Effects of Vitamin B₁₂-Deficiency on Testes Tissue in Rats

Tetsunori KAWATA,¹ Tomomi TAKADA,¹ Fusako MORIMOTO,¹ Naoko FUJIMOTO,¹ Nobuo TANAKA,² Kazuhiro YAMADA,³ Masahiro WADA,³ Tadahiro TADOKORO,³ and Akio MAEKAWA³

¹Faculty of Education, Okayama University, Tsushimanaka, Okayama 700, Japan
²Department of Internal Medicine, The Jikei University of Medicine, Katsushika-ku, Tokyo 124, Japan
³Department of Agricultural Chemistry, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156, Japan

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Summary The state of vitamin B₁₂-deficiency in rats was evaluated by determination of hepatic vitamin B₁₂-dependent enzyme activities after the animals had fed on a vitamin B₁₂-deficient soybean protein diet for 150 days. The effect of vitamin B₁₂-deficiency on testicular tissue was also studied by morphological observations. Growth of vitamin B₁₂-deficient rats was retarded and marked increase in urinary methylmalonic acid was observed. Vitamin B₁₂ contents in the organs were depressed distinctly by the deficiency, especially in testes, vitamin B₁₂ content decreased to 2.5 ng/g. Hepatic methionine synthase and methylmalonyl-CoA mutase activities showed striking depression to 5% of the control rats and extreme vitamin B₁₂-deficiency was confirmed. Testes weight also showed marked decrease together with their relative weight per 100 g body weight. Morphological observations of testes of vitamin B₁₂-deficient rats revealed atrophy of the seminiferous tubules and aplasia of sperms and spermatids. The above results proved that vitamin B₁₂-deficiency affected rat testes, and suggested that the rat could be the animal model for elucidation of the mechanism of B₁₂ action on testicular functions.

Key Words vitamin B₁₂-deficient rats, methionine synthase, testicular morphology

There are many reports on the result of B₁₂ administration not only in cases with human B₁₂-deficiency but also in some other diseases in the medical standpoint. Sharp and Witts (1) found in 1962 that B₁₂ administration in the patients of pernicious anemia improved sperm parameters and suggested that B₁₂ would affect male reproductive function. Since then, results of B₁₂ administration, in particular

Abbreviations used: B₁₂, vitamin B₁₂; CN-B₁₂, cyanocobalamin.
CH$_3$-B$_{12}$, in male infertility have been accumulated in Japan, and the therapeutic effects of B$_{12}$ have been confirmed (2–4). Further, it was reported that CH$_3$-B$_{12}$ administration to the model mice with experimentally disturbed spermatogenesis by doxorubicin (adriamycin), an anticancer agent, improved the symptom (5, 6). Those reports have suggested that B$_{12}$ may play an important role in maintaining normal testicular functions. However, the effects of B$_{12}$-deficiency on the testicular function and its detailed mechanism of B$_{12}$ have not been clarified.

While B$_{12}$-deficient animal is useful for elucidating in vivo B$_{12}$ functions, it is generally said that it is difficult to keep severe dietary B$_{12}$-deficient rats (7). The cases of dietary B$_{12}$-deficiency in which changes in testicular morphology and sperm parameters were observed have been reported only in Japan (8). This has caused a disturbance in the elucidation of the mechanism of B$_{12}$ action on the testicular functions.

The authors observed the testicular morphology in B$_{12}$-deficient rats in which B$_{12}$-deficiency was judged from B$_{12}$-dependent enzyme activities as a clue for investigating the effect and the mechanism of B$_{12}$ action on the testicular functions. Moreover, the authors further discussed the possibilities of dietary B$_{12}$-deficient rat used as the animal model for elucidation of the mechanism of B$_{12}$ action.

MATERIALS AND METHODS

Animals and diets. Male Wistar weanling rats (about 25 g) were used in experiment. They were born from dams which were fed on a B$_{12}$-deficient diet during pregnancy and lactation. Newborn rats produced in this way were weaned on the 23th day after birth, and divided into experimental groups. The B$_{12}$-deficient diet contained the following: (in %) soybean protein*$_1$ 18.0; glucose anhydrous*$_2$ 66.6; lard*$_3$ 10.0; salt mixture*$_4$ 5.0; vitamin mixture*$_4$ 0.25; choline chloride*$_5$ 0.15. All rats were fed on the B$_{12}$-deficient diet for 150 days. B$_{12}$-supplemented rats received 1 ìg of CN-B$_{12}$ per day by oral administration. Diet and water were given ad libitum. Rats were individually housed in stainless steel screen-bottom cages under the room conditions of constant temperature (22±3°C), and a 12 h cycle of light (7 a.m.–7 p.m.) and dark (7 p.m.–7 a.m.).

