

Article

Effects of Betaine on DNA Methylation Level, Expression Level, and Fat Synthesis of *VNN1* in Goose Hepatocytes

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Simple Summary

This study demonstrates for the first time in goose hepatocytes that betaine ameliorates fat accumulation by epigenetically modulating the *VNN1* gene. The novel mechanism involves betaine's ability to increase DNA methylation levels in the *VNN1* promoter region, which is associated with the downregulation of *VNN1* expression. This epigenetic regulation occurs alongside the suppression of key lipogenic genes (*FAS*, *SCD*, *SREBPQ*), establishing a direct link between betaine-induced hypermethylation of *VNN1* and the reduