Testicular Injury to Rats Fed on Soybean Protein-Based Vitamin B<sub>12</sub>-Deficient Diet Can Be Reduced by Methionine Supplementation

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Summary We have previously reported that rats fed on a vitamin B<sub>12</sub> (B<sub>12</sub>)-deficient diet containing 180 g soybean protein per kg diet showed marked histologic damage in their testes. In this paper, we report the effect of B<sub>12</sub>-deficiency on B<sub>12</sub>-dependent methionine synthase in the rats’ testes and the effect of methionine supplementation of the diet on testicular damage. Rats were fed the soybean protein-based B<sub>12</sub>-deficient diet for 120 d. We confirmed that those rats were in serious B<sub>12</sub>-deficiency by measuring urinary methylmalonic acid excretion and B<sub>12</sub> content in tissues. Methionine synthase activity in the testis of the B<sub>12</sub>-deficient rats was less than 2% of that in B<sub>12</sub>-supplemented (control) rats. To complement disrupted methionine biosynthesis, methionine was supplied in the diet. A supplement of 5 g D,L-methionine per kg diet to the B<sub>12</sub>-deficient diet did not affect urinary methylmalonic acid excretion of B<sub>12</sub>-deficient rats. The testicular histology of rats fed the methionine-supplemented B<sub>12</sub>-deficient diet was almost indistinguishable from that of control rats. Thus, we conclude that the lowered testicular methionine synthase activity is the primary cause of the histologic damage due to B<sub>12</sub>-deficiency and that methionine supplementation to the diet can reduce the damage. These findings would indicate the importance of the methionine synthase activity, especially for testicular function.

Key Words vitamin B<sub>12</sub>-deficiency, rat, testis, methionine synthase

In mammals, vitamin B<sub>12</sub> (B<sub>12</sub>) acts as a cofactor for two enzymes, methylmalonyl-CoA mutase and methionine synthase. Adenosylcobalamin is required for methylmalonyl-CoA mutase, which catalyzes a reaction that interconverts succinyl-CoA and methylmalonyl-CoA. Dysfunction of methylmalonyl-CoA mutase causes methylmalonic aciduria. Methylcobalamin (MeB<sub>12</sub>) binding to methionine synthase serves as an intermediate in the transfer of methyl from methyltetrahydrofolate to homocysteine, donating the methyl group to homocysteine to form methionine or deoxyuridine. The C1-group also is precursor in de novo purine biosynthesis. Methionine is one of the amino acids that are building blocks for protein. In addition, methionine is activated to S-adenosylmethionine (AdoMet), which is well known as a major methyl donor for numerous substrates and an allosteric regulator for methylenetetrahydrofolate reductase (MTHFR) and cystathionine-β-synthase. While causal of symptoms related with dysfunction of methionine synthase, such as megaloblastic anemia and neuropathy, might be explained by the “Methyl trap” hypothesis (1–4), the biochemical mechanism has not yet been fully understood.

There are two established animal models for study of the biochemical and nutritional functions of B<sub>12</sub>. One is the nitrous oxide (N<sub>2</sub>O, an anesthetic gas) exposed animal model (5, 6). Methionine synthase can be rapidly inactivated by N<sub>2</sub>O exposure both in vivo (5, 6) and in vitro (7–9). N<sub>2</sub>O exposure does not affect the activity of methylmalonyl-CoA mutase (6). Thus, the N<sub>2</sub>O-exposed animal system has been used for many investigations related to inactivation of methionine synthase. The other model is a B<sub>12</sub>-deficient animal, which would be a

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Abbreviations: AdoMet, S-adenosylmethionine; B<sub>12</sub>, vitamin B<sub>12</sub>; CN-B<sub>12</sub>, cyanocobalamin; MeB<sub>12</sub>, methylcobalamin; MMA, methylmalonic acid; MTHFR, methylenetetrahydrofolate reductase; N<sub>2</sub>O, nitrous oxide

* * *
model for inadequate vitamin intake. This model is suitable for studies of dysfunction of both B₁₂-dependent enzymes.

