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Original Article

The effects of biotin administration to pregnant mice with biotin deficiency on fetal development

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Summary

To clarify the role of biotin in palatal development, we examined the effects of biotin administration to pregnant mice with biotin deficiency at midgestation on the incidence of cleft palate. Pregnant mice were fed a biotin-deficient diet or biotin-supplemented (control) diet. Several mice in the biotin-deficient group were administered biotin solution via oral gavage at 0900 on day of gestation (dg) 11. After biotin administration, they were fed the biotin-supplemented diet until dg 17. The incidence of cleft palate was lower in the dg 11-supplemented group than in the biotin-deficient group. There was no significant difference in the incidence of cleft palate between the dg 11-supplemented group and the control group. This suggests that biotin is necessary for the normal development of the palatal process in the mouse fetus on dg 11 and earlier, which may be an important time point. Biotin may play a role in the initial stage of palatogenesis.

Introduction

Biotin is a water-soluble B-group vitamin. In mammals, biotin is an essential cofactor for four carboxylases in fatty acid synthesis, branched-chain amino acid (BCAA) metabolism and gluconeogenesis[1]. Biotin deficiencies are rare in humans because biotin is well distributed in many types of food. The potential cases of biotin deficiency are intestinal malabsorption in individuals with long-term use of drugs, such as antibiotics, certain antiseizure medications and lipoic acid, excessive alcohol consumption and continuous consumption of raw egg whites[2-4]. It was recently demonstrated that the decreased urinary excretion of biotin in the late stage of gestation is observed even in normal pregnancy, suggesting that pregnant women experience mild biotin deficiency[5-6]. We previously found that maternal biotin deficiency causes severe malformations in mouse fetuses[7-8]. The external malformations are mainly cleft palate, micrognathia and micromelia in the ICR and A/Jax strains. However, there are strain and species differences in the teratogenic effects of biotin deficiency in rodents[9-10]. Biotin deficiency severe enough to cause hair loss or dermatitis has never been reported in human pregnancy. A relationship between marginal biotin deficiency in pregnant women and the incidence of birth defects has yet to be established.

In mice, palatal outgrowths are first detectable by day of gestation (dg) 11.5 and palatal fusion is complete by dg 17[11]. We examined the effects of biotin deficiency on palatal development in fetuses on dg 12 using the organ culture system[12]. The addition of biotin in the culture medium did not affect the fusion of palatal processes dissected from biotin-deficient fetuses *in vitro*. However, palatal processes from the biotin-supplemented fetuses were fused even in biotin-free medium. Furthermore, in an *in vivo* study, when the mouse dams were fed the biotin-deficient diet since dg 0, normal palate fusion progressed when the day of biotin supplementation was earlier[13]. However, fu-

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sion of the palatal process was not recovered in the dg 12- to dg 14-supplemented groups. This suggested that biotin is necessary for the palatal processes to develop and fuse in mouse fetuses at midgestation.

Sodium-dependent multivitamin transporter (SMVT), holocarboxylase synthetase (HCS) and biotinidase (BTD) play essential roles in biotin homeostasis by regulating biotin absorption and recycling[14]. SMVT transports the water-soluble vitamins biotin, pantothenate and liponate[15]. HCS catalyzes the biotinylation of carboxylases and histones[16], and BTD is the enzyme responsible for the recycling of biotin, the transport of biotin in plasma and the regulation of histone biotinylation[17]. It remains unclear how these three proteins influence biotin metabolism and palatal development during pregnancy.

The mechanism by which biotin deficiency interferes with palatal development is not yet known. Therefore, to clarify the role of biotin in palatal development, the present study was designed to examine the effects of biotin administration to pregnant mice with biotin deficiency at midgestation on the incidence of cleft palate.

Materials and Methods

Animals

Nulliparous female ICR mice, aged 7 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 22 ± 1℃. The female mice were mated with healthy males for a short period in the morning (0900–1100). The day when a copulation plug was detected at the end of mating was designated as dg 0 (Fig. 1). Pregnant females were randomly divided into two groups: a biotin-deficient group fed a biotin-deficient diet (Oriental Yeast Co., ltd., Tokyo, Japan) and a control group fed a biotin-supplemented diet (biotin-deficient diet supplemented with 5 mg of biotin/kg). Several mice in the biotin-deficient group were orally gavaged with 250 μL of biotin solution (0.1 mg/mL) at 0900 on dg 11. After biotin administration, they were fed the biotin-supplemented diet until dg 17 (defined as the dg 11-supplementated group). Mice were housed in stainless steel cages with a wire-bottomed floor, and given the diets and distilled water *ad libitum* during the experimental periods.

