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FTO-dependent function of N6-methyladenosine is involved in the hepatoprotective effects of betaine on adolescent mice

Jingqing Chen · Xihong Zhou · Weiche Wu · Xinxia Wang · Yizhen Wang

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Abstract Nonalcoholic fatty liver disease (NAFLD) is now the most common cause of chronic liver disease among children and adolescents in the developed world. Betaine, as a methyl donor, recently has been demonstrated to exert its hepatoprotective effects through rectifying the genomic DNA hypomethylation state. However, whether betaine supplementation affects N6-methyladenosine (m^6A) mRNA methylation in NAFLD is still unknown. We conducted the current study to investigate the effects of betaine supplementation during adolescence on high-fat diet-induced pathological changes in liver of mice, and we further identified the effects of betaine supplementation on expression of the fat mass and obesity-associated gene (FTO) and hepatic m^6A mRNA methylation. Our results showed that betaine supplementation across adolescence significantly alleviated high-fat-induced impairment of liver function and morphology as well as ectopic fat accumulation. Surprisingly, no significant effects on serum TG and NEFA level, as well as fat mass, were observed in mice supplemented with betaine. We also found that high-fat diet upregulated ACC1 and FAS gene expression and downregulated HSL and ATGL gene expression. However, these alterations were rectified by betaine supplementation. Moreover, an m^6A hypomethylation state and increased FTO expression were detected in mice fed with high-fat diet, while betaine supplementation prevented these changes. Our results suggested that betaine supplementation during adolescence could protect mice from high-fat-induced NAFLD by decreasing de novo lipogenesis and increasing lipolysis. Furthermore, a novel FTO-dependent function of m^6A may involve in the hepatoprotective effects of betaine.

Keywords Betaine · Adolescence · FTO · N6-methyladenosine · Methylation

Jingqing Chen and Xihong Zhou contributed equally to this work.

J. Chen · X. Zhou · W. Wu · X. Wang · Y. Wang (✉)
Key Laboratory of Animal Nutrition and Feed Science, Ministry of Agriculture, Zhejiang Provincial Laboratory of Feed and Animal Nutrition, Institute of Feed Science, Zhejiang University, 866 Yuhangtang Road, Hangzhou, Zhejiang Province 310058, People's Republic of China
e-mail: yzwang321@zju.edu.cn

X. Zhou
Chinese Academy of Science, Institute of Subtropical Agriculture, Changsha, Hunan 410125, People's Republic of China

Introduction

Nonalcoholic fatty liver disease (NAFLD) has been rapidly emerging among children and adolescents and is now the most common cause of chronic liver disease in the developed world [2, 15, 26]. Adolescents with NAFLD also tend to be more insulin resistant and dyslipidemic, but the extent to which these associations are independent of total body fatness is not clearly understood [1, 5, 27]. These children are potentially at a life-long risk of developing NAFLD-related complications [21].

In recent decades, the hepatoprotective effects of betaine have been reported in many animal models of

liver disease, especially alcoholic liver disease and NAFLD [12, 14]. Betaine (trimethylglycine) serves as a substrate for the formation of methionine and could be further converted to *S*-adenosylmethionine (SAM) [16]. SAM is the major methyl group donator for DNA methylation and N6-methyladenosine (m^6A) mRNA methylation [4, 19]. So far, the effects of betaine on the modulation of gene expression through modifying epigenetic marks such as DNA methylation have been reported [7]. However, the effects of betaine on m^6A mRNA methylation have not been examined. Interestingly, the role of betaine in the rectification of genomic DNA methylation has been suggested as a possible mechanism of the improvements in NAFLD upon betaine supplementation. Furthermore, maternal betaine could protect offspring against hepatic fat accumulation [8] and affect hepatic gene expression through epigenetic mechanism which may include DNA methylation [4]. However, whether betaine could also exert the same protective effects on NAFLD during adolescence has not been reported.

Consequently, the current study was conducted to investigate the effects of betaine supplementation on hepatic fat accumulation in an adolescence mouse model of NAFLD induced by high-fat diet. To further explore the potential changes in epigenetic mechanism, we also determined the status of m^6A mRNA methylation and expression of the fat mass and obesity-associated gene (FTO), which exerted an oxidative demethylation activity targeting m^6A residues in RNA.

