Effects of Mild and Severe Vitamin B_1 Deficiencies on the Meiotic Maturation of Mice Oocytes

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ABSTRACT: We investigated the effects of vitamin B_1 deficiency on the meiosis maturation of oocytes. Female Crl:CD1 (ICR) mice were fed a 20% casein diet (control group) or a vitamin B_1–free diet (test group). The vitamin B_1 concentration in ovary was approximately 30% lower in the test group than in the control group. Oocyte meiosis was not affected by vitamin B_1 deficiency when the deficiency was not accompanied by body weight loss. On the contrary, frequency of abnormal oocyte was increased by vitamin B_1 deficiency when deficiency was accompanied by body weight loss (referred to as severe vitamin B_1 deficiency; frequency of abnormal oocyte, 13.8% vs 43.7%, $P = .0071$). The frequency of abnormal oocytes was decreased by refeeding of a vitamin B_1–containing diet (13.9% vs 22.9%, $P = .503$). These results suggest that severe vitamin B_1 deficiency inhibited meiotic maturation of oocytes but did not damage immature oocytes.

KEYWORDS: Vitamin B_1, deficiency, oocyte quality, mouse, oocyte meiosis

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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 B_1 is a water-soluble vitamin. Vitamin B_1 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 B_1–dependent enzyme, its activity in vitamin B_1–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 B_1 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 B_1 in the maturation of oocytes. Here, we investigated the effect of a vitamin B_1 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),[15] vitamin B_1–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 B_1–free diet, is shown in Table 1.

Table 1. Diet compositions.

	CONTROL DIET, G/KG DIET	VITAMIN B_1–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 B_1–free vitamin mixture (AIN-93)[a]	—	12

[a]Composition 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 B_1 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 B_1 (Table 1) for 62 days. The test group was initially fed a vitamin B_1–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% paraformaldehyde 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 goat anti-mouse IgG with Alexa Fluor-488 and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Dojindo Laboratories, Kumamoto, 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 *PDHα1* mRNA in isolated oocytes were assessed by RT-qPCR with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA as an internal loading control for

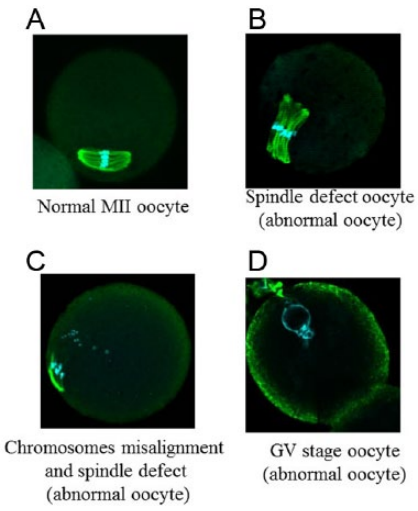

Figure 1. Normal oocyte and abnormal oocyte: MII oocyte α-tubulin and chromosomal misalignment were stained by immunofluorescence method: (A) Normal MII oocyte, (B) spindle defect oocyte, (C) chromosomal misalignment and spindle defect oocyte, and (D) GV stage oocyte. Representative examples of meiotic spindles in oocytes from indicated mice after labeling α-tubulin antibody (green) and counter-staining DNA with DAPI (aqua blue). DAPI indicates 4′,6-diamidino-2-phenylindole dihydrochloride; GV, germinal vehicle; MII, metaphase II.

standardization. Glyceraldehyde-3-phosphate dehydrogenase mRNA expression has been used as a housekeeping gene in reports about oocyte quality.[2,16] Pan et al[17] 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 (Qiagen, 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 solutions were stored at −30°C until analysis.

