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Study on Teratogenicity of Biotin Deficiency in Mice at Midgestation

Ayumi TANIGUCHI, Yoshiko NAGAI and Toshiaki WATANABE
Laboratory of Dietary Environment Analysis
School of Human Science and Environment
Himeji Institute of Technology, University of Hyogo, Himeji, Japan

Summary

It is known that biotin is intrinsic for maintaining reproductive function. Biotin deficiency induces both external and skeletal malformations in the embryos of fowl, and maternal biotin deficiency is severely teratogenic in mammals. In mice, maternal biotin deficiency results in cleft palate, micrognathia and limb hypoplasia. However, the relationship between morphogenesis and biotin is not sufficiently clear. This study was conducted to elucidate the mechanism of biotin transport from dams to embryos and the nutritional roles of biotin in ICR mice. It became obvious that biotin was supplied from dams growing embryos during morphogenesis. In particular, a large amount of biotin was transported to palates and mandibles on days 12 - 15 of gestation. In fetuses, the transportation of biotin to embryos differed among embryonic growth periods and organs, and biotin may play a principal role in the formation of tissues and organs.

These results suggest that biotin is an essential nutrient and may play an important role in reproductive performance.

Introduction

Biotin, a water-soluble vitamin, serves as a covalently bound coenzyme for various mammalian carboxylases used in fatty acid synthesis, glucose metabolism and amino acid metabolism[1-3]. One of these, methylcrotonyl CoA carboxylase (MCC), catalyzes an essential step in the intermediary metabolism of the branched-chain amino acid leucine. Decreased activity of MCC shunts the substrate 3-methylcrotonyl CoA to an alternate metabolic pathway, producing 3-hydroxyisovaleric acid (3-HIA). Biotin also plays unique roles in cell signaling, epigenetic control of gene expression and the chromatin structure[4].

The importance of biotin for normal pregnancy and the development of embryos and offspring has been demonstrated in birds and mammals. It is known that biotin deficiency induces both external and skeletal malformation in domestic fowls[5, 6]. In mammals, maternal biotin deficiency causes severe teratogenic effects such as cleft palate, micrognathia and limb hypoplasia in mice[7-10]. Recently, evidence has shown that increased urinary excretion of 3-HIA was observed in the normal pregnancy of mammals and human[11, 12].

However, more information is needed to determine the requirements of biotin during pregnancy. Therefore, the authors examined the mechanism of biotin transport from dams to fetuses and the nutritional roles of biotin in mice.

Address correspondence to Toshiaki Watanabe, PhD, DSc, Laboratory of Dietary Environment Analysis, School of Human Science and Environment, University of Hyogo, 1 - 1 - 12 Shinzaike Honcho, Himeji 6700092, Japan. E-mail: watanabe@shse.u-hyogo.ac.jp
Present address (A.T.): Institute for Environmental and Gender Specific Medicine, Graduate School of Medicine, Juntendo University of Medicine

Materials and Methods

1. Animals

Virgin female and proven breeder male JCL: ICR mice aged 8 weeks and weighing about 30 g average were purchased from CLEA Japan, Inc. (Tokyo). They were allowed tap water and standard rodent diet (CE-2; CLEA Japan, Inc.) and kept for at least 2 wks under regular conditions. Animal rooms were maintained at a constant temperature (23–25℃) and humidity under a 12-h light/dark cycle (light from 7:00 to 19:00 hours). Females were housed together in plastic cages, and males were housed individually.

For breeding, five virgin female mice were placed with a male for 2 hours in early morning (from 7:00 to 9:00). The females were examined for the presence of a vaginal plug and this day was designated as day 0 of gestation (GD 0). Throughout the gestational period, all dams were housed in plastic cages with stainless-mesh bottoms.