Materials and methods

Animal model and experimental protocol

Eight adult female C57BL/6 mice from the same pool of litters were housed at 22±1 °C with 12-h light cycle and were mated with their male siblings. The female mice were fed with a low-fat diet supplemented with betaine from mating and throughout gestation and lactation. Litters were culled to five males per dam. Thirty offspring weaned at the age of day 21 and then were randomly assigned to three groups fed with either a low-fat diet (LF), high-fat diet (HF), or high-fat diet supplemented with betaine (HB) for 6 weeks. Betaine was supplemented in the drinking water at a concentration of 2 % (*w*/*v*) (anhydrous; sigma). The low-fat diet are consisted of 10 % (kcal%) fat from lard and soybean oil, 20 % protein, and 70 % carbohydrate, and the high-fat diet are consisted of 45 % (kcal%) fat, 20 % protein, and 35 % carbohydrate. At the end of the experiment (all the mice at the age of day 63), blood was taken from the retro-orbital sinus after 4 h fasting and after cervical dislocation. Liver, epididymal fat, and inguinal fat were separated and weighted and then were either immediately fixed in paraformaldehyde solution for morphology observation or snap-frozen in liquid nitrogen and stored at −80 °C until analysis. All experiments were approved by the Committee of Experimental Animal Care, Zhejiang University (Hangzhou, China).

Serum analysis

The serum biochemical assays were performed with commercially available kits: glucose, triglyceride (TG) and total cholesterol (TC) (Applygen Technologies Inc. Beijing, China), and nonesterified fatty acid (NEFA) (Beijing Strong Biotechnologies, Inc. Beijing, China).

Measurement of SAM, SAH, and hepatic steatosis

Serum alanine aminotransferase (ALT) and alanine aminotransferase (AST) were measured using a commercially available kit (Cusabio Biotech Co., Ltd, Wuhan, China). Hepatic SAM and SAH (*S*-adenosylhomocysteine) were measured according to the method described by Wang et al. [28]. Fresh liver section was fixed with 4 % paraformaldehyde and paraffin embedded. Sections of 8 μm were stained with hematoxylin-eosin (H&E) to evaluate the pathologic structure of the hepatocytes. Fresh liver section was also directly embedded in O.C.T. compound and flash-frozen in dimethyl butane at −80 °C, and then cryostat sections were sliced (10 μm) and stained with Oil Red O to examine the lipid droplets.

Quantitative real-time PCR

Total RNA was extracted from the tissues using the TRIzol Reagent protocol (Invitrogen Life Technologies). Reverse transcription (RT) was performed to synthesize cDNA using the First Strand cDNA Synthesis Kit (Fermentas Life Science, St. Leon-Rot, Germany). Primers of target genes are described in Table 1. The PCR analysis was then carried out using SYBR Green PCR technology with the StepOne Plus real-time PCR system (Applied Biosystems) in a 10-μL reaction volume containing 5-μL SYBR Green Master Mix, 1 μL

Table 1 Primer sequence for RT-PCR analysis

Gene name	Primer sequences (5′–3′)	Amplicon length (bp)
18s rRNA	F: TAACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG	105
ACC1	F: GGGCACAGACCGTGGTAGTT R: CAGGATCAGCTGGGATACTGAGT	150
FAS	F: ATCCTGGAACGAGAACACGATCT R: AGAGACGTGTCACTCCTGGACTT	140
ATGL	F: AACACCAGATCCAGTTCAA R: GGTTCAGTAGGCCATTCCTC	144
C/EBPβ	F: GGACAAGCTGAGCGACGAGTA R: CCGTCAGCTCCAGCACCTT	122
HSL	F: GCCGGTGACGCTGAAAGTGGT R: CGCGCAGATGGGAGCAAGAGGT	197

10 mM each of the forward and reverse primers, and 1 μL of diluted cDNA. The thermal profile for the SYBR Green real-time RT-PCR was 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles at 95 °C for 10 s, and 60 °C for 30 s. To calculate the mRNA expression of selective genes, the ΔCt values were used for detection of their mRNA related to internal control 18s rRNA expression using the $2^{-\Delta\Delta}$Ct method.

