In ovo injection of betaine promotes adrenal steroidogenesis in pre-hatched chicken fetuses

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ABSTRACT Corticosterone is critical for the maturation and survival of chicken fetus around hatching. Betaine is used as a feed additive in poultry industry to promote growth and mitigate stress. However, it remains unknown whether betaine could affect adrenal corticosterone synthesis in pre-hatching chicken fetuses. In this study, betaine (2.5 mg/egg) was injected into developing chicken fetuses at d 11 of incubation (E11) and its impact on adrenal steroidogenesis was investigated at day 19 (**E19**). Plasma corticosterone concentration was significantly ($P < 0.05$) elevated in betaine-treated fetuses, together with increased adrenal expression of melanocortin 2 receptor and steroidogenic acute regulatory protein. Accordingly, the corticosterone biosynthetic enzymes, such as cytochrome P450 family 11 subfamily A member 1, 3β-hydroxysteroid dehydrogenase and cytochrome P450 family 21 subfamily A member 2, as well as cholesterol biosynthesis or regulation-related genes, such as sterol regulatory element-binding protein 1, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and low-density lipoprotein receptor, were all significantly ($P < 0.05$) upregulated in betaine group. Meanwhile, steroidogenic factor-1 and glucocorticoid receptor were significantly ($P < 0.05$) enhanced, whereas expression of dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome gene, a nuclear receptor known as a repressor of adrenal steroidogenesis, was significantly ($P < 0.05$) downregulated. Betaine significantly ($P < 0.05$) increased adrenal expression of genes involved in one-carbon metabolism and DNA methylation, such as S-adenosyl homocysteine hydrolase, betaine-homocysteine-methyltransferase, methionine adenosyl transferase and DNA methyltransferases, yet the promoter regions of most steroidogenic genes were significantly ($P < 0.05$) hypomethylated. These results indicate that in ovo injection of betaine promotes adrenal glucocorticoid synthesis in chicken fetuses before hatching, which involves alterations in DNA methylation.

Key words: chicken, adrenal, betaine, cholesterol, steroidogenesis

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INTRODUCTION

Adrenal glands are the principal steroid-producing organ that initiates steroidogenic function in chicken embryos even before the maturation of the hypothalamus-pituitary-adrenal (**HPA**) axis (Carsia, 2015). Corticosterone (**CORT**), the primary glucocorticoid hormone in birds, plays critical roles in the developmental and hatching process of embryos (Tong et al., 2013). Plasma CORT concentration of chicken embryos was reported to drop around d 10 of incubation and to rise dramatically prior to hatching (Kanda et al., 2000).

Steroidogenesis in the chick embryo is present as early as d 4 of incubation. By d 7 of embryogenesis, the mRNA for the enzyme cytochrome P450scc (***CYP11A1***) is already expressed in the adrenal glands (Kanda et al., 2000). CYP11A1 that causes transformation of cholesterol to pregnenolone is a rate-limiting enzyme in CORT biosynthesis in the chicken adrenal glands in the first term of embryogenesis (Siegel and Gould, 1976; Mashaly, 1991). The nuclear receptor, steroidogenic factor-1 (***SF-1***) is the earliest indicator of adrenocortical activity and is required for developing the steroidogenic cells (Smith et al., 1999). *SF-1* also governs cholesterol conveyance to steroidogenic reactions, by transactivating steroidogenic acute regulatory protein (***StAR***) that shuttles cholesterol from the outer to the inner mitochondrial membrane (Val et al., 2003).

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Betaine is used as a feed additive in poultry nutrition, which can serve as a methyl donor and spare part of the methionine in the diet (Alagawany et al., 2021). The methyl groups are involved in many vital biological processes including DNA methylation, DNA synthesis and maintenance (Lever and Slow, 2010), as well as protein synthesis (Sun et al., 2008). Betaine supplementation in poultry diet has been shown to promote feed efficiency (Nutautaitė et al., 2020), improve growth performance (Yang et al., 2021), and attenuate heat stress (Ratriyanto and Mosenthin, 2018; Alagawany et al, 2021). Previously, we reported that in ovo betaine exposure modulates cholesterol metabolism in liver (Hu et al., 2015) or hypothalamus (Idriss et al., 2017) of newly hatched chicks and cockerels, respectively. Also, increased CORT deposition in the egg yolk was also reported in laying hens following dietary betaine supplementation, which was associated with activation of adrenal *StAR* expression (Abobaker et al., 2017). However, the effects of in ovo betaine exposure on the adrenal steroidogenesis of late-stage chicken fetuses before hatching remain unknown.

Therefore, this study was aimed to investigate the effect of in ovo injection of betaine on adrenal steroidogenesis in chicken fetuses: first to see whether there is an effect, second, whether it is related to steroidogenic gene regulation, third, whether it involves one-carbon metabolism and DNA methylation.

MATERIALS AND METHODS

Ethics Statement

The Animal Ethics Committee of Nanjing Agricultural University approved the experimental protocol, with the project number 31972638. The sampling procedures complied with the "Guidelines on Ethical Treatment of Experimental Animals'' (2006) No. 398 sets by the Ministry of Science and Technology, China.

Animals and Treatment

A total of 300 inseminated chicken eggs at embryonic E9 were obtained from a native farm and artificially incubated at 37.5°C and 60% relative humidity. Eggs were randomly assigned to 2 groups, control and betaine group. On E11, betaine (B2629, Sigma−Aldrich, St Louis, MO) dissolved in saline was injected into the yolk sac at the dose of 2.5 mg per egg in a volume of 100 μL. The dose was determined according to the range of betaine concentrations in chicken eggs reported previously (Zeisel et al., 2003, Hu et al., 2015; Idriss et al., 2017). The incubator was set according to our previous publications. On E19, female embryos were identified and removed from the eggs, euthanized by decapitation which is approved by the American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2013 Edition. Then blood samples were taken, and plasma was separated and stored at −20°C. Adrenal

Table 1. Nucleotide sequences of specific primers used for real-time PCR.