2. Diets

The pregnant mice were assigned to a biotin-deficient group or biotin-supplemented group at random. Biotin deficiency was induced by feeding a basal diet, the composition of which is given in Table 1. This diet contained egg white as a protein source. Avidin, a component of egg white, forms an unabsorbable complex with biotin in the alimentary tract, consequently producing biotin deficiency in animals. The biotin-supplemented diet was prepared in the same manner, except that 5.0 mg biotin/kg diet was added. This amounts is much more than the usual biotin requirement of these species of animals. Food and distilled water were supplied *ad libitum*.

Table 1 Components of the biotin-deficient basal diet

Ingredient	Contents (%)
Egg white, spray dried	20
D-glucose	63.7
Corn oil	10
Cellulose powder	2
Vitamin mix	1.17
Mineral mix	3.13

3. Experimental protocols

The experimental procedure is shown in Fig. 1. On GDs 12 or 15, the dams were killed by diethyl ether anesthesia. Blood samples were collected directly from the maternal heart at the time of laparotomy, and serum was obtained. The livers ware collected and weighed.

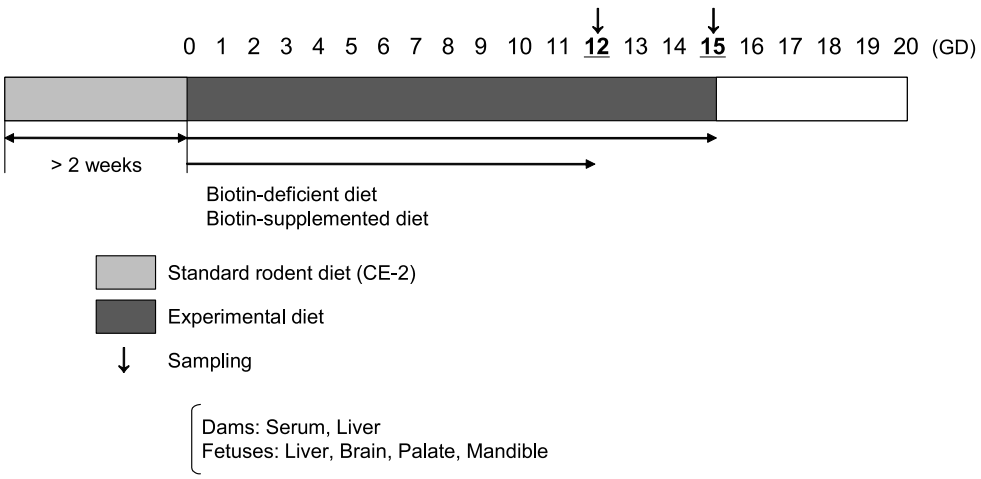

Fig. 1 Experimental procedure.

The embryos were removed and the following observations recorded: the number of fetuses, fetal body weight and any external malformations. A half of fetuses was placed in Bouin's fixative for observation. Livers, brains, palates and mandibles were collected from the remaining half of fetuses. These samples were frozen at −40℃ until analysis.

4. Biotin content

The biotin content of samples was measured by a bioassay using *Lactobacillus plantarum* ATCC 8014[13-15]. Samples were incubated in standard medium for biotin determination (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with *L. plantarum* suspension for 18 hours at 37℃ and the absorbance was read at 610 nm with a microplate reader. Biotin concentration is expressed as pmol/g (mL).

Since some biotin existing in the samples was in the protein-bound form, protein-bound biotin was hydrolyzed before biotin measurement. Samples of 100 μL were transferred into a tube and 100 μL of 4.5 N H_2SO_4 was added. After hydrolysis of the samples by heating in an autoclave for 60 min at 121℃, samples were neutralized by adding about 100 μL of 4.5 N NaOH.

5. Biotinidase activity

Biotinidase, a biotin-binding protein, is known to have two functions[16-18]. Biotin cannot be used *in vivo* without release from binding protein. Protein-bound biotin becomes the free form by biotinidase activity. Recently, biotinidase has been shown to act as a biotin-carrier protein.

Biotinidase activity was determined with the colorimetric method by measuring *p*-aminobenzoate liberation from N-biotinyl-*p*-aminobenzoate[16]. Biotinidase activity is expressed as nmol/min/g (mL).