Identification of vitamin B$_{12}$-deficiency. Every 30th day during the experimental period, the rats were transferred to metabolic cages and their urine was collected for 24 h. The amount of methylmalonic acid (MMA) was determined by the method of Giorgio and Plaut (10). B$_{12}$ contents in organs were determined by microbiological assay with Lactobacillus leichmannii (ATCC 4797). Extraction

*$_1$ "Ajipron SU" (crude protein content 85.4%).
*$_2$ Produced by Ajinomoto Co.
*$_3$ Produced by Nihon Yurou Yakuhin Co.
*$_4$ Formulated according to reference (9). CN-B$_{12}$ was omitted from vitamin mixture. All chemicals were purchased from Wako Pure Chemicals Industries Co.
*$_5$ Produced by Tokyo Kasei Industries Co.
procedures of B₁₂ from rat organs were followed as previously described by Hayashi et al. (11). After the rats had fed for 150 days as the experimental period, hepatic B₁₂-dependent enzyme activities were determined. Their liver was removed after perfusion with cold saline and homogenized with 4 volumes of 0.25 M sucrose containing 40 mM potassium phosphate buffer, pH 7.5, per gram of liver. The homogenate was centrifuged at 600×g for 10 min at 4°C; an aliquot of the supernatant used for determination of methylmalonyl-CoA mutase (MMCoA mutase) activity. Moreover, the supernatant fluid of 600×g was centrifuged at 105,000×g for 60 min at 4°C. The supernatant fluid was collected and used for determination of methionine synthase (Met synthase) activity. The determination of Met synthase activity was carried out according to the method described by Matsuno et al. (12), except that the authors used 0.2 mM NADH instead of FMNH₂. The determination of MMCoA mutase activity was carried out according to the method described by Kolhouse and Allen (13). Protein was determined by the method of Lowry et al. with bovine serum albumin as standard (14).

Light micrographs of testes. The testes of rats were isolated and then the testes' adipose tissue and blood were removed. The testes were fixed with 10% formalin neutral buffer solution, pH 7.4, for 24 h, at room temperature, and were stained with hematoxylin and eosin.

RESULTS

State of vitamin B₁₂-deficiency

Growth curves of rats are shown in Fig. 1. Growth retardation of the B₁₂-deficient rats was already observed after 30-day feeding. Final body weight in the B₁₂-supplemented control rats at the end of 150 days was 45% of the B₁₂-supplemented control rats. And decreases of food intake and food efficiency

![Fig. 1. Growth curves of the vitamin B₁₂-supplemented and -deficient rats. *Each point presented the weight of the rats and showed mean±SD of five rats. □, B₁₂-supplemented; ○, B₁₂-deficient.](image-url)
Fig. 2. Urinary methylmalonic acid contents of vitamin B\textsubscript{12}-deficient rats during experimental period. *Each point presented the methylmalonic acid content of B\textsubscript{12}-deficient rats and showed the mean±SD of five rats. Methylmalonic acid in urine of vitamin B\textsubscript{12}-supplemented rats was not detected throughout the experimental period. ●, B\textsubscript{12}-deficient.

Fig. 3. Vitamin B\textsubscript{12} contents in various organs of vitamin B\textsubscript{12}-supplemented and -deficient rats. *Each bar presented the vitamin B\textsubscript{12} content in organ of rats and showed mean±SD of five rats.
Table 1. Hepatic vitamin B12-dependent enzymes activities of vitamin B12-supplemented and -deficient rats.

<table>
<thead>
<tr>
<th></th>
<th>B12-supplemented</th>
<th>B12-deficient</th>
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<tr>
<td>Methionine synthase*</td>
<td>186.6±23.9</td>
<td>9.2±1.6*</td>
</tr>
<tr>
<td>Methylmalonyl-CoA mutase*</td>
<td>194.1±25.6</td>
<td>10.8±4.3*</td>
</tr>
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Data presented as mean±SD of five rats. a) Significantly different from means of the vitamin B12-supplemented group; p<0.01.

Table 2. Organ weight and organ weight/100 g body weight of vitamin B12-supplemented and -deficient rats.