Target genes	GenBank accession	Primer sequences (5′ to 3′)	PCR product (bp)
MC2R	NM_001031515.2	F: TAGCATTGCCATTGCCCTCT R: GATGGACTGCACTGGTTGG	167
TSPO	NM_001278057.1	F: CCCACGCAGGAGGAGTTTTA R: AGCAAACACCCAGTTAGGGG	107
VIM	AH002482.2	F: CTTTGCCCAGTGCTGTAGTC R:AAACACGGGCTGTCAGTCA	171
StAR	NM_204686.2	F:ATGGCATCCAAGGAGTGA R: GGGAGACAGAAGGGAACAG	103
CYP11A1	NM_001001756.1	F: AGCACTTCAAGGGACTGAGC R:ACTTGGTCCCAACTTCCACC	147
3BHSD2	XM_417988.5	F: CTGGAAGAAGATGAGGCGCT R: ACCTGTCACGTTGACTTCCC	249
CYP21A2	>NM_001099358.1	F: CTTTGAGGCGTTCACGTTCC R: CTGGGACTCCACAAAGGCAT	169
CYP19A1	NM_001364699.1	F: CATGCACCCAATAGAAAGGCA R: GCATTTCTTAAAGTGACTGCAAAC	130
SF-1	XM_015279334.1	F: TCTTCCTGAATTTCCCTT R: TGAACATCCCATCTAGTGA	151
GR	NM_001037826.1	F: CTTCCATCCGCCCTTCA R: TCGCATCTGTTTCACCC	
CREB	XM_015294628.2	F: GTCAGACACACCAGAGCCTT R: CATTCCTGCTCCCCTTCCTC	128
BHMT	XM_414685.3	F: CGAGTGGGACGGCTTCTT R: AGGCGATAGGTGTCAGGGA	144
AHCYL1	NM_001030913.1	F:TGGTGTTGTTGGGGGAGAT R:CCCCATCAATACTCATCCAAC	227
DNMT1	NM_206952.1	F:TGATAYGTTGGATGAGTATTGATGG R:AAAAAAACTCTCACTCAACTCCAC	264
GNMT1	XM_015283546.1	F: GGAGGAGGGCTTCCAAGTGA R: GCTCCAGCGTCAGCCAGTT	140
β-actin	NM_205518.1	F: ATG GCTCCGGTATGTGCAA R: TGTCTTTCTGGCCCATACCAA	120

samples were collected, rapidly frozen in liquid nitrogen and stored at −80°C for further analysis.

Histological Analysis of Adrenal Tissue

Adrenal glands were collected immediately after euthanizing chickens. Tissue samples were immediately fixed in 4% paraformaldehyde and Bouin's solution and were then routinely processed for light microscopy. Tissue sections of the adrenal gland, 5-μm thick, were stained with Harris' Hematoxylin and Eosin (**H&E**). Images were taken using an Olympus BX-63 microscope with attached digital camera.

Determination of Plasma Total Cholesterol

The plasma concentration of total cholesterol was measured with an automatic biochemical analyzer (Beckman coulter, AU2700) using a commercial kit purchased from Maccura Biology Co., Ltd. (ECH0103152, Chengdu, China). The intra-assay coefficient of variation was less than 3.0%.

Plasma Corticosterone Assay

Corticosterone concentration in the plasma was measured with a commercial Enzyme Immunoassay (**EIA**) kit (No.ADI-900-097, Enzo, New York, NY). The detection limit was 26.99 pg/mL and the intra-assay coefficient of variation was 8%.

RNA Isolation and Real-Time PCR

Ten mg adrenal gland samples were used to isolate total RNA by using 500 mL of TRIzol reagent (Invitrogen, Carlsbad, CA) and 2 μg of total RNA was subjected to RT-PCR as formerly described (Idriss et al., 2017). Beta-actin was chosen as an internal control. Samples were run in duplicates. Primers for real-time PCR (Table 1) were synthesized by Genewiz (Suzhou, China). Data were analyzed using the method of $2^{-\triangle\triangle CT}$ (Livak and Schmittgen, 2001), and the results are presented as the fold change relative to the average value of the control group.

Protein Extraction and Western Blot Analysis

Total protein was extracted from 10 mg frozen adrenal samples, as previously described (Abobaker et al., 2019). BCA Protein Assay kit (No. 23225, Thermo Scientific, Waltham, MA) was used to measure protein concentrations following manufacturer's instructions. Fifty micrograms of protein were used for electrophoresis on a 10% SDS-PAGE gel. Western blot analysis was performed following the protocols provided by the manufacturers, using the primary antibodies for StAR (BS6960, Bioworld, Bloomington, IN, diluted 1:1,200), CYP11A1 (13363-1-AP, Proteintech, Chicago, IL, diluted 1:1,400), HMGCR (BS6625, Bioworld, Bloomington, IN, diluted 1:1,000), SREBP1 (SC-366, Santa Cruz, Dallas, TX, diluted 1:200), GR (Custom made for chickens by Genecreate Biotech Co., Wuhan, China, diluted 1:1,000), CREB (AB31387, Abcam, Cambridge, UK, diluted 1:1,000), BHMT (15965-1-AP, Proteintech, Chicago, IL, diluted 1:1,000), GNMT (18790-1AP, Proteintech, Chicago, IL, diluted 1:1,000), AHCYL1 (10658-3AP, Proteintech, Chicago, IL, diluted 1:1,000), and DNMT1 (24206-1-AP, Proteintech, Chicago, IL, diluted 1:1,000). β-actin (AP0060, Bioworld, Bloomington, IN, diluted 1:10,000) and Tublin-β (AP0064, Bioworld , Bloomington, IN, diluted 1:5,000) were used as internal references. All antibodies were verified to work with chicken samples previously in the lab. VersaDoc 4000MP system (Bio-Rad, Hercules, CA) was employed to capture the images and Quantity One software (Bio-Rad, Hercules, CA) was used to analyze the band density.

Table 2. Nucleotide sequences of primers used for MeDIP.

Target genes	Primer sequences (5′ to 3′)	PCR products, bp
VIM		
Segment 1	F: GTGGGGACGCCGCTCTT	181
	R: GGGTGCTGGACGTGATGTAG	
Segment2	F: GGGACGCCGCTCTTCTT	110
	R: GAGGAGTTCTTGCTGCTGGT	
StAR		
Segment 1	F: CAGGGACACCTCGGTTCTTC	177
	R: GCTGTTATCCCAATGGAGCG	
Segment 2	F: CTCGGGGTCTTTCATTGCCA	129
	R: CAGGGAGCAGGCGATAAGAT	
Segment3	F: GGCGGTTTCTGTTCAGAGGT	160
	R: : CAGAGCAACACCCCAAACAC	
CYP11A1		
Segment 1	F: TAAGGGCCGTGTTTTGGAGG	194
	R: TGGGGACTCAGCAGATTTCG	
Segment 2	F: AACTGACAGCGTAATGCCCA	164
	R: AAAGAGGGGGTTGGAAACGG	
Segment3	F: CAGGGTATGGGTTGCAGGTT	116
	R: GTGGAAAACCCCCATCGTCT	
SF-1		
Segment 1	F: CATTTACCCCCGCAAACACC	187
	R: CTCTAGCAGGTTCAAGGTCCC	
Segment 2	F: TATCGCCAAAGTCCTCACCG	193
	R: CTCCATCCACGCGGCTTATC	
GR		
Segment 1	F: ACAGGGTTTTGCCATGTTGC	147
	R: CCACCTCTCAACTCCGCTTT	
Segment 2	F: TGATTCTGTAACCCGGCACC	
	R: CTGCTAGGCCTAAGCGAGTGGT	198
Segment3	F: CACACTAACTGGGTCCCGC	
	R: AACGTGGATTGGGTTTTGCG	144
DAX1		
Segment 1	F: AAGTGATCTGCCTGTCTCGG	170
	R: TTGAGGCTGGGAGCAAATGT	
Segment 2	F: GCAATAGCTTCGCACAGGAAA	178
	R: GCAAGCAGCTTTCTGTACCTG	
Segment3	F: CATCGTTTCACCCACAGGAC	185
	R: TCTTGGTTCAGAGGGTTGGT	