We found that B₁₂-deficient rats and mice fed a B₁₂-deficient diet in which soybean protein was used as a protein source showed severe damage of testicular tissue accompanying weight loss (10–12). In fact, there are independent reports about male infertility accompanying B₁₂-deficiency, and improvement of sperm motility upon B₁₂ administration (13–17). These clinical observations are supported by reports which show that experimental disturbance of spermatogenesis in experimental animals can be alleviated by the administration of B₁₂ (18, 19). In addition, we have reported that rats fed a B₁₂-deficient diet containing casein instead of soybean protein did not show testicular weight loss (20). The casein diet contains more methionine than soybean protein does, because a primary limiting amino acid of soybean protein, generally known as legume protein, is methionine. Interestingly, the N₂O-exposed rats' testes showed loss of dry weight and reduced spermatogenesis (21). Together, these lines of evidences imply that methionine synthase plays an important role in maintaining testicular function. However, to the best of our knowledge, changes in testicular methionine synthase activities of B₁₂-deficient animals and effects of a methionine supplement to the soybean protein-based B₁₂-deficient diet on the testicular damage have not yet been reported.

In this paper, we describe methionine synthase activity in the testis of the B₁₂-deficient rat. We also examine the effects of a supplement of methionine to the B₁₂-deficient diet on the testicular damage.

**MATERIALS AND METHODS**

**Animals.** Wistar strain rats, 10 wk old male and female, were purchased from Clea, Tokyo, Japan. In accordance with our previous report (22, 23), male rats (approximately 25 g of body weight) born to dams that had been fed on a B₁₂-deficient diet during pregnancy and lactation were used in the following experiments. These male rats were divided into a B₁₂-supplemented (control) group and a B₁₂-deficient group immediately upon weaning and individually housed in stainless steel screen-bottom cages under conditions of constant temperature (22±3°C) and a 12 h cycle of light and dark. All rats were allowed to access food and water freely. The protocols complied with the Guideline for Animal Experimentation (24). Urine was collected using metabolic cages for 20 h, filtered, and stored at −30°C until use.

**Diets.** A B₁₂-deficient diet containing 180 g soybean protein per kg diet was prepared as in our previous reports (22, 23). A methionine-enriched diet was prepared by adding 5 g dl-methionine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) per kg of the B₁₂-deficient diet. The diet composition is shown in Table 1. Rats in both the control and the B₁₂-deficient group were fed B₁₂-deficient diets, but rats in the control group were orally administered 1 μg of cyanocobalamin (CN-B₁₂, Wako Pure Chemical Industries, Ltd.) per day throughout the feeding period, and those of the B₁₂-deficient group were given a similar amount of distilled water for the same period.

<table>
<thead>
<tr>
<th>Component</th>
<th>Vitamin B₁₂-deficient diet</th>
<th>Methionine-enriched vitamin B₁₂-deficient diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean protein isolate¹</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>Glucose, anhydrous²</td>
<td>673.5</td>
<td>668.5</td>
</tr>
<tr>
<td>Lard¹, (containing fat-soluble vitamins⁴)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mineral mixture³</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Water-soluble vitamin mixture without B₁₂⁶</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline chloride⁷</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>dl-methionine⁷</td>
<td>—</td>
<td>5</td>
</tr>
</tbody>
</table>

¹ Ajinpon SU, crude protein content 85.4%, Ajinomoto, Tokyo, Japan.
² Sanei Toka, Ehime, Japan.
³ Hayashi Chemicals, Tokyo, Japan.
⁴ Fat-soluble vitamins in 1 kg of diet: dl-tocopheryl acetate, 35 mg; retinyl acetate, 4000 IU; and cholecalciferol, 1000 IU. These vitamins were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.
⁵ Composition of mineral mixture (g/kg of mixture): calcium phosphate, dibasic, 500; sodium chloride, 74; potassium citrate, monohydrate, 220; potassium sulfate, 52; magnesium oxide, 24; manganese carbonate, 3.5; ferric citrate, 6; zinc carbonate, 1.6; cupric carbonate 0.3; potassium iodate, 0.01; sodium selenite, 0.01; and chromium potassium sulfate, 0.55. Weight of the mixture amounted to 1 kg by addition of glucose (anhydrous). All mineral salts were purchased from Wako Pure Chemical Industries, Ltd.
⁶ Composition of water-soluble vitamin mixture (mg/kg of mixture): thiamin hydrochloride, 600; riboflavin, 600; pyridoxine hydrochloride, 700; nicotinic acid, 3000; calcium pantothenate, 1600; folic acid, 200; biotin, 20; and menadione, 5. Weight of the mixture amounted to 1 kg after addition of glucose (anhydrous). All of the vitamins were purchased from Wako Pure Chemicals.
⁷ Wako Pure Chemical Industries, Ltd.

