Note

Influences of Maternal B₁₂ and Methionine Intake during Gestation and Lactation on Testicular Development of Offspring in Rats

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Summary The influence of maternal vitamin B₁₂ malnutrition on testicular development of offspring was examined using soy protein-based B₁₂-deficient diets with or without 0.5% DL-methionine supplementation. Dams were fed the B₁₂-deficient diet throughout gestation and lactation, whereas dams in a control group were fed a control diet which contained cyanocobalamin in the B₁₂-deficient diet without methionine. Offspring born to dams fed the B₁₂-deficient diet without methionine showed poor testicular development, e.g. decreased numbers of seminiferous tubules containing healthy spermatocytes and a high ratio of apoptotic cells per all germ cells. The abnormality was rarely observed in the group fed the B₁₂-deficient diet with methionine. It was likely that the testicular abnormality of offspring was caused by B₁₂-deficiency post partum and was prevented by the methionine supplementation. These observations suggested that maternal B₁₂ nutritional status during the pre-weaning period is quite important for spermatogenesis of male offspring and that the requirement of B₁₂ for testicular development is to produce active B₁₂-dependent methionine synthase.

Key Words vitamin B₁₂, methionine synthase, folate and methionine metabolism, testicular development, maternal nutrition

Vitamin B₁₂ (B₁₂) is thought to be the animal protein factor per se or a closely related substance (1), and its daily requirement is the smallest of all vitamins. The biochemical role of B₁₂ is to be a cofactor for several enzymes. In mammals, methylcobalamin and adenosylcobalamin work as cofactors for methionine synthase (MS) and methylmalonyl-CoA mutase, respectively. Insufficient intake of B₁₂ results in dysfunction of these enzymes, which causes characteristic B₁₂-deficient symptoms, such as megaloblastic anemia, homocysteinemia, and methylmalonic aciduria. To successfully incorporate the very limited dietary sources of B₁₂ into its target enzymes requires specific binding proteins and receptors for absorption, transportation, and activation. Disruption of this system by dysfunction of proteins due to gene mutations can cause similar symp-

toms to B₁₂-deficiency (2). More recently, B₁₂ and folate attracted attention as homocysteine-lowering vitamins since hyperhomocysteinemia is an independent risk factor for cardiovascular disease. In addition, these vitamins have been also recognized as important factors for mammalian reproduction since the early 1960s; Sharp and Witts (3) and Lillie (4) reported an association between megaloblastic anemia (pernicious anemia in their reports) and human infertility in 1962.

Recently, many researchers have turned their attention to epigenetics. Chemical modification of DNA and histone with methyl groups may dramatically alter gene expression, which can induce cellular differentiation. These methyl groups are donated from S-adenosylmethionine (AdoMet), active methionine. While methionine is one of the essential amino acids in mammals, it can be re-synthesized by methylation of homocysteine using a methyl group on methyltetrahydrofolate (CH₃-H₄folate).

The reaction catalyzed by MS (Eq. (1)) is responsible for two pathways: production of tetrahydrofolate for a

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Fig. 1. Experimental design. Parent rats were fed with commercial lab chow and mated. After confirming development of a vaginal plug on each female rat, they were individually caged and divided into three groups. Maternal rats received a B₁₂-deficient diet without (–B₁₂) or with methionine (–B₁₂+Met) until weaning. Rats in the “+B₁₂” group, as a control group, were fed a diet containing cyanocobalamin (1 µg CN-B₁₂/kg diet).

reduced-folate cycle by de-methylation of CH₃-H₄folate and re-generation of methionine by methylation of homocysteine. Methionine is activated to AdoMet by a reaction catalyzed by methionine adenosyltransferase. Although the biochemical bases of several B₁₂-deficiency symptoms have been poorly understood, considering the effect of these metabolic malfunctions on epigenetics may be critical for rational understanding.

To examine the action of B₁₂, we have established a severely B₁₂-deficient animal model by feeding rodents a soy protein-based B₁₂-deficient diet (5–8). The B₁₂-deficient rats and mice show remarkable damage to the testis (9–12), which can be almost completely prevented by supplementation of methionine to the diet (13). These findings suggest that the tissue injury is caused by dysfunction of MS, rather than methylmalonyl-CoA mutase. Methylmalonyl-CoA mutase is the other B₁₂-dependent enzyme in mammals, and this enzyme catalyzes the rearrangement of methyl-malonyl CoA to succinyl-CoA. Thus methionine supplementation would not complement deficiencies of this enzyme activity. Recently, Watanabe et al. have suggested that dietary B₁₂-deficiency during gestation induces irreversible damage to spermatogenesis and sperm maturation is impaired by further feeding of a B₁₂-deficient diet after weaning (14, 15). The hypothesis that impaired testicular development could be rescued by a methionine-enriched diet has not yet been examined. Here, we report effects of maternal intake of B₁₂ and methionine on testicular morphology of offspring.