Methylated DNA Immunoprecipitation Analysis

Methylated DNA immunoprecipitation (**MeDIP**) analysis was performed according to our previous publication (Abobaker et al., 2019). Briefly, high-quality genomic DNA was isolated from adrenal

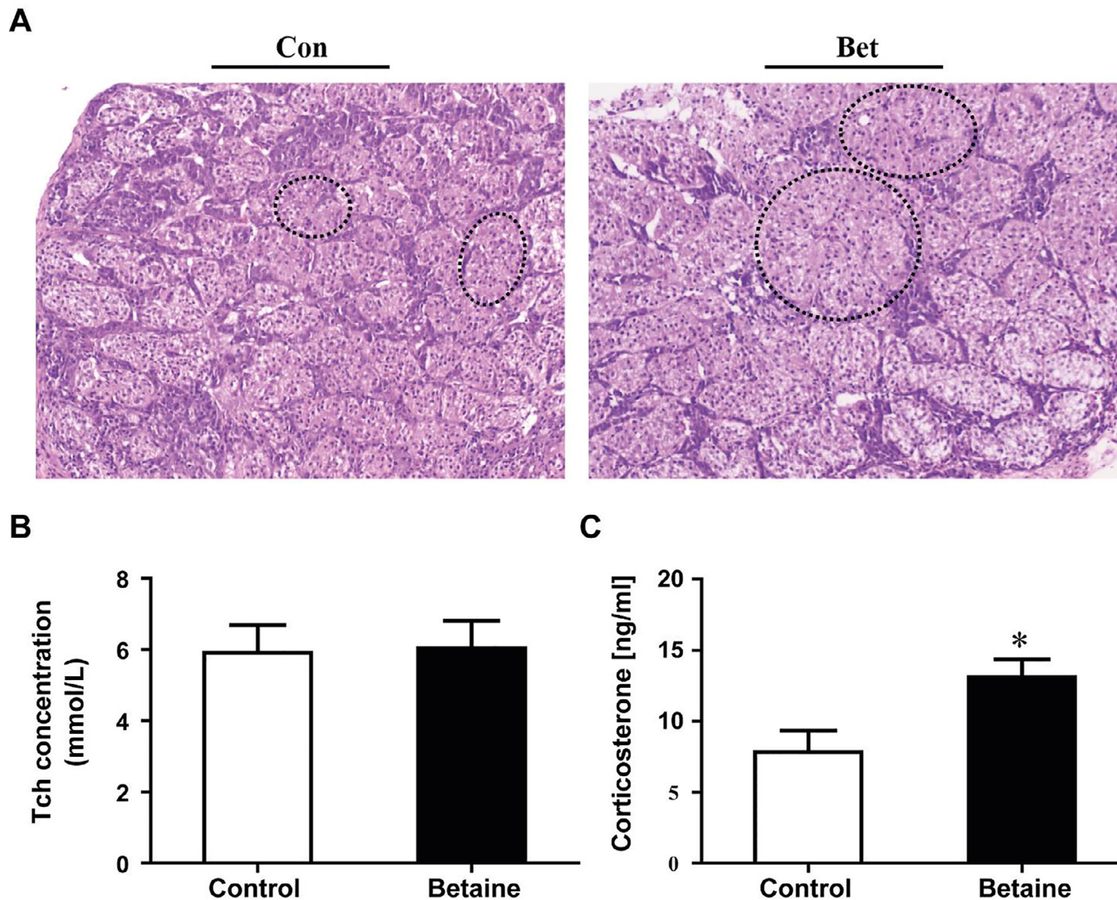

Figure 1. Effect of betaine on plasma concentration of corticosterone. (A) Hematoxylin and eosin staining; (B) total plasma cholesterol; (C) plasma corticosterone. Values are means ± SEM, $*P < 0.05$, compared with control (n = 9). Control = egg injected with 100 μL saline only; Betaine = egg injected with 2.5 mg betaine dissolved in 100 μL saline.

glands and sonicated to produce small fragments ranging from 300 to 1,000 bp. Two micrograms of fragmented DNA were heat-denatured to produce single-stranded DNA, and a portion of the denatured DNA was stored as input DNA. The immunoprecipitation was performed over-night at 4°C with 2 μg antibody against 5-methyl cytosine (ab10805, Abcam, Cambridge, UK). Pretreated protein A/G agarose beads (40 mL, 50% slurry, sc-2003, Santa Cruz, Dallas, TX) were used to capture the antibody/DNA complexes. The beads bound to immune complexes were washed to eliminate nonspecific binding and resuspended in 250 mL digestion buffer containing proteinase K. Finally, the MeDIP DNA was purified. A small aliquot of MeDIP DNA and control input DNA was used to amplify the proximal promoter sequence of chicken corticosterone biosynthesis genes by real-time PCR with specific primers listed in Table 2. Data were normalized to the input and presented as the fold change relative to the average value of control group.

Statistical Analysis

Data are presented as means ± SEM. Comparisons were performed using independent-samples *t*-test with SPSS v18.0 for Windows. The data was considered statistically significant when the *P*-value was less than 0.05.

RESULTS

Effect of Betaine on Adrenal Histological Analysis and Plasma Concentration of Corticosterone

In ovo injection of betaine at E11 led to an increase in the number of corticoid cells stained with Hematoxylin and Eosin in the adrenal glands of the betaine group compared to the control (Figure 1A). Concurrently, higher concentration ($P < 0.05$) of corticosterone was determined in the plasma of betaine group compared to the control (Figure 1C).

