Folic Acid Deficiency Does Not Adversely Affect Oocyte Meiosis in Mice

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Summary Spindle defect and chromosome misalignment occurring in oocyte meiosis induce nondisjunction. Nondisjunction causes Down syndrome, also known as trisomy 21. Folic acid (FA) is an essential nutrient composition for fetal growth and development. It has been reported that FA nutritional status is associated with the risk of Down syndrome. However, to our knowledge, little is known about the effect of FA deficiency on abnormal oocytes (spindle defects, chromosome misalignments and immature oocyte) in vivo. In the present study, we investigate the effects of FA deficiency on oocyte meiosis in female mice. In order to induce FA deficiency in mice, female Crl:CD1 mice were fed a FA-free diet for 58 d. The diet also contained an antibiotic which has functions on limiting FA formation by intestinal microorganisms. The level of FA deficiency was determined by measuring the concentration of FA in the liver, hemocyte, uterus, ovary, and urine. FA concentrations in these samples from the FA-deficient group were 50–90% lower. Despite this, the frequency of abnormal oocytes was no different between the FA-deficient and control groups (20.0% vs 14.6%). According to the past research, FA transporter was strongly expressed in oocytes. Hence, it is possible that FA-free diets may not affect the concentration of oocyte FA in mice. To sum up these data, our study concluded that FA deficiency did not adversely affect oocyte meiosis.

Key Words folic acid, deficiency, oocyte meiosis, mouse, oocyte quality

Spindle defects and chromosome misalignments which occur in oocyte meiosis induce nondisjunction (1). Nondisjunction of chromosome 21 in particular is known as Down syndrome, which easily causes infertility, abortion and chromosomal abnormalities (1–3). Folic acid (FA) is a water-soluble vitamin, which is an essential nutrient for normal cell growth, fetal development (4), methylation (5) and DNA stability (6). FA deficiency accompanied by DNA methylation (5) and DNA damage (6). Recently, some reports indicated that abnormal methylation on DNA was associated with nondisjunction in oocyte meiosis (7, 8). Wang et al. (9) reported that when human lymphocytes were incubated in a low-FA medium, there was an increase in aneuploidy of chromosome 21. Meanwhile Takamura et al. (2) and Oliver et al. (3) reported that mothers of Down syndrome children have lower serum and plasma FA levels than mothers of non-Down syndrome children. James et al. (10) reported that there were increased levels and frequencies of homocysteine in plasma and methylene tetrahydrofolate reductase (MTHFR) 677CT and 677TT genotypes in mothers of Down syndrome children, respectively. These reports also suggested that maternal FA deficiency increased the risk of spindle defects and chromosome misalignments during oocyte meiosis. However, as far as we know there is no paper directly demonstrating the relationship FA deficiency between the frequency of oocyte spindle defects and chromosome misalignments in vivo. In this study, we investigate the effects of FA deficiency on maturation of oocytes in female mice.

MATERIALS AND METHODS

Chemicals. Vitamin-free milk casein and gelatinized cornstarch, mineral mixture (AIN-93G-MX) (11), dextrin and cellulose were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Corn oil was obtained from Ajinomoto Co., Inc. (Tokyo, Japan). L-Methionine, pteroylmonoglutamic acid, nicotinic acid, calcium pantothenate, pyridoxine hydrochloride, thiamin hydrochloride, riboflavin, dl-α-tocopherol, cholecalciferol, vitamin K1, cyanocobalamin and D-biotin were provided by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Vitamin A acetate and succinylsulfathiazole were from MP Biomedicals, Inc. (Tokyo, Japan).

Mice and diets. The care and treatment of experimental animals were approved under the guidelines set by the University of Shiga Prefecture (Shiga, Japan) for the ethical treatment of laboratory animals (approval number, No. 26–6).

Female 3-wk-old Crl:CD1 mice (n=20) were purchased from Charles River Laboratories (Tokyo, Japan). They were randomly divided into two groups of 10
mice; one group was fed a nutritionally complete diet (control group) and the others fed an FA-free diet (FA-deficient group) (Table 1). To suppress the FA supply from intestinal microbioflora, succinylsulfathiazole (an antibiotic) was added to each diet at 1% (w/w) (12). In the preliminary experiment, we confirmed that the addition of succinylsulfathiazole to the control diet would not adversely affect either the food intake, body weight gain, and FA concentrations in the body and urine or the oocyte quality (data not shown).

Animals had ad libitum access to food and water. Body weight was measured once every other day and food intake of the mice was daily measured. The temperature of the animal room was maintained at 22 ± 2°C, and the humidity maintained at around 60 ± 5%.

Urine collection and storage. Twenty-four-hour urine samples were collected from mice in metabolic cages into amber bottles containing 1 mL of 1 mol/L HCl. To each 0.9 mL of collected the urine, 0.1 mL of 1 mol/L ascorbic acid was added to prevent FA degradation. Urine samples were stored at −30°C until analysis.

