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ORIGINAL ARTICLE

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Enhanced hepatic cholesterol accumulation induced by maternal betaine exposure is associated with hypermethylation of CYP7A1 gene promoter

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Abstract

Purpose Betaine contains three methyl groups and plays a critical role in regulating glucose and lipid metabolism via epigenetic modifications. However, it is unclear whether prenatal betaine intake could affect cholesterol metabolism of progeny through DNA methylation.

Methods Hence, pregnant rats were randomly divided into control and betaine groups fed standard diet or 1% betaine supplementation diet, respectively, throughout gestation and lactation.

Results Maternal betaine exposure significantly ($P < 0.05$) increased serum and hepatic cholesterol contents but not triglyceride levels in offspring rats. Accordantly, maternal intake of betaine markedly downregulated ($P < 0.05$) hepatic cholesterol 7 alpha-hydroxylase (CYP7A1) expression at both the mRNA and protein level, while the protein content of low-density lipoprotein receptor (LDLR) was upregulated in the liver of betaine-exposed rats. In addition, prenatal betaine supplementation extremely increased ($P < 0.05$) hepatic betaine-homocysteine methyltransferase (BHMT) expression at the mRNA and protein level but not affected the expression of other key enzymes involved in methionine metabolism. Furthermore, hepatic hypermethylation of CYP7A1 gene promoter was observed in progeny rats derived from betaine-supplemented dams.

Conclusions Our results provide evidence that maternal betaine supplementation significantly enhances hepatic cholesterol contents accompanied with alterations of cholesterol metabolic genes and hypermethylation in offspring rats at weaning.

Keywords Maternal · Betaine · DNA methylation · Cholesterol

Introduction

Cholesterol is an essential constituent of cell membranes and is required to maintain cell membrane integrity and fluidity [1]. Under normal condition, hepatic cholesterol homeostasis plays a vital role in maintaining physiological process via converting into various hormones and bile acids [2]. However, abnormal cholesterol metabolism is associated with metabolic syndrome disease, such as cardiovascular diseases and nonalcoholic fatty liver disease (NAFLD) [3, 4]. Thus it is critical to maintain cholesterol homeostasis via biosynthesis, conversion, and transport. 3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) is a rate-limiting enzyme for cholesterol biosynthesis, which can be regulated by a transcription factor sterol regulatory element-binding protein 2 (SREBP2). In addition, cholesterol 7 alpha-hydroxylase (CYP7A1) and cholesterol 27 alpha-hydroxylase (CYP27A1) are responsible for bile acid synthesis from hepatic cholesterol, while low-density lipoprotein receptor (LDLR) predominantly exists in cell membrane to mediate plasma cholesterol influx [5].

A number of literatures clearly demonstrate that maternal nutrition supplementation during pregnancy and lactation,

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which are considered as critical windows of development, plays an important role in regulating metabolic consequences [6–8]. For instance, Vieira et al. reports that maternal intake of soybean changes cholesterol and triglyceride (TG) content in offspring [9]. Rodrigo and colleagues also describe that dietary fructose supplementation can influence, in a gender-dependent manner, plasma parameters of cholesterol metabolism in adult progeny associated with the alteration of hepatic mRNA gene expression [10]. Maternal high folate intake increases blood total TG and cholesterol levels and accelerates the development of obesity in male offspring [11]. Thus it is important to clarify the effect of early life environment on fetal growth and development.

Betaine (also called as trimethyl glycine) is used as feed additive to improve growth performance, skeletal muscle development, and meat quality in livestock and chickens [12]. Betaine is also critical for embryonic and fetal development [13]. Numerous studies have shown that dietary betaine supply can alleviate alcoholic fatty liver disease or NAFLD [14, 15] and atherosclerosis [16]. Previously, we have reported that prenatal betaine exposure affects hepatic and hypothalamic cholesterol metabolism in neonatal piglets [17] and chickens [18], respectively. However, the underlying mechanism of maternal betaine exposure on offspring hepatic cholesterol homeostasis in weaning stage is still elusive.

Accumulating evidences demonstrate that epigenetic regulation may link early life nutrition to long-term alteration in metabolism and phenotype [19, 20]. One of the most relevant epigenetic modification is DNA methylation, which affects the gene promoter and generally inhibits gene expression [21]. Numerous studies indicate that maternal methyl donor supplementation modulates offspring DNA methylation and its gene expression [22–24]. Interestingly, betaine, a highly efficient methyl donor, acts as a substrate of methionine cycle involving one-carbon metabolism and donates methyl groups for methylation reactions (such as DNA and histone). It has been reported that prenatal dietary betaine supplement affects histone methylation (H3K4me3) enrichment on the promoter of cholesterol metabolic genes in weaning offspring [25]. Thus question arises whether DNA methylation is involved in cholesterol metabolism in weaning offspring from betaine-exposed mother.

Therefore, we use rats as model to investigate whether prenatal betaine exposure during pregnancy and lactation affects hepatic cholesterol metabolism in weaning offspring. To explore the potential mechanisms, we determined whether serum and hepatic cholesterol contents as well as hepatic cholesterol metabolic gene expression together associated with DNA methylation status on the promoter of the affected genes.

Materials and methods

Animals and experimental design

A total of 20 Sprague-Dawley female rats aged 3 months were obtained from the laboratory animal center of Jiangsu University. Animals were maintained in a temperature (22 ± 0.5 °C)- and humidity (50 ± 5%)-controlled room with 12 h:12 h dark–light cycle. All rats had free access to food and water throughout experiments.

After mating and confirming pregnancy, female rats were randomly allocated to standard diet as Control group or 1% betaine supplementation diet as Betaine group during gestation and lactation (Table S1). Betaine was purchased from Sigma-Aldrich Co., LLC., USA (B2629). After birth, litter size was adjusted to five males and five females. After weaning on postnatal day 21, all pups were killed with pentobarbital sodium. Serum samples were collected immediately and centrifuged at 3000 rpm for 20 min at 4 °C and stored at −80 °C. Liver tissue was removed, snap-frozen in liquid nitrogen, and stored at −80 °C.

Serum and hepatic biochemical analysis

Serum concentration of glucose, total TG, total cholesterol (Tch), high-density lipoprotein cholesterol (HDLC), and low-density lipoprotein cholesterol (LDLC) were measured by biochemical automatic analyzer (Hitachi 7020, HITACHI, Tokyo, Japan). Hepatic total TG and Tch were measured using commercial assay kits (E1013 and E1015, respectively; Applygen Technologies, Inc. Beijing) according to the manufacturer's instructions. Hepatic S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) levels were determined using high-performance liquid chromatography (ACQUITY UPLC H-Class system, Waters, MA, USA) according to a previously published research with modification [26].

RNA isolation and real-time PCR

Thirty milligrams of liver samples were used to isolate total RNA by TRIzol reagent (Invitrogen, USA). Next, 1 μg of total RNA samples was reverse transcribed according to the manufacturer's protocol (Vazyme Biotech, Nanjing, China). Diluted cDNA (2 μL, 1:25) was used for real-time PCR by Mx3000P System (Stratagene, USA). Amplification reactions of selective genes were performed using 2×SYBR Green qPCR Master Mix purchased from Bimake. β-Actin was chosen as a reference gene. All primer sequences are described in Table S2. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative gene expression.

Protein extraction and western blot assay

Total protein was extracted from 50 mg of frozen liver samples as previously described. Briefly, liver samples were homogenized with RIPA buffer (50 mM pH 8.0 Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, and 0.1% sodium dodecyl sulfate), incubated on ice for 20 min, and then were centrifuged at 12,000 rpm for 20 min at 4 °C. Protein concentrations were measured using the BCA Protein Assay Kit (NO.23225, Thermo Scientific) according to the manufacturer's instructions. Western blot analysis was carried out to detect target proteins depending on the protocols provided by the manufacturers. GAPDH was selected as an internal control. All antibodies used are listed in Table S3.

Methylated DNA immunoprecipitation (MeDIP)

MeDIP analysis was carried out as previously described. First, purified genomic DNA was sheared to a length of approximately 300–500 bp. And then 1 μg fragmented DNA was heat denatured for 10 min at 95 °C and kept in ice water bath to maintain single-strand DNA. Second, 5-mc antibody (ab10805, Abcam, UK) and protein A/G agarose beads (sc-2003, Santa Cruz Biotechnology) were used to immunoprecipitate and capture DNA and antibody/DNA complexes, respectively. Finally, the immunoprecipitated DNA was purified, diluted with 20 μL ddH_2O, and used to amplify target gene promoter with specific primers (Table S2).

Statistical analysis

All data are presented as means ± SEM. Student's *t* test was used to compare the difference between the control and betaine groups by SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Values of mRNA and protein are presented as the fold change relative to the mean value of the control group. The differences were considered statistically significant at *P* value <0.05.

Results

Maternal betaine intake increased serum and hepatic cholesterol levels in weaning offspring rats

Maternal betaine supplementation tended to decrease ($P = 0.085$) the weaning body weight compared to the control group, yet liver weight and the ratio of liver to body weight did not show significant difference in both the groups (Table 1). Serum concentrations of Tch and HDLC were significantly increased at weaning in offspring of betaine-supplemented rats ($P < 0.05$). However, serum glucose, TG, and LDLC contents showed no obvious difference between the betaine and control groups. In addition, hepatic Tch content but not TG was remarkably ($P < 0.05$) enhanced in progeny rats derived from betaine-supplemented dams (Table 1).

Table 1 Effects of maternal betaine on body weight, liver weight, and serum and hepatic biochemical parameters in weaning offspring rats

Parameters	Control	Betaine	*P* value
Body weight (g)	69.94 ± 1.84	64.01 ± 2.61	0.085
Liver weight (g)	3.06 ± 0.08	2.86 ± 0.15	0.254
Liver index (%)	4.38 ± 0.09	4.46 ± 0.10	0.560
Glucose (mmol/L)	7.42 ± 0.27	7.85 ± 0.26	0.267
Total cholesterol (mmol/L)	2.82 ± 0.07	3.10 ± 0.11	0.039
Triglyceride (mmol/L)	0.85 ± 0.07	0.78 ± 0.09	0.547
HDLC (mmol/L)	0.93 ± 0.03	1.07 ± 0.03	0.003
LDLC (mmol/L)	1.27 ± 0.03	1.37 ± 0.08	0.221
Triglyceride (mg/g liver)	10.05 ± 1.16	9.70 ± 1.09	0.826
Total cholesterol (mg/g liver)	1.61 ± 0.14	2.08 ± 0.13	0.030

The values are presented as means ± SEM. $n = 8$–10

HDLC high-density lipoprotein cholesterol, *LDLC* low-density lipoprotein cholesterol

Maternal betaine exposure changed hepatic cholesterol metabolic gene expression in weaning offspring rats

Compared to the control group, maternal betaine diet decreased the HMGCR mRNA expression, yet the protein expression was not altered. The expression of SREBP2 was did not show significant difference between the two groups at the mRNA and protein levels (Fig. 1a, b). In addition, maternal betaine exposure extremely ($P < 0.05$) down-regulated the CYP7A1 expression at mRNA and protein levels, also tended to ($P = 0.09$) decrease CYP27A1 protein expression. Interestingly, we found that the mRNA expression of LDLR tended to be lower ($P = 0.06$), whereas the protein expression was significantly upregulated in betaine-exposed weaning rats (Fig. 1c, d).

In addition, we also found that there were no significant differences in the protein level of hepatic lipid metabolism (such as ACC1, FASN, PPARγ, PPARα, and CD36) (data not shown).

Maternal betaine supplementation upregulated hepatic betaine-homocysteine methyltransferase (BHMT) expression in offspring rats at weaning

Maternal betaine supplementation did not affect offspring rat hepatic SAM and SAH contents ($P > 0.05$; Fig. 2b, c). However, the key gene involved in methionine metabolism BHMT was extremely ($P < 0.05$) enhanced at both the

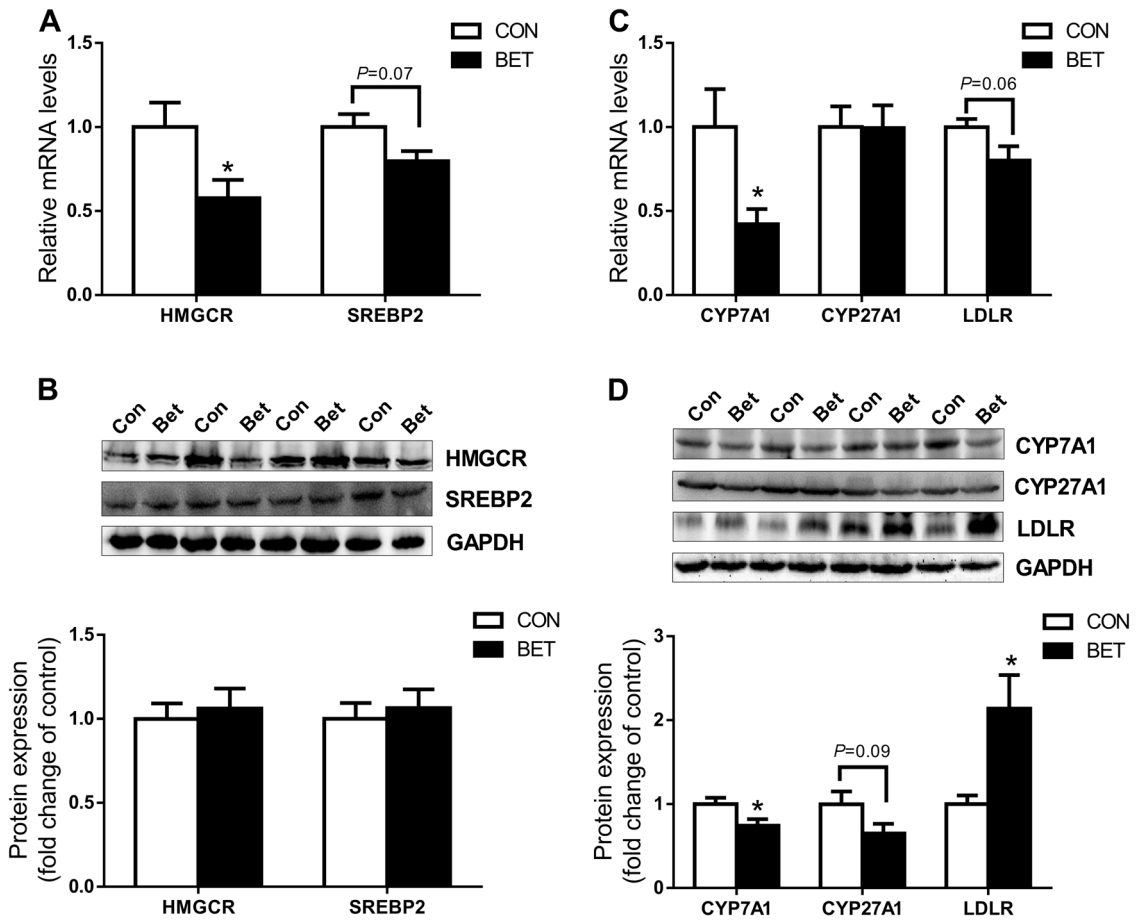

Fig. 1 Hepatic cholesterol metabolic gene expression. **a** 3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) and sterol regulatory element-binding protein 2 (SREBP2) mRNA expression; **b** hepatic HMGCR and SREBP2 protein level; **c** cholesterol 7 alpha-hydroxylase (CYP7A1), cholesterol 27 alpha-hydroxylase (CYP27A1), and low-density lipoprotein receptor (LDLR) mRNA expression; **d** hepatic CYP7A1, CYP27A1, and LDLR protein level. Data presented are means ± SEM, $n = 8$, $^*P < 0.05$ compared with control

mRNA and protein levels in betaine-supplemented rats, while GNMT, AHCYL1, MAT2b, and DNMTs were not altered either at the mRNA or at the protein level (Fig. 2d–f).

Maternal betaine supplementation led to hypermethylation of CYP7A1 gene promoter in offspring rats at weaning

Next, we detected the DNA methylation status of affected genes by MeDIP method. We found that there was a tendency for a higher methylation ($P = 0.09$) in HMGCR gene promoter (Fig. 3a). It was also observed that CYP7A1 was hypermethylated ($P < 0.05$) in the liver of betaine-exposed rats compared with the control group (Fig. 3b).