Effect of Betaine on Adrenal Expression of Steroidogenic Genes

In ovo injection of betaine significantly ($P < 0.05$) enhanced *MC2R* mRNA expression at E19. Concurrently, genes linked to cholesterol trafficking such as translocator protein (***TSPO***) and *StAR* that facilitate the entrance of cholesterol to the mitochondria were significantly ($P < 0.05$) upregulated at the level of both mRNA and protein (Figures 2A and 2B). Moreover, the downstream enzymes, such as cytochrome P450 family 21 subfamily A member 1 (***CYP11A1***), 3β-hydroxysteroid dehydrogenase (***3βHSD2***) and cytochrome P450 family 21 subfamily A member 2 (***CYP21A2***), were also activated at the

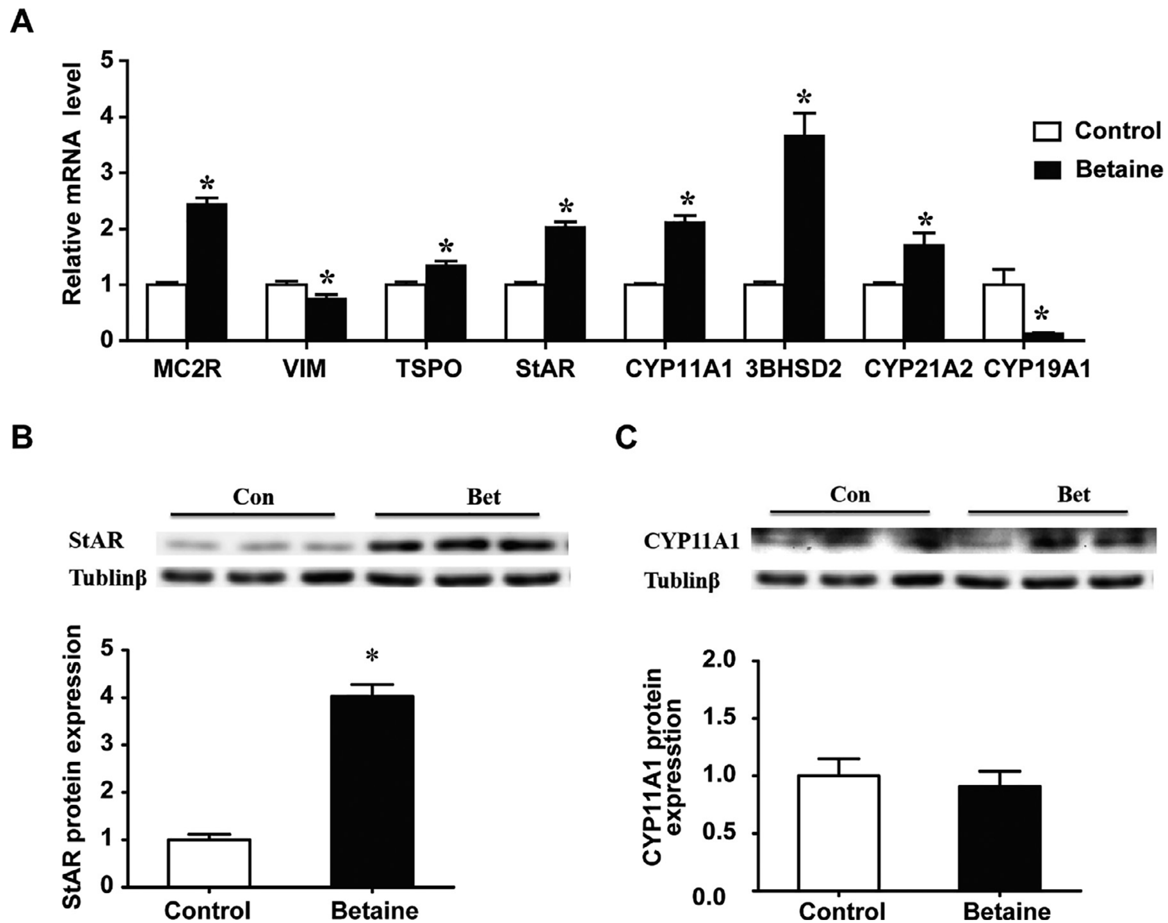

Figure 2. Effect of betaine on adrenal expression of steroidogenic genes. (A) Steroidogenic genes mRNA expression; (B) StAR protein level; (C) CYP11A protein level. Values are means ± SEM, $*P < 0.05$, compared with control (n = 5 for control group and 4 for the betaine group) for the mRNA and (n = 4) for the protein. Control = egg injected with 100 μL saline only; Betaine = egg injected with 2.5 mg betaine dissolved in 100 μL saline.

level of mRNA. Nevertheless, the protein expression of CYP11A1 did not change (Figure 2C).

Effect of Betaine on Adrenal Expression of Cholesterol Metabolic Genes

The adrenal expression of cholesterol biosynthetic genes, such as *SREBP1* and *SREBP2*, 3-hydroxy-3-methylglutaryl-CoA synthase 1 (***HMGCS***), 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (***HMGCR***), low density lipoprotein receptor (***LDLR***) and acetyl-CoA acetyltransferase 2 (***ACAT2***), were significantly ($P < 0.05$) elevated, while *ABCA1* remained unchanged in the betaine group compared to the control (Figure 3A). In addition, the protein expression of HMGCR and LDLR were significantly ($P < 0.05$) increased, while SREBP1 did not show significant difference between the 2 groups (Figure 3B).

Effect of Betaine on Adrenal Expression of Transcription Factors Regulating Steroidogenesis

SF-1 was significantly increased at mRNA level ($P < 0.05$) in the adrenal glands of E19 female fetuses in the betaine group compared to the control group (Figure 4A), whereas mRNA expression of dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome gene 1 (*DAX1*) was significantly decreased in the betaine group in comparison with the control group (Figure 4B). In addition, the protein content of GR and CREB was significantly ($P < 0.05$) enhanced (Figures 4D and 4F), despite unaltered mRNA levels (Figures 4C and 4E).

Effect of Betaine on Adrenal Expression of Methionine Metabolic Genes

The mRNA abundance of methionine metabolic genes, *AHCYL, BHMT, MAD2B*, and *DNMT1*, was significantly ($P < 0.05$) higher in betaine group compared with the control, while *GNMT1* was downregulated significantly (Figure 5A). The protein expression of AHCYL (Figure 5B), GNMT1 (Figure 5D) and DNMT1 (Figure 5E) was significantly ($P < 0.05$) increased in the betaine group compared to the control group.

