9. Trebukhina AV, Ostrovsky YM, Petushok VG, Velichko MG, Tumanov VN. Effect of thiamine deprivation on thiamine metabolism in mice. *J Nutr.* 1981;111:505–513.

10. Johnson MT, Freeman EA, Gardner DK, Hunt PA. Oxidative metabolism of pyruvate is required for meiotic maturation of murine oocytes in vivo. *Biol Reprod.* 2007;77:2–8.

11. Jankowska-Kulawy A, Bielarczyk H, Pawelewska M, Szutowicz A. Acetyl-CoA deficit in brain mitochondria in experimental thiamine deficiency encephalopathy. *Neurochem Int.* 2010;57:851–856.

12. Anzalone S, Vetreno RP, Ramos RL, Savage IM. Cortical cholinergic abnormalities contribute to the amnesic state induced by pyrithiamine-induced thiamine deficiency in the rat. *Eur J Neurosci.* 2010;32:847–858.

13. El-Hindi HM, Amer HA. Effect of thiamine, magnesium, and sulfate salts on growth, thiamine levels, and serum lipid constituents in rats. *J Nutr Sci Vitaminol.* 1989;35:505–510.

14. Meikle AW, Wittek PJ, Klain GJ. An aberration of glucose metabolism and steroidogenesis in adrenals of thiamin-deficient rats. *Biochem J.* 1989;268:317–323.

15. Shibata K, Shimizu A, Fukuwatari T. Vitamin B1 deficiency does not affect the vitamin A status of growing oocytes. *J Nutr.* 1981;111:505–513.

16. Shibata K, Kobayashi R, Fukuwatari T. Vitamin B1 deficiency inhibits the in vitro oxidation of pyruvate and glucose by thiamin-deficient oocytes. *Biosci Biotechnol Biochem.* 1981;4:187–191.

17. Pan H, Ma P, Zhu W, Schultz RM. Age-associated increase in aneuploidy and changes in gene expression in mouse eggs. *Dev Biol.* 2008;316:397–407.

18. Iwata H, Matsuda T, Tomonura H. Improved high-performance liquid chromatographic determination of thiamine and its phosphate esters in animal tissues. *J Chromatogr.* 1988;468:317–323.

19. Vikesvanes S, Hikker DM, Nakornchai S, Rungruangsak K, Dhanamitta S. Effects of betel nut and fermented fish on the thiamin status of northeastern Thais. *Am J Clin Nutr.* 1975;28:1458–1463.

20. Baines M. Detection and incidence of B and C vitamin deficiency in alcohol-related illness. *Ann Clin Biochem.* 1978;15:307–312.

21. Thomson AD, Jeyasingham MD, Pratt OE, Shaw GK. Nutrition and alcoholic encephalopathies. *Acta Med Scand.* 1977;17:55–65.

22. Strumilo S, Czernecki J, Dobrzyn P. Regulatory effect of thiamin pyrophosphate on pig heart pyruvate dehydrogenase complex. *Biochem Biophys Res Commun.* 1999;256:341–345.

23. Chen L, Shu Y, Liang X, et al. OCT1 is a high-capacity thiamine transporter that regulates hepatic steatosis and is a target of metformin. *Proc Natl Acad Sci U S A.* 2014;11:9983–9988.

24. Zhang X, Wu XQ, Li S, Guo YL, Mu X. Deficit of mitochondria-derived ATP during oxidative stress impairs mouse MII oocyte spindles. *Cell Res.* 2006;16:841–850.

25. Pekovich SR, Martin PR, Singleton CK. Thiamine deficiency decreases steady-state transketolase and pyruvate dehydrogenase but not alpha-ketoglutarate dehydrogenase mRNA levels in three human cell types. *J Nutr.* 1998;128:683–687.

26. Shibata K, Shimizu A, Fukuwatari T. Vitamin B1 deficiency does not affect the liver concentrations of the other seven kinds of B-group vitamins in rats. *Nutr Metab Insights.* 2013;6:1–10.

27. Shibata K, Kobayashi R, Fukuwatari T. Vitamin B1 deficiency inhibits the increased conversion of tryptophan to nicotinamide in severe food-restricted rats. *Biosci Biotechnol Biochem.* 2015;79:103–108.

28. Liu M, Altimov AP, Wang H, et al. Thiamine deficiency induces anorexia by inhibiting hypothalamic AMPK. *Neurosci. 2014;267:102–113.

29. Rathanaswami P, Sundaresan R. Effects of insulin secretagogues on the secretion of insulin during thiamine deficiency. *Biochem. 1988;17:523–528.

30. Liang CC. Metabolic changes in rats during developing thiamin deficiency. *Biosci. 1975;146:739–740.

31. Valsangkar D, Downs SM. A requirement for fatty acid oxidation in the hormone-induced meiotic maturation of mouse oocytes. *Biol Reprod.* 2013;89:43.

32. Khokhlova IS, Kam M, Gonen S, Degen AA. Level of energy intake affects the estrous cycle in Sundevall’s jird (Meriones crassus). *Physiol Biochem Zoed.* 2000;73:257–263.

33. Yoo J, Zhou B, Yang J, et al. Glucose can reverse the effects of acute fasting on mouse ovulation and oocyte maturation. *Reprod Fertil Dev.* 2008;20:703–712.

34. Camporeale G, Shubert EE, Sarath G, Cerny R, Zempleni J. K8 and K12 are biotinylated in human histone H4. *Eur J Biochem.* 2004;271:2257–2263.

35. Chew YC, Camporeale G, Korthapalli N, Sarath G, Zempleni J. lysine residues in N-terminal and C-terminal regions of human histone H2A are targets for biotinylation by biotinidase. *J Nutr.* 2006;126:255–233.

36. Kobza K, Camporeale G, Rueckert B, et al. K4, K9, and K18 in human histone H3 are targets for biotinylation by biotinidase. *FEBS J.* 2005;272:4249–4259.