Sequence of *Pdha1*and *Gapdh* was obtained by Ensembl. Primer synthesis was ordered from Hokkaido System Science Co., Ltd. (Hokkaido, Japan) Primer sequences were *Pdhα1* primers 5′-AGTCACCACAGTGCTCACCA-3′ and 5′-AGGC TTCCTGACCATCACAC-3′, and *Gapdh* primers 5′-TAA AGGGCATCCTGGGCTACACT-3′ and 5′-TTACTCCT TGGAGGCCATGTAGG-3′. The cDNA solution was 2-fold diluted with Milli-Q water (Merck Millipore Co., Tokyo, Japan). To 1 μL of diluted cDNA solution, we added 0.8 μL of 10 μmol/L forward primer or reverse primer, 10 μL of KAPA SYBR Fast qPCR Mater Mix (catalog number KK4611, Kapa Biosystems, Inc., Tokyo, Japan) with a LightCycler 480 II (Roche Ltd., Tokyo, Japan), and 8.2 μL of H_2O. Final volume was about 20 μL. The mixtures were used to analyze mRNA expression with the LightCycler 480 (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.).

Standard curves of *Gapdh* and *Pdhα1* were produced using cDNA of ICR mouse liver, and a standard curve was produced for each experiment. mRNA expressions were calculated by standard curve of each gene, then *PDHα1* mRNA expression was corrected by *GAPDH* mRNA expression. *PDHα1* mRNA expression in the test group was shown relative to the control group.

Measurement of urinary vitamin B_1

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 10 000*g* for 10 minutes at 4°C. The supernatant was retained and used to measure thiamin, which is a major form of vitamin B_1 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 10 000*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 B_1–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 KH_2PO_4-K_2HPO_4 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 B_1 in liver, ovary, and uterus

To examine vitamin B_1 nutrient status in the body, vitamin B_1 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

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 B_1 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 10 000*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 10 000*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 B_1" section. For measurement of tissue thiamin diphosphate, 0.1 mol/L KH_2PO_4-K_2HPO_4 buffer (pH 7.0) containing 1.5% acetonitrile was used as a mobile phase.[18] Vitamin B_1 values in the tissues are the sum of thiamin, thiamin monophosphate, and thiamin diphosphate.

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 H_2O 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).[19] 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 oocyte 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 B_1–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 B_1 deficiency and severe vitamin B_1 deficiency on frequency of abnormal oocyte

Ovulated oocyte numbers did not differ between the control and test groups (Supplementary Table 1).

Frequencies of abnormal oocytes are shown in Figure 3. 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 B_1 deficiency status; the vitamin B_1 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 B_1 deficiency status; the vitamin B_1 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 B_1 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 B_1–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 B_1–containing diet for 20 days) and day 62 (after the mice had been refed the vitamin B_1–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.

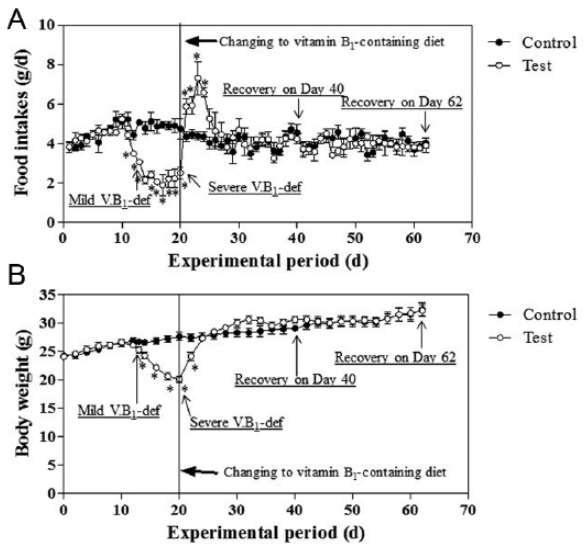

Figure 2. Food intake and body weight change during experimental period: (A) Food intake and (B) body weight. Control mice were fed a V.B_1-containing diet (control diet) during the experimental period. Test mice were initially fed a V.B_1-free diet from days 0 to 20 and then a control diet from days 21 to 62. Data are shown as mean ± SE. Data were analyzed by Student *t* test on each day. **P* < .05. Mild V.B_1 deficiency was until day 13, which on body weight did not differ between the 2 groups. Severe V.B_1 deficiency was until day 20, by which point body weight was markedly lower in the test mice than in the control mice. GV indicates germinal vesicle; V.B_1, vitamin B_1.

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; 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 ± 0.39 and 0.82 ± 0.22, respectively (Figure 4A). *PDHα1* mRNA expressions in the control and test groups on day 20 were 1.01 ± 0.17 and 0.92 ± 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 B_1–free diet and refeeding of the vitamin B_1–containing diet on the vitamin B_1 concentrations in urine, liver, uterus, and ovary

The vitamin B_1 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).

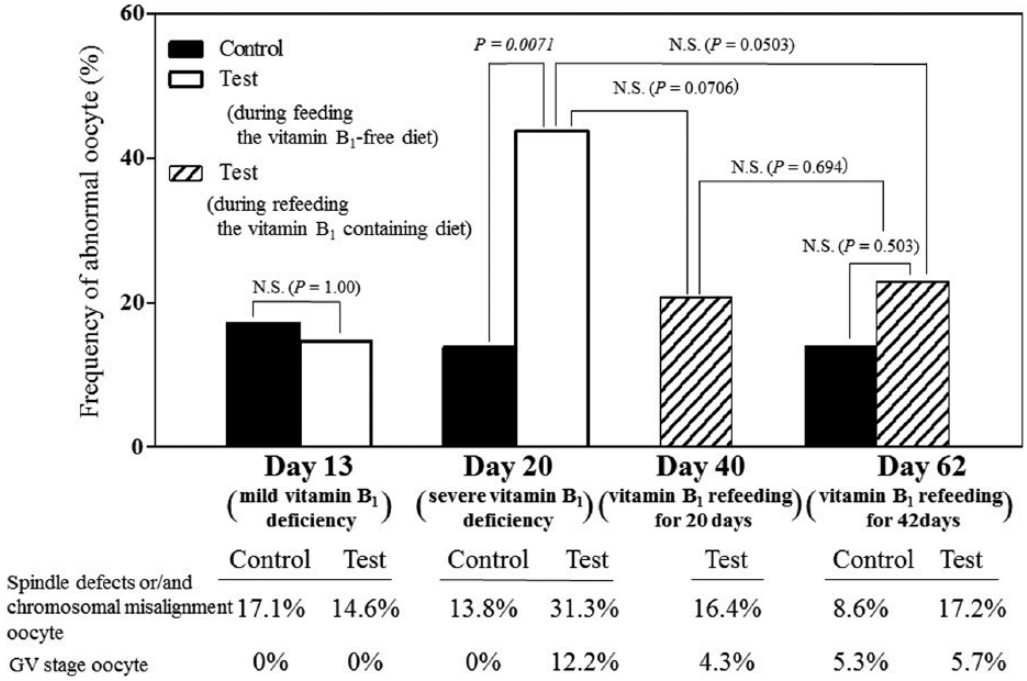

Figure 3. Frequency of abnormal oocytes in test group during vitamin B_1–free diet. Frequency of abnormal oocytes on day 13 (mild vitamin B_1 deficiency), day 20 (severe vitamin B_1 deficiency), day 40, and day 62. Control mice were fed vitamin B_1–containing diet (control diet) during the experimental period. Test mice were initially fed a vitamin B_1–free diet from days 0 to 20 and then a control diet from days 21 to 62. The data of the control group are shown in closed bar. The data of the test group are shown in open bar (feeding the vitamin B_1–free diet) and in strip bar (refeeding the vitamin B_1–containing diet). Percentages under the graph show a break down of abnormal oocytes at each point and in each group. Abnormal oocyte number is the sum of those at the GV stage and with chromosome alignment and spindle defects in MII. Oocytes (n = 29-48 per group) were analyzed. Data were analyzed by chi-square test. *P* values are shown in the figure. GV indicates germinal vehicle; MII, , metaphase II; N.S., not significant.

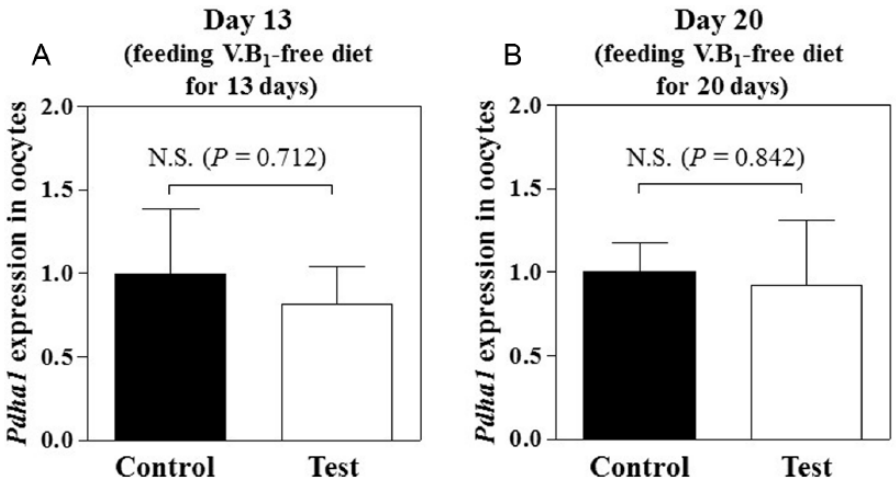

Figure 4. Pdhα1 mRNA expression in oocytes: Pdhα1 mRNA expression in oocytes taken from test group mice fed the $V.B_1$-free diet for (A) 13 days or (B) 20 days. Control mice were fed $V.B_1$-containing diet (control diet) during the experimental period. mRNA expression in test group mice during the $V.B_1$-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; Pdhα1, pyruvate dehydrogenase E1 alpha 1; $V.B_1$, vitamin B_1.

Table 2. $V.B_1$ concentrations in urine, liver, ovary, and uterus.

		CONTROL GROUP (FEEDING CONTROL DIET FOR 63 D)		TEST GROUP (FEEDING $V.B_1$–FREE DIET ON DAY 0-20 → CONTROL DIET ON DAYS 21-63)	
		MEAN	SE	MEAN	SE
$V.B_1$–deficient experiment					
Day 13	Urine, nmol/d[a]	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[a]	32.6	3.6	1.31	0.09***
	Liver, nmol/g	38.0	2.5	0.9	0.2***
Recovery experiment					
Day 40 (refeeding $V.B_1$ for 20 d)	Urine, nmol/d[a]	—	—	38.0	4.0
	Liver, nmol/g	—	—	35.8	4.8
Day 62 (refeeding $V.B_1$ for 42 d)	Urine, nmol/d[a]	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; $V.B_1$, vitamin B_1.
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]): *$P < .05$, **$P < .01$, ***$P < .001$.
[a]We considered the effect of superovulation on urinary excretion of $V.B_1$, 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.

After the vitamin B_1–deficient mice had been refed on a vitamin B_1–containing diet for 20 days, vitamin B_1 concentrations in the urine and the liver were restored (Table 2). On day 62, the vitamin B_1 concentrations in the liver, ovary, and uterus were similar in both groups (Table 2).

Effects of feeding the vitamin B_1–free diet and refeeding the vitamin B_1–containing diet on plasma estradiol levels

Plasma estradiol concentration did not change even when mice were fed the vitamin B_1–free diet for 13 days

(Supplementary Table 2). Refeeding of the vitamin B_1–containing diet to the vitamin B_1–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 B_1–free diet and refeeding of the vitamin B_1–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 B_1–free diet, estrus was arrested from around day 14 (days 11-17). However, when the vitamin B_1–deficient mice were refed the vitamin B_1–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 B_1 deficiencies on oocyte maturation. Mild vitamin B_1 deficiency was induced by feeding mice a vitamin B_1–free diet for 13 days. Severe vitamin B_1 deficiency was induced by feeding mice a vitamin B_1–free diet for 20 days. Mild vitamin B_1 deficiency did not affect the quality of oocytes. On the contrary, severe vitamin B_1 deficiency caused an increase in the frequency of abnormal oocytes. However, this frequency was returned to the normal range by refeeding the mice with a vitamin B_1–containing diet.

