〈Original Article〉

Low level of maternal biotin intake changes the expression of biotin transporter in dams and fetuses in mice

Hiromi Sawamura[1, 2], Yoshie Ishii[3], Ryoko Shimada[3], Masahiro Yuasa[3], Munetaka Negoro[4] and Toshiaki Watanabe[2, 3]

Summary To clarify the effects of maternal biotin deficiency on biotin homeostasis in mammals, we examined whether a low level of maternal biotin intake affects the expression of any gene that plays an important role in maintaining biotin homeostasis in mice. Pregnant mice were fed a biotin-deficient diet or a biotin-supplemented (control) diet for 14 days of gestation. The biotin concentration was significantly decreased in all tissues examined, except maternal kidney of biotin-deficient mice, compared with the control. In the placenta, the ratios of sodium-dependent multivitamin transporter (SMVT) mRNA and protein expression in the biotin-deficient group were significantly higher than those in the control. However, the expression of holocarboxylase synthetase and biotinidase mRNA was not significantly different between the two dietary groups. We first confirmed that a low level of maternal biotin intake changes the expression of SMVT and might affect biotin homeostasis in both dams and fetuses.

Key words: Biotin deficiency, Maternal nutrition, Fetal development, Transporter expression, Mice

1. Introduction

Biotin is a water-soluble vitamin that is classified as a B-group vitamin. In mammals, biotin serves as an essential cofactor for four carboxylases in fatty acid synthesis, branched-chain amino acid (BCAA) metabolism and gluconeogenesis[1]. It is known that biotin deficiency causes the dysfunction of these metabolic pathways, and the resulting biochemical and physiological impairments induce skin disorders such as dermatitis, hair loss, neuritis and susceptibility to infections[1]. Biotin deficiencies are rare in humans, as biotin is well distributed in various kinds of food. However, biotin deficiency can be induced by

[1]Department of Molecular Nutrition, School of Human Science and Environment, University of Hyogo, Himeji 670-0092, Japan
[2]Reserch Institute for Food and Nutrition Sciences, University of Hyogo, Himeji 670-0092, Japan
[3]Department of Dietary Environment Analysis, School of Human Science and Environment, University of Hyogo, Himeji 670-0092, Japan
[4]Department of Chemical and Biological Engineering, Ube National College of Technology, Yamaguchi 755-8555, Japan
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Correspondence: Hiromi Sawamura, MS
Department of Molecular Nutrition, School of Human Science and Environment, University of Hyogo, Shinzaike Honcho 1-1-12, Himeji 670-0092, Japan
TEL/FAX: +81-79-292-9421
E-mail: sawamura@shse.u-hyogo.ac.jp

consuming large amounts of raw egg whites, which contain high levels of avidin. Avidin is known to inhibit the absorption of biotin from the intestinal tract and to produce biotin deficiency[1]. It is also reported that biotin deficiency is induced in patients provided anticonvulsants[2] and in infants fed with special therapeutic infant formulas in Japan[3].

Biotin is essential for reproduction and fetal development in mammals. We first detected that maternal biotin deficiency causes severe malformations in mouse fetuses[4, 5]. The external malformations are mainly cleft palate, micrognathia and micromelia in the ICR and A/Jax strains. We previously detected that there are strain and species differences in the teratogenic effects of biotin deficiency in rodents[6, 7]. It has been demonstrated that decreased urinary excretion of biotin in the late stage of gestation is observed even in normal pregnancy, suggesting that pregnant women may suffer mild biotin deficiency[8, 9]. In pregnant mice, biotin excretion in urine decreased on day of gestation (dg) 4 in biotin-deficient dams and on dg 16 in biotin-supplemented dams[10, 11]. The requirement for biotin may increase during gestation and/or fetal development at the specific stages. A large amount of biotin compared to the normal stage is necessary for maintaining normal reproductive performance during the late stage of gestation. The relationship between biotin and fetal development is not well known.