Materials and Methods

Animals and diet. As parent rats, 10-wk-old Wistar strain male and female rats (CLEA Japan, Inc., Tokyo, Japan) were purchased. Rats were bred with free access to food and water under constant temperature (22±3°C) and a 12-h cycle of light and dark. All protocols complied with the Guideline for Animal Experimentation (16). A B₁₂-deficient diet was prepared according to our previous reports (5, 7, 8, 13). Briefly, diet composition (per kg) was as follows: soy protein (Ajipron SU, crude protein content 85.4%, Ajinomoto, Tokyo, Japan), 180 g; anhydrous glucose (Sanei Toka, Ehime, Japan), 673.5 g; lard containing fat-soluble vitamins (8, 13), 100 g; a mineral mixture (8, 13), 35 g; a water-soluble vitamin mixture without B₁₂ (8, 13), 10 g; choline

Table 1. Effects of maternal vitamin B₁₂ and methionine intake during gestation and lactation on testis weight of offspring.

Groups	Dam <i>n</i> ¹	Offspring		
		<i>n</i> ²	Testis weight (g)	Testis weight (g)/ 100 g body weight
–B ₁₂	10	49	0.12±0.02 ^{3b}	0.49±0.05 ^a
–B ₁₂ +Met	9	52	0.14±0.04 ^a	0.49±0.05 ^a
+B ₁₂	6	28	0.15±0.04 ^a	0.49±0.04 ^a

¹ Total numbers of dams in each group.

² Total numbers of male offspring in each group.

³ Average±SD.

Values in a column of each experiment with different superscript letters are significantly different, *p*<0.05.

chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 1.5 g. A methionine-enriched B₁₂-deficient diet contained 0.5% DL-methionine (Wako Pure Chemical Industries, Ltd.), which was prepared by adding 5 g DL-methionine per 1 kg of the B₁₂-deficient diet. Total amount of the diet was balanced by reducing the amount of anhydrous glucose to 668.5 g (8, 13). Control rats were fed a control diet, which contained cyanocobalamin (CN-B₁₂, Wako Pure Chemical Industries, Ltd., 1 µg CN-B₁₂/kg diet) in the B₁₂-deficient diet.

Experimental design. The experimental schedule is summarized in Fig. 1. After copulation, maternal rats were divided into three groups; “–B₁₂,” “–B₁₂+Met,” and “+B₁₂.” Offspring born to dams fed the B₁₂-deficient diet and the methionine-enriched B₁₂-deficient diet were named “–B₁₂” and “–B₁₂+Met,” respectively. As a control group, maternal rats who were fed the B₁₂-deficient diet but who received CN-B₁₂, were designated as “+B₁₂.” They were individually housed and fed the same diets throughout gestation and lactation for 45 d. Male offspring were used for testicular morphology at the end of lactation and their body weight was measured at that time. Testes were fixed in neutralized formalin and then embedded in paraffin. Sections at 4 µm thickness were cut and stained with hematoxylin and eosin.

Statistical analysis. One-way ANOVA and Kruskal-Wallis tests using Stat View (version 5.0; SAS Institute,

Fig. 2. Effects of maternal intake of the B₁₂-deficient diet with or without methionine supplementation on testicular morphology of offspring at the end of the lactation period. Typical results of testicular morphology of the $-B_{12}$ group (A and B) and the $-B_{12} + \text{Met}$ group (C and D) are shown. Formalin-fixed testes were embedded in paraffin, then sliced and stained by hematoxylin and eosin. Panel E shows a testicular section of the $+B_{12}$ group, which was prepared by the same method except that the testis was fixed in Bouin's solution. Scale bars indicate 50 μm for panels A, C and E, and 100 μm for panels B and D. An asterisk marks a seminiferous tubule lacking spermatocytes and an arrow points at a pachytene spermatocyte. An apoptotic cell, possibly derived from a spermatogonium or sertoli cell, is indicated by a filled arrow, whereas an open arrowhead shows a likely apoptotic spermatocyte.