Table 1. Composition of experimental diets.
**Experimental design.** Two independent experiments (Experiment 1 and 2) were carried out. The first experiment, Experiment 1, was designated to elucidate changes in methionine synthase activity of rats’ testes upon B12-deficiency and effects of MeB12 administration to B12-deficient rats on methionine synthase activity. In this experiment, the soybean diet was used throughout the breeding period of 120 d. A short term recovery test was also done as follows: 0.1 μg of methylcobalamin (MeB12, Eizai, Tokyo, Japan) per g of body weight was intraperitoneally injected into B12-deficient rats at 20 or 40 h before ending the breeding period. The second experiment, Experiment 2, was done to examine effects of a methionine supplement to the diet on testicular histologic changes. In this experiment, rats were fed either the soybean diet or the methionine-enriched soybean diet throughout the breeding period of 120 d. In Experiment 1 and 2, food and water intake was not limited, so-called ad libitum feeding. At the end of the breeding period, blood was collected from hearts of rats under anesthesia (Somnopentyl; pentobarbital sodium, Pitman-Moore, Mundelein, IL), and then tissues were removed. Those tissues were frozen in liquid nitrogen immediately and stored at −80°C, except tissues that were used for the histologic study. The tissues for the histologic study were soaked in formalin neutral buffer, pH 7.4, for 24 h at room temperature, and were stained with hematoxylin and eosin. Group names were defined as follows: Control and B12-deficient rats were for rats fed the B12-deficient diet and receiving CN-B12, or water, respectively. Rats fed the methionine-enriched B12-deficient diet and receiving CN-B12 were named Met+ control, while those fed the same diet without CN-B12 were called Met+B12-deficient rats.

**Determination of urinary methylmalonic acid (MMA).** MMA excretion in urine was determined by the colorimetric method of Giorgio and Plaut (25). MMA excretion was expressed as mol MMA/mol creatinine. Urinary creatinine was determined by the colorimetric method of Cook (26).

**Assay of testicular B12.** Tissue B12 was extracted by boiling in the presence of potassium cyanide, and then determined by a microbiological assay using *Lactobacillus delbrueckii* lactis ATCC 7830 (27, 28).

**Methionine synthase activity.** Methionine synthase assay was carried out as described in our previous report (23). Briefly, methyl transfer from methyltetrahydrofolate to homocysteine was measured using [methyl-14C]-tetrahydrofolate (Amersham Pharmacia Biotech, Uppsala, Sweden). The reaction was carried out under anaerobic conditions using an H2 atmosphere and Ti(III) citrate. To measure the apomethionine synthase/holo methionine synthase ratio, the assay was done in the presence or absence of MeB12 in the reaction mixture. The enzyme activities in the presence and absence of MeB12 were expressed as total enzyme activity and holoenzyme activity, respectively. Protein was assayed by the colorimetric method of Bradford (29) using bovine serum albumin as a standard.

**Statistical analysis.** Data shown are means±SD. In Experiment 1, the data were analyzed by a one-way ANOVA and Kruskal-Wallis test using Stat View (Version 5.0; SAS Institute, Cary, NC) or Excel Tokei (Version 5.0; Esumi Co., Tokyo, Japan), and then differences among groups were evaluated by the Scheffe and Steel-Dwass tests. Differences with p<0.05 were considered significant. In Experiment 2, the overall effects and interactions of B12-deficiency and methionine supplementation were determined with two-way ANOVA using Stat View (Version 5.0; SAS Institute, Cary, NC) or Excel Tokei (Version 5.0; Esumi Co., Tokyo, Japan). Post hoc analysis using the Scheffe test was conducted when the main effects were identified by the two-way ANOVA.