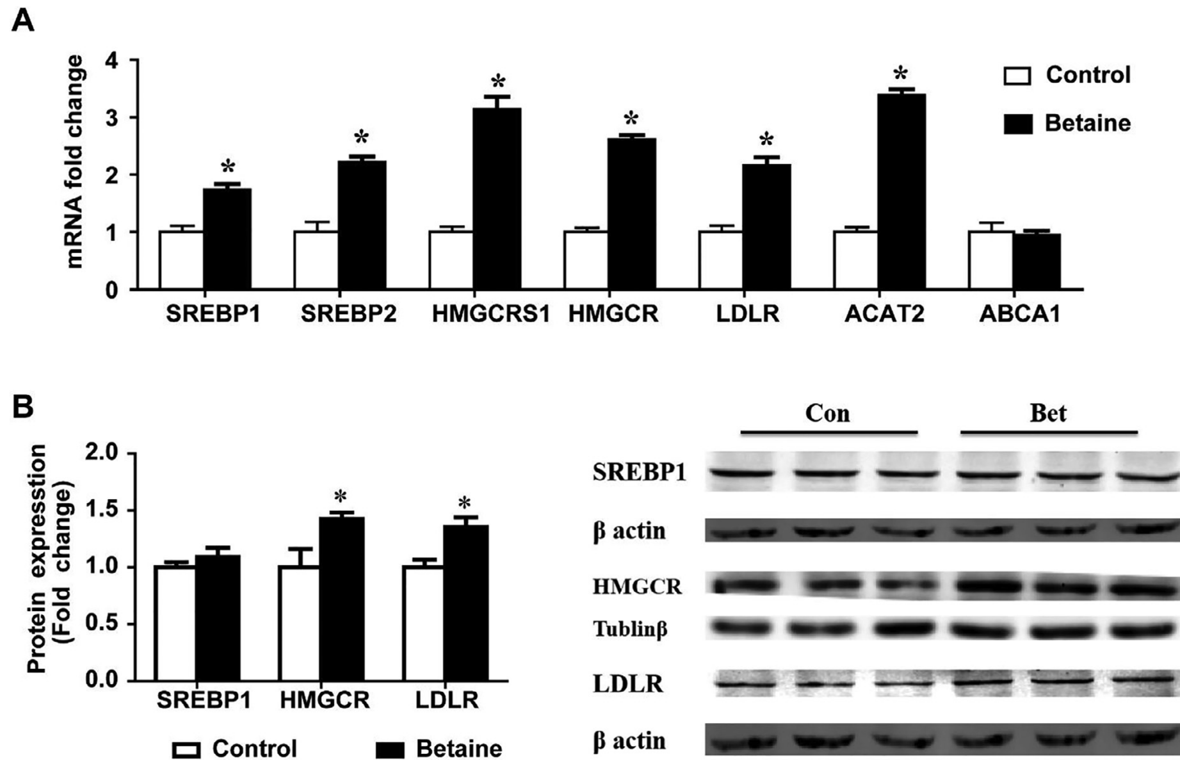

Figure 3. Effect of betaine on adrenal expression of cholesterol metabolic genes. (A) Cholesterol biosynthetic genes mRNA expression; (B) SREBP1, HMGCR, and LDLR protein level. Values are means ± SEM, $*P < 0.05$, compared with control (n = 5 for control group and 4 for the betaine group) for the mRNA and (n = 4) for the protein. Control = egg injected with 100 μL saline only; Betaine = egg injected with 2.5 mg betaine dissolved in 100 μL saline.

Effect of Betaine on DNA Methylation on the Promoter of Steroidogenic Genes

Different segments (S) of the promoter sequences of chicken Vimentin (***VIM***), *StAR* and *CYP11A1* (Figures 6A, 6C, and 6E) were analyzed with MeDIP-PCR technique. In ovo injection of betaine at E11 resulted in significantly ($P < 0.05$) decreased level of DNA methylation on the S2 of *VIM* and *StAR* gene promoters (Figures 6B and 6D), together with significantly ($P < 0.05$) hypomethylated S1, S2, and S3 on *CYP11A* gene promoter (Figure 6F).

Effect of Betaine on DNA Methylation on the Promoter of Transcription Factors

Moreover, the promoter sequences of genes coding for relevant transcription factors, including *SF-1, GR*, and *DAX1*, were also analyzed with MeDIP-PCR (Figures 7A, 7C, and 7E). There was no difference in the level of DNA methylation detected for *SF-1* gene promoters (Figure 7B), while some sequences in the promoter *of GR* and *DAX1* genes showed significant ($P <$ 0.05) hypomethylation in betaine group (Figure 7F).

DISCUSSION

Chicken eggs contain a significant quantity of betaine which is perceived as indispensable nourishment to developing embryo (Hu et al., 2015). Plasma CORT is vital to embryonic development, and relatively higher level of CORT is required to start up the chick's hatching process (Mashaly, 1991). A previous study pointed out that serum CORT concentration is low in chicken embryos before E9, which remains low until it rises around E14-E16 (Zheng et al., 2008). In this study, betaine injected at E11 in ovo increased plasma CORT concentrations in female fetuses at E19. It is likely that enhanced plasma CORT contributes to the growth promoting effects of betaine in the chicken via improving the fetal development and the hatching process.

In the present study, the CORT elevating effects of betaine in prehatching chicken fetuses were allied with modified expression of *MC2R* which modulates adrenal glands growth and function. This result is in line with previous findings that ACTH acts on *MC2R* mostly within the Zona fasciculate, to increase adrenal cell volume and drive glucocorticoid production (Chida et al., 2007; Ferreira et al., 2007). The observed increase in *MC2R* at E19 is associated with improved adrenal growth which is confirmed by the increased number of the adrenal corticoid cell in the betaine group. This is also in line with the study of Schulte et al. (2007) who found increased MC2R mRNA levels in mouse adrenal glands during fetal development, from E11 to birth.

Cholesterol is the prerequisite substrate for adrenal synthesis of steroid hormones, where *SF-1* acts as a trigger for both cholesterol biosynthetic and steroidogenic genes (Inoue et al., 2016; Baba et al., 2018). In this