Cary, NC) or Excel Tokei (Version 5.0; Esumi Co., Tokyo, Japan) were used for data analysis. Scheffe and Steel-Dwass tests were employed for evaluation of differences among groups. Differences were considered significant when p values were less than 0.05. Data are expressed as mean $\pm$ SD.

Results

Testicular weight of offspring born to dams fed the B₁₂-deficient diet with ($-B_{12} + \text{Met}$) or without methionine ($-B_{12}$) at the end of lactation is shown in Table 1. Although the testicular weight of offspring in the $-B_{12}$ group was lighter than that in the other two groups, there is no difference in three groups when they were compared with ratios of testicular weight per 100 g body weight. This was because offspring in the $-B_{12}$ group did not grow as well as those in the two other groups.

Testicular morphology in the $-B_{12}$ and $-B_{12} + \text{Met}$ groups is shown in Fig. 2. In the $-B_{12}$ group (Fig. 2A and B), approximately 25% of the seminiferous tubules lacked healthy spermatocytes. The number of spermatogonia was greatly reduced, although some pachytene spermatocytes, which can be found in the very early stage of meiosis, were observed. The ratio of apoptotic cells (likely derived from spermatogonia and/or sertoli cells) to all germ cells was very high. In contrast, the ratio was greatly reduced in the $-B_{12} + \text{Met}$ group (Fig. 2C and D). Moreover, the diameters of seminiferous tubules in the $-B_{12} + \text{Met}$ group were seemingly larger than those in the $-B_{12}$ group. Indeed, the testicular morphology of the $-B_{12} + \text{Met}$ rats was more similar to that of normal rats (Fig. 2E). These observations indicate that methionine supplementation to the diet pre-

vented the testicular damage, although full remediation was not achieved.

Discussion

Clarifying the contributions of folate and methionine metabolism to human health is quite difficult because various environmental conditions including nutritional and genetic diversities could affect this metabolism and its regulation. Folate fortification is likely to lower the concentrations of homocysteine in blood. However, why elevated levels of homocysteine are associated with cardiovascular disease and how adequate folate prevents neural tube defects are poorly understood. Here, we demonstrated for the first time roles of B₁₂ and methionine on maternal nutrition using classic nutritionally deficient rats as a model. In this and our previous (13) studies, we showed the strong remedial effect of methionine on B₁₂-deficiency, indicating that the function of MS in the testis would be important for tissue development. In fact, activity of the enzyme in the testis is higher than those in most of the other organs (13). Since B₁₂ is the essential cofactor for MS and the diets contained sufficient folate, we focus on maternal nutrition of B₁₂ and methionine in the following discussion.

The biological half-life of B₁₂ is approximately 1 y (17–19), and this is the reason why raising animals in B₁₂-deficiency is difficult, especially in a short period. We have learned that it takes more than 90 d to induce B₁₂-deficiency in mature rats by feeding the soy protein-based B₁₂-deficient diet after weaning (5, 10). However we found that the offspring of rats fed a B₁₂-deficient diet for only the 45 d of gestation and lactation showed significant damage to the testes in this experiment, suggesting an increased-requirement of B₁₂ during ges-

tation and lactation. Clear observation of nutritional complementation by methionine to prevent the testicular damage indicated that methionine deficiency was the primary causal factor of the testicular abnormalities in B₁₂-deficient rats.

Interestingly, rat milk contains B₁₂ in much higher concentrations (10–100 times) than human breast milk (20), which would imply the importance of B₁₂ transportation from dam to neonate in rats. B₁₂ concentration in human breast milk ranges from 0.2–0.8 ng/mL, and the blood B₁₂ level in humans (~0.5 ng/mL) is not different from that in rats. Rat milk contains 10–20 ng/mL (20), while a specific active-transport system of B₁₂ in rats' mammary glands has not been reported. The body weight of the rat neonate increases 5–10 times during lactation (in 3 wk) (21), whereas the human baby generally takes more than 3 mo to gain doubled body weight. Since the body weight of rats and humans at delivery is certainly different, such direct comparison could be inaccurate. However, it can be presumed that higher content of B₁₂ in rat milk during lactation is needed for neonates to undergo massive gains in body weight and full development of the testis in a short period.

