

Full Length Research Paper

Effect of dietary betaine supplementation on mRNA level of lipogenesis genes and on promoter CpG methylation of fatty acid synthase (*FAS*) gene in laying hens

Jinyi Xing¹ and Yunliang Jiang^{2*}

¹College of Life Science, Linyi University, Linyi 276005, People's Republic of China;

²College of Animal Science and Veterinary Medicine, Shandong Agricultural University, Tai'an 271018, People's Republic of China

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Our aims are to determine the effects of dietary betaine supplementation on growth performance, percentage of abdominal fat, ratio of liver to body weight, mRNA expression levels of acetyl-CoA carboxylase (*ACC*), fat acid synthase (*FAS*) and adipose differentiation-related protein (*ADRP*) genes in the abdominal fat of hens. In addition, promoter DNA methylation patterns of *FAS* gene were also analyzed by bisulfite sequencing. A total 120 of Hy-Line White laying hens were randomly divided into four diet groups with supplemented betaine of 0, 0.04, 0.06 and 0.08%, respectively. At 180 old days of age, the significant difference in egg production was observed between the control and 0.08% betaine diet group ($P < 0.05$). The *FAS* mRNA abundance was increased in the abdominal adipose of hens receiving 0.08% betaine compared to the hens receiving control diet with no betaine ($P < 0.05$). However, supplementation of betaine had no significant effect on *FAS* expression level in 165-day-old hens. The *ACC* and *ADRP* gene expression levels were not significantly affected by betaine at either 165 or 180-day-old hens ($P > 0.05$). Furthermore, the overall CpG methylation patterns at the *FAS* gene promoter region ranging from -961 to -749 were not significantly different among all groups in 180-day-old hens ($P > 0.05$), CpG methylation mainly occur from 1st to 5th sites. This study suggests that betaine could regulate lipogenesis of laying chickens by affecting the transcription of *FAS* genes, and that DNA methylation at some CpG dinucleotides may play a role in regulating *FAS* gene expression.

Key words: Betaine, CpG methylation, gene expression, laying hen, lipogenesis genes.

INTRODUCTION

Nowadays, excessive fat accumulation in laying hens has become a worldwide problem, such as causing fatty liver

hemorrhagic syndrome, decreasing egg production and egg quality. As a product of choline degradation, betaine acts as a methyl donor to homocysteine to form methionine, or upon complete demethylation results in glycine formation (Lawrence et al., 2002). The betaine is now widely used in poultry diets due to its effects in sparing methionine, reducing abdominal fat content and minimizing the impact caused by challenges like heat stress and coccidiosis (Kettunen et al., 2001; Jahanian and Rahmani., 2008). Betaine is also reported to prevent excessive fat accumulation in the liver (Craig, 2004).

The effects of betaine on decreasing fat accumulation were significant as reported in pigs (Fernandez-Figares

Corresponding author. E-mail: ylijiang723@yahoo.com.cn. Tel: +86 538 824 1593. Fax: +86 538 824 1419.

Abbreviations: **ACC**, Acetyl-CoA carboxylase; **ADRP**, adipose differentiation-related protein; **FAS**, fat acid synthase; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **qRT-PCR**, quantitative reverse transcription polymerase chain reaction; **EP**, egg production; **EW**, egg weight; **PAF**, percentage of abdominal fat; **RLBW**, ratio of liver to body weight.

Table 1. Composition of experimental basal diets.

Ingredient	%	Composition by calculation (%)
Corn (CP, 8.5%)	65.6884	Crude Protein 15
Soybean meal (CP, 43%)	21.7873	ME (MJ/kg) 12.13
Shell powder	8.9392	Ca 3.25
Soybean oil	2.2083	Available Phosphate 0.25
Dicalcium phosphate	0.6086	Salt 0.34
Salt	0.3469	Methionine 0.3
choline chloride (55%)	0.09	Lysine 0.6435
DL-Methionine 99%	0.0813	
Vitamin promix	0.05 ^a	
Mineral promix	0.2 ^b	