Liver, uterus, ovary, plasma and hemocyte collection. On Day 58 of the experiment, mice were sacrificed. Blood samples were collected into tubes containing EDTA-2K (Terumo Co., Ltd., Tokyo, Japan). The liver, uterus and ovaries were removed and stored at −80°C until analysis. Blood samples were centrifuged at 1,700 × g for 30 min at room temperature to obtain separate plasma and hemocyte samples (12), which were then stored at −80°C until analysis.

Measurement of FA. The pretreatment method for samples was previously described (13, 14). FA compounds (pteroylmonoglutamic acid + dihydropteroylmonoglutamic acid + tetrahydropteroylmonoglutamic acid + 5-formyltetrahydropteroylmonoglutamic acid + 10-formyltetrahydropteroylmonoglutamic acid + 5,10-methyl-entetrahydropteroylmonoglutamic acid + 5-methyltetrahydropteroylmonoglutamic acid) in the plasma, hemocyte, liver, uterus, ovary and urine were measured using a microbiobioassay method with Lactobacillus rhamnosus ATCC 27773 (15).

Oocyte quality check; superovulation, collection of oocytes and immunofluorescence. The methods of oocyte collection through injection of steroid hormones and immunofluorescence have been described previously (16). The collected oocytes were immunostained using mouse anti-α-tubulin antibody (Cell Signaling Technology, Inc., Danvers, MA) followed by a goat anti-mouse IgG with Alexa Flour-488. Chromosomes in the oocytes were stained using 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Figure 1 shows the normal oocyte and abnormal oocyte. Normally-ovulated oocytes were mature metaphase II (MII) oocytes. We counted the number of MII oocytes with 1) spindles exhibiting serious malformations, and 2) chromosomes failing to align on normal meiotic spindles as abnormal oocytes.

Measurement of plasma estradiol. Estradiol was measured in plasma collected on Day 58 from mice housed in plastic cages. An enzyme-linked immunosorbent assay (ELISA) kit (catalog no. KB30-H1; Arbor Assay LLC., Ann Arbor, MI) was used for estradiol measurement, as per the manufacturer’s instructions.

Statistical analyses. The values in figures and tables are expressed as mean ± SE. Significant differences in urinary excretion of FA were analyzed using a two-way ANOVA followed by Bonferroni’s post hoc test. When two-way ANOVA indicated the presence of a day-group interaction, one-way ANOVA was conducted followed by Tukey’s multiple-comparison test, *p < 0.05; vs control group in the same day. Significant differences in frequency of abnormal oocytes was tested using Student’s t-test. *p < 0.05. p values were calculated by Graph Pad Prism version 5.0 (Graph Pad Software).

Table 1. Diet composition.

<table>
<thead>
<tr>
<th></th>
<th>Control diet (Control group)</th>
<th>FA-free diet (FA-deficient group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin-free milk casein</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Gelatinized cornstarch</td>
<td>367.9</td>
<td>367.9</td>
</tr>
<tr>
<td>Sucrose</td>
<td>183.6</td>
<td>183.7</td>
</tr>
<tr>
<td>Corn oil</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Dextrin</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mixture (AIN-93-G-MX) &lt;sup&gt;1&lt;/sup&gt;</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>Vitamin mixture (folic acid free) &lt;sup&gt;1&lt;/sup&gt;</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Folic acid &lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.0024</td>
<td>—</td>
</tr>
<tr>
<td>Succinylsulfathiazole &lt;sup&gt;3&lt;/sup&gt;</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>1</sup> Ref. 11.
<sup>2</sup> Pteroylmonoglutamic acid was added to the diet as folic acid.
<sup>3</sup> Succinylsulfathiazole was used as an antibiotic compound.
RESULTS

Body weight, food intake, the weights of the liver, uterus and ovary were no different between the control and FA-deficient groups (Table 2). The FA concentrations in the liver, hemocyte, uterus, ovary and the urinary excretion were reduced in the FA-deficient group compared with the control group (Table 2). The urinary excretion of FA in the FA-deficient group was almost zero from Day 1 (Fig. 2A). Oocyte meiosis occurs by luteinizing hormone (LH) surge. In our study, LH surge was caused by steroid hormones. Together, these results indicated that the mice of the FA-deficient group were in FA deficient status during oocyte meiosis. There was no difference in the frequency of abnormal oocytes between the control and FA-deficient groups ($p=0.661$; Table 2).

Table 2. Nutritional variables and FA concentrations in various tissues.