Under the conditions of our experiments, reserve pools of B₁₂ in the dam could be used to supply the fetus in utero, even when the dam is on a B₁₂-deficient diet. For instance, in humans, blood B₁₂ concentration in the fetus is ~3 times higher than that in the mother (22). After delivery, nursing is the only way to provide nutrients from dam to neonate. It would be difficult to provide such a high concentration of B₁₂ in milk (see above) without adequate intake of B₁₂. Both exhaustion of B₁₂ and subsequently resulting low-production of methionine due to dysfunction of MS in the dam would result in the failure of testicular development of the neonate. Methionine supplementation could be beneficial for increasing free methionine and producing milk protein, consequently improving protein nutrition of dam and neonate. Similar to the testicular damage of the mature rat on a B₁₂-deficient diet, disrupted transmethylation would explain the neonatal testicular damage, as discussed in our previous paper (13).

We have previously reported that damaged testicular morphology in B₁₂-deficient mature rats cannot be completely recovered by administration of CN-B₁₂ for 30 d (11). Similarly, damage to spermatogonia due to B₁₂-deficiency in a pre-weaning period could be critical for further development of the tissue. If the damage were irreversible in the testis of the infant, this could yield individuals infertile for the rest of their lives. Watanabe et al. (14) found impaired sperm quantity and quality in mature male rats born to dams fed the B₁₂-deficient diet from gestation, although no apparent damage in testicular histology was found in the neonate at 0 d postpartum (15). As we mentioned above, B₁₂ functions in quite small amounts and has a long biological half-life. The fetus could drain B₁₂ from dam during gestation (see above), implying that it will be quite difficult to raise a fetus in B₁₂-deficiency within 22 d (the gestation

period). Thus, it is likely that the testicular injury of offspring chiefly resulted from methionine deficiency in the lactation period, which was caused by dysfunction of MS due to B₁₂-deficiency. In fact, the *Mtr* (gene name for MS)-deficiency (*Mtr*^{-/-}) mouse is an embryonic lethal (23), indicating absolute requirement of MS in the gestation period for fetal survival.

Offspring born to the B₁₂-deficient dam were grown under a condition expected to lead to suppressed transmethylation, which might lead to hypomethylation of DNA, protein, and other methyl group acceptors in every organ. Extremely low levels of methionine would be difficult to achieve even if methionine (or methyl donor)-derived diets were prepared, because the pathway of methionine re-synthesis by methylenetetrahydrofolate reductase and MS would be activated under these conditions. Therefore, offspring in this study could be a novel model to investigate effects of a lowered-level of methionine in the pre-weaning period on development of infants, although biochemical parameters have to be determined. In our routine protocol, we use a casein-based B₁₂-deficient diet instead of the soy protein diet during gestation and lactation (5). The casein diet contains a comparable amount of methionine to the methionine-enriched soy protein diet (13), suggesting that testicular damage before weaning would be minimized. Thus, the post-weaning young B₁₂-deficient rat raised by our method would be a suitable model to investigate the biochemical basis of infertility caused by B₁₂-deficiency.

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REFERENCES

- 1) Ott WH, Rickes EL, Wood TR. 1948. Activity of crystalline vitamin B₁₂ for chick growth. *J Biol Chem* **174**: 1047.
- 2) Froese D, Gravel R. 2010. Genetic disorders of vitamin B₁₂ metabolism: eight complementation groups—eight genes. *Expert Rev Mol Med* **12**: e37.
- 3) Sharp A, Wits L. 1962. Seminal vitamin B₁₂ and sterility. *Lancet* **280**: 779.
- 4) Lillie E. 1962. Obstetrical aspects of megaloblastic anaemia of pregnancy. *BJOG: Int J Obstet Gynaecol* **69**: 736–738.
- 5) Kawata T, Maekawa A, Suzuki T. 1981. The change of urinary methylmalonic acid excretion and the effect of vitamin B₁₂-deprivation in the rat. *Vitamins (J Vitam Soc Jpn)* **55**: 141–146 (in Japanese).
- 6) Doi T, Kawata T, Tadano N, Iijima T, Maekawa A. 1989. Effect of vitamin B₁₂ deficiency on S-adenosylmethionine metabolism in rats. *J Nutr Sci Vitaminol* **35**: 1–9.
- 7) Doi T, Kawata T, Tadano N, Iijima T, Maekawa A. 1989. Effect of vitamin B₁₂-deficiency on the activity of hepatic cystathionine beta-synthase in rats. *J Nutr Sci Vitaminol* **35**: 101–110.
- 8) Yamada K, Kawata T, Wada M, Isshiki T, Onoda J, Kawanishi T, Kunou A, Tadokoro T, Tobimatsu T, Maekawa A, Toraya T. 2000. Extremely low activity of methionine