^aProvided the following amount per kilogram of diet: vitamin A, 12000 IU; vitamin D3, 2000 IU; vitamin E 25 IU; menadione, 1.2 mg; riboflavin, 5.0 mg; pyridoxine, 8.0 mg; vitamin B12, 0.08 mg; biotin, 0.3 mg; folic acid, 0.7 mg; D-pantothenic acid, 18.0 mg; niacin, 30.0 mg; ^bProvided the following amount per kilogram of diet: Mn, 100.0 mg; Fe, 60.0 mg; Zn, 80.0 mg; Cu, 10.0 mg; Se, 0.66 mg; I, 1.2 mg.

et al., 2002; Huang et al., 2008). The lipotropic effect of betaine was also detected in poultry. Saunderson and Mackinlay (1990) indicated that betaine was effective in reducing chicken body fat. Wang et al. (2004) showed that betaine supplementation significantly decreased abdominal fat in meat ducks. For 41-day-old broilers fed diets containing 0.06% betaine, both abdominal fat and percent abdominal fat were decreased, although the effect was not significant (Esteve-Garcia and Mack, 2000; McDevitt et al., 2000).

In laying hen, a study conducted by Zou and Lu (2002) found that 0.06% betaine supplementation in diets significantly decreased percentage of abdominal fat at 50 and 70 weeks of age. Moreover, betaine supplementation decreased the activities of acetyl-CoA carboxylase (ACC), fat acid synthase (FAS) and malic enzyme as well as the FAS mRNA level in abdominal adipose tissue in finishing pigs with less fat deposition (Huang et al., 2008) and betaine suppressed carnitine palmitoyltransferase I in skeletal muscle but not in liver of finishing pigs (Huang et al., 2009).

In laying hen, although the effect of betaine on fat deposition was investigated, the molecular mechanism by which abdominal adipose is down-regulated by betaine is poorly understood. In this experiment, the effects of dietary betaine supplementation on the production traits and the mRNA expression of ACC, ADRP (adipose differentiation-related protein) and FAS genes, and the role of DNA methylation at FAS promoter region in regulating FAS expression were investigated.

MATERIALS AND METHODS

Chicken and diets

One hundred and twenty 130-day-old Hy-Line White laying hens were randomly allocated to four dietary treatments ranging in betaine concentration, that is, 0% (C group), 0.04% (B1 group),

0.06% (B2 group) and 0.08% (B3 group). Each of the treatments had three replicate cages with ten birds per replicate cage. The basal diets contained corn, soybean meal and were fed as mash, and the composition of the basal diet was formulated to contain all nutrients to meet the nutritional requirements for hens (NRC, 1994; Table 1). Water and mash feed were provided *ad-libitum*, with conventional vaccination and natural lighting. Daily egg records were kept individually, and egg production (EP) was calculated on a hen-day basis. Egg weights (EW) were analyzed on an individual hen basis. Animal experimentations were carried out according to internationally accepted guidelines.

Performance evaluation and sample collection

Laying hens were weighed individually at the start and end of the feeding trial after fasting for 12 h. At 165 and 180 days of age, hens were sacrificed by bleeding from jugular veins. Fifteen birds per group were randomly chosen for tissue sampling. Abdominal fat pad and liver were removed and weighed, and percentage abdominal fat (PAF) and ratio of liver to body weight (RLBW) were calculated for each group at 180 days of age. Meanwhile, abdominal adipose samples were collected and snap-frozen in liquid nitrogen and stored at -80°C for total RNA extraction.

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from abdominal adipose using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. Before RT-PCR, samples were treated with RNase-free Dnase I (Promega, Madison, WI) to remove contaminating genomic DNA. Total RNA (2 μg) was reverse-transcribed using Oligo (dT)₁₈ primers and M-MLV Reverse Transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. Quantitative RT-PCR was performed using SYBR Premix Ex Taq™ (TaKaRa) according to the manufacturer's instructions in LightCycler 480 (Roche Applied Science, Germany). The sequences of the primers used for qRT-PCR are given in Table 2. Expression levels for all genes were normalized to GAPDH. The thermal cycling conditions composed of an initial denaturation step at 95°C for 10 min followed by 40 cycles of PCR using the following profile: 94°C , 30 s; $T^{\circ}\text{C}$ (Table 2), 30 s;

Table 2. Primers for mRNA expression and *FAS* promoter CpG methylation assay.