**RESULTS**

**Experiment 1: The effect of B12 deficiency on testicular methionine synthase activity**

**Growth and MMA excretion.** Rats in the B12-deficient group showed significant growth retardation as com-

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**Table 2. Summary of Experiment 1: Effects of B12-deficiency and MeB12 administration to B12-deficient rats on body weight, testis weight, testicular B12 levels, urinary methylmalonate excretion, and testicular methionine synthase activity of rats.**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Gain in body weight g/120 d</th>
<th>Testes weight wet g</th>
<th>MMA (pmol/g testis)/creatinine mol/mol</th>
<th>B12 content pmol/g testis</th>
<th>Methionine synthase activity holo total nmol/(min·mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats</td>
<td>7</td>
<td>251.6±22.4a</td>
<td>2.61±0.19a</td>
<td>&lt;0.2c</td>
<td>58.7±9.7a</td>
<td>0.87±0.04a</td>
</tr>
<tr>
<td>B12-deficient rats</td>
<td>5</td>
<td>140.5±13.7b</td>
<td>0.80±0.2b</td>
<td>13.7±2.5a</td>
<td>2.1±0.7c</td>
<td>&lt;0.01c</td>
</tr>
<tr>
<td>MeB12-administered rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MeB12 (0.1 μg/g body weight) intraperitoneally injected to B12-deficient rats</td>
<td>5</td>
<td>143.7±11.2b</td>
<td>0.80±0.12b</td>
<td>2.7±1.0b</td>
<td>12.3±4.5b</td>
<td>0.07±0.03b</td>
</tr>
<tr>
<td>at 20 h before killing</td>
<td>5</td>
<td>147.3±14.5b</td>
<td>0.73±0.06b</td>
<td>0.2±0.1c</td>
<td>18.6±4.6b</td>
<td>0.11±0.01b</td>
</tr>
<tr>
<td>at 40 h before killing</td>
<td>5</td>
<td>147.3±11.2b</td>
<td>0.80±0.12b</td>
<td>2.7±1.0b</td>
<td>12.3±4.5b</td>
<td>0.07±0.03b</td>
</tr>
</tbody>
</table>

1Results are means±SD, n=7 or 5. Values in a column of each experiment with different superscript letters are significantly different, p<0.05.2
pared to the control rats (Table 2). Urinary MMA excretion in B12-deficient rats on day 120 of the feeding period was remarkably high (Table 2). Although MeB12 is a cofactor for methionine synthase, MMA excretion following MeB12 injection clearly decreased to the control level within 40 h.

Testicular B12 content and methionine synthase activity. B12 content in the testis was lowered by feeding the B12-deficient diet for 120 d (Table 2). The testicular B12 level of the B12-deficient group was ~4% that of control group. The administration of MeB12 to the B12-deficient rats increased the testicular B12 content to 32% that of control within 40 h. Both total- and holomethionine synthase activities in rat testis in the B12-deficient group were near or less than the detection limit and were depressed to less than 2% of those in the control group (Table 2). No stimulation of the enzyme activity by addition of MeB12 to the reaction mixture was observed.

Table 3. Summary of Experiment 2: Effect of dietary methionine supplement on body weight, testis weight, testicular B12 levels, and urinary methylmalonate excretion of control and B12-deficient rats.1

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Gain in body weight g/120 d</th>
<th>Testes weight wet g</th>
<th>MMA/creatinine mol/mol</th>
<th>B12 content pmol/g testis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats</td>
<td>10</td>
<td>237.8±20.7b</td>
<td>2.57±0.21a</td>
<td>&lt;0.2b</td>
<td>57.8 ± 7.8a</td>
</tr>
<tr>
<td>B12-deficient rats</td>
<td>7</td>
<td>133.0±18.0c</td>
<td>0.76±0.18b</td>
<td>14.3 ± 2.3a</td>
<td>2.98 ± 0.9b</td>
</tr>
<tr>
<td>Met+control rats</td>
<td>10</td>
<td>335.0±10.5a</td>
<td>2.69±0.15a</td>
<td>&lt;0.2b</td>
<td>61.8 ± 15.9a</td>
</tr>
<tr>
<td>Met+B12-deficient rats</td>
<td>10</td>
<td>225.8±18.3b</td>
<td>2.79±0.12a</td>
<td>12.3 ± 2.2a</td>
<td>2.3 ± 1.2b</td>
</tr>
</tbody>
</table>

Two-way ANOVA

B12 <0.0001 <0.0001 <0.0001 <0.0001
Methionine <0.0001 <0.0001 0.0548 0.5715
B12×Methionine 0.6977 <0.0001 0.0572 0.4665

1Results are means±SD, n=7 or 10. Values in a column of each experiment with different superscript letters are significantly different, p<0.05.

Fig. 1. Effects of dietary methionine supplement on testicular histologic change: Light micrographs of testis of control (photo A), B12-deficient (photo B), Met+control (photo C), and Met+B12-deficient (photo D) rats. Testes were fixed with neutral buffered formalin, and were stained with hematoxylin and eosin. The bar in A represents 0.2 mm for all panels.
administration of MeB12 to B12-deficient rats was effective in increasing both total- and holoenzyme activity, although the activities were low at approximately 10% of control.