The present study aimed to clarify the effects of biotin deficiency during pregnancy on three genes related to biotin homeostasis in mammals. Sodium-dependent multivitamin transporter (SMVT), holocarboxylase synthetase (HCS) and biotinidase (BTD) play crucial roles in biotin homeostasis by regulating biotin absorption and recycling[12]. SMVT, which transports some water-soluble vitamins, biotin, pantothenate and liponate, is expressed in various tissues such as placenta, intestine, liver and kidney[13, 14]. Ghosal et al. reported that conditional knockout of the SMVT gene in mouse intestine caused growth retardation and decreased bone density and length[15]. There are no reports about the effect of biotin deficiency on SMVT expression *in vivo*. Meanwhile, HCS catalyzes the biotinylation of carboxylases[16] and histones[17], and BTD is the enzyme responsible for the recycling of biotin, the transport of biotin in plasma[18] and the regulation of histone biotinylation[19]. In humans, the biotin cycle was shown to be disrupted by genetic deficiency of HCS or BTD[20], but it remains unclear whether these three proteins directly affect biotin metabolism during pregnancy. Therefore, we examined whether a low intake of biotin during pregnancy affects the expression of these proteins in maternal and fetal tissues in mice.

2. Materials and methods

2.1 Animals and diet

Nulliparous female ICR mice, aged 6 weeks, were obtained from CLEA Japan Inc. (Tokyo, Japan). All animals, including males used for mating, were housed for 2 weeks before mating in an animal room maintained under 12 h light-dark cycle conditions of 0900-2100 and at a constant room temperature of 23 ± 2℃. The female mice were mated with healthy males for a short mating period in the morning (0900-1100). The day when a copulation plug was detected at the end of mating was designated as day 0 of gestation (dg 0). Pregnant females were randomly divided into two groups: a biotin-deficient group (n=11) fed a biotin-deficient diet (Table 1) and a control group (n=10) fed a biotin-supplemented diet (biotin-deficient diet supplemented with 5 mg biotin/kg). These mice were housed in stainless steel cages with a wire-bottomed floor and given the diets and distilled water ad *libitum* for 14 days (full term = 19 days). Diet consumption had been confirmed to be approximately the same in the two dietary groups in our previous study[4]. All experimental procedures including the care and treatment of mice described in this paper were approved by the Institutional Animal Care and Use Committee of the School of Human Science and Environment, University of Hyogo (#038, 087).

2.2 Collecting samples

Pregnant mice were killed on dg 14 because, in normal murine craniofacial development, the secondary palates undergo major organogenesis on dg

12-15[21]. Serum was collected to measure biotin concentration and biotinidase activity, and was stored at -20℃ until needed. Fetuses were collected from the uterus and immersed in phosphate-buffered saline (PBS). The placenta was removed from the fetuses and the number of fetuses was confirmed. Maternal tissues (brain, liver, kidney and placenta) and fetal tissues (liver and palatal process) were collected. Palatal processes were carefully dissected from the head in fetuses under a dissecting microscope using a technique described previously[21]. These samples were immediately stored at -80℃ until analysis.

2.3 Measurements of biotin concentration and biotinidase activity

Blood was centrifuged for 10 min at 3,000 rpm and serum was collected. Tissues were lysed with solubilization buffer (1% Triton-X100 and 0.02% protease inhibitor in PBS). These tissue samples were homogenized on ice using a sonicator. The sonicated samples were centrifuged at 15,000 rpm for 15 min at 4℃ and the supernatant was collected.

Biotin concentration in tissues was determined using a microtiter plate adaptation of a microbiological assay with *Lactobacillus plantarum* ATCC 8014[22-24]. This bacterium was obtained from American Type Culture Collection, which is generally used for determining the quantity of some vitamins, cultured in a microtiter plate for 24 h and the cell density was determined at 610 nm. As biotin in tissues partially existed in a protein-binding form, for the determination of total biotin, 100 μL of sample solution was pretreated with 2.25 M H_2SO_4 for 121℃ for 60 min and neutralized with 4.5 M NaOH. Biotin concentrations are expressed as pmol/mL or nmol/g protein. The protein concentration of samples was determined with a BCA Assay kit (Thermo Fisher Scientific Inc., Kanagawa, Japan). Biotinidase activity was measured using the colorimetric method by measuring the liberation of *p*-aminobenzoate from *N*-biotinyl-*p*-aminobenzoate[25]. Biotinidase activity is expressed as nmol/min/mL or nmol/min/g protein.