Gene ^a	Name Primer	Primer sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
<i>ACC</i>	F	CCAAACAACAACAACACTATGC	215	57
	R	GCAGTCTGAGCCACTATT		
<i>ADRP</i>	F	CAAAGTGAGAGATGCTAAAC	281	57
	R	TTGTAACCAGTGTGAGGC		
<i>FAS</i>	F	TGAAGGAGGAAGTCAACG	196	55
	R	GATGGTGAGGAGTCCGAT		
<i>GAPDH</i>	F	CTACACACGGACACTTCAAG	244	59
	R	ACAAACATGGGGGCATCAG		
For <i>FAS</i> CpG methylation analysis	M-F1	AAATGGAGGAGGTGAATTTTTAT	393	55
	M-R1	AATTACCACAAATCAACRACAC		
	M-F2	TTAAAATATTAATAAATAGTTTATTGATTT	213	
	M-R2	AAAAACCCACCTACTATAAAAC		

^aGenBank accession no. *ACC*, NM_205505; *ADRP*, NM_001031420 XM_424822; *FAS*, NM_205155; *GAPDH*, K01458. *FAS*, Fatty acid synthase; *ACC*, acetyl-coA carboxylase, *ADRP*, adipose differentiation-related protein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

and 72°C, 30 s. The relative expressions of genes were analyzed according to the 2^{-ΔΔCT} method (Livak and Schmittgen, 2001) using the *GAPDH* gene for normalization.

Bisulfite sequencing

Genomic DNA was extracted from abdominal adipose of 180-day-old hen using Genomic DNA Purification Kit (Fermentas Life Science, EU) according to the manufacturer's protocol. Four DNA pools were prepared based on *FAS* expression (high and low). For each group of three pen replicates, equal quantity of DNA from 12 samples was mixed together according to its concentration. Sodium bisulfite treatment of DNA pool was performed with minor modifications to the previously described method (Hajkova et al., 2002). In brief, 1 μg DNA was digested with 15 U restriction enzyme Hind III (TaKaRa, Dalian, China) at 37°C for 8 h in a 20 μl mixture containing 2 μl 10×M buffer, denatured by adding freshly prepare 3 M NaOH and incubated for 20 min at 37°C. Aliquots of 10 mM of freshly prepared hydroquinone (Sigma, USA) and 3 M of sodium bisulfite (pH 5.0, 520 μl; Sigma, USA) were added and the solution was incubated at 50°C for 16 h for modification. The DNA samples were then purified with the Wizard[®] SV Gel and PCR Clean-Up System (Promega, Madison, WI) and eluted with 100 μl TE buffer (100 mM Tris-Cl, 10 mM EDTA, pH 8.0). The modification was completed with 20 μl 3.0 M NaOH treatment for 20 min at 37°C, and then precipitated with ethanol, resuspended in TE (pH 8.0). Modified DNA was stored at -20°C and used as template for subsequent amplification.

The *FAS* gene promoter region (GenBank accession no. X77339) ranging from -961 to -749 was amplified by nested PCR. The primer sequences for the amplification of *FAS* are listed in Table 2. The PCR reaction was performed in 25 μl reactions containing 2.5 μl 10×PCR buffer, 2 μl of 10 mM dNTPs, 10 pmol primer, 1 unit rTaq DNA polymerase (TaKaRa, Dalian, China), 2 μl MgCl₂ (25 mM), 1 μl bisulfite-modified genomic DNA (equivalent to 50 ng). The first

round PCR (using primers M-F1 and M-R1, Table 2) conditions for the reaction were 94°C for 3 min, 12 cycles of 94°C for 30 s, 56°C for 30 s (the annealing temperature was lowered 0.5°C every cycle beginning at 55.5°C and ending at 50°C), 72°C for 30 s; followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s; finally 7 min at 72°C. The second round PCR used 0.5 μl of the first round PCR product as template with primer pair M-F2 and M-R2 (Table 2) to amplify the 213 bp including 9 CpGs. Amplification conditions consisted of 94°C for 5 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s, with a final extension at 72°C for 7 min.

PCR products were gel purified, cloned into the pMD18-T vector (TaKaRa, Dalian, China) and sequenced using BigDye Terminator v.3.1 Cycle Sequencing Kit (PE Applied Biosystems) according to the recommendations of the manufacturer. The DNA sequences were run on a 3730xl DNA Analyzer (PE Applied Biosystems). For each sample, ten clones were randomly picked up. Amplified plasmid DNA samples containing the *FAS* gene promoter region from each sample were sequenced by Sangon Biotech (Shanghai, China) Co. Ltd. At each CpG site, when the sequence obtained was TpG, the CpG was considered as methylated.

Statistical analysis

Group data for multiple comparisons were analyzed by ANOVA using the GLM procedure, followed by Duncan's multiple range test to test for differences. Statistical significance was set at $P < 0.05$. Data were expressed as means ± Standard Error.

RESULTS

Effect of dietary betaine supplementation on partial traits

The effect of betaine supplementation to the basal diet on

Table 3. The effect betaine on partial traits in laying hens at 180 days of age.

Trait	Betaine (%)				P-value
	0	0.04	0.06	0.08	
EP (%)	70.800±5.622 ^b	75.000±3.171 ^{ab}	76.267±5.359 ^{ab}	89.333±2.645 ^a	0.0269
EW (g)	45.271±0.439	46.551±0.785	47.522±0.662	46.118±1.021	0.2096
PAF (%)	2.713±0.387	3.437±0.367	2.858±0.362	2.938±0.166	0.4575
RLBW (%)	2.316±0.099	2.224±0.115	2.110±0.052	2.189±0.082	0.4298

The different lowercase in the same row indicate differences ($P < 0.05$). No superscripts in the same row mean no significant difference ($P > 0.05$). EP=Egg production; EW= Egg weight; PAF=Percentage of abdominal fat; RLBW=Ratio of liver to body weight.

production traits is shown in Table 3. In 180-day-old hens, the egg production in birds fed a 0.08% betaine diet was significantly higher than those fed a control diet ($P=0.0269$), and a trend of increase in egg weight was observed in all betaine groups. However, the ratio of liver to body weight showed a decrease trend.

Effect of dietary betaine supplementation on mRNA expression of *ACC*, *ADRP* and *FAS* gene

The mRNA expression levels of *ACC*, *ADRP* and *FAS* genes in 165-day-old and 180-day-old hens were analyzed and shown in Figures 1 and 2. In 165-day-old hens, no differences among groups in *ACC*, *ADRP* and *FAS* mRNA expression were found ($P > 0.05$), although *ACC* mRNA expression decreased 59.91% (1.5528 ± 0.4206 in C group vs. 0.6226 ± 0.2083 in B1 group) in the group of 0.04% betaine supplementation as compared to the C group. In 180-day-old hens, the effect of betaine on the mRNA expression levels of both *ACC* and *ADRP* were also not significant ($P > 0.05$). The mRNA level of *FAS* expression increased with supplementation of 0.08% betaine compared with the control ($P < 0.05$).

Effect of dietary betaine supplementation on DNA methylation of *FAS* promoter

To verify whether betaine regulate the expression of *FAS*, mRNA and DNA methylation, bisulfite sequencing was performed in *FAS* gene promoter region from -961 to -749 including 9 CpG dinucleotides. The result (Figure 3) demonstrates that the overall GpG methylation patterns at the *FAS* gene promoter region ranging from -961 to -749 were not significantly different among all groups in 180-day-old hens ($P > 0.05$), CpG methylation mainly occur from 1st to 5th sites. The CpG methylation percentages (33.33 and 23.33%) were higher in C and B2 groups in which *FAS* mRNA expression levels were lower. Finally, in ten clones for C group, the overall percentage of methylation in the third CpG site were 100%, but there was no methylation of the third CpG site in 10 clones for B1 and

B3 group.