**Testicular histologic changes.** We observed testicular histologic changes in the B12-deficient rat. The administration of MeB12 did not reverse the histologic changes (data not shown).

**Experiment 2: Effect of methionine supplementation on testicular changes induced by B12-deficiency**

**Growth and testes weight.** The body weight gain of the B12-deficient rats was 133 g, which was 56% of that of the control (Table 3). Feeding of the methionine-enriched diet resulted in more body weight gain. The body weight gain of rats in Met+B12-deficient group was 67% of the body weight of the Met+control group. Testis weight of rats in B12-deficient group was lower than that in Met+control group (Table 3). However, testis weight of rats in Met+B12-deficient group was the same as that in both control and Met+control groups.

**MMA excretion and B12 content in testis.** Extensive MMA excretion was observed in both B12-deficient groups (Table 3). Testicular B12 content in both B12-deficient groups was remarkably low (Table 3).

The methionine supplement did not affect the tissue B12 content. Rats’ testes in the B12-deficient group contained approximately 3 pmol B12/g tissue. This was 5% of the corresponding control group.

**Effect of the methionine supplement on the testicular histologic change.** The effect of dietary methionine supplementation on the testicular histologic change was dramatic (Fig. 1). Atrophy of seminiferous tubules and aplasia of sperms and spermatids, which was observed in testes of the B12-deficient rats (photo B), was not observed in the tissue of Met+B12-deficient rats (photo D). The tissue of Met+B12-deficient rats was almost indistinguishable from that of both groups of control rats using a light microscope (photo A and C).

**DISCUSSION**

The testicular histologic changes in N2O-exposed rats are similar to those seen in dietary B12-deficient rats, as follows: B12-deficient rats had a reduced wet weight in their testes (10, 11) and the dry weight of testes from the N2O-exposed rats was also lower after 32 d of N2O exposure (21). Both rats’ testes show abnormal spermatogenesis, such as aplasia of sperm and spermatoids. Methionine synthase activities in testes were lowered by B12-deficiency (this study). Brodsky et al. (30) have reported that only 1 h exposure of 50% N2O could cause 63% inhibition of the enzyme activity in the testes of rats. N2O-exposed rats needed at least 72 h to recover methionine synthase activity to the control level and B12-deficient rats may need more than 40 h (see below). These results suggest that the testicular injury is responsible for the inactivation of methionine synthase, not for dysfunction of methylmalonyl-CoA mutase because N2O-exposure does not affect methylmalonyl-CoA mutase activity (6).

The mechanism of methionine synthase inactivation by N2O has been extensively investigated using purified Escherichia coli MetH, a homologue of mammalian B12-dependent methionine synthase (7–9). Human and rat methionine synthase have significantly high homology to the E. coli enzyme. These identities in deduced amino acid sequences from their cDNAs are 55% for human enzyme (31) and 53% for rat enzyme (32). N2O can destroy the E. coli enzyme in vitro by irreversible oxidation, i.e. protein modification (9). Based on the high homology, it is likely that the same irreversible inactivation would happen to other mammalian methionine synthases. Methionine synthase activity in the testes of the B12-deficient group is also clearly low. Our previous study revealed that the low activity of methionine synthase in the liver of B12-deficient rats is caused by the low enzyme level (23). This may be because of extreme instability of apomethionine synthase whose half life was estimated as approximately 2 min at 37˚C (33). It is likely that the low activity of testicular methionine synthase can be explained in the same way. Because of the lack of intact apo- and holoenzyme, both N2O-exposed and B12-deficient rats would need newly synthesized methionine synthase protein to recover enzyme activity. This may be the reason why it takes a while to restore the enzyme activity after intraperitoneal administration of B12. Methylmalonyl-CoA mutase, however, rapidly recovered its activity after the administration of MeB12 (Table 2), as judged by decreased MMA excretion. This observation suggests that the conversion from MeB12 to adenosylcobalamin and the formation of holoenzyme occurred rapidly.