- synthase in vitamin B₁₂-deficient rats may be related to effects on coenzyme stabilization rather than to changes in coenzyme induction. *J Nutr* **130**: 1894–1900.
- 9) Kawata T, Takada T, Morimoto F, Fujimoto N, Tanaka N, Yamada K, Wada M, Tadokoro T, Maekawa A. 1992. Effects of vitamin B₁₂-deficiency on testes tissue in rats. *J Nutr Sci Vitaminol* **38**: 305–316.
 - 10) Kawata T, Tashiro A, Tamiki A, Suga K, Kamioka S, Yamada K, Wada M, Tadokoro T, Maekawa A. 1995. Utilization of dietary protein in the vitamin B₁₂-deficient rats. *Int J Vitam Nutr Res* **65**: 248–254.
 - 11) Kawata T, Tamiki A, Tashiro A, Suga K, Kamioka S, Yamada K, Wada M, Tanaka N, Tadokoro T, Maekawa A. 1997. Effect of vitamin B₁₂-deficiency on testicular tissue in rats fed by pair-feeding. *Int J Vitam Nutr Res* **67**: 17–21.
 - 12) Kawata T, Funada U, Wada M, Matsushita M, Sanai T, Yamada H, Kuwamori M, Arai K, Yamamoto Y, Tanaka N, Tadokoro T, Maekawa A. 2004. Breeding severely vitamin B₁₂-deficient mice as model animals. *Int J Vitam Nutr Res* **74**: 57–63.
 - 13) Yamada K, Kawata T, Wada M, Mori K, Tamai H, Tanaka N, Tadokoro T, Tobimatsu T, Toraya T, Maekawa A. 2007. Testicular injury to rats fed on soybean protein-based vitamin B₁₂-deficient diet can be reduced by methionine supplementation. *J Nutr Sci Vitaminol* **53**: 95–101.
 - 14) Watanabe T, Ohkawa K, Kasai S, Ebara S, Nakano Y, Watanabe Y. 2003. The effects of dietary vitamin B₁₂ deficiency on sperm maturation in developing and growing male rats. *Congenit Anom* **43**: 57–64.
 - 15) Watanabe T, Ebara S, Kimura S, Maeda K, Watanabe Y, Watanabe H, Kasai S, Nakano Y. 2007. Maternal vitamin B₁₂ deficiency affects spermatogenesis at the embryonic and immature stages in rats. *Congenit Anom* **47**: 9–15.
 - 16) Japanese Association for Laboratory Science. 1987. Guideline for animal experimentation. *Exp Anim* **36**: 285–288 (in Japanese).
 - 17) Schlosser LL, Deshpande P, Schilling RF. 1958. Biologic turnover rate of cyanocobalamin (vitamin B₁₂) in human liver. *AMA Arch Intern Med* **101**: 306–309.
 - 18) Reizenstein PG. 1959. Body distribution, turnover rate, and radiation dose after the parenteral administration of radio-vitamin B₁₂. *Acta Med Scand* **165**: 467–479.
 - 19) Glass GB, Mersheimer WL. 1960. Metabolic turnover of vitamin B₁₂ in the normal and diseased liver. *Am J Clin Nutr* **8**: 285–292.
 - 20) Meyer ML, Thompson HT, Elvehjem CA. 1951. The effect of vitamin B₁₂ on reproduction and lactation in rats receiving pork or beef diets. *J Nutr* **45**: 551–565.
 - 21) Pass D, Graham F. 1993. The rat. *ANZCCART News* **6**: 1–4.
 - 22) Okuda K, Helliger AE, Chow BF. 1956. Vitamin B₁₂ serum level and pregnancy. *Am J Clin Nutr* **4**: 440–443.
 - 23) Swanson D, Liu M, Baker P, Garrett L, Stitzel M, Wu J, Harris M, Banerjee R, Shane B, Brody L. 2001. Targeted disruption of the methionine synthase gene in mice. *Mol Cell Biol* **21**: 1058–1065.