6. Total protein assay

Protein concentration was measured by modified Lowry's method using a BCA protein assay kit (PIERCE).

7. Holocarboxylase

Biotinylated proteins PC, PCC, MCC and ACC in the maternal liver and fetal organs were separated by SDS-PAGE in NuPAGE 3-8 % Tris-acetate gels (Invitrogen Japan Co., Ltd., Tokyo, Japan) and determined by Western blotting using horseradish peroxidase (HRP) as follows for both diet groups[19-21].

8. Statistical analysis

Values are the means ± SD. Statistical analysis of these data was performed using StatView Ver. 5.5 (SAS Institute Inc., Cary, NC). Differences were considered significant at a probability level of $p < 0.05$ in all analyses.

Results

1. Effects of maternal biotin deficiency on reproduction in mice

The effects of dietary biotin deficiency on the reproduction of female mice and their embryos are presented in Table 2. Maternal reproduction success and fetal viability were not affected by the biotin-deficient diet. There was no appreciable difference in mean maternal weight gain during gestation between groups. Pregnant mice in the biotin-deficient group did not show any known signs of deficiency such as hair loss, dermatitis, or nervous irritability.

The mean numbers of live fetuses per female and the body weight of fetuses were not different between dietary groups. However, almost all of the live fetuses from dams in the biotin-deficient group showed malformations such as cleft palate, micrognathia and micromelia (Table 3). Cleft palate occurred in 97.1 % of fetuses per litter affected in the biotin-deficient group on GD 15.

Table 2 Effects of maternal biotin deficiency on reproduction in mice

Dietary groups	GD 12		GD 15	
	Deficient	Supplemented	Deficient	Supplemented
Body weight dam (g)	42.5 ± 1.51[a]	42.0 ± 2.12	51.3 ± 3.95	51.5 ± 3.45
No. of fetuses	13.4 ± 2.33	14.3 ± 2.49	14.6 ± 2.26	14.0 ± 2.45
Fetal weight (g)	0.08 ± 0.01	0.09 ± 0.01	0.45 ± 0.07	0.45 ± 0.05

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

Table 3 Indication of external malformations produced by maternal biotin deficiency in mice

Dietary groups	GD 15	
	Deficient	Supplemented
cleft palate	97.1*	16.1
micrognathia	74.5*	22.5
micromelia	63.8*	17.5

*$p < 0.05$, compared with the biotin-supplemented group (Mann-Whitney's U test).

2. Changes in biotin and biotinidase in dams

The effects on changes in biotin and biotinidase activity of dams are presented in Fig. 2 and Table 4. Maternal serum biotin content was lower in the biotin-deficient group than in the biotin-supplemented group on both GDs 12 and 15. Biotinidase activity was the same in the two dietary groups, but the serum biotin level and biotinidase activity decreased on GD 15 in both dietary groups. Hepatic biotin content was lower in the biotin-deficient group than in the biotin-supplemented group. In the biotin-supplemented group, biotin content was the same on GDs 12 and 15, but in the biotin-deficient group, biotin content decreased on GD 15.

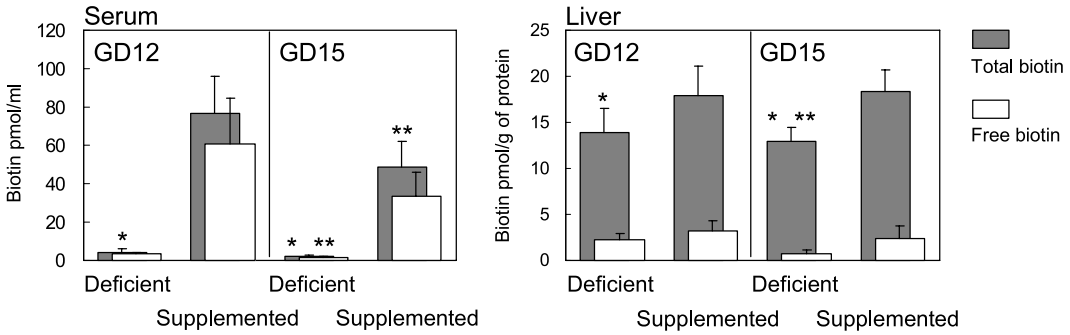

Fig. 2 Biotin concentrations in serum and liver in dams.
means ± SD, n = 8.
*$p < 0.05$, compared with the biotin-supplemented group (Mann-Whitney's U test).
**$p < 0.05$, compared with GD 12 (Mann-Whitney's U test).