Discussion

Betaine plays an important role in regulating glucose and lipid metabolism and protects hepatic abnormal fatty deposition in animal models [12, 27]. However, it is unclear whether maternal betaine intake can affect offspring cholesterol homeostasis. It has been reported that dietary methionine intake increased hepatic cholesterol content in pig [28]. Similar results were observed that dietary betaine modified cholesterol and lipid profiles in broilers [29] and human subjects [30, 31]. Previously, we have reported that in ovo betaine injection increased serum and hepatic Tch in chicks [18]. Also, prenatal betaine diet enhanced or decreased hepatic cholesterol and TG contents, respectively, while serum concentrations of TG, Tch, LDLC, and HDLC were not affected in piglets [17]. In the present study, we found that serum and hepatic Tch levels but not TG levels were markedly evaluated in weaning offspring rats from betaine-exposed dams. The above results indicated that prenatal betaine supplementation can enhance hepatic cholesterol accumulation in offspring. Currently, it is still controversy about the "good or bad" role of cholesterol [32, 33]. However, in our study, we believed that the upregulation of cholesterol contributes to the development at the early weaning period.

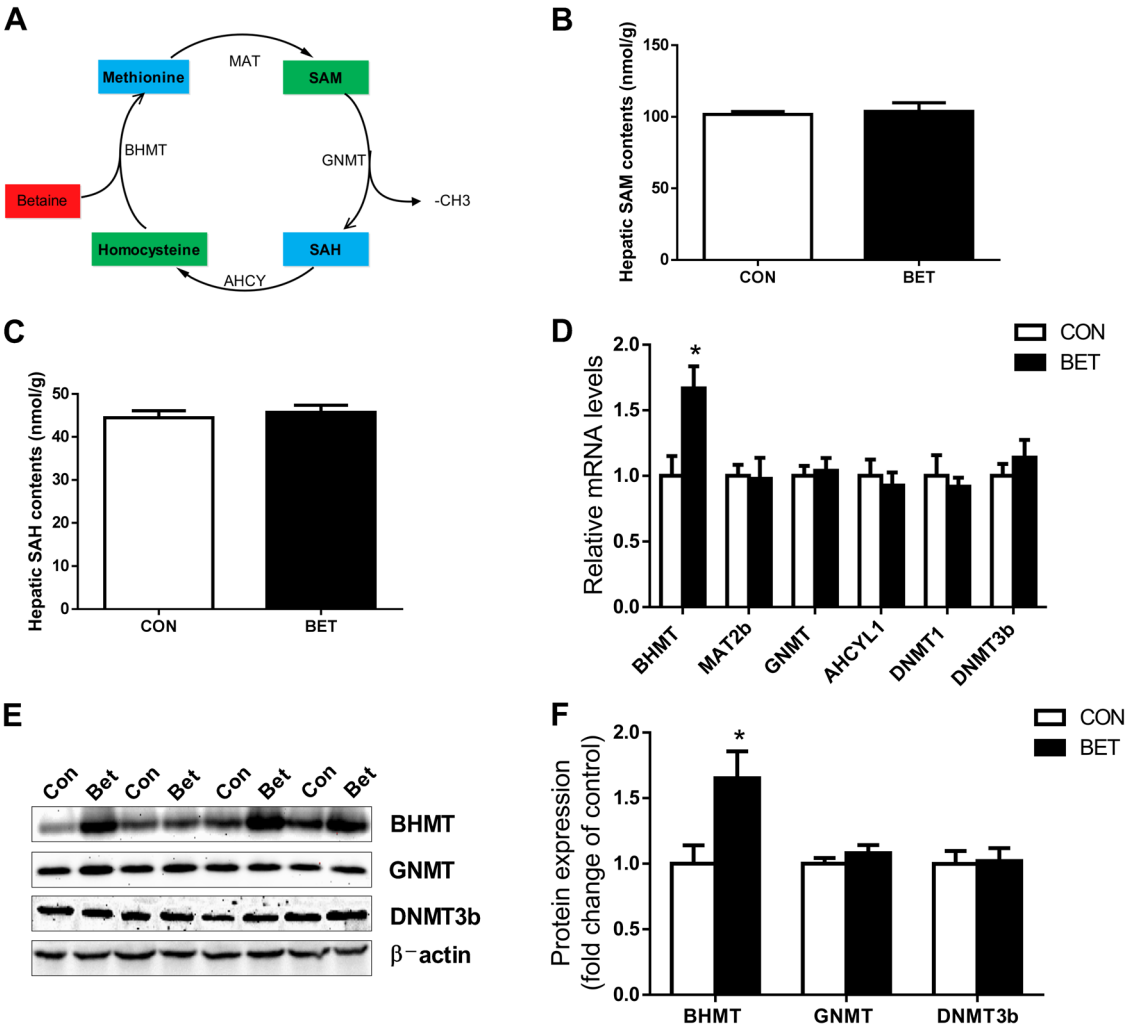

Fig. 2 Hepatic one-carbon metabolism. **a** Schematic diagram of one-carbon metabolism; **b**, **c** hepatic S-adenosylmethionine and S-adenosylhomocysteine content; **d**–**f** hepatic expression of key enzymes involved in methionine metabolic cycle at the mRNA (**d**) and protein (**f**) level. Data presented are means ± SEM, $n = 8$, $^*P < 0.05$ compared with control