Mice were orally gavaged because it was necessary for the recovery time from the biotin-deficient state to be short. The biotin dosage was calculated to be 25 μg based on the daily biotin intake of the biotin-supplemented diet (5 g on average).

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 (#205).

Sample preparation

Pregnant mice were sacrificed on dg 17. Fetuses were collected from the uterus and immersed in phosphate-buffered saline (PBS). The placenta was removed from the fetus and the number of fetuses was confirmed. Maternal liver 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[18]. These samples were immediately stored at −80℃ until analysis.

Biotin measurement

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

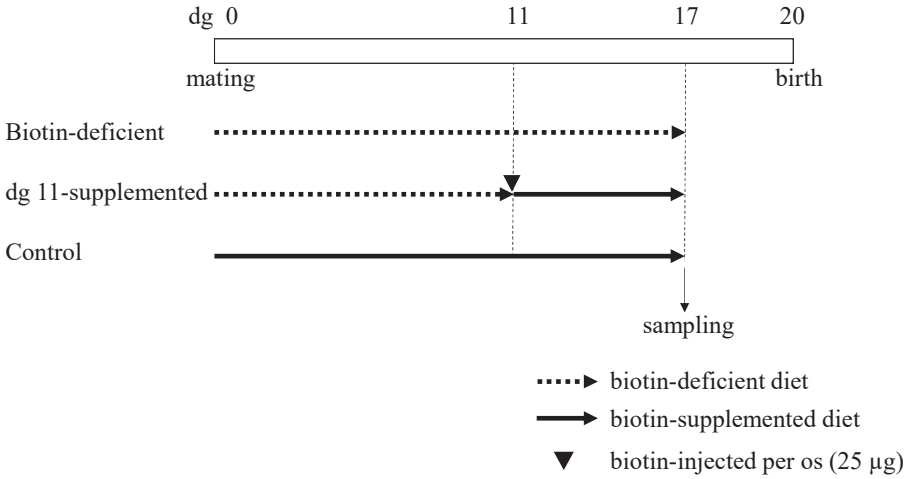

Fig. 1 Experimental protocol.

PBS). These tissue samples were homogenized on ice. The homogenate samples were centrifuged at 15,000 rpm for 15 min at 4℃ and the supernatant was collected.

The biotin concentration in samples was measured using a microtiter plate adaptation of a microbiological assay with *Lactobacillus plantarum* ATCC 8014[19–21]. This bacterium, which is generally used for assessing the quantity of vitamins, was obtained from American Type Culture Collection and cultured in a microtiter plate for 24 h. The cell density was measured at 610 nm. As biotin in samples partially existed in a protein-binding form, to measure total biotin, sample solutions were pretreated with 2.25 M H_2SO_4 at 121℃ for 60 min and neutralized with 4.5 M NaOH. Total and free-form biotin concentrations in samples were expressed as pmol/mL or nmol/g.

Quantitative real-time PCR

Total RNA from the palatal processes was isolated using TRIzol reagent (Life Technologies Japan Ltd., Tokyo, Japan) and complementary DNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies Japan, Ltd., Tokyo, Japan). 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'-CATCCATCGGTCCTGAGC-3' and reverse 5'-TAATCTGCACACCCTTCTGG-3'; for β-actin, forward 5'-CTAAGGCCAACCGTGAAAAG-3' and reverse 5'-ACCAGAGGCATACAGGGACA-3'. Quantitative RT-PCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) on a StepOne Real-time PCR System (Applied Biosystems). The results were normalized to *β*-actin. Fold changes in expression were calculated using threshold cycle (Ct) values and calculated via the 2-ΔΔCT method[22].

Statistical analysis

Values are expressed as the mean ± SD. Statistical comparison of means among experimental groups was conducted by one-way ANOVA and Tukey-Kramer's tests. Incidences of cleft palate were compared between the groups using the χ^2 test. Statistical analysis of the data was performed using SPSS 24 (IBM Corporation). Differences were considered significant if *P*-values were less than 0.05 in all analyses.

Results

Body weight and fetal growth

No significant differences were observed among the three dietary groups in body weight gain or fetus number (Table 1). There were no clinical signs of biotin deficiency in dams from these groups. The fetal body weight in the dg 11-supplemented group was significantly lower than that in the control group.

The incidence of fetuses with cleft palate was 7.6% in the dg 11-supplemented group, which was significantly lower than the 43.1% in the biotin-deficient group. There was no significant difference in the incidence of cleft palate between the dg 11-supplemented group and the control group.