Table 4 Biotinidase activity in the serum and liver in dams

Dietary groups	GD 12		GD 15	
	Deficient	Supplemented	Deficient	Supplemented
Serum (nmol/min/ml)	7.1 ± 0.7[a]	6.5 ± 0.5	4.3 ± 0.3**	4.8 ± 0.6**
Liver (nmol/min/g protein)	45.3 ± 10.3	36.8 ± 6.9	26.6 ± 4.3**	26.7 ± 4.8**

means ± SD.
*$p < 0.05$, compared with the biotin-supplemented group (Mann-Whitney's U test).
**$p < 0.05$, compared with GD 12 (Mann-Whitney's U test).

3. Changes in biochemical markers in fetuses

The effects on changes in biotin and biotinidase activity of the fetal liver, brain, palate and mandible are presented in Fig. 3 and Table 5. Biotin content was lower in the biotin-deficient group than in the biotin-supplemented group in all tissues analyzed on GDs 12 and 15. In both groups, the biotin content of the four tissues increased on GD 15. On GD 12, hepatic biotindase activity was twice the level on GD 15. In the brain of biotin-supplemented fetuses, biotinidase activity on GD 15 was higher than on GD 12. Biotinidase activity in the palate decreased on GD 15 in both groups, but activity in the mandible did not change. The expression of holocarboxylases in the maternal liver and fetal tissues is shown in Fig. 4. In the maternal liver, the expression of carboxylases was shown to be unchanged in the dietary groups or the gestation days. In fetal tissues, the expression of ACC was lower and remained unchanged, but PC, PCC and MCC were highly expressed in biotin-supplemented fetuses. Moreover, in the biotin-deficient group, the expression level of carboxylases decreased on GD15.

