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Urinary Excretion of 3-Hydroxyisovaleric Acid Increases and Biotin Decreases during Pregnancy in Mice

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Summary

The effects of biotin deficiency on maternal metabolism and embryonic development in pregnant mouse dams were studied. In the biotin-deficient group, the inhibition of embryonic development and external malformations such as cleft palate (100 %), micrognathia (100 %) and micromelia (91.4 %) were detected in embryos. Biotin excretion in urine was decreased on day 4 of gestation (dg) and subsequently not detected, and the urinary concentration of 3-hydroxyisovaleric acid (3-HIA) was increased after dg 12. In contrast, the biotin concentration in urine was significantly increased on dgs 4, 8 and 12 in the biotin-supplemented group, and decreased on dg 16 in biotin-supplemented and biotin-control groups. Pyruvic acid in the biotin-deficient group was higher than that in the biotin-supplemented group throughout gestation. These findings demonstrated that the requirement of biotin increases at conception and/or during embryonic development, and a large amount of biotin is necessary for embryonic development in the late stage of gestation.

Introduction

As biotin is well distributed among various foodstuffs, spontaneous biotin deficiency rarely occurs in animals and humans. However, biotin deficiency can be induced by giving large amounts of raw egg white containing avidin. Avidin is known to inhibit the absorption of biotin from the intestinal tract and to produce biotin deficiency. The characteristic pathological signs of biotin deficiency such as dermatitis, alopecia and neurological abnormalities develop in humans as well as rodents. The importance of biotin for normal reproduction and the development of embryos and offspring have also been demonstrated in mice. Maternal biotin deficiency during gestation produces external malformations such as cleft palate, micrognathia and micromelia in mice[1-3]. However, no embryonic effects have been observed in biotin-deficient rats. Thus, there are species and strain differences in the teratogenic effects of biotin deficiency[4].

Biotin is an essential cofactor for normal function of the carboxylases involved in the fixation of carbon dioxide. The major biotin-containing enzymes are 3-methylcrotonyl CoA carboxylase (MCC), propionyl CoA carboxylase (PCC), pyruvate carboxylase (PC), and acetyl CoA carboxylase (ACC). These enzymes occupy an important position in such metabolic pathways as gluconeogenesis, fatty acid synthesis, the catabolism of amino acids and the regulation of carbohydrate metabolism. MCC catalyzes an essential step in the degradation of leucine, which converts

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3-methylcrotonyl CoA to 3-methylglutaconyl CoA. The reduced activity of MCC leads to elevated excretion of 3-methylcrotonic acid, the product of its hydration (3-hydroxyisovaleric acid: 3-HIA), and 3-methylcrotonylglycine, formed by conjugation with glycine. The increased urinary excretion of these abnormal metabolites reflects the reduced activity of MCC or is due to dietary biotin depletion in genetically normal individuals.

The concentrations of these organic acids and biotin, and the activity of carboxylases in the serum and urine are generally used as indicators of biotin status[5, 6]. Mock *et al.*[7-9] demonstrated that decreased urinary biotin and increased urinary 3-HIA are sensitive indicators of early biotin deficiency, but methylcrotonylglycine and isovalerylglycine, which are also produced due to the decreased activity of MCC, are not. 3-HIA is detected in urine before the appearance of clinical signs of biotin deficiency. Thus, it is expected to be a useful indicator of early biotin deficiency. The authors have recently developed the HPLC method for 3-HIA, which can be a useful tool clinically as well as in the research laboratory.

In humans, Mock *et al.*[8] obtained the evidence that the biotin status decreases during pregnancy in women and urinary 3-HIA excretion is increased in both early and late pregnancy. It is suggested that some pregnant women may suffer marginal biotin deficiency during gestation. However, the mechanism by which marginal biotin deficiency is induced by pregnancy is unclear. Therefore, we studied biochemical changes in biotin-deficient mouse dams and their embryos during the gestational period.