Western blotting analysis for FTO

The liver protein was extracted using the Tissue Protein Extraction Reagent (KeyGENBioTECH, Nanjing, China). The total protein content was quantified using the BCA Protein Assay Kit (KeyGENBioTECH, Nanjing, China), and identical amounts of proteins (50 μg/lane) were separated through a 10 % SDS-polyacrylamide gel followed by electrotransfer to nitrocellulose membranes (Millipore, Bedford, MA, USA). After blocking in defatted milk, the membranes were incubated with an anti-rabbit FTO antibody (Epitomics, California, USA) and GAPDH antibody (Boster, Wuhan, China) followed by an incubation in the presence of a peroxidase (HRP)-conjugated secondary antibody (Pierce, Thermo Fisher Scientific, USA). The signals were detected using the ECL Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ, USA).

Analysis of m^6A level using dot-blot assay

m^6A level was analyzed according to Jia et al. [13]. Briefly, isolated mRNA (100 ng) was first denatured at 95 °C for 3 min, followed by chilling on ice immediately. Then, the denatured mRNA was spotted on an NC membrane optimized for nucleic acid transfer. After UV cross-linking in a Stratagene Stratalinker 2400 UV Crosslinker, the membrane was washed by 1× Tris-buffered saline and Tween 20 (TBST) buffer, blocked with 5 % of nonfat milk in TBST for 1 h, and incubated with anti-m^6A antibody (1:2000; Synaptic Systems) overnight at 4 °C. After being incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody, the membrane was visualized by ECL Western Blotting Detection Kit. To ensure that an equal amount of mRNA was spotted on the membrane, the same blot was stained with 0.02 % methylene blue in 0.3 M sodium acetate (pH 5.2).

Statistical analysis

Data are expressed as the mean±SEM. The significance of the differences between the groups in all of the experiments was determined by one-way analysis of variance (ANOVA) with Tukey's post hoc test using SPSS 16.0. Significance was set at $P<0.05$.

Results

Effects of betaine supplementation during adolescence on body weight gain in mice

Although food intake was slightly decreased in HB group when compared with those in control and HF

group, no significant difference was observed in food intake among all three diet groups during the experiment (Fig. 1a). At the end of the experiment, body weight in HF group was significantly higher than the control group. Surprisingly, betaine supplemented in the drinking water did not alleviate HFD-induced body weight gain (Fig. 1b).

Effects of betaine supplementation during adolescence on serum parameters in mice

According to Table 2, glucose and TC concentrations in serum were significantly increased in response to a high-fat diet, while these increases were corrected in mice supplemented with betaine. High-fat diet also significantly increased TG and NEFA concentration in serum. Surprisingly, betaine supplementation did affect TG and NEFA concentration in mice fed with high-fat diet. To examine the effects of betaine on liver function, we detected AST and ALT levels in serum. Our results showed that ALT and AST levels in serum were significantly increased in response to a high-fat diet, while betaine supplementation significantly decreased ALT and AST levels in serum.

Effects of betaine supplementation during adolescence on liver weight, morphology, and fat mass in mice

Liver weight, as well as inguinal and epididymal fat weight in mice upon high-fat diet, was significantly higher than those in mice fed with a low-fat diet, while betaine supplementation only significantly decreased liver weight (Fig. 2a). Our results suggested that betaine supplementation across adolescence did not affect fat deposition in adipose tissue induced by high-fat diet (Fig. 2b, c). To further investigate the effects of betaine on liver morphology and lipid accumulation, we carried out the H&E and Oil Red O staining on liver sections. Our results showed that the normal morphology of the liver was damaged (Fig. 3e) and lipid accumulation was increased (Fig. 3h) in mice subjected to high-fat diet across adolescence, while betaine supplementation across adolescence could alleviate these damages (Fig. 3f, i).

Effects of betaine supplementation during adolescence on lipid metabolism, FTO expression, and m^6A mRNA methylation in mice

To identify whether betaine supplementation during adolescence decreased lipid accumulation through inhibiting lipogenesis in the liver, we detected gene expression of fatty acid synthetase (FAS), acetyl-CoA carboxylase 1 (ACC1), and CCAT/enhancer binding protein β (C/EBPβ), as well as gene expression of adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL). Our results showed that expression of genes (FAS, ACC1, and C/EBPβ) involved in fatty acid synthesis were significantly higher (Fig. 5a) while expression of genes (ATGL and HSL) involved in lipolysis was significantly lower (Fig. 5b) in mice fed with high-fat diet while compared with those in mice upon low-fat diet. However, when mice were supplemented with betaine, expressions of these genes were all normalized to the level of the control group (Fig. 5a, b).

Our results also showed that although HF diet significantly reduced hepatic SAM concentration, betaine supplementation rectified this decrease (Fig. 4a).

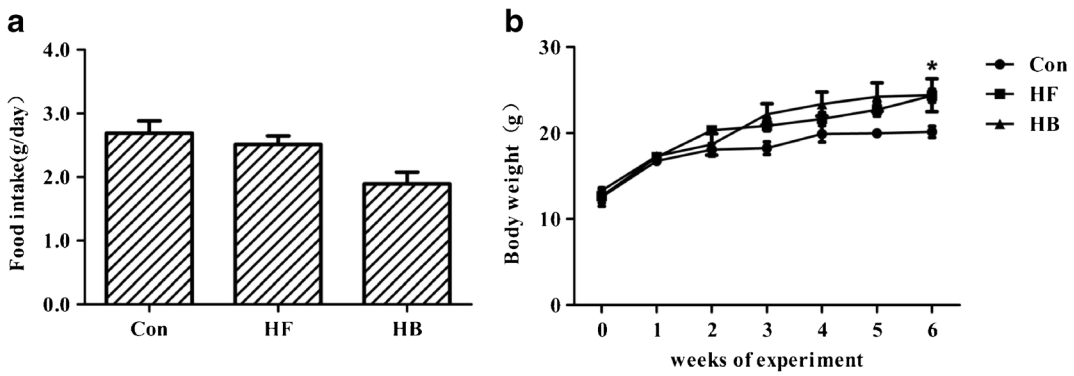

Fig. 1 Betaine supplementation during adolescence reduced HF-induced body weight gain in mice. *Con* mice with low-fat diet, *HF* mice with high-fat diet, *HB* mice with high-fat diet and betaine supplemented in the drinking water. **a** Food intake and **b** body weight during the experiment. Data are means±SEM, $n=10$; *$P<0.05$

Table 2 Effects of betaine supplementation during adolescence on serum parameters in mice

	Glucose (mM)	TC (mM)	TG (mM)	NEFA (mM)	AST (U/L)	ALT (U/L)
Con	4.77±0.37^{b}	2.64±0.16^{b}	1.13±0.1	1.66±0.19	195±26^{b}	52.33±7.8^{b}
HF	9.04±0.56^{a}	4.15±0.57^{a}	1.11±0.07	1.24±0.14	245±15^{a}	126.5±9.5^{a}
HB	6.5±0.85^{b}	3.32±0.17^{ab}	0.84±0.07	1.17±0.05	220±10^{b}	52.5±12.5^{b}

Data are means±SEM, $n=10$

^{a,b} Groups that share the same superscript letters are not significantly different from each other ($P<0.05$)

TC total cholesterol, *TG* triglyceride, *NEFA* nonesterified fatty acid, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *Con* mice with low-fat diet, *HF* mice with high-fat diet, *HB* mice with high-fat diet and betaine

Moreover, there was a parallel increase in the hepatic SAM/SAH ratio with SAM concentration by betaine supplementation (Fig. 4b). Accordingly, to further explore whether m^{6}A mRNA methylation was affected since SAM/SAH ratio was increased, we detected hepatic m^{6}A methylation status and FTO expression. Our results showed that m^{6}A mRNA methylation was significantly decreased (Fig. 5c), while FTO expression was significantly increased in mice fed with high-fat diet (Fig. 5d). However, betaine supplementation prevented these changes in the liver.

Discussion

NAFLD is pathogenically related to obesity and insulin resistance and has been identified as a predictor of type 2 diabetes mellitus and metabolic syndrome in adolescence [23]. In the present study, a model of NAFLD by feeding mice with high-fat diet across adolescence was used to investigate the potential effects of betaine on ectopic fat accumulation in the liver. Our results showed that betaine supplementation not only significantly decreased ALT and AST levels (two blood-based indicators of liver function) but also attenuated hepatic injury and abnormal lipid deposition. These results were in line with previous studies which demonstrated the same effects of betaine on adult animal models of NAFLD [24, 29]. In addition, we also found that liver weight was significantly decreased, although the whole body weight, inguinal, and epididymal fat mass were not affected by betaine supplementation.

Surprisingly, we did not observe any significant changes in TG and cholesterol concentration in serum. These results were different from those in the experimental model of adult mice as they declared that betaine supplementation significantly decreased TG and cholesterol level [29, 30]. However, other studies even found an elevation in plasma TG and cholesterol after betaine supplementation [25, 31]. Studies suggested that the effects of betaine on TG concentration may depend on diet and on the presence of other clinical conditions [16]. Our results suggested that age may also alter the response of plasma lipid markers to betaine supplementation. Previous studies have demonstrated that increased rates of hepatic fatty

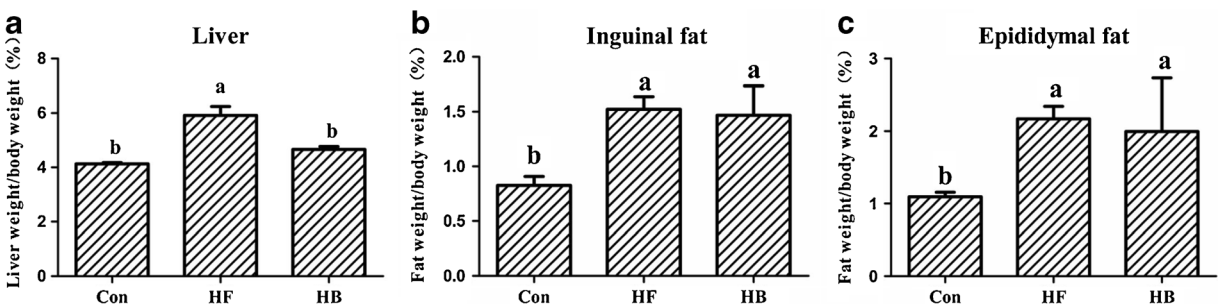

Fig. 2 Betaine supplementation during adolescence reduced HF-induced liver weight gain in mice. *Con* mice with low-fat diet, *HF* mice with high-fat diet, *HB* mice with high-fat diet and betaine supplemented in the drinking water. **a** The ratio of liver weight to body weight, **b** the ratio of inguinal fat to body weight, and **c** the ratio of epididymal fat to body weight. Data are means±SEM, $n=10$. Groups that share the same superscript are not significantly different from each other ($P<0.05$) (*a* and *b*)