Biotin concentration

The biotin concentrations in the samples are shown in Table 2. In biotin-deficient mice, total and free-form biotin concentrations were significantly lower than those in the control group. In particular, the biotin level was markedly decreased in palatal processes. On the other hand, in the dg 11-supplemented group, biotin levels did not differ from those in the control group.

Table 1 The effects of biotin administration to pregnant mice with biotin deficiency on fetal development

	Dietary groups		
	Control	dg11-supplemented	Biotin-deficient
Dams			
No. dams examined	5	5	5
Body weight gain during dg 0–17 (g)	25.1 ± 5.3	23.6 ± 2.2	23.5 ± 3.3
Fetuses			
No. fetuses examined	62	71	71
Mean	12.4 ± 3.2	14.2 ± 1.9	14.2 ± 0.8
Body weight on dg 17 (g)	1.09 ± 0.07[a]	0.88 ± 0.17[b]	1.00 ± 0.07[a]
Cleft palate (%)	0[a]	7.6[a]	43.1[b]

Values are the mean ± SD.
Different superscript letters indicate a significant difference between groups ($P < 0.05$).

Table 2 Biotin concentration in maternal and fetal samples

		Biotin concentration	Dietary groups		
			Control	dg11-supplemented	Biotin-deficient
Dams					
	Serum[1]	total	520.1 ± 158.2	717.5 ± 250.6	118.9 ± 30.7**
		free	96.2 ± 23.0	151.1 ± 74.5	19.8 ± 4.9*
	Liver [2]	total	6.9 ± 1.4	5.2 ± 1.4	4.1 ± 1.4*
		free	0.6 ± 0.2	0.6 ± 0.2	0.5 ± 0.2
Fetuses					
	Liver [2]	total	3.0 ± 0.9	3.8 ± 1.2	0.6 ± 0.1*
		free	1.9 ± 0.5	2.2 ± 0.5	0.3 ± 0.1**
	Palatal process[2]	total	0.7 ± 0.1	0.8 ± 0.1	0.1 ± 0.0***
		free	0.8 ± 0.3	0.9 ± 0.3	0.1 ± 0.0***

[1] pmoL/L, [2] nmol/g
Values are the mean ± SD.
Difference from the control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