Materials and Methods

1. Animal care and diets

The mature 8-week-old male and female ICR strain mice used in this study were purchased from CLEA Japan, Inc. (Tokyo, Japan). These animals were given commercial pellets (CE-2) and distilled water *ad libitum* for at least 3 weeks. They were kept at controlled room temperature (23 ± 3℃), relative humidity (50 ± 10 %), and photoperiod (light on 7: 00–19: 00) throughout the experiment. Females were exposed to males for 2 hours early in the day (from 7: 00 to 9: 00). Pregnancy was confirmed by the presence of a vaginal plug, and this day was designated as day 0 of gestation (dg 0). Pregnant animals were randomly assigned to three groups and given biotin-deficient diet, biotin-supplemented diet or biotin-control diet during gestation (Fig. 1). The animals were placed in individual plastic cages with stainless steel wire bottoms to prevent coprology. They were given free access to these diets and to distilled water during the experiment period.

The biotin-deficient diet used in this study contained 25 % of dried egg white. The biotin-supplemented diet was identical to the biotin-deficient diet, except that 5.0 mg/kg biotin was included. Diet consumption and weight gain of

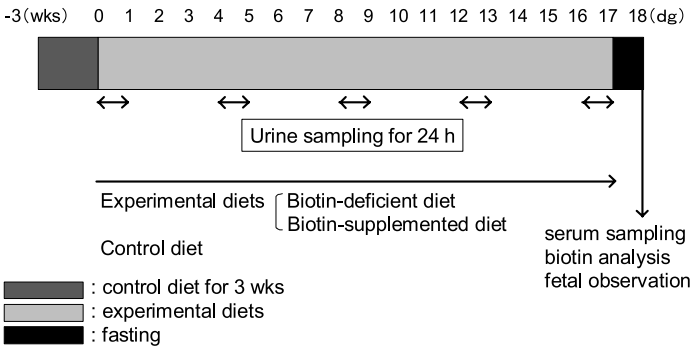

Fig. 1 Experimental protocol.

animals were checked at regular intervals. There was no difference in diet consumption between biotin-deficient and biotin-supplemented groups during gestation. Biotin-control mice were given CE-2 diet, which was purchased from CLEAR Japan (Tokyo, Japan).

All animal procedures were performed in accordance with the standards related to the care and management of experimental animals of the Japanese Prime Minister's Office (Notification No. 6, March 27, 1980).

2. Urine collection

Urine was sampled for 24 hours (9: 00 to 9: 00) in individual metabolic cages on dgs 0, 4, 8, 12 and 16, and the mice were weighed before and after urinary collection. They were given free access to diets and distilled water during urinary collection.

On dg 17, mice were not fed and only water was given. On dg 18, blood was directly collected from the heart of pregnant mice shortly after anesthesia with diethyl ether, centrifuged at 3,000 rpm for 10 min and serum was collected. After collecting blood for a whole day, the uterus was removed and weighed.

Urine and serum were stored at −40℃ shortly before biotin determination.

3. Analysis of 3-HIA

Organic acids, including 3-HIA in urine, were determined by HPLC with an organic acid system (Shimadz Co., Ltd., Kyoto, Japan). The column used was Shim-pack SPR-H, an elution system of 4 mM *p*-toluensulfonic acid, elution speed of 0.8 mL/min, and temperature of 40℃ and 50 μL sample was injected. The reaction reagents were 4 mM *p*-toluensulfonic acid, 16 mM Bis-Tris, and 80 μM EDTA. The elution speed was 0.8 mL/min, and the reaction temperature was 40℃. The detector was Electric Detector CDD-10Avp[10].

Urine was pretreated using a 0.45 μm filter (Minisart). 3-hydroxyisovaleric acid, purchased from Tokyo Kasei Industry, Co., Ltd. (Tokyo, Japan), was used as the standard. The area was determined at a retention time of 21.4 min and the urinary concentration of 3-HIA was determined.

4. Biotin determination

Biotin in serum and urine was determined by a bioassay. Biotin concentration in the urine was quantified using a microtiter plate adaptation of a microbiological assay with *Lactobacillus plantarum* ATCC 8014, which was cultured on a microtiter plate for 18 hours and determined at 610 nm. Biotin concentration expressed as μmol/mL. Biotin determination ranged from 0.4−8.2 nmol/mL (0.1−2.0 ng/mL).

As biotin in serum existed in a protein-bound form, in which biotin is bound to protein such as globulin and peptide, protein-bound biotin and free biotin were determined separately. For the determination of total biotin, 100 μL of serum was pretreated with 4.5 N H_2SO_4 for 121℃ for 60 min and neutralized by 4.5 N NaOH. For free biotin, serum was pretreated with FRIGEN® II (Dade Behring Inc.,) before determining. The percentage of free biotin per total biotin concentration was the "free biotin percentage". Urinary biotin was determined as only total biotin.