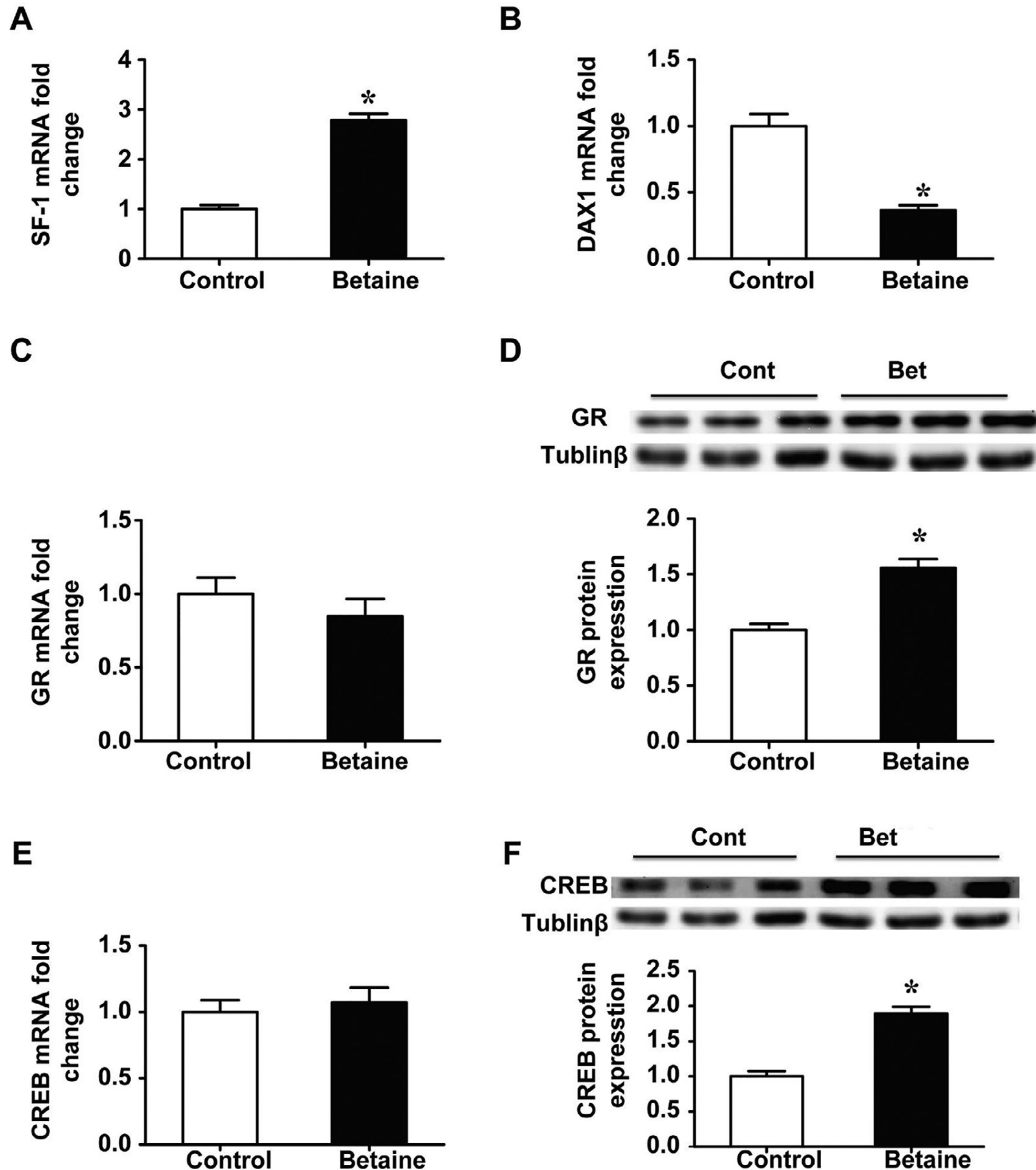

Figure 4. Effect of betaine on adrenal expression of transcription factors regulating steroidogenesis. (A) SF-1 mRNA expression; (B) DAX1 mRNA level; (C) GR mRNA expression; (D) GR protein expression; (E) CREB mRNA expression; (F) CREB protein level. Values are means ± SEM, $^{*}P < 0.05$, compared with control (n = 5 for control group and 4 for the betaine group) for the mRNA and (n = 4) for the protein. Control = egg injected with 100 μL saline only; Betaine = egg injected with 2.5 mg betaine dissolved in 100 μL saline.

study, we found that the expression of cholesterol metabolic genes was markedly upregulated in the adrenal gland of betaine-treated fetuses coupled with increased expression of SF-1. Moreover, higher expression of *SREBP1* and 2, together with higher *HMGCR*, indicate enhanced cholesterol biosynthesis in the adrenal glands. Alongside corticosterone generating enzymes such as TSOP, *StAR, CYP11A, CYP21A*, and *3BHSD3* were also more abundantly expressed in adrenal glands of betaine-treated fetuses at E19. These results imply that the genes coding for cholesterol biosynthesis and steroidogenesis are synegistically regulated in the adrenal glands of chicken fetuses in response to betaine exposure. Nevertheless, such synergistic regulation appears to be age dependent. In an earlier study, we reported that pullets derived from betaine-supplemented laying hens show suppressed CORT production despite increased cholesterol biosynthesis in the adrenal glands via inhibition of cholesterol shuttling to mitochondria for CORT biosynthesis (Abobaker et al., 2019). This contradiction in the findings may be due to difference in the age of chicken at time of sampling or betaine exposure.

Adrenal organogenesis and functionality are known to be regulated by *SF-1* as enhancer and *DAX-1* as the repressor in reciprocal genetic cascades (Hammer et al., 2005). We found that in ovo betaine treatment at E11 repressed gene expression of *DAX1*. This is concurrent with a study using adrenocortical tumor cell line treated with ACTH, in which reduced expression of *DAX1* was associated with up regulation of all the corticoid synthetic genes (Ragazzon et al., 2006). It is also consistent with studies in hormone

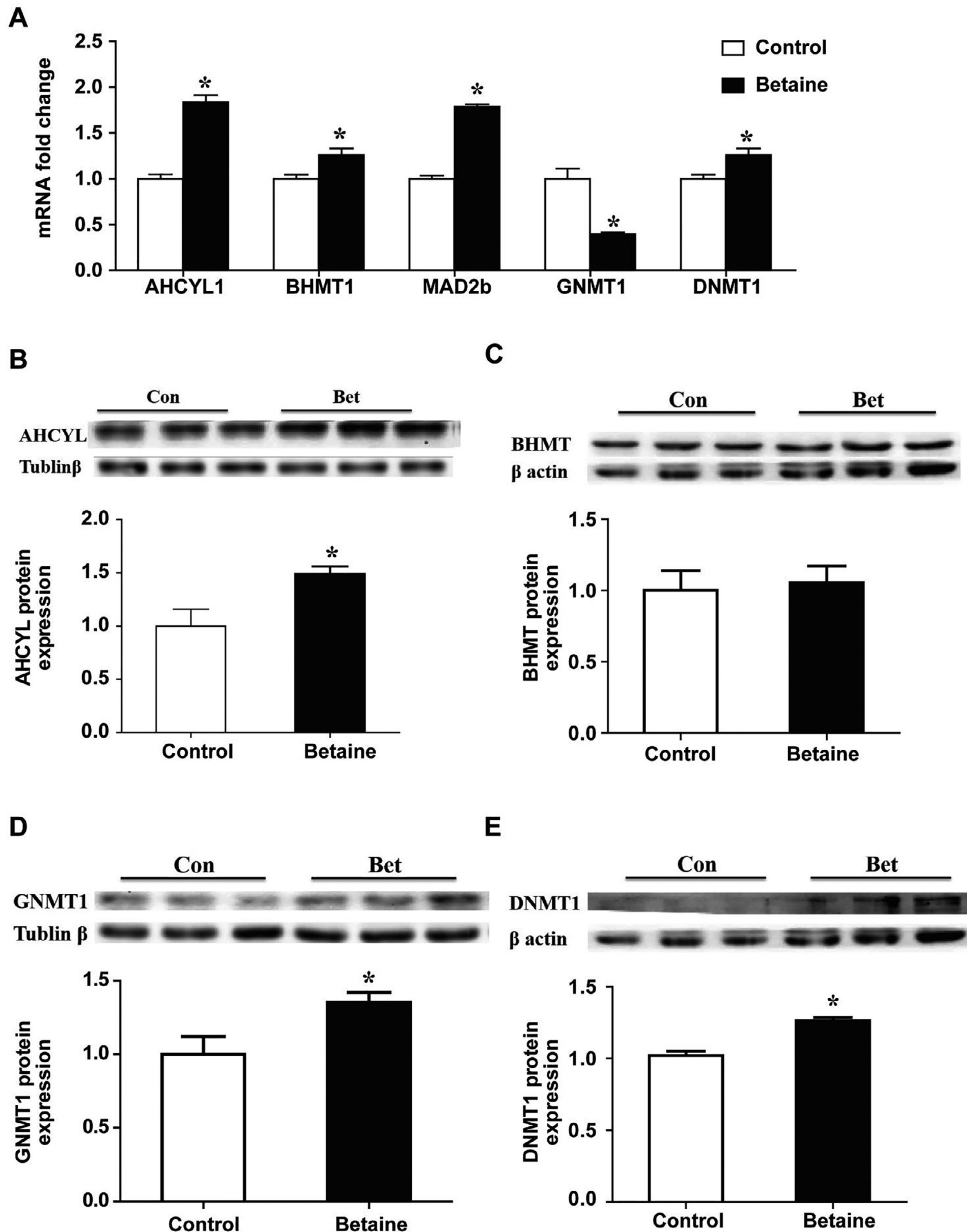

Figure 5. Effect of betaine on adrenal expression of methionine metabolic genes. (A) Methionine metabolic gene expression; (B) AHCYL protein expression; (C) BHMT protein expression; (D) GNMT1 protein expression; (E) DNMT1 protein expression. Values are means ± SEM, $^{*}P < 0.05$, compared with control (n = 5 for control group and 4 for the betaine group) for the mRNA and (n = 4) for the protein. Control = egg injected with 100 μL saline only; Betaine = egg injected with 2.5 mg betaine dissolved in 100 μL saline.

stimulated primary cultures of adrenal zona glomerulosa (Jo and Stocco, 2004), ovarian granulosa cells (Osman et al., 2002), and MA-10 Leydig cell lines (Yazawa et al., 2003). However, the detailed mechanism eliciting the suppression of *DAX-1* expression is obscure. Our data proposed a crucial role of betaine on the equilibrium between the *SF1* and *DAX1* levels during the steroidogenesis in the adrenal glands of pre-hatched chicken fetuses. However, further in-depth study is needed to understand the regulatory

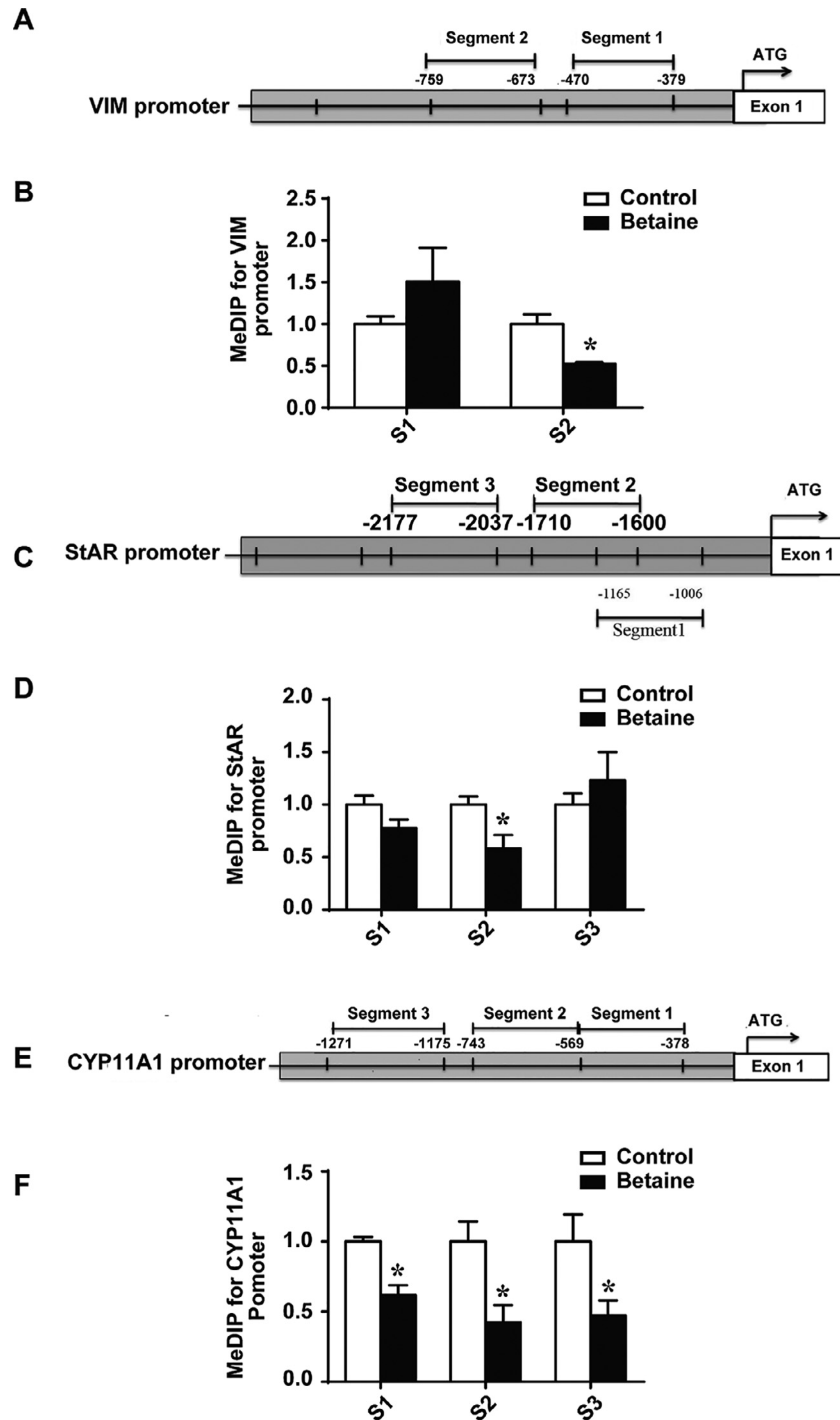

Figure 6. Effect of betaine on DNA methylation at the promoter of steroidogenic gene. (A) Schematic diagram showing the amplified segments (S) on the promoter sequence of VIM. (B) DNA methylation status on the promoter of VIM; (C) schematic diagram showing the amplified segments (S) on the promoter sequence of Star; (D) DNA methylation status on the promoter of StAR; (E) schematic diagram showing the amplified segments (S) on the promoter sequence of CYP11A1 (S); (F) DNA methylation status on the promoter of CYP11A1 Values are means ± SEM, $*P < 0.05$, compared with control (n = 4). Control = egg injected with 100 μL saline only; Betaine = egg injected with 2.5 mg betaine dissolved in 100 μL saline.

mechanisms of betaine in the interplay between *DAX1* and *SF-1* during the developmental stage of adrenal glands in the chicken.