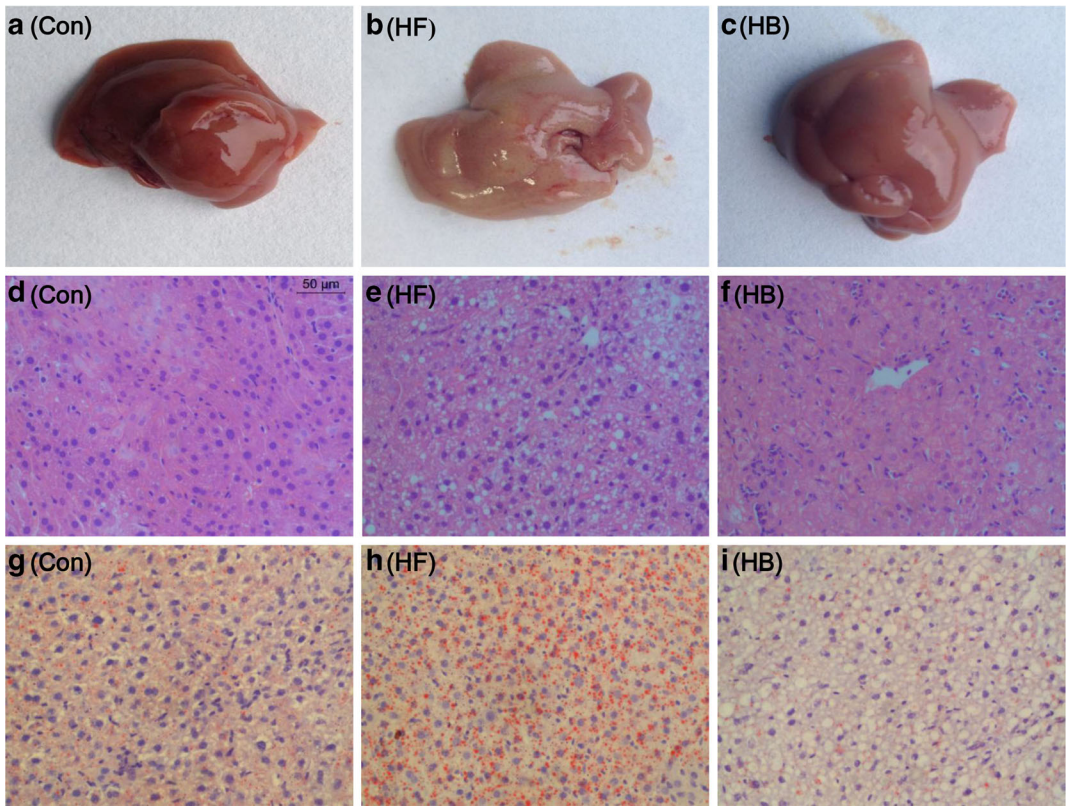

Fig. 3 Betaine supplementation during adolescence alleviated hepatic fat accumulation in HF-fed mice. *Con* mice with low-fat diet, *HF* mice with high-fat diet, *HB* mice with high-fat diet and betaine supplemented in the drinking water. **a**, **b**, **c** Liver shape, **d**, **e**, **f** H&E staining of histologically sectioned liver, and **g**, **h**, **i** Oil Red O staining of histologically sectioned liver

acid synthesis contributed to the development of fatty livers in rodent models of obesity [3, 17]. In the current study, our results showed that betaine supplementation decreased fatty acid synthesis and increased TG breakdown, which led to a decrease of hepatic lipid accumulation. These results are in line with previous studies which showed decreased TG content in the liver upon increasing betaine availability on adult mice [9, 11]. In addition, studies also suggested that the effects of betaine on hepatic lipid deposition might also be through its ability to increase endogenous carnitine, as carnitine might increase carnitine palmitoyltransferase I-mediated fatty acid translocation into the mitochondria and β-oxidation [6, 22].

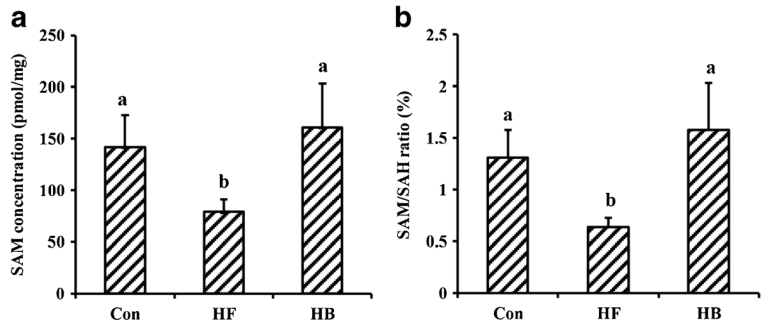

Fig. 4 Betaine supplementation during adolescence increased hepatic SAM concentration in mice. *Con* mice with low-fat diet, *HF* mice with high-fat diet, *HB* mice with high-fat diet and betaine supplemented in the drinking water. **a** SAM concentration in the liver and **b** the ratio of SAM concentration to SAH concentration in the liver. Data are means±SEM, $n=10$. Groups that share the same superscript are not significantly different from each other ($P<0.05$) (*a* and *b*)