5. Biochemical analyses

For the determination of creatinine, the kit "Creatinine Test Wako" (Wako Pure Chemical Industries, Co. Ltd., Tokyo, Japan) was used on the basis of Jeff reaction. The reaction solution was determined at 520 nm. The concentrations of urinary biotin, 3-HIA, lactic acid and picric acid were expressed as μmol/mol creatinine and mmol/mol creatinine, respectively.

6. Observation of fetuses

Females were killed by diethyl ether at 13: 00 hours on dg 18. After laparotomy, the uterine horns were removed and fetuses were then dissected free from the uterine horns and extraembryonic tissues were removed. The number of live and dead or resorbed fetuses was determined. Live fetuses were weighed and examined for morphologi-

cal development under a dissecting microscope, and any external malformations such as micrognathia or micromelia. After these fetuses were washed with PBS solution and fixed in Bouin's solution for more than 10 days, the incidence of cleft palate was recorded. Fetal malformations could not be assessed in an entirely blind fashion among the three dietary groups, since their body size and weight differed.

7. Statistical analysis

Statistical comparisons of the means among experimental groups were conducted by one-way ANOVA and Tukey-Kramer. Differences of $p < 0.05$ were considered significant. The values in the text and Figures are the means ± standard deviation (SD). All analyses were performed with StatView for Windows (Version 5.5; SAS Institute Inc., Cary, NC).

Results

1. Effects of dietary biotin deficiency on reproduction of female mice

On dg 18, there was no appreciable difference in the mean body weight gain of dams during gestation among the three dietary groups (Table 1). Pregnant mice from the biotin-deficient group did not show any known signs of deficiency such as alopecia or dermatitis. The total biotin concentration of serum in the biotin-deficient group was 3.0 pmol/mL on average, which was significantly decreased compared to 22.5 pmol/mL in the biotin-supplemented group (Fig. 2). The change of free biotin concentration in the biotin-deficient group was in accord with that in the biotin-supplemented group. The percentage of free biotin was 70.8 % and 74.5 % in respective groups.

Table 1 Effects of maternal biotin deficiency on reproduction in mice

Dietary groups	Biotin-deficient	Biotin-supplemented	Control
No. of dams examined	5	5	5
Body weight gain of dam, *g*	19.0 ± 2.4[1]	20.2 ± 4.7	20.1 ± 2.1
No. of live fetuses	62	64	67
	12.4 ± 1.5	12.8 ± 3.0	13.4 ± 1.5
Fetal weight, *g*	1.03 ± 0.13*	1.25 ± 0.12	1.20 ± 0.10

[1] Values are means ± SD.
*$p < 0.05$, compared with the biotin-supplemented group (Mann-Whitney's U test).

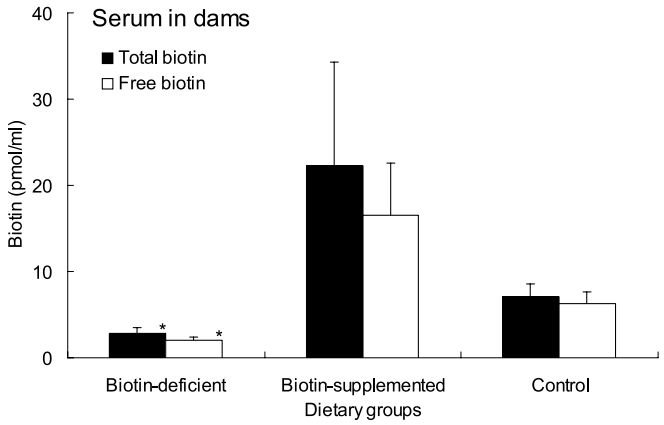

Fig. 2 Biotin concentration in serum in biotin-deficient and biotin-supplemented dams. means ± SD. *$p < 0.05$, compared with the biotin-supplemented group (one-way ANOVA).