<table>
<thead>
<tr>
<th></th>
<th>B12-supplemented</th>
<th>B12-deficient</th>
</tr>
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<tbody>
<tr>
<td>Brain</td>
<td>1.71±0.04</td>
<td>1.56±0.02*</td>
</tr>
<tr>
<td>(wet organ g)</td>
<td>0.98±0.06</td>
<td>0.67±0.03*</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.45±0.03</td>
<td>0.15±0.03*</td>
</tr>
<tr>
<td>Kidneys</td>
<td>1.74±0.10</td>
<td>1.77±0.19</td>
</tr>
<tr>
<td>Adrenals*</td>
<td>0.940±0.002</td>
<td>0.935±0.003</td>
</tr>
<tr>
<td>Testes</td>
<td>2.43±0.06</td>
<td>0.63±0.08*</td>
</tr>
</tbody>
</table>

Data presented as mean±SD of five rats. a) Significantly different from means of the vitamin B12-supplemented group; p<0.01.
Fig. 4. Light micrographs of testis in vitamin B₁₂-supplemented and -deficient rats. A, B₁₂-supplemented ×40; B, B₁₂-supplemented ×200; C, B₁₂-deficient ×40; D, B₁₂-deficient ×200.
were observed in B12-deficient rats, compared to B12-supplemented rats.

Urinary MMA excretion by rats fed on the B12-deficient diet during the experimental period increased markedly (Fig. 2). The amount of MMA in urine of 30 days was significantly increased by B12-deficiency, and it continued to increase throughout the experimental period of 150 days. In the B12-supplemented rats, urinary MMA was not detected.

The contents of B12 in the organs is shown in Fig. 3. The contents of B12 in the brain, heart, liver, spleen, kidneys and testes of rats fed on the B12-deficient diet decreased extremely, compared to the B12-supplemented rats given oral administration of 1 μg/day CN-B12. B12 content in the testes of B12-deficient rats declined to 2.5 ± 0.5 ng/g of wet tissues.

Then, the authors determined hepatic B12-dependent enzyme activities to evaluate the B12-deficiency. Table 1 shows the activities of hepatic Met synthase and MMCoA mutase in rats. Hapatic Met synthase and MMCoA mutase activities in B12-deficient rats decreased to about 5% of the B12-supplemented rats.

Rat testicular morphology

Table 2 shows the wet weight and relative weight/100g body weight of the organs. The relative weight/100 g body weight of the brain, heart, liver, kidneys and adrenals increased by B12-deficiency. However, the relative weight of testes of rats fed on the B12-deficient diet decreased markedly to 56% of the control, i.e., the values were 0.50 ± 0.05 and 0.89 ± 0.04, respectively. Similarly, it was shown that B12-deficiency depressed testes weight in rats to about 26% of the control rats. The testes weights in B12-deficient and B12-supplemented rats were 0.63 ± 0.05 g and 2.43 ± 0.06 g, respectively.

The light microscopic observations of rat testicular morphology revealed distinct changes due to B12-deficiency. The testicular morphology of the B12-supplemented and B12-deficient rats is shown in Fig. 4. Production of sperms and spermatids was observed in the B12-supplemented rats given oral administration of 1 μg/day CN-B12 (Photos A and B). However, contrary to the normal testicular functions maintained in the control rats (Photos A and B), severe atrophy of the seminiferous tubules, aplasia of sperms and spermatids as well as fibrotic change of the seminiferous tubules were observed in the B12-deficient rats (Photos C and D).

DISCUSSION

Although rats are the most popular experimental animals for studies of B12, it is generally said that development of severe dietary B12-deficiency in rats is difficult to be induced (7). The following reasons are given for this: biological half-life of B12 is long and feeding of the B12-deficient rats extends over a long period; rats show a tendency of coprophagy; strict control is needed for breeding as well as preparation of B12-deficient diet. On the other hand, it was found that nitrous oxide would inactivate Cob(I)alamin and reduce Met synthase activity (15, 16). Kondo et al.
(17) reported that Met synthase activity decreased markedly after the start of exposure to nitrous oxide, reaching to about 15% of the control in the rats having been exposed to 50% nitrous oxide for 23 days. From this, nitrous oxide-exposed rats are used as experimental B12-deficient animal in which B12-deficiency can be reproduced in a short time and relationship of B12 to folate and Met metabolism is being investigated.