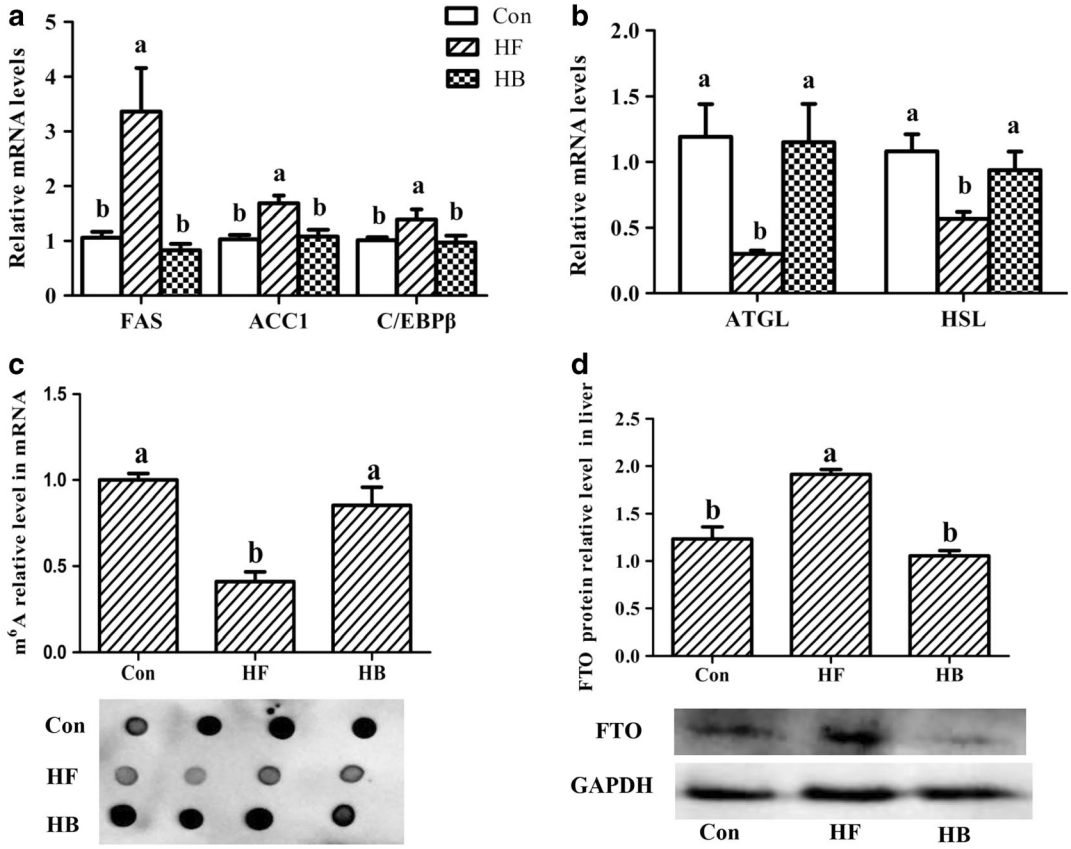

Fig. 5 Betaine supplementation during adolescence increased lipid catabolism and m^{6}A methylation and decreased FTO expression in HF-fed mice. *Con* mice with low-fat diet, *HF* mice with high-fat diet, *HB* mice with high-fat diet and betaine supplemented in the drinking water. **a** mRNA expression of fatty acid synthesis related genes, **b** mRNA expression of ATGL and HSL, **c** m^{6}A level in mRNA in liver, and **d** protein expression of FTO in the liver. Data are means±SEM, n=10. Groups that share the same superscript are not significantly different from each other (P<0.05) (*a* and *b*)

Recently, the effects of betaine on the rectification of DNA methylation have also been declared to be a major mechanism accounting for the hepatoprotective effects of betaine [9, 30]. These effects of betaine were mostly due to its role in one-carbon metabolism as a methyl donor. Betaine provides methyl group for the transformation of homocysteine to methionine, which serves as a substrate for the synthesis of SAM. Importantly, SAM is the major methyl group donator not only for DNA methylation but also for mRNA m^{6}A methylation [4, 19]. Similar to methyl modification in DNA, mRNA also undergoes posttranscriptional methylation to regulate its fate and function [18]. Importantly, m^{6}A is the most prevalent methylated nucleoside in mRNA. As a result, many studies have demonstrated the regulatory role of m^{6}A in RNA metabolism, including mRNA transcription, splicing, nuclear export, and translation ability and stability [20]. In addition, m^{6}A could serve as a docking site for RNA binding proteins, and abnormal m^{6}A methylation levels may lead to dysfunction of RNA and cause diseases [20]. FTO is the first identified RNA-modifying enzyme, demethylating m^{6}A on mRNA [10]. Recently, FTO-dependent m^{6}A demethylation has been evidenced to play a critical role in the regulation of adipogenesis [32]. In the current study, we found that betaine supplementation rectified the decreased m^{6}A status and the increased FTO expression in response to high-fat diet. These results suggested a potential role of FTO-dependent function of m^{6}A in the effects of betaine on hepatic fat accumulation.

In all, our results demonstrated that betaine supplementation across adolescence could protect mice from

high-fat-induced lipid accumulation in the liver. Moreover, FTO-dependent m^6A demethylation may be affected by betaine. The reversible m^6A modification in mammalian mRNA may represent a novel epigenetic mechanism involved in the hepatoprotective effects of betaine.

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