Table 1 Component of the biotin-deficient deiet

Ingredient	Amount (%)
Egg white, spray dried	20
L-cystine	0.3
Corn starch	39.7486
a-Corn starch	13.2
Sucrose	10
Soybean oil	7
Cellulose powder	5
Mineral mix (AIN-93G)	3.5
Biotin-free vitamin mix (AIN-93G) †	1
Choline bitartrate	0.25
tert-Butylhydroquinone	0.0014

† Component of the biotin-free vitamin mix	
Vitamin A (All-trans-retinyl paimitate) [500,000U/g]	0.08
Vitamin D_3 (Cholecalciferol) [400,000IU/g]	0.025
Vitamin E (All-rac-a-Tocopheryl Acetate) [50%]	1.5
Vitamin K_1 (Phylloquinon)	0.0075
Vitamin B_1 (Thiamine hydrochloride)	0.06
Vitamin B_2 (Riboflavin)	0.06
Vitamin B_6 (Pyridoxine Hydrochloride)	0.07
Vitamin B_{12} (Cyanocobalamin) [0.1%]	0.25
Folic acid	0.02
Calcium pantothenate	0.16
Nicotinic acid	0.3
Sucrose	97.4675

2.4 Quantitative real-time PCR

Total RNA from tissues was isolated using TRIzol reagent (Life Technologies Japan Ltd., Tokyo, Japan), and complementary DNA was synthesized using a ReverTra Ace qPCR RT Master Mix (TOYOBO Co. Ltd., Osaka, Japan). Quantitative mRNA expression was assessed via SYBR Green qPCR assay. The gene-specific primer sequences were as follows: for SMVT, forward 5'-ACGCAAGGCAAGCAGAAC-3' and reverse 5'-GCACCGACTGATTCTGTGAGTA-3'; for HCS, forward 5'-TCCAGCATTTGATGTCCTTG-3' and reverse 5'-TATCGTTGGGCCACTTCACT-3'; for BTD, forward 5'-CATCCATCGGTCCT-GAGC-3' and reverse 5'-TAATCTGCACACCCT-TCTGG-3'; for β-actin, forward 5'-CTAAGGC-CAACCGTGAAAAG-3' and reverse 5'-ACCAGAGGCATACAGGGACA-3'. The mRNA levels were assessed with β-actin as an internal control under the following conditions: pre-incubation at 98℃ for 2 min, followed by 40 cycles of 98℃ for 10 s, 60℃ for 10 s, and 68℃ for 30 s. All qPCR was performed in KOD SYBR qPCR mix (TOYOBO Co. Ltd., Osaka, Japan) on a StepOne Real-time PCR System (Applied Biosystems Inc., Japan, Tokyo, Japan). The results were normalized to β-actin. Fold change expression was calculated using threshold

Table 2 Effect of maternal biotin deficiency on maternal tissue weights

	Brain	Liver	Kidney	Placenta
Control	0.49 ± 0.05	2.73 ± 0.18	0.24 ± 0.01	0.08 ± 0.01
Biotin-deficient	0.47 ± 0.02	2.36 ± 0.27	0.22 ± 0.01	0.08 ± 0.01

Each value is expressed as the mean ± SD (n=3).

Table 3 Biotin concentration in maternal and fetal tissues

			Dietary groups	
			Control	Biotin-deficient
Dams	Serum	total biotin (pmol/mL)	132.9 ± 53.3	29.2 ± 11.2**
		free biotin (pmol/mL)	109.8 ± 18.4	14.6 ± 0.9**
		The ratio of free biotin (%)	54.4 ± 10.2	53.8 ± 19.3
	Liver	total biotin (nmol/g of protein)	26.4 ± 5.8	15.5 ± 2.3*
		free biotin (nmol/g of protein)	3.5 ± 0.3	2.6 ± 0.2*
		The ratio of free biotin (%)	13.5 ± 1.7	16.9 ± 1.2*
	Kidney	total biotin (nmol/g of protein)	27.6 ± 8.1	26.1 ± 2.4
		free biotin (nmol/g of protein)	1.0 ± 0.3	0.7 ± 0.0
		The ratio of free biotin (%)	4.0 ± 2.5	2.6 ± 0.3
	Placenta	total biotin (nmol/g of protein)	11.8 ± 8.1	1.9 ± 0.4*
		free biotin (nmol/g of protein)	4.6 ± 1.8	0.4 ± 0.1*
		The ratio of free biotin (%)	40.5 ± 15.5	24.4 ± 8.9
Fetuses	Liver	total biotin (nmol/g of protein)	86.1 ± 25.4	2.3 ± 0.5**
		free biotin (nmol/g of protein)	72.5 ± 18.7	1.3 ± 0.2**
		The ratio of free biotin (%)	86.3 ± 16.2	57.2 ± 8.6
	Palatal process	total biotin (nmol/g of protein)	23.0 ± 20.6	2.3 ± 1.0
		free biotin (nmol/g of protein)	14.4 ± 10.1	0.5 ± 0.4
		The ratio of free biotin (%)	73.1 ± 17.5	18.8 ± 11.3*

Each value is expressed as the mean ±SD (n=3-11).

*$P < 0.05$, **$P < 0.01$, compared with the control group.

Table 4 Biotinidase activity in maternal and fetal tissues

		Dietary groups	
		Control	Biotin-deficient
Dams			
	Serum (nmol/min/mL)	7.3 ± 1.3	7.9 ± 1.4
	Liver (nmol/min/g protein)	357.9 ± 109.9	378.1 ± 73.9
	Kidney (nmol/min/g protein)	508.6 ± 44.8	541.2 ± 68.1
	Placenta (nmol/min/g protein)	164.3 ± 61.0	198.7 ± 95.0
Fetuses			
	Liver (nmol/min/g protein)	76.1 ± 31.7	62.6 ± 39.9
	Palatal process (nmol/min/g protein)	117.5 ± 66.2	191.5 ± 118.1

Each value is expressed as the mean ± SD (n=3).

cycle (Ct) values and determined via the $2^{-\Delta\Delta CT}$ method[26].

2.5 Western blot analysis

Tissues samples were homogenized as described above. The protein concentration of samples was determined with a BCA Assay kit (Thermo Fisher Scientific Inc., Kanagawa, Japan). Each sample was adjusted to the same concentration of protein and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Extracted protein was heated at 100℃ for 5 min before loading. Samples were then separated on 8% gels, transferred onto polyvinylidene difluoride (PVDF) membranes (Pall

Fluoro Trans W membrane, NIPPON Genetics Co., Ltd., Tokyo, Japan) and blocked for 1 h at room temperature in 1% bovine serum albumin. Monoclonal anti-β-actin (1:3000; Sigma-Aldrich Co. Ltd., Tokyo, Japan) and polyclonal anti-SMVT (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, USA) were obtained to detect these antigens. Membranes were incubated with these antibodies overnight at 4℃ and were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature. Target protein was detected using the enhanced chemiluminescence system (GE Healthcare Japan Co., Ltd., Tokyo, Japan).

2.6 Statistical analysis

The values in the text are expressed as mean ± SD. To assess the effects of biotin deficiency, statistical comparison of means of two experimental groups was conducted by Student's *t*-tests. Statistical analysis of the data was performed on a personal computer using a standard statistical package (Statcel Ver. 3, Tokyo, Japan). Differences were considered statistically significant if *P* values less than 0.05 in all analyses.

3. Results

3.1 Body weight and growth

No significant differences were observed between the two dietary groups for food intake, body weight gain and fetal number (data not shown). These results are consistent with our previous studies of the effect of biotin deficiency during pregnancy on mice[4, 27]. No clinical signs of biotin deficiency in dams were observed. Maternal tissue weights were also not significantly different between the two dietary groups (Table 2). Incidence of cleft palate of fetuses in the biotin-deficient group was 97.4%.

3.2 Biotin concentration and biotinidase activity

The biotin contents in the tissues are shown in Table 3. In biotin-deficient mice, total biotin concentration was significantly decreased in the maternal serum (22% of control), liver (59%), placenta (16%), fetal liver (3%) and palatal process (10%) compared with the control group. In particular, the biotin contents were markedly decreased in maternal serum and fetal liver. These results consistent with our previous study[27]. Meanwhile, in maternal kidney, biotin contents did not differ between the two dietary groups. The ratio of free biotin was significantly increased in the maternal liver of biotin-deficient mice (125% of control), while a significant decrease was observed in fetal palatal process (26%). Biotinidase activity showed no significant difference in these tissues between the two dietary groups (Table 4).

3.3 SMVT, HCS and BTD gene expression

In terms of the expression of mRNA in the tissues, quantitative RT-PCR analysis showed that the relative level of SMVT mRNA was significantly increased in the placenta and fetal liver of the biotin-deficient group compared with the control group (Fig. 1A). Meanwhile, no significant differences were observed in maternal liver and fetal palatal process. The expression of HCS and BTD mRNA was not significantly different in all tissues examined between the two dietary groups (Fig. 1B, C).

3.4 Biotin transporter protein expression

Western blot analysis demonstrated that the expression of SMVT protein was significantly elevated in the placenta of biotin-deficient mice compared with control group (Fig. 2B). In maternal liver, SMVT protein expression showed a pattern to decrease in biotin-deficient mice (p=0.100) (Fig. 2A). There was no significant difference in fetal liver between the two dietary groups (Fig. 2C).

4. Discussion

We suggested in previous studies that biotin deficiency during pregnancy in mice causes a remarkably high incidence of congenital malformations such as cleft palate, micromelia and micrognathia in fetuses[4-6, 28-29]. Levin et al. also suggested that rat fetuses from dams given a biotin-deficient diet throughout gestation had some obvious dysmorphic features[30]. These studies suggested that biotin may be required to

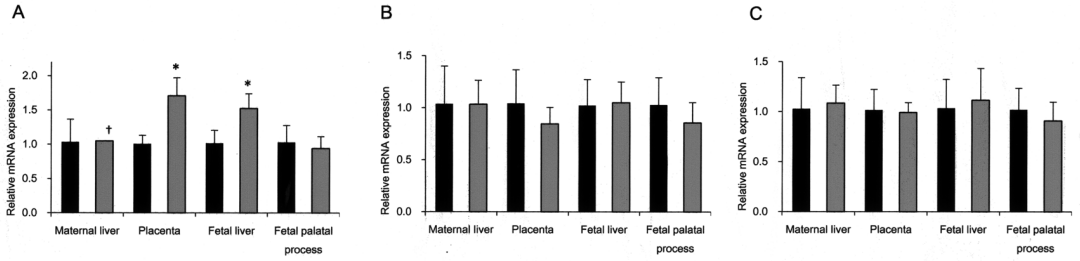

Fig. 1 Effects of biotin deficiency on gene expression of SMVT (A), HCS (B) and BTD (C) in maternal and fetal tissues. Ct value were normalized with β-actin as a housekeeping gene. Relative expression of mRNA is represented as fold change in comparison to the control group. Black bar, control; gray bar, biotin-deficient. Each value is expressed as the mean ± SD (n=3-6). *$P < 0.01$, compared with the control group.
One value of the data of SMVT mRNA expression in the maternal liver of the biotin-deficient group was deleted since this value was unusually high (about 5 times higher than in the control group). When this value is included, the mean value becomes 2.47 ± 2.44 (n=3).

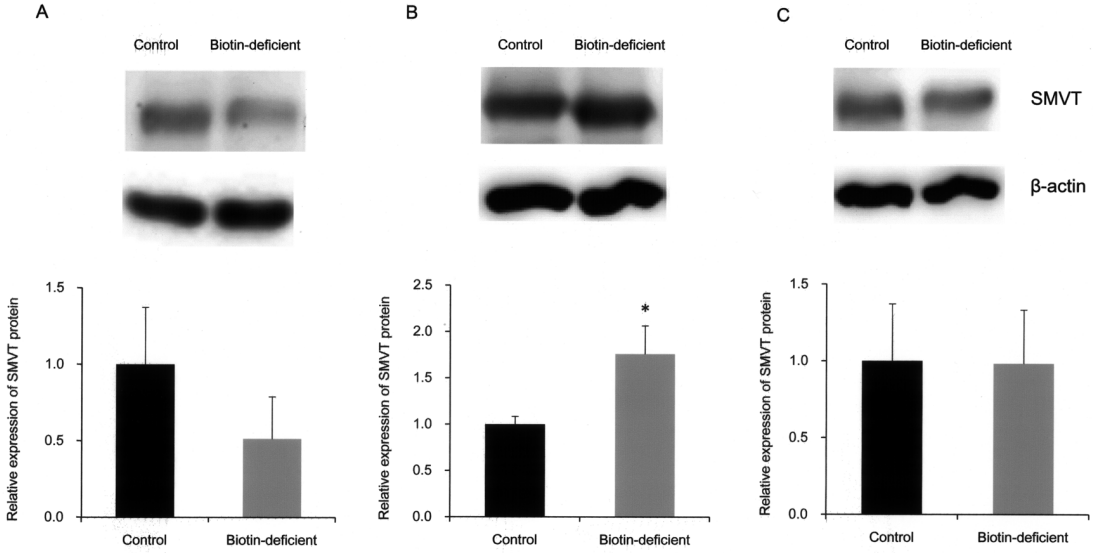

Fig. 2 Effects of biotin deficiency on SMVT protein expression in maternal and fetal tissues: (A) maternal liver, (B) placenta, (C) fetal liver. SMVT protein in tissues was detected by Western blot analysis. A 69-kDa band corre sponding to SMVT protein was detected. The intensity of individual bands was quantified using Image J densito metry software. As an internal control, β-actin (42 kDa) was used for normalization. The ratio for the control group was assigned a value of 1. The results for one representative sample and the normalized average values for the 3 samples studied, compared with the control group, are shown. Each value is expressed as the mean ± SD (n=3). *$P < 0.05$, compared with the control group