DISCUSSION

In this study, betaine supplementation decreased the ratio of liver to body weight in laying hens, however, there was not significant difference. As a methyl donor, betaine provides the one-carbon units that can spare the amount of dietary methionine and choline required for optimal nutrition. When diet contains sufficient methyl, it can decrease significant amount of liver fat (Xu et al., 2001). Therefore, betaine may prevent fatty liver. Saunderson and MacKinlay (1990) presented data suggesting that chicks given diets betaine and methionine had lower liver weights than those given diet choline and control, so they speculated that betaine may be a more effective lipotropic agent than choline for poultry.

By now, there are some reports that betaine reduced body fat in chicks and pigs (Esteve-Garcia and Mack, 2000; Fernandez-Figares et al., 2002; Huang et al., 2008). However, there are contradictive conclusions. Rostagno and Pack (1996) and Garcia et al. (2000) concluded that betaine reduced neither carcass fat nor abdominal pad size in chickens. Recently, Sun et al. (2008) found that supplementation of betaine to replace up to 25% of total dietary methionine did not affect the growth performance but improved the carcass quality of the broilers.

Abdominal fat deposition is affected by many factors, such as age, sex and genetics, which affect the capacity for lean accretion and the amount of fat deposition and consequently might interfere with the mode of action of betaine (Eklund et al., 2005). Neto et al. (2000) found that the initial degree of fatness of the birds is a potentially important factor for the activity of any lipotropic agent, lipotropic agents (for example betaine) should be more effective in fat birds. We found that betaine addition increased egg production and egg weight in hens, and B3 group displayed the best effect in egg production for 180 days of age, which is consistent with the findings of Zou and Lu (2002) and Lu and Zou (2006). As for the underlying mechanism of betaine in increasing egg

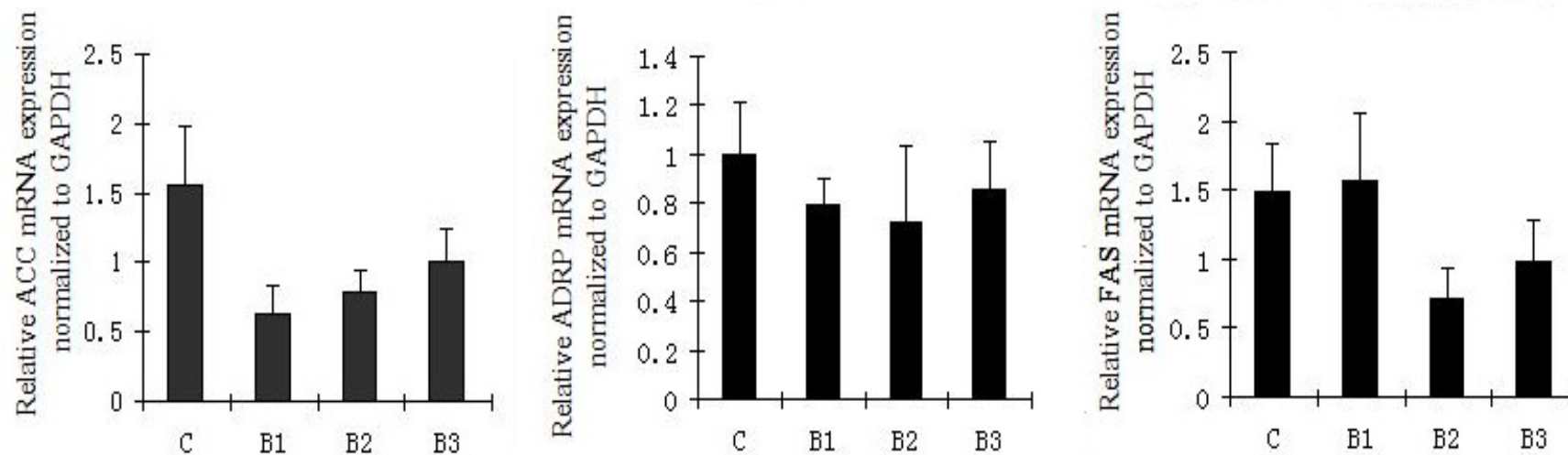


Figure 1. Different dietary betaine supplementation influences expression of *ACC*, *ADRP*, *FAS* mRNA normalized to *GAPDH* in abdominal adipose tissue in 165-day-old hens. The bars represent the mean \pm Std Error, no superscript represents $P>0.05$, N=5.

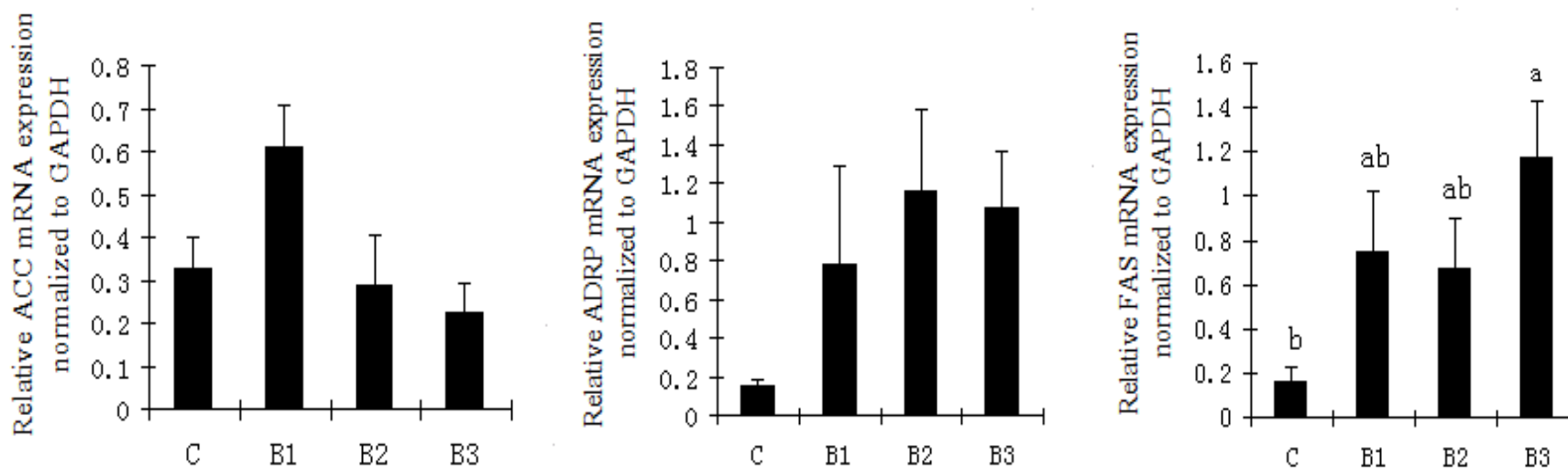


Figure 2. Different dietary betaine supplementation influences expression of *ACC*, *ADRP*, *FAS* mRNA normalized to *GAPDH* in abdominal adipose tissue in 180-day-old hens. The bars represent the mean \pm Std Error, different lowercase represents $P<0.05$, N=5.

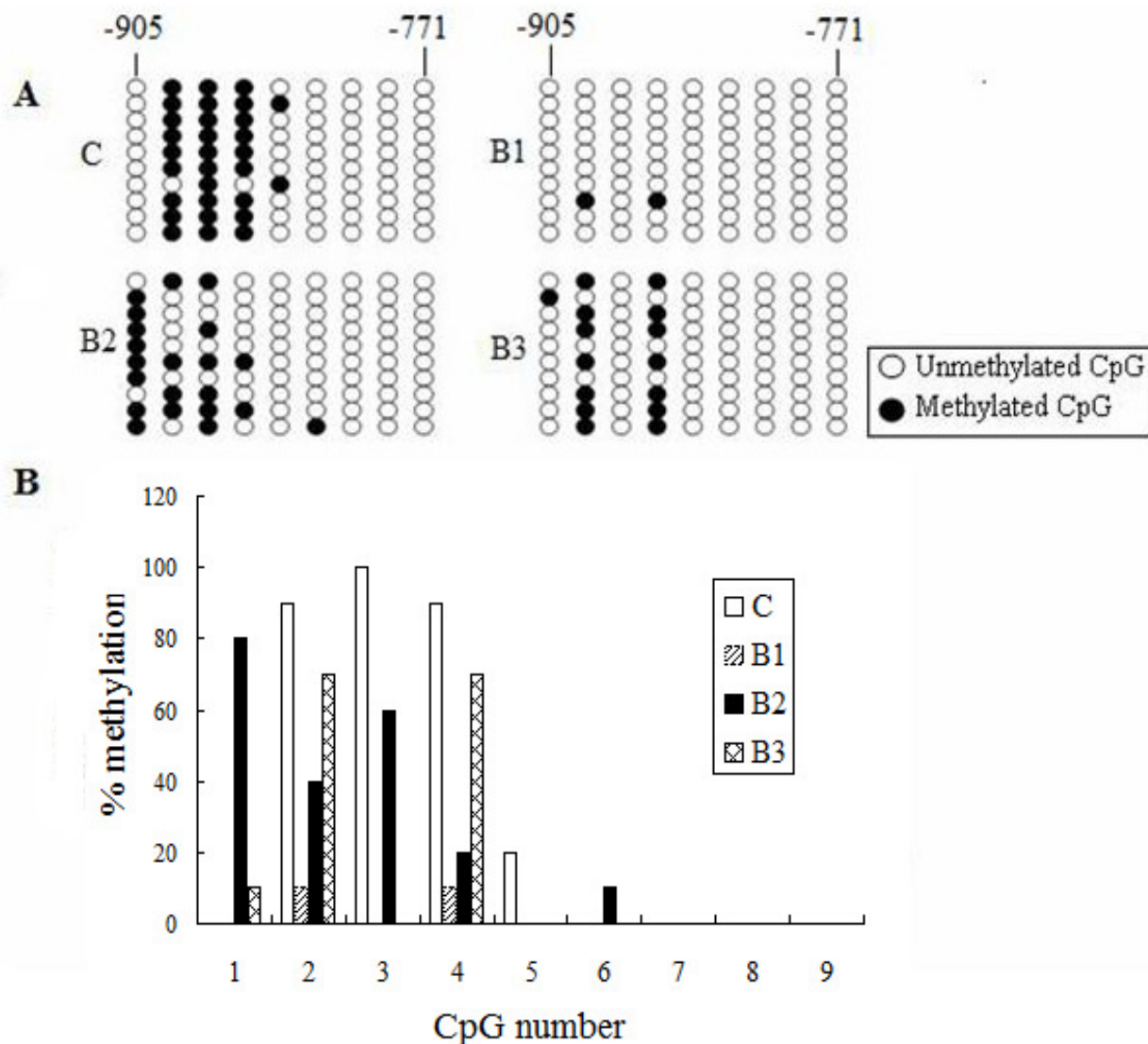


Figure 3. Bisulfite sequencing analysis of *FAS* promoter CpG methylation in 180-day-old laying hens. (A) CpG methylation profiles at *FAS* promoter region. (B) Percentage of each CpG methylation of *FAS*. Number 1-9 refer to the CpG dinucleotide. C represents control, B1 represents basal diets +0.04% betaine, B2 represents basal diets +0.06% betaine, B3 represents basal diets +0.08% betaine.

production, betaine can stimulate anterior pituitary to secrete and release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). And then FSH and LH promote follicle growth and ovulation, inhibit follicular atresia and subsequently increase egg cell and egg production. Triiodothyronine (T3) and thyroxine (T4) can improve ovary function and ovulation, and they are also helpful for improving egg production (Zou, 2001; Lu and Zou, 2006).