Liver plays an important role in regulating cholesterol homeostasis determined by cholesterol biosynthesis, conversion, and transport. Hu et al. found that in ovo injection with betaine markedly enhanced hepatic cholesterol accumulation associated with the upregulation of HMGCR and SREBP1 proteins [18]. Similar results were observed in neonatal piglets [17]. In our study, we found that maternal betaine exposure downregulated CYP7A1, which stimulates cholesterol catabolism to bile acid, at both the mRNA and protein levels. Moreover, LDLR, which mediates cholesterol transport, was significantly upregulated at the protein level in the liver of betaine-exposed rats, which is in agreement with previous finding in weaning piglets [25]. Interestingly, other studies also suggested that maternal malnutrition evaluated cholesterol accumulation associated with decrease of CYP7A1 protein [34–36]. Together, our results indicate that cholesterol accumulation in the liver of betaine-treated rats may be caused by suppression of cholesterol conversion and enhancement of cholesterol uptake. These different mechanisms are due to the differences of animal species, age stage, and dose of betaine.

Betaine can act as a substrate involved in one-carbon metabolism, which plays an important role in maternal–fetal interaction [37]. It has been well documented that betaine can cross the placenta [38] and be transmitted to the milk [39]. However, we have previously reported that newborn or weaning piglets derived from betaine-supplemented sows have significantly higher betaine concentration in the plasma and liver [17, 25]. In our study, hepatic SAM or SAH contents and the expression of key enzymes involved in methionine metabolism were not changed between the two groups. BHMT plays a vital role in catalyzing the transfer of a methyl group from betaine to homocysteine to produce methionine. Interestingly, we found that BHMT expression at both the mRNA and protein levels was evaluated in the liver of betaine-supplemented rats, which is in