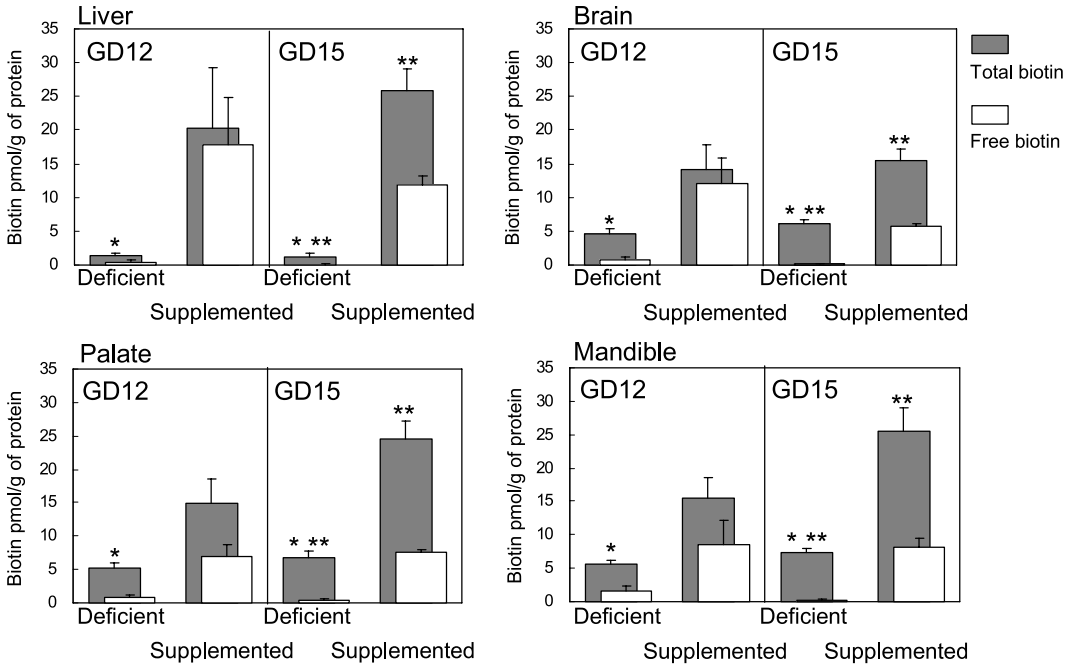

Fig. 3 Biotin concentrations in liver, brain, palate and mandible in fetuses.
means ± SD, n = 8.
*$p < 0.05$, compared with the biotin-supplemented group (Mann-Whitney's U test).
**$p < 0.05$, compared with GD 12 (Mann-Whitney's U test).

Table 5 Biotinidase activity in the liver, brain, palate and mandible in fetuses

Dietary groups	GD 12		GD 15	
	Deficient	Supplemented	Deficient	Supplemented
Liver	155.1 ± 29.0[a]*	79.9 ± 26.5	72.9 ± 29.2**	72.6 ± 20.1
Brain	10.6 ± 2.5	11.9 ± 2.8	11.8 ± 2.8*	24.6 ± 3.5**
Palate	26.8 ± 4.6	28.3 ± 4.6	33.1 ± 4.6**	38.7 ± 5.2**
Mandible	52.1 ± 8.8	48.3 ± 13.0	56.3 ± 7.4	54.9 ± 13.4

[a]means ± SD. unit : nmol/min/g of protein.
*$p < 0.05$, compared with the biotin-supplemented group (Mann-Whitney's U test).
**$p < 0.05$, compared with GD 12 (Mann-Whitney's U test).

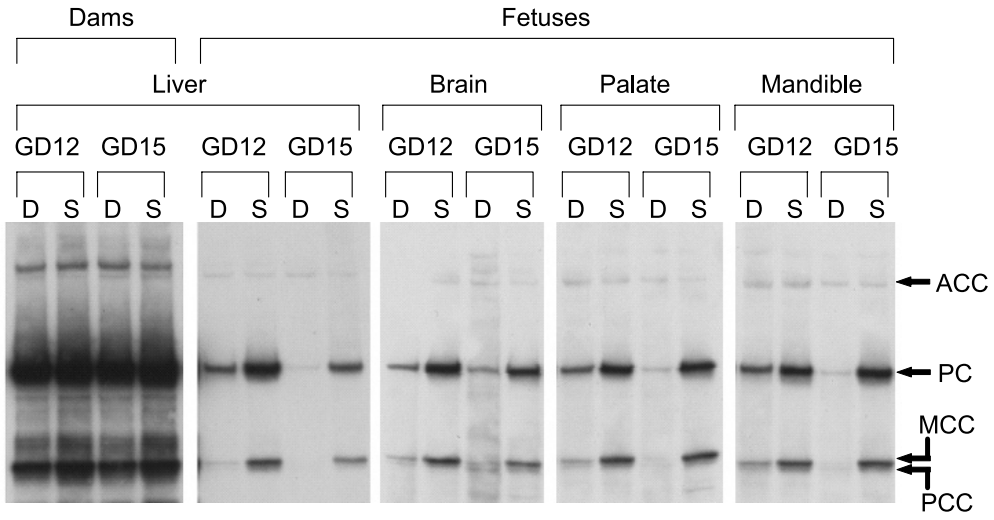

Fig. 4 Carboxylases in dams and fetuses.

Discussion

For the reproductive effects of maternal biotin deficiency in mammals, Giroud *et al.*[22] reported that biotin deficiency is not teratogenic in rats. Although Cooper and Brown[23] observed histological abnormalities such as a poor cell structure, enlarged and irregular nuclei and vacuolated cytoplasm of the cells in the organs of the heart, liver and kidney of newborn biotin-deficient rats, no presence of macroscopical abnormalities in these newborns is mentioned. Subsequently, the authors found that maternal biotin deficiency causes severe malformations in mouse embryos of ICR and C57BL strains. The malformations produced by biotin deficiency are mainly cleft palate, micrognathia, micromelia and open eyelids[8, 24]. In A/Jax strain mice with a high incidence of spontaneous cleft palate, the incidence of cleft palate was not so high in biotin-deficient embryos. Levin *et al.*[25] also demonstrated that rat fetuses from dams given a biotin-deficient diet throughout gestation have obvious dysmorphic features.

In rats, biotin deficiency induced no malformation and, in hamsters, biotin deficiency during gestation induced the inhibition of embryonic growth and increased the number of resorptions and dead embryos[9]. It was also demonstrated that biotin supplementation during gestation increased the number of newborns and slightly recovered dermatitis of the extremities in swine. Therefore, there were strain and species differences in the induction of external malformation by maternal biotin deficiency in mammals. It is suggested that biotin is an essential nutrient and plays an important role for maintaining the growth and development of mammalian embryos.

For embryonic development, in the present study there was no difference in the body weight of embryos between GDs 12 and 15 at midgestation. However, the body weight of biotin-deficient embryos on GD 18 decreased less than that of biotin-supplemented embryos in previous studies. In these biotin-deficient embryos, external malformations such as cleft palate, micrognathia and micromelia were observed. Also, biotin concentration in dams and their embryos in the biotin-deficient group was significantly decreased from the biotin-supplemented group on GDs 12 and 15. Thus, biotin may be required to maintain normal pregnancy and embryonic development in the middle and late stages of gestation.

Biotinidase may release biotin from protein-bound biotin in the yolk, and this free biotin may be transferred into the embryo, although the relationship between biotin concentration and biotinidase activity is unclear. In dams, the activity of biotinidase and serum biotin concentration on GD 15 decreased less than on GD 12. In embryos, the total biotin concentration of the liver, brain, palatal process and mandible on GD 15 was increased, but free biotin was

decreased, compared with that on GD 12. It was demonstrated that biotin is required for embryos during morphogenesis. In particular, the percentage of protein-bound biotin was high in the liver and brain of embryos, showing that biotin is stored in these organs at midgestation. On the other hand, free biotin concentration in the palatal process and mandible was not changed on GDs 12 and 15. Biotinidase activity was also high in these organs. A large amount of biotin is demonstrated to be needed for formation of the palate and mandible during morphogenesis. Therefore, when biotin concentration is decreased in these organs, biotin-related metabolism is inhibited and may cause a cleft plate and micrognathia.

Biotin is a coemzyme for 4 types of carboxylases, methylcrotonyl CoA carboxylase (MCC) in the leucine catabolism pathway, acetyl CoA carboxylase (ACC) as a rate-limiting enzyme for lipogenesis, pyruvate carboxylase (PC) as a rate-limiting enzyme for gluconeogenesis, and propionyl CoA carboxylase (PCC), which is present in the catabolism pathway of amino acids such as leucine. The expressions of these carboxylases in liver did not differ between biotin-deficient and biotin-supplemented dams. ACC was expressed only slightly in biotin-supplemented embryos, but PC, PCC, and MCC were present, although their expressions were decreased in biotin-deficient embryos. Thus, gluconeogenesis and fatty acid synthesis may be inhibited in biotin-deficient embryos. Also there was a difference in the expression of these carboxylases between on GDs 12 and 15. From these findings, it is suggested that biotin may be associated with the expression and production of these carboxylases.

In this study, it became obvious that biotin was supplied from dams to embryos as the embryos grew, and the biotin is an essential nutrient, even in embryos. In particular, a large amount of biotin was transported to the palatal process and mandible on GDs 12–15. From these findings, it was suggested that biotin is important to maintain of reproductive performance in mammals.

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