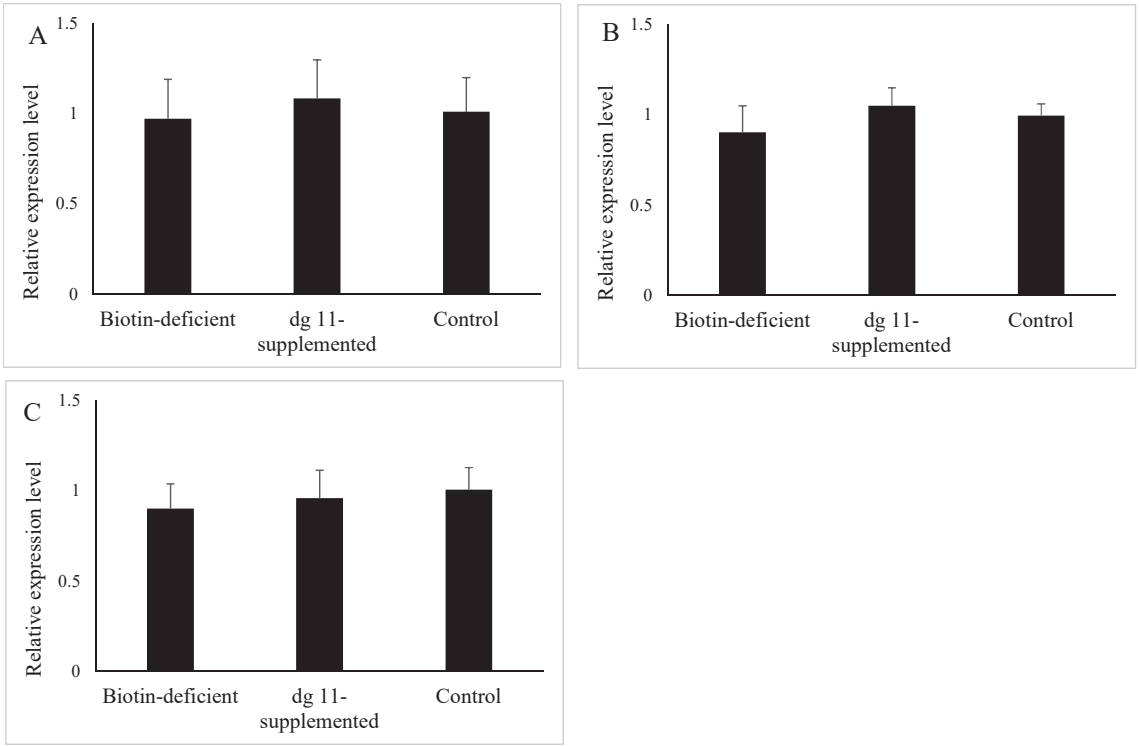

Fig. 2 Effects of biotin administration to pregnant mice with biotin deficiency on gene expression of SMVT (A), HCS (B) and BTD (C) in palatal processes.
Ct values were normalized to β-actin as a housekeeping gene. The relative expression of mRNA is represented as fold change in comparison with the control group.

SMVT, HCS and BTD gene expression in the palatal processes

Biotin administration to pregnant mice with biotin deficiency did not affect mRNA expression of any of the genes examined. SMVT, HCS and BTD mRNA expression levels in palatal processes in the biotin-deficient and dg 11-supplemented groups did not differ from those in the control group (Fig. 2). Similarly, maternal biotin deficiency did not affect biotin-dependent carboxylase (pyruvate carboxylase, propionyl-CoA carboxylase and acetyl-CoA carboxylase) mRNA expression in palatal processes (data not shown).

Discussion

We demonstrated in previous studies that biotin deficiency during pregnancy in mice causes a markedly high incidence of congenital malformations, such as cleft palate, micromelia and micrognathia, in fetuses[7–9]. When pregnant mice were fed a biotin-deficient diet before biotin administration in the dg 12- to dg 14-supplemeted groups, the biotin concentration in palatal processes on dg 15 recovered to the same level as that in the control group[13]. This suggested that the biotin concentration in the palatal processes rapidly increased to the control level after

changing to a biotin-supplemented diet. Although an increase in palatal fusion was observed in the dg 12- to dg 14-spplemented groups, it was significantly lower than that in the group receiving biotin since dg 0. In the present study, the incidence of cleft palate was lower in the dg 11-supplemented group than in the biotin-deficient group. There was no significant difference in the incidence of cleft palate between the dg 11-supplemented group and the control group. This suggests that biotin is necessary for the normal development of the palatal process in the mouse fetus on dg 11 and earlier.

SMVT is essential for mediating and regulating biotin uptake into mammalian cells. In the present study, mRNA levels of the biotin transporter SMVT did not change in the palatal processes on dg 17. HCS and BTD gene expression was also unchanged by biotin deficiency. These results are consistent with our previous studies on the effects of biotin deficiency during pregnancy on dg 15[23]. In biotin deficiency, a significant upregulation in biotin uptake associated with induction in the level of expression of SMVT protein and mRNA were reported[24].

We speculated that biotin requirements increase during palatogenesis, and therefore biotin requirements after palatal fusion are low. Further studies are needed to investigate the effects of SMVT, HCS and BTD expression in the palatal processes before palatal fusion is complete on dg 15.

Cleft lip and/or palate are the most common craniofacial malformations, with a prevalence ranging from 1/500 to 1/2500 births[25]. Most cases of cleft lip and cleft palate are caused by a complex interaction of genetic and environmental factors. In mice, secondary palate development starts at embryonic day 11.5 (E11.5) and palatal process fusion is completed at E15[11]. Failure of the palate development process leads to cleft palate. A recent study suggested that environmental factors interfere with histone acetylation, thereby leading to cleft palate[26]. The expression of HCS, which catalyzes the covalent binding of biotin to histones, depends on biotin[16]. Cross-talk between histone biotinylation and methylation marks was demonstrated to be required for maintaining genome stability[27]. These studies suggested that a change in histone biotinylation by maternal biotin deficiency affects murine palatogenesis.

In conclusion, biotin administration to pregnant mice with biotin deficiency from dg 11 to dg 15 reduced the incidence of cleft palate. This study suggests that biotin plays a role in the initial stage of palatogenesis (E11.5). Further studies are needed to clarify the role of biotin in palatal development.