There is no rule for mandatory addition of vitamin B_1 in cereals in Japan. Vitamin B_1 is still an important vitamin for Japanese because main staple is rice in Japan. Vitamin B_1 deficiency can be accelerated by some of the reasons, such as increased energy requirement, the ingestion of raw fish and seafood containing anti-thiamin components,[19] and chronic alcoholics.[20,21] Pyruvic acid is a vital energy source during meiotic maturation. Pyruvate dehydrogenase and its coenzymes play critical roles in energy production.[6–8] K_m value of pig heart PDH for thiamin diphosphate is 0.2 µmol/L.[22] In the present experiment, PDH activity in the mildly vitamin B_1–deficient mice may be low compared with the value of control group on day 13, because the vitamin B_1 concentration in the ovary was lower than the K_m value of 0.2 µmol/L. Adenosine monophosphate (AMP)/ATP ratio in the liver was reported to increase in mice fed a vitamin B_1–free diet.[23] Oocyte meiosis needs more energy than the usual metabolic state. Adenosine triphosphate deficiency causes spindle defects in oocyte meiosis.[24] In the present experiment, the ATP concentration in oocytes under mild vitamin B_1 deficiency might be lower than that in the control group oocytes. Contrary to our expectations, frequency of abnormal oocytes did not increase in mild vitamin B_1 deficiency. We hypothesize that the decrease of ATP concentration in the mild vitamin B_1–deficient mice was not enough to induce oocyte meiosis failure. The decrease of ATP concentration in severely vitamin B_1–deficient mice was enough to induce oocyte meiosis failure. We did not measure ATP concentration in oocytes because their amounts are too small to measure ATP. We measured PDHα1 mRNA expression. It has been reported that PDHα1 mRNA expression was not affected by vitamin B_1 deficiency in human cells.[25] In the present study, PDHα1 mRNA expression was affected by both mild and severe vitamin B_1 deficiencies.

Anorexia is often observed as a symptom of severe vitamin B_1 deficiency.[26,27] Also, in the present study, food intake was reduced by feeding a vitamin B_1–free diet. A recent study showed that vitamin B_1 deficiency induces anorexia by inhibiting the phosphorylation of AMP kinase (AMPK) in the hypothalamus.[28] Hypothalamic AMPK is regarded as a mediator of food intake.[28] Therefore, the following mechanism might be considered: reduced ATP concentration → reduced AMPK activity → negative feedback control of food intake.

Accumulation of lactic acid, increase of blood glucose, and decrease of elevated serum insulin have been reported in severe vitamin B_1–deficient animals.[29] Liang[30] reported that the accumulation of nonesterified fatty acids in plasma was observed in vitamin B_1–deficient animals. Valsangkar and Downs[31] revealed that administration of a β-oxidation inhibitor was stopped to resume from GV stage oocyte meiosis to GV break down. Zhang et al[24] reported that high quantities of ATP are needed for making spindle structure. In the present study, frequency of abnormal GV stage oocytes under the mildly vitamin B_1–deficient mice was not observed to be different compared with the control mice. However, the frequency of abnormal GV stage oocytes in the severely vitamin B_1–deficient mice was higher in the test group than in the control group. These results indicated that available body fat still remained in the mildly deficient mice but was completely depleted in the severely deficient mice. We considered that depletion of fatty acid and a resulting ATP insufficiency in the severely vitamin B_1–deficient mice caused an increase in the frequency of abnormal GV stage oocytes, spindle defect oocytes, and oocytes with chromosome misalignment. Selesniemi et al[4] 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