Concerning mRNA studies in abdominal adipose, *ACC* gene expression was not significant in the betaine group as compared to the C group for 165-day-old hens or 180-day-old hens. *ACC* catalyze the generation of malonyl-CoA, and this malonyl-CoA product can be used in diverse biological processes. The most important role of

ACC is in the biosynthesis of long-chain fatty acids. In fact, *ACC* catalyzes the committed and rate-limiting step in fatty acid biosynthesis, and is an essential enzyme in many organisms (Tong, 2005). In the current study, *ACC* gene expression level was the lowest in B1 groups in 165-day-old hens, but it was the highest in 180-day-old hens in all groups. This result suggests that *ACC* gene expression level seems to relate to chicken age.

Regarding ADRP, it is a lipid droplet-associated protein that has been ascribed a role in cellular fatty acid uptake and storage (Tobin et al., 2006). ADRP may play a significant role in regulating the intracellular distribution of phospholipids and lipids in general (McIntosh et al., 2010). However, in this study, we did not found statistical differences of the mRNA expression levels of both *ACC*

and *ADRP* in 165 or 180-day-old hens. We speculate that *ADRP* gene mRNA expression was slight affected by betaine.

In relation to *FAS*, it plays an important role in de novo lipogenesis and is physiologically regulated by energy balance (Menendez et al., 2007; Kuhajda, 2006). High-carbohydrate/low-fat diets up-regulate *FAS* whereas exercise and energy restriction down-regulate *FAS* (Motoshima et al., 2006). In our trial, compared with other groups, in 180-day-old hens, the C group had the lowest ($P < 0.05$) mRNA expression level of *FAS*, but it was the highest in B3 group. However, this result was inconsistent with Huang et al. (2008). In general, *FAS* catalyzes the last step in the fatty acid biosynthetic pathway during lipogenesis. The lower expression of *FAS* mRNA is supposed to be one of the major causes leading to less fat deposition. In addition, although the mRNA level of *FAS* was decreased in C group in 180-day-old hens, the percentage of abdominal fat was not significantly affected which may indicate that chicken abdominal fat accumulation is affected by many factors, and the influence of *FAS* gene is only partial.

DNA methylation at the 5'-position of cytosine of CpG dinucleotide is an epigenetic process influenced by the environment in which heritable changes in the coding pattern of DNA occurs (Klutke et al., 2010). Our finding suggests that betaine may affect on *FAS* promoter methylation states because betaine (three methyl glycine), a product of choline degradation acts as a methyl donor to homocysteine to form methionine, or upon complete demethylation will result in glycine formation (Lawrence et al., 2002). At present, only one work has reported no relevant correlations between methylation status and *FAS* gene expression (Lomba et al., 2010). Also, in our previous study, two distinct CpG methylation profiles at the promoter region of chicken lipoprotein lipase (LPL) gene can be classified by betaine supplement to dietary of chickens (Xing et al., 2009). Recently, Su et al. (2009) reported that the DNA methylation pattern in the S14 α gene transcription start site may not be related to the expression of Spot144 α transcript in response to betaine supplementation in Landes goose liver. In our case, a total of 9 CpG sites were analyzed in the transcription start region of the *FAS* gene (-961 to -749). No significant change of methylation was observed among all groups. However, methylation distribution patterns at the 9 CpGs sites were different, for example, in C group, CpG methylation occurs mainly at 2st to 5th, the remaining sites were unmethylated. But, it was interesting that the third CpG site was complete methylation in ten clones in C group, the same CpG site in B1 and B3 groups was unmethylated. Moreover, we noticed that *FAS* gene expression level was significant between C group and B3 group. This result suggests that the third CpG site probably contributed to *FAS* gene expression. Certainly, further studies should be carried out to verify correlations between the *FAS* gene

expression and the methylation profiles.

In conclusion, the present study demonstrated that betaine affected some production traits and lipogenesis gene expressions in adipose tissues. CpG methylation distribution patterns of *FAS* in the promoter distal region were also analyzed by bisulfite sequencing. The results implied that betaine could regulate lipogenesis of laying chickens by affecting the transcription of genes involved in lipogenesis, and that gene expression may be regulated by gene methylation status. Therefore, an understanding of the intimately correlated methylation changes for *FAS* gene may be of importance for betaine-induced epigenetic mechanisms on regulation of adipose deposition.

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