maintain normal pregnancy and fetal development in the middle stage of gestation. However, it remains unclear how maternal biotin deficiency affects biotin homeostasis during pregnancy through the regulation of biotin transfer. In order to clarify the effect of maternal biotin deficiency on the maintenance of biotin homeostasis during pregnancy, we studied the expression of three genes related to biotin homeostasis in mammals.

SMVT (product of the *SLC*5A6 gene) is essential for mediating and regulating biotin uptake into mammalian cells. In the present study, we showed that mRNA levels of biotin transporter SMVT were increased in accordance with biotin deficiency during

pregnancy in the placenta and fetal liver. SMVT protein expression was also increased in the placenta, but there was no significant difference in the fetal liver. Meanwhile, in maternal liver, mRNA levels of SMVT did not differ between the two dietary groups, but SMVT protein expression showed a pattern of decrease in biotin-deficient mice. Absorptive tissues, such as the intestinal mucosa and kidney, and the placenta have very high levels of SMVT-specific mRNA[31]. The placenta plays a key role in the maternal-fetal relationship, maintaining fetal homeostasis through the regulation of nutrient transfer. It has been reported that biotin deficiency decreased the level of hSMVT in human liver HepG2 cells[32] and leukocytes[33]. On the other hand, Reidling et al. demonstrated that biotin deficiency leads to an increase in the protein and mRNA levels of hSMVT in human intestinal epithelial cells[34]. In addition, Crisp et al. suggested that biotin concentration correlates negatively with the expression of SMVT in human choriocarcinoma cells[35]. These studies indicated that the effects of biotin deficiency on SMVT expression might differ between tissues that have high levels of SMVT, such as intestine and placenta, and other tissues. We suggested that maternal biotin deficiency leads to changes in the expression of SMVT in the placenta in an attempt to maintain the homeostasis of biotin.

In the present study, the expression of SMVT mRNA was increased in accordance with biotin deficiency in the fetal liver, whereas its protein expression was not modified. The protein expression of SMVT was not consistent with mRNA expression, implying the existence of post-transcriptional regulation of SMVT. MicroRNAs (miRNAs) are a class of small non-coding functional RNAs that mediate post-transcriptional regulation of gene expression[36]. miRNAs reduce the translation and/or stability of that mRNA, leading to a reduction in protein levels. A recent study showed that the expression of miR-539 depends on biotin's regulation of the expression of HCS in human kidney cells[37]. miRNA may play roles in the regulation of SMVT expression.

HCS and *BTD* gene expression in maternal liver, placenta, fetal liver and palatal process was unchanged by biotin deficiency in the present study. Biotinidase activity in these tissues also did not differ between the two dietary groups. Rodríguez-Meléndez et al. suggested that HCS mRNA was significantly decreased in the liver, kidney, muscle and brain in biotin-deficient male rats[38]. A recent study showed that HCS acts as a biotin sensor, which may be involved in post-translational regulation of SMVT expression in Jurkat cells[39]. On the other hand, it has been demonstrated that hepatic mRNA for HCS did not change significantly in either dams or fetuses in mice[40], which is consistent with our findings. These studies indicate that the effects of biotin deficiency on HCS expression may be specific to the gender and the type of cell. In terms of BTD expression, gene expression was not affected by biotin supply in human choriocarcinoma cells[35]. We suggested that the biotin recycling system does not act to maintain the homeostasis of biotin when dams become biotin-deficient.

In conclusion, we detected that a low intake of biotin during pregnancy changes SMVT gene and protein expression. There are no reports about the effect of biotin deficiency on SMVT expression *in vivo*. We first confirmed that a low level of maternal biotin intake changes the expression of SMVT and might affect the biotin homeostasis in both dams and fetuses. Further studies about the relationship between maternal biotin deficiency and fetal development are needed.

Conflicts and interest

The authors have declared no conflict of interest.

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