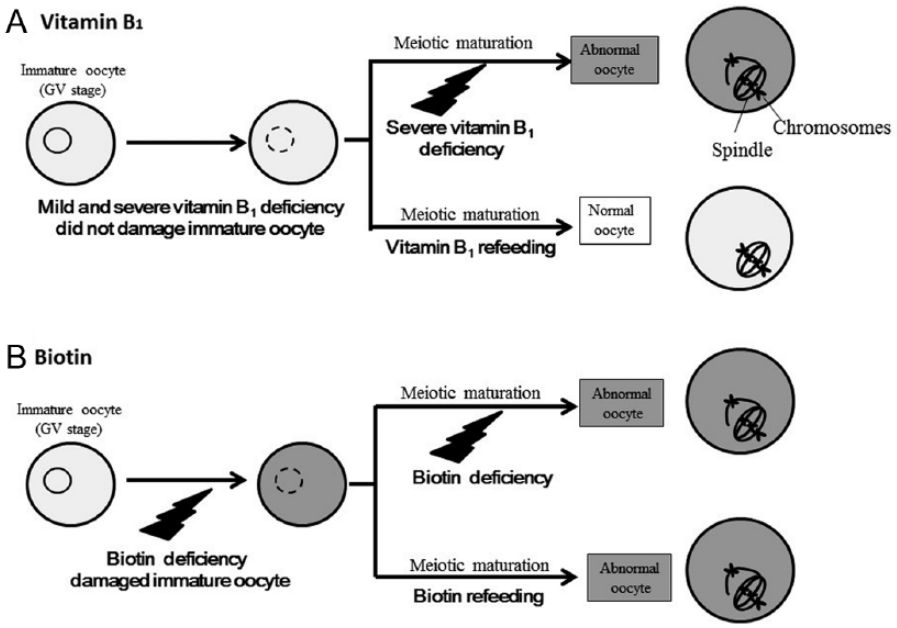

Figure 5. Characteristic effects of vitamin B_1 deficiency and biotin deficiency on oocyte maturation: (A) Severe vitamin B_1 deficiency (top oocyte) inhibited normal oocyte meiosis maturation. Mild and severe vitamin B_1 deficiencies did not damage immature oocytes in primary follicle (second oocyte from the left), and the oocyte quality was recovered by refeeding vitamin B_1–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).[5] GV indicates germinal vehicle.

(Figure 5A). However, damage to oocytes in the primary follicle cannot be repaired (Figure 5B).[32,33] The frequency of abnormal oocytes in the severely vitamin B_1–deficient mice decreased from 43.5% to 20.7% upon refeeding of vitamin B_1. The result suggested that vitamin B_1 deficiency inhibits meiotic maturation, and its deficiency does not damage oocytes in the primary follicle (Figure 5A).

We previously reported that the frequency of abnormal oocytes increased with biotin deficiency.[5] The frequency of abnormal oocytes in biotin-deficient mice increased 2.5-fold. Then, the frequency of abnormal oocytes was not restored by refeeding a biotin-containing diet for 20 days, although the restoration of biotin nutrient status was clearly observed.[5] Thus, immature oocytes in biotin deficiency were damaged in the primary follicle (Figure 5B). This damage to oocytes might be due to biotin being related to gene expression via biotinylation of histones H2A, H3, and H4.[34-36] To our knowledge, however, the relationship between oocyte quality and biotin at the molecular level is not known in detail. Taken together, these findings suggest that severe vitamin B_1 and energy deficiency might affect normal meiotic maturation of oocytes, but the deficiency does not damage the oocytes in the primary follicle, and that biotin might be required to maintain good quality oocytes in the primary follicle. The relationships between oocyte quality and the status of vitamin nutrition differ for each vitamin. These differences might be due to the different roles of vitamins in oocytes.

In the present study, we demonstrated the relationship between vitamin B_1 nutrition and oocyte maturation. Considering the different effects of vitamin B_1 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.

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