On dg 0, the biotin concentration in urine was 70.2 and 51.6 μmol/mol creatinine in the biotin-deficient and biotin-control groups, respectively, which were different from 251.1 μmol/mol creatinine in the biotin-supplemented group (Fig. 3). Urinary biotin concentration in the biotin-supplemented group was significantly higher than that in biotin-control and biotin-deficient groups throughout gestation. The biotin concentration in urine was significantly increased on dgs 4, 8, 12 in the biotin-supplemented group, and was decreased on dg 16. In contrast, biotin excretion in the biotin-deficient group was decreased on dg 4, and was subsequently under the lower limit. In the biotin-control group, urinary biotin excretion did not change before dg 12 but was significantly decreased on dg 16.

2. Effects of maternal biotin deficiency on embryonic development

The mean numbers of live fetuses and implantation sites per female were not different among the three experimental dietary groups. However, live fetuses had a mean body weight of 1.03 g in the biotin-deficient group, as compared to 1.25 g in the biotin-supplemented group and 1.20 g in the biotin-control group. Fetal growth retardation was observed in biotin-deficient mice.

As for the teratogenic effect of biotin deficiency on live fetuses, high incidences of various types of gross congenital malformations are typical in mice. The most frequently occurring malformations in the biotin-deficient group were micrognathia (100 %), cleft palate (100 %) and micromelia (forelimb hypoplasia, 92.3 %; hindlimb hypoplasia, 73.1 %) (Fig. 4). More fetuses had short tails (69.2 %) and low-set ears (38.5 %). On the other hand, almost no malformations of any kind were observed in the biotin-supplemented and biotin-control groups.

3. Effects of maternal biotin deficiency on the excretion of organic acids

The urinary concentration of 3-HIA on dg 0 was 33.3 and 5.2 mmol/mol creatinine in the biotin-control and biotin-deficient groups, respectively (Fig. 5). These values were under the limit of the reference range. These did not change and were not different between biotin-supplemented and biotin-control groups throughout the entire period of gestation. In contrast, in the biotin-deficient group, urinary 3-HIA excretion increased gradually but not significantly from dgs 0 to 8. However, 3-HIA concentration was 195.4 and 204.7 mmol/mol creatinine on dgs 12 and 16, respectively, which were significantly increased compared with dg 0.

As for the urinary concentration of other organic acids, the urinary excretion of pyruvic acid in the biotin-deficient group was significantly higher than that in the biotin-supplemented group throughout gestation (Fig. 6). These

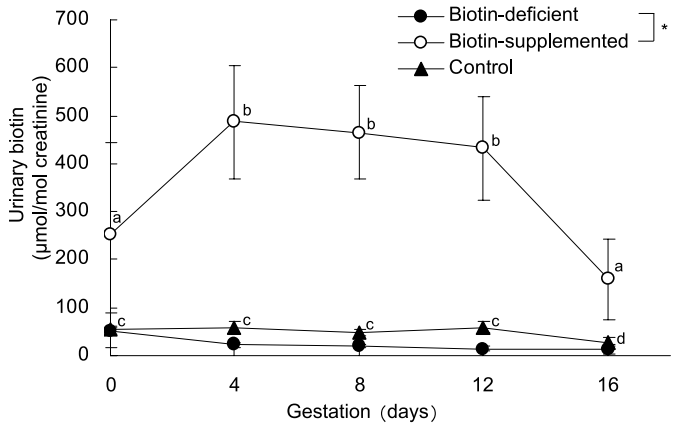

Fig. 3 Effect of the biotin-deficient diet on urinary excretion of biotin in pregnant mice. Biotin excretion decreased significantly with second half of gestation in the biotin-supplemented group. means ± SD. $^{*}p < 0.05$ (two-way ANOVA). $^{a-b,\,c-d}p < 0.05$ (Tukey-Kramer).

concentrations in urine were significantly increased in both biotin-deficient and biotin-supplemented groups on dg 16, compared to dg 0. However, there was no change in the urinary concentration of pyruvic acid in the biotin-control group. On the other hand, the lactic acid excretion did not change in these dietary groups.

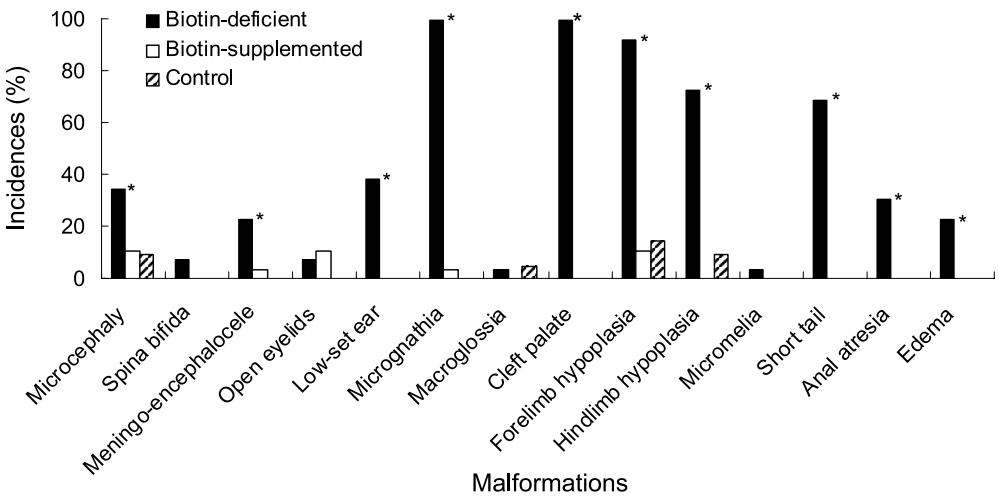

Fig. 4 Incidences of gross congenital malformations in fetuses of biotin-deficient mice.
$^{*}p < 0.05$, compared with the biotin-supplemented group (χ^2-test).

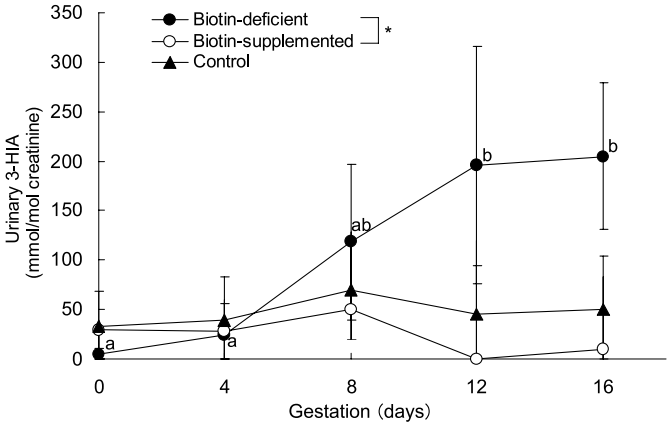

Fig. 5 Effect of the biotin-deficient diet on urinary excretion of 3-hydroxyisovaleric acid (3-HIA) in pregnant mice. On dg 0, 3-HIA excretion did not differ among the diet any groups. 3-HIA excretion increased significantly with second half of gestation in the biotin-deficient group. Biotin excretion had no effect for biotin-supplemented group and control group.
means ± SD. $^{*}p < 0.05$ (two-way ANOVA).
$^{a-b}p < 0.05$ (Tukey-Kramer).

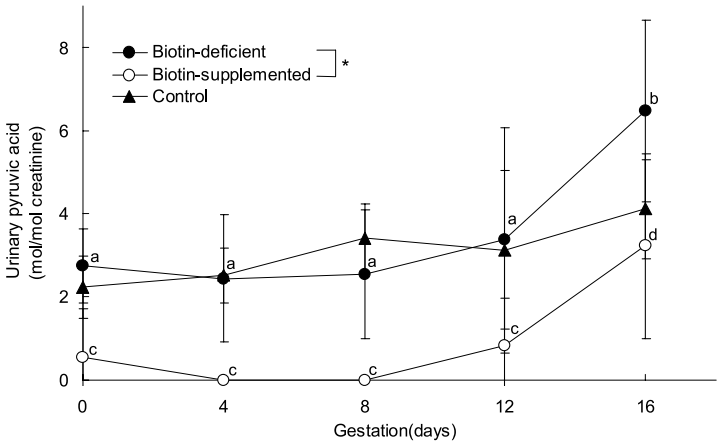

Fig. 6 Effect of the biotin-deficient diet on urinary excretion of pyruvic acid in pregnant mice.
means ± SD. $^{*}p < 0.05$ (two-way ANOVA).
$^{a-b,\ c-d}p < 0.05$ (Tukey-Kramer).

Discussion

These findings confirmed previous findings in which maternal biotin deficiency affected embryonic growth and development during gestation, and was related with the induction of gross external malformations such as cleft palate, micrognathia and micromelia in mice. The induction mechanism of cleft palate by biotin deficiency is unknown. We demonstrated using organ culture of palates in mice that organic acids such as 3-HIA acid inhibited the development of palatal processes, but did not affect the fusion between palatal shelves[11]. It is unknown in detail how 3-HIA affects the development of palatal processes in biotin-deficient dams. One possibility is that biotin is an essential nutrient factor to maintain the proliferation of mesenchymal cells in the palatal process. In addition, a high incidence of short tail, microcephaly and edema was detected in biotin-deficient mice in this study. It is suggested that biotin deficiency may affect the physiological function and inhibit normal proliferation in various kinds of cells in embryos nonspecifically.

We have recently studied using fertilized eggs the transportation of biotin from yolk to embryos in domestic fowls[12]. In the maxillofacial area, biotin concentration markedly increased at the embryonic age of 11 days, and the rate of free biotin, which is an active form, was 92.0 %. This may be because a large amount of biotin is necessary for palatal formation in chicken embryos and is actively incorporated during organogenesis. We also studied the relationship between biotin concentration and biotinidase activity in mice. In dams, the activity of biotinidase and serum biotin concentration on dg 15 decreased less than on dg 12. On the other hand, in embryos, total biotin concentration of the palatal process and mandible on dg 15 was increased. In particular, concentration of free biotin in the palatal process, mandible and brain remained constant on dgs 12 and 15, and biotinidase activity continued to be high in these organs. Thus, it is demonstrated that a large amount of biotin is necessary for the formation of orofacial processes during organogenesis in birds and rodents.

Mock and Stadler[13] studied the biotin nutritional status during normal gestation in humans, and demonstrated that 3-HIA is high in early and late gestation. It is suggested that pregnancy impaired the renal reclamation of biotin, bisnorbiotin and biotin sulfoxide, or pregnancy caused metabolic or renal effects that increased 3-HIA excretion nonspecifically. We reported that the concentration of 3-HIA was not detected in non-pregnant mice for 4 weeks after eating a biotin-deficient diet. However, in this study, the urinary 3-HIA concentration was already increased on dg 16 in biotin-deficient mice. Also, biotin excretion in urine was decreased on dg 16 in biotin-deficient mice. These findings indicate that the requirement of biotin is increasing by the time of conception and/or embryonic development, resulting in a lack of biotin in dams. In this study, a large variation in urinary excretion of 3-HIA and biotin was observed in biotin-supplemented and biotin-deficient groups, respectively (Figs. 3, 5). This may be due to the small number of mice used in each experimental group and that one dam showed an extremely high value in urinary excretion of 3-HIA and biotin.

Urinary organic acids indicate the deficiency of several biotin-dependent carboxylases. Mardach *et al.*[14] reported that a child with a novel genetic defect in biotin transport had a high concentration of plasma and urinary organic acids such as lactic and pyruvic acids, in addition to 3-HIA. Abnormally increased urinary excretion of these organic acids is induced by nutritional biotin deficiency or inherited enzymatic deficiencies of biotin-dependent carboxylases. In this study, pyruvic acid in biotin-deficient mice was higher than in biotin-supplemented mice during gestation and increased in the late gestation. However, lactic acid did not differ among dietary groups and was not changed during gestation. A clear relationship between the increased excretion of organic acids and biotin deficiency is not evident from these findings. It is suggested that the increased concentration of organic acids in urine in late gestation may be caused by the increased requirement of biotin in growing embryos, not by the disturbance of renal function.

The relation between the induction of cleft palate and maternal biotin deficiency is not apparant from previous findings. Cleft palate is a common anomaly in humans. In Japan, the incidence of cleft palate is 1: 2,500 in newborns, and it is more common in America and Europe. Mock *et al.*[15, 16] reported that biotin in pregnant women decreased in the late stage of gestation compared with the early stage. The present study showed the induction potential of biotin deficiency in the late stage of gestation, even in mice given the biotin-control diet during gestation. From these findings, some pregnant women may suffer from a lack of biotin in the late stage of gestation. Also, as biotin is a water-soluble vitamin, toxicity and adverse effects of biotin have not been reported in physiological and pharmacological doses. Therefore, it is recommended that pregnant women take more biotin throughout gestation.

Further studies are needed to clarify the relationship between the biotin status and pregnancy in rodents as well as humans.

Acknowledgments

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