Reference

1) Zempleni J, Wijeratne SSK, Kuroishi T (2012) Biotin, Present Knowledge in Nutrition, 10th Edition. International Life Sciences Institute, Washington DC: pp. 359–374.
2) Mock DM, Dyken ME (1995) Biotin deficiency results from long-term therapy with anticonvulsants. Gastroenterology 108: A740.
3) Mock DM, Dyken ME (1997) Biotin catabolism is accelerated in adults receiving long-term therapy with anticonvulsants. Neurology 49: 1444–1447.
4) Zempleni J, Trusty TA, Mock DM (1997) Lipoic acid reduces the activities of biotin-dependent carboxylases in rat liver. J Nutr 127: 1776–1781.
5) Mock NI, Malik MI, Stumbo PJ, Bishop WP, Mock DM (1997) Increased urinary excretion of 3-hydroxyisovarelic acid and decreased urinary excretion of biotin are sensitive early indicators of decreased biotin status in experimental biotin deficiency. Am J Clin Nutr 65: 951–958.
6) Mock DM, Stadler DD, Stratton SL, Mock NI (1997) Biotin status assessed longitudinally in pregnant women. J Nutr 127: 710–716.
7) Watanabe T (1983) Teratogenic effects of biotin deficiency in mice. Teratology 113: 574–581.
8) Watanabe T, Endo A (1984) Teratogenic effects of avidin-induced biotin deficiency in mice. Teratology 30: 91–94.
9) Watanabe T, Endo A (1989) Species and strain differences in teratogenic effects of biotin deficiency in rodents. J Nutr 119: 255–261.
10) Watanabe T (1993) Dietary biotin deficiency affects reproductive function and prenatal development in hamsters. J Nutr 123: 2101–2108.
11) Bush JO, Jiang R (2012) Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development. Development 139: 231–243.
12) Watanabe T, Dakshinamarti K, Persaud TVN (1995) Biotin influences palatal development of mouse embryos in organ culture. J Nutr 125: 2114–2121.
13) Watanabe T, Suzuki A, Ebara S, Negoro M, Fukui T (2010) Biochemical alterations in the palatal processes in fetuses of biotin-deficient mice. Congenit Anom 50: 21–28.
14) Zempleni J, Hassan YI, Wijeratne SS (2008) Biotin and biotinidase deficiency. Expert Rev Endocrinol Metab 3: 715–724.
15) Wang H, Huang W, Fei YJ, Xia H, Yang-Feng, TL, Leibach FH, Devoe LD, Ganapathy V, Prasad PD

(1999) Human placental Na+-dependent multivitamin transporter. Cloning, functional expression, gene structure, and chromosomal localization. J Biol Chem 274: 14875–14883.
16) Narang MA, Dumas R, Ayer LM, Gravel RA (2004) Reduced histone biotinylation in multiple carboxylase deficiency patients: a nuclear role for holocarboxylase synthetase. Hum Mol Genet 13: 15–23.
17) Hymes J, Fleischhauer K, Wolf B (1995) Biotinylation of histones by human serum biotinidase: assessment of biotinyl-transferase activity in sera from normal individuals and children with biotinidase deficiency. Biochem Mol Med 56: 76–83.
18) Watanabe T, Endo A (1990) Teratogenic effects of maternal biotin deficiency in mouse embryos examined at midgestation. Teratology 42: 295–300.
19) Fukui T, Iinuma K, Oizumi J (1994) Agar plate method using *Lactobacillus plantarum* for biotin determination in serum and urine. J Nutr Sci Vitaminol 40: 491–498.
20) Ronald RE, Landen WO (1998) Biotin, Vitamin Analysis for the Health and Food Sciences, CRC Press, Boca Raton: pp. 478–487.
21) Ball GMF (2005) Microbiological Methods for the Determination of the B-group Vitamins, Vitamins in Foods, CRC Press, Boca Raton: pp. 339–368.
22) Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25: 402–408.
23) Sawamura H, Ishii Y, Shimada R, Yuasa M, Negoro M, Watanabe T (2014) Low level of maternal biotin intake changes the expression of biotin transporter in dams and fetuses in mice. Int J Anal Bio-Sci 2: 126–134.
24) Reidling JC, Nabokina SM, Said HM (2007) Molecular mechanisms involved in the adaptive regulation of human intestinal biotin uptake: A study of the hSMVT system. Am J Physiol Gastrointest Liver Physiol 292:G275–81.
25) Dixon MJ, Marazita ML, Beaty T H, Murray J C (2011) Cleft lip and palate: understanding genetic and environmental influences. Nat Rev Genet 12: 167–178.
26) Yuan X, Qiu L, Pu Y, Liu C, Zhang X, Wang C, Pu W, Fu Y (2016) Histone acetylation is involved in TCDD-induced cleft palate formation in fetal mice. Mol Med Rep 14:1139–1145.
27) Pestinger V, Wijeratne SSK, Rodriguez-Melendez R, Zempleni J (2011) Novel histone biotinylation marks are enriched in repeat regions and participate in repression of transcriptionally competent genes. J Nutr Biochem 22:328–333.