In the present study, the state of B12-deficiency in dietary B12-deficient rats was evaluated. Weanling rats were used, which were weaned from the dams fed on the B12-deficient diet throughout the period of pregnancy and lactation to keep B12 transport through the placenta to the minimum. And B12-deficient rats were kept under the strict feeding conditions to minimize the risk of contamination of B12 during the experimental period. The B12-deficient rats produced under the strict feeding conditions showed remarkable growth retardation and extreme increase in urinary MMA excretion as well as exhaustion of in vivo B12 compared to previous reports (7, 18–23). Severe B12-deficiency was confirmed by the growth, urinary MMA excretion, and B12 contents in organs of rats. Moreover, the state of B12-deficiency in the rats was also assessed by hepatic B12-dependent enzyme activities. Hepatic MMCoA mutase and Met synthase activities in the B12-deficient rats decreased markedly to about 5% of the B12-supplemented rats. The activities in the rats fed on the B12-deficient diet for 150 days were lower than the previous reports (24, 25). Thus, it was considered that dietary B12-deficient rats in the present experiment were useful in elucidation of secondary metabolic changes induced by a marked decrease in B12-dependent enzyme activities.

Dryden and Hartman (26) reported that the relative weight/100 g body weight of the heart, liver, kidneys, spleen, adrenals and testes increased in the B12-deficient rats fed on the 20% casein diet ad libitum for 189–301 days. Although Dryden and Hartman (26) reported an increase in the relative weight of testes, B12-deficiency depressed distinctly the relative weight of testes in the present experiment. Similarly, the wet weight of the testes in B12-deficient rats decreased to 50% of the control rats. The authors already reported that decreases in the wet weight and relative weights of the testes were not observed in rats fed on the casein diet but only observed in the B12-deficient rats fed on the soybean protein diet (27). It is suspected that the decrease in testes weight has some correlations to amino acid composition, sulfur amino acid composition in particular, in dietary protein. Further investigation of Met metabolism in testes of B12-deficient rats is needed.

In relation to human reproductive functions, there are many reports on the effectiveness of B12 administration for the treatment of male infertility. Sharp et al. (1) administered B12 to a male patient with pernicious anemia accompanied by oligozoospermia in 1962 and improved sperm parameters leading to conception 6 months later. Then, Blair (28) reported in 1968 that B12 administration was effective even in the hematologically normal patient with oligozoospermia. At present, B12, CH3-B12 in particular, is being used as a therapeutics for male infertility and its clinical effects have been proved (2–4). These reports indicate that B12
exhibits positive effects on male reproductive functions. Meanwhile, it was reported that doxorubicin, an anticancer agent, would inhibit nucleic acid synthesis in rat testes (29–31). Oshio et al. (5) and Ozaki et al. (6) reported that changes of the testicular morphology and of the parameter of sperms in the rats administered doxorubicin, such as decreases in the number of sperms and sperm motility, were alleviated by CH$_3$-B$_{12}$. From the results of studies with a model animal of experimentally disturbed spermatogenesis, it was assumed that B$_{12}$ was participating in DNA synthesis in some way to maintain normal testicular functions. However, there are many unknown points in the mechanism of B$_{12}$ action on testicular functions. To elucidate this, not only the animal model with experimentally disturbed spermatogenesis but also that of dietary B$_{12}$-deficiency are considered extremely useful. The rats in the present study, which were judged as being severely B$_{12}$-deficient from B$_{12}$-dependent enzyme activities, showed distinct changes including extreme atrophy of the seminiferous tubules, aplasia of sperms and spermatids, and fibrotic change of the seminiferous tubules. Up to now, cases of dietary B$_{12}$-deficient rats with changes in the testicular morphology and sperm parameters have been reported only in Japan. Namely, Umemura (32) reported in 1964 that decreased number of mature sperms and atrophic organization of the seminiferous tubules were observed in B$_{12}$-deficient rats, although testicular morphology was not given. In 1991, Oshio et al. (8) reported that reduced diameter of the seminiferous tubules, change in testicular morphology including decreased number of spermatids and their irregular arrangement, as well as decreased number of spermatids in the epididymides and active sperms were observed in mice fed on the B$_{12}$-deficient diet containing 0.3 ng/g diet of B$_{12}$ for 10 weeks. Together with the present results, the above findings not only clarified that B$_{12}$-deficiency induces changes in the testicular morphology and sperm parameters in rats or mice but also suggested the possibility that dietary B$_{12}$-deficient rats serve as the animal model for investigating the mechanisms of B$_{12}$ action on testicular functions. Moreover, our rats showed remarkable changes in the testicular morphology and are considered useful as a more precise model animal.

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REFERENCES


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