Genes involved in methyl transfer are important for maintaining appropriate DNA methylation which is critical for normal development of organisms (Chen and Li, 2006). Betaine is a primary methyl provider for the epigenetic regulation of gene expression through DNA methylation (Anderson et al., 2012; Day and Kempson, 2016). Our data showed that in ovo injection of betaine enhances the expression of methionine cycle enzymes such as *DNMT1, AHCYL1, BHMT* and *MAT2b,* which coincided with promoter hypomethylation of some target genes like *VIM, StAR, CYP11A1*, and *GR* in the adrenal glands. Our results are consistent with previous reports in which exposure to betaine led to hypomethylation on promoters of some functional genes in the chicken

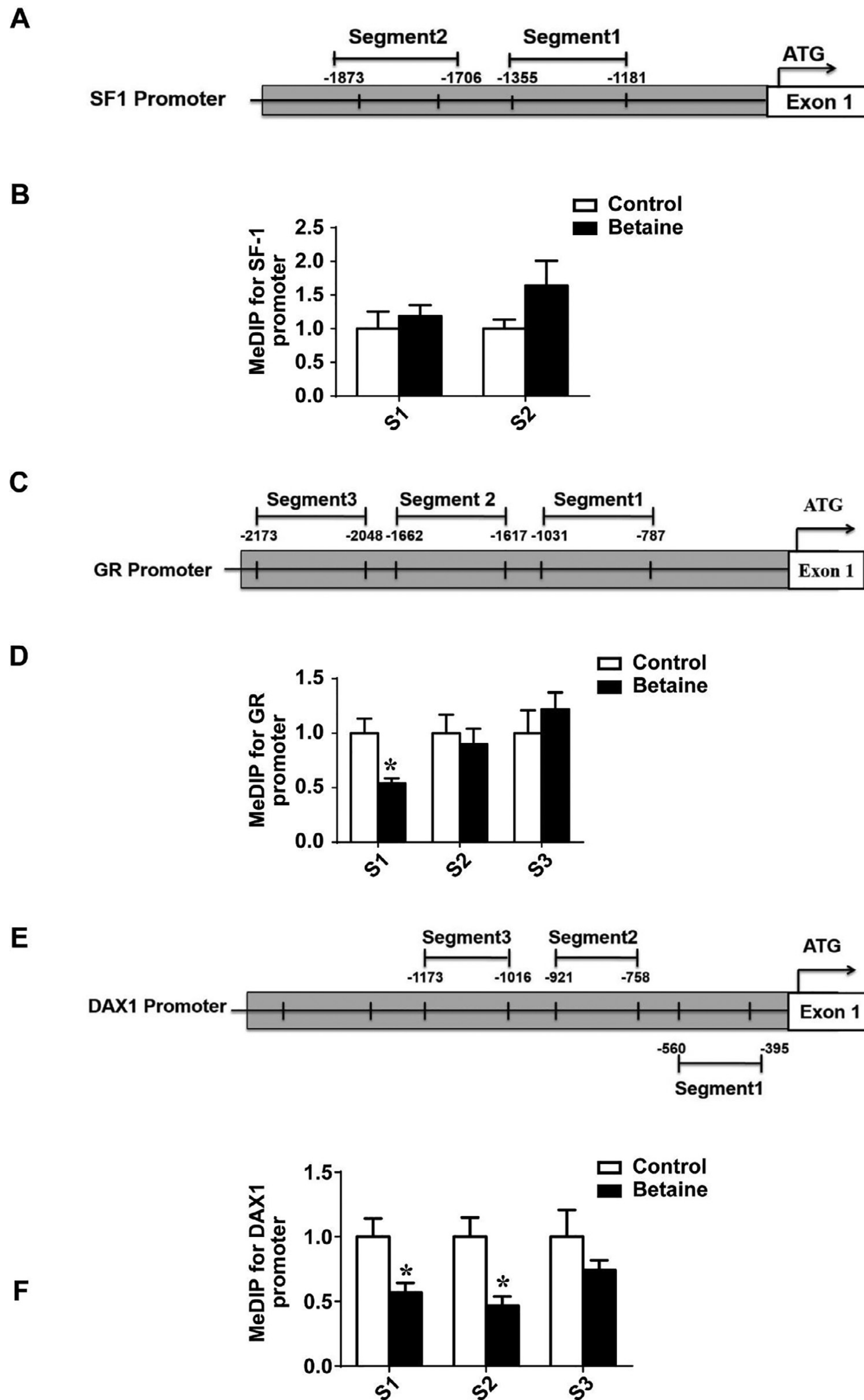

Figure 7. Effect of betaine on DNA methylation at the promoter of transcription factors. (A) Schematic diagram showing the amplified segments (S) on the promoter sequence of SF-1. (B) DNA methylation status on the promoter of SF-1; (C) schematic diagram showing the amplified segments (S) on the promoter sequence of GR; (D) DNA methylation status on the promoter of GR; (E) Schematic diagram showing the amplified segments (S) on the promoter sequence of DAX 1; (F) DNA methylation status on the promoter of DAX1; Values are means ± SEM, $*P < 0.05$, compared with control (n = 4). Control = egg injected with 100 μL saline only; Betaine = egg injected with 2.5 mg betaine dissolved in 100 μL saline.

(Omer et al., 2018; Hu et al., 2020). Interestingly, in this study *DAX1* promoter region was hypomethylated although its expression was downregulated. Obviously, more in-depth investigations are required to reveal the mechanisms by which betaine induces the locus-specific modulation of DNA methylation and age-dependent regulation of steroidogenic gene expression in the adrenal glands of chickens.

CONCLUSIONS

This study indicates that in ovo injection of betaine at E11 activates adrenal expression of steroidogenic genes in E19 fetuses, possibly by regulation of *SF-1* and *DAX1* through epigenetic mechanisms, mainly DNA methylation. Pre-hatch alterations in adrenal functional genes may cause long-lasting impacts on performances at hatching and later in life. Follow-up studies are needed to evaluate the fetal programming induced by in ovo injection of betaine on the adrenal function and stress-coping characteristics later in life.

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DISCLOSURES

None of the authors have any conflicts of interest to declare.

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