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>FA-deficient group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight on Day 58 (g)</td>
<td>29.4±0.8</td>
<td>28.2±1.4</td>
</tr>
<tr>
<td>Total food intake (g/58 d)</td>
<td>249±9</td>
<td>254±10</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.21±0.08</td>
<td>1.21±0.16</td>
</tr>
<tr>
<td>Uterus weight (g)</td>
<td>0.130±0.026</td>
<td>0.095±0.019</td>
</tr>
<tr>
<td>Ovary weight (g)</td>
<td>0.0210±0.0052</td>
<td>0.0226±0.0022</td>
</tr>
<tr>
<td>Urinary excretion of FA on Day 55 (nmol/d)</td>
<td>4.77±0.92</td>
<td>0.03±0.01***</td>
</tr>
<tr>
<td>FA in liver (nmol/g)</td>
<td>24.9±0.6</td>
<td>5.6±1.1***</td>
</tr>
<tr>
<td>FA in hemocyte (nmol/mL)</td>
<td>1.15±0.05</td>
<td>0.11±0.02***</td>
</tr>
<tr>
<td>FA in uterus (pmol/g)</td>
<td>137±7</td>
<td>40.2±4.0***</td>
</tr>
<tr>
<td>FA in ovary (pmol/g)</td>
<td>16.8±3.0</td>
<td>7.5±2.6*</td>
</tr>
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</table>

Values are means±SE; $n=5$. *$p<0.05$, ***$p<0.0001$ according to Student’s $t$ test.

Fig. 1. Oocyte quality check: normal oocyte and abnormal oocyte. MII oocyte α-tubules and chromosomes were stained by an immunofluorescence method. (A) normal MII oocyte, (B) spindle defect oocyte, (C) GV stage oocyte. Representative examples of meiotic spindles (Spi) in oocytes from indicated mice after labeling α-tubulin antibody and counter-staining DNA (Chr) with DAPI. Spi indicates spindle, Chr indicates chromosomes, GV indicates germinal vehicle in the pictures.
control, 14.6±3.0%; FA-deficient, 20.0±11.4%) (Fig. 2B). Nor did estradiol, a biomarker of ovarian function, present differently between groups (p=0.097; control, 27.5±2.1 pg/mL; FA-deficient, 34.3±2.9 pg/mL), suggesting that FA deficiency did not affect ovarian function.

DISCUSSION

In our study, FA deficiency did not affect oocyte meiosis in mice. Some studies reported that FA concentration had been decreased in the tissues of animals who were fed a FA-free diet for at least 3 wk (16, 17). In our study, FA level in the plasma, homocyte, liver and uterus was markedly lower in the FA-deficient group than in the control group at Day 36 (data are not shown). FA level in the ovary was not significantly different between the FA-deficient group and the control group (control group, 25.8±5.7 pmol/g; FA-deficient group, 14.6±3.7 pmol/g; p=0.134). Thus the FA-deficient group was considered to have low FA status for at least 24 d; however, the term of low FA status in the ovary was considered shorter than 24 d. It has been reported that maternal nutritional status, such as biotin deficiency (18), zinc deficiency or obesity (19) results in poor oocyte quality. FA deficiency decreases DNA methylation (5). Immature oocytes (GV stage oocytes) in primary follicles resume meiosis after LH surge. The GV oocytes in primary follicles mature to the MII oocytes. DNA methylation increased during the maturation stage (7). A low methylation level was observed in abnormal oocytes, such as nondisjunction (7, 8). Some epidemiology studies reported that the ability of FA metabolism is lower, which increases the risk of nondisjunction, which especially happen in Down syndrome (2, 20). In contrast, our study suggested that FA deficiency did not affect oocyte meiosis in vivo. Generally speaking, susceptibility to FA deficiency is different among tissues. In actuality, the effects of the FA deficient diet on the FA concentrations in the brain and spleen in mice was light (21). FA transporter expressions in the brain, spleen and ovary are known to be higher than in the other tissues such as liver and kidney (22, 23). FA transporter expressions in the ovary and cumulus-oocyte complex are high, too (22, 24). FA is transported by two major transport systems: the proton-coupled folate transporter (PCFT) and the reduced folate carrier (RFC1) (25). FA and reduced FA are absorbed by PCFT and metabolized to 5-methyltetrahydrofolate (5-methyl-THF) in the small intestine. RFC1 expresses in peripheral tissues and RFC1 has high affinity for the reduced FA such as 5-methyl-THF, 5-formyl-THF (25). Interestingly, the RFC1 expression level is promoted in an FA-deficient environment to maintain the FA homeostasis (26). RFC1 expression is strong in the ovary of hens (22) and also in the cumulus-oocyte complex of mice (24). In our study, we showed that the FA concentration in the ovary was markedly lower in the FA-deficient group than the control group. Although we confirmed that the ovary FA concentration in our FA-deficient mice was very low during oocyte meiosis, frequency of abnormal oocytes did not increase in the FA-deficient group. This result suggests that feeding the FA-deficient does not affect the oocyte FA concentration, because the RFC1 expression in HEK293 cells, liver and intestine is known to be increased by FA deficiency (26–28). However, there is no report about effect of FA deficiency on RFC1 expression in the ovary or oocyte. We were unable to measure the oocyte FA concentration due to the mass of the oocytes being too small. In conclusion, our study suggested that FA deficiency does not affect oocyte meiosis, despite causing a very low FA concentration in the ovary.

Acknowledgments

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Authors’ contributions

AT, TN and KS designed the study. AT and KS drafted the manuscript. TN critically read the draft paper and gave valuable comments. AT and RN performed the experiments. All authors read and approved the final manuscript.

REFERENCES