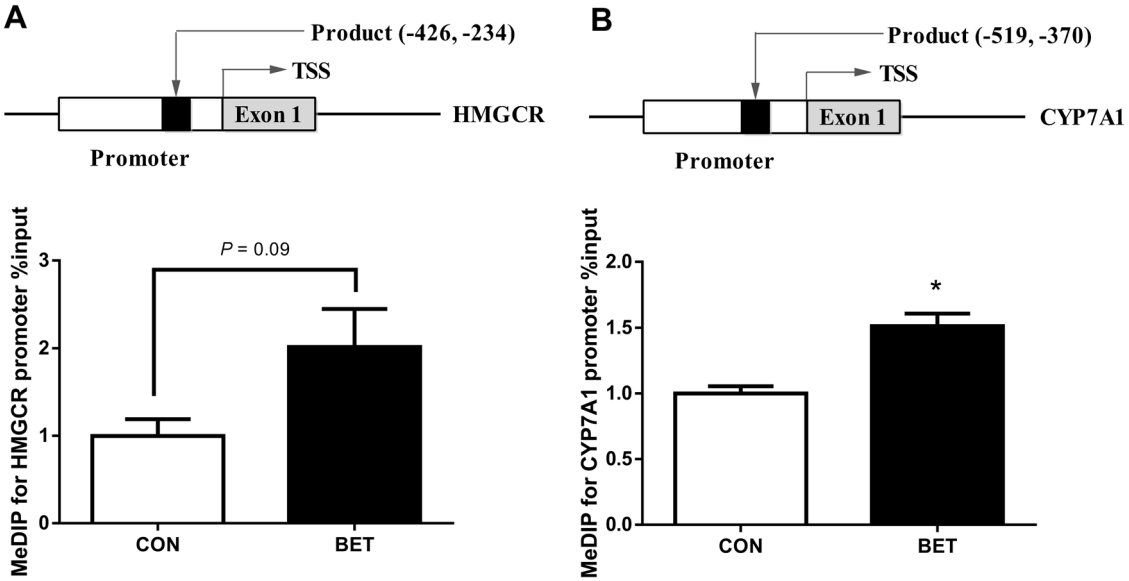

Fig. 3 DNA methylation status on the promoter of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) and cholesterol 7 alpha-hydroxylase (CYP7A1). **a** DNA methylation status on the promoter of HMGCR; (**b**) DNA methylation status on the promoter of CYP7A1. Data presented are means ± SEM, $n = 3$, $^*P < 0.05$ compared with control

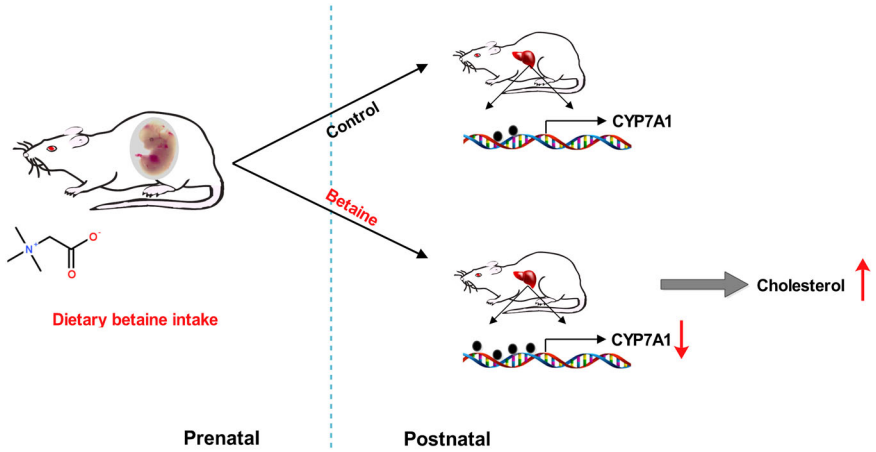

Fig. 4 Schematic representation of the effect of maternal betaine on hepatic cholesterol metabolism in weaning offspring rats

line with previous literatures [40, 41]. We speculated that the increase of BHMT may be caused by a higher betaine content in the liver or elevated plasma betaine concentrations. Thus this indicated that epigenetic modifications may be involved in cholesterol metabolism. DNA methylation is the most commonly epigenetic regulation occurring in gene promoter region without alteration of gene sequences. Growing evidences showed that maternal methyl supplements at conception increased offspring DNA methylation status [42, 43]. In the present study, maternal betaine supplementation led to hepatic CYP7A1 hypermethylation in weaning rats, which was the complete opposite of its mRNA expression. It has also been provided that histone-marker H3K4me3 can be activated on LDLR promoter in betaine-treated weaning piglets [25]. In addition, other researchers reported that maternal fructose diet markedly affected DNA methylation of LXRα gene, an important transcriptional factor of cholesterol metabolism, in adult progeny in a gender-dependent manner, associated with change of hepatic cholesterol content [10]. Therefore, these results indicate that one-carbon metabolism may play a vital role in maintaining offspring hepatic cholesterol homeostasis.

In conclusion, our results indicate that maternal betaine exposure during gestation and lactation enhances hepatic cholesterol accumulation in weaning offspring rats by modifying cholesterol metabolic gene expression through DNA hypermethylation of CYP7A1, which is beneficial for offspring growth and development in infancy stage (Fig. 4).

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Author contributions N.Z. contributed to hormone and gene assays, data analysis, and drafting of the manuscript. S.Y. was responsible for animal care, breeding, and sampling. Y.F. and B.S. provided technical support. R.Z. contributed to conception, experimental design, data interpretation, and critical revision of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The authors declare that handling of animals and all experimental procedures were according to "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set by the Ministry of Science and Technology, China and approved by the Animal Ethics Committee of Nanjing Agricultural University.

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