In Experiment 1, we demonstrated lowered methionine synthase activity in the B12-deficient rat testis. The specific activity of the enzyme in the testes of the control group was 2.8 times higher than that in the liver (23). This may indicate the importance of methionine synthase function in rat testes. In the diet used in Experiment 1, methionine content can be calculated to be 2.2 g/kg (1.2 g methionine/100 g soybean protein (34)). We have reported the effect of casein on testis weight of B12-deficient rats. Methionine content in casein is 2.8% of total amino acids (34). If the diet contained casein instead of soybean protein, the methionine content would be 5 g/kg diet. Thus, we decided to examine the effect of a methionine supplement in the B12-deficient diet on the testicular injury. Since added D,L-methionine was 5 g/kg diet, i.e. 2.5 g L-methionine/kg diet, methionine content in the diet was approximately doubled by supplementation. As shown in Experiment 2, effects of the methionine supplement in the diet were dramatic (Table 3 and Fig. 1). Based on the low B12 content, activities of testicular methionine synthase in both B12-deficient groups can be assumed to be as low as those in the B12-deficient group in the Experiment 1. Thus, we concluded that the methionine supplement to the diet was effective to reduce the testicular damage even during severe B12-deficiency.

Odd-chain fatty acids and blanched-chain amino acids can be precursors for MMA when methylmalonyl-CoA mutase is inactive. In addition, methionine is also
metabolized to succinyl-CoA via propionyl-CoA, suggesting that methionine supplementation could result in elevated MMA excretion. However, the difference in MMA excretion of the Met+B12-deficient group and B12-deficient group was statistically insignificant (Table 3). We previously reported that protein utilization was reduced in B12-deficient rats (35). The depressed protein utilization might be improved by the methionine supplement. Then, other amino acids, such as valine and isoleucine, could be used to synthesize protein rather than wasted. According to this argument, at least in part, we may explain that the excretion of MMA in the Met+B12-deficient rat was not increased. In the B12-deficient rats, methionine deficiency appears to be the major factor for the testicular damage.

While a complex process is needed for spermatogenesis, at least in this case, it is likely that impaired methionine biosynthesis due to the dysfunction of methionine synthase is the key factor causing the histologic changes in the B12-deficient rats’ testes. The methionine supplementation can improve protein utilization (see above). Moreover, AdoMet production can be increased by the methionine supplementation, thus the AdoMet/S-adenosylhomocysteine ratio might be changed. The ratio is known as an important factor not only to regulate MTHFR activity but also to determine efficiency of AdoMet-dependent transmethylation (36). Because the methionine supplement should affect many important factors, it is hard to specify the biochemical mechanism of the testicular damage by our experiments. Recently, some reports related to folate and methionine metabolism and testicular morphology have been published. An MTHFR gene knock-out mouse has recently been developed (37). Interestingly, homozygote Mthfr−/− mice showed similar abnormal spermatogenesis (38). Theoretically, methylenetetrahydrofolate cannot be trapped in tissues of Mthfr-deficient mice, because MTHFR is the only known enzyme to convert methylenetetrahydrofolate to methylenetetrahydrofolate in mammals. Thus, the “Methyl trap” hypothesis may not explain testicular injury due to B12-deficiency. The relation between severe testicular damage and hypo-DNA methylation has been reported by Hata et al. (39) and Webster et al. (40) using a Dnmt3L−/− mouse model, indicating that the ratio is important for testicular function. AdoMet content might be low in testes of B12-deficient rats due to the imparted methionine biosynthesis; hence, hypo-methylation of DNA could be one of the most likely explanations for the testicular injury. We have to measure methionine metabolites, i.e. methionine, homocysteine, AdoMet, adenosyl-homocysteine, polyamine, methyl-group acceptors (for example DNA and proteins), and methylenetetrahydrofolate, and protein turn-over in testes to fully elucidate the mechanism.

Though our experiment was done under extreme conditions, dietary B12-deficiency could occur due to inadequate B12 intake. In addition, dysfunction of the absorbing or transporting systems of B12 could cause inactivation of B12-dependent enzymes in cells, even if the diet contains adequate B12. The testis damage due to B12-deficiency can be reduced by high methionine intake, so that it can be simply interpreted as methionine deficiency. Our data would indicate how important and powerful the re-methylation of homocysteine is. If a lowered concentration of methionine caused by B12-deficiency is a real factor, betaine supplementation in the diet would be expected to reduce the testis damage. Although betaine: homocysteine methyltransferase is a liver specific enzyme, the enzyme is still active in the liver of the B12-deficient rat (41). In fact, abnormal spermatogenesis in the Mthfr-deficient mouse testis could be improved by betaine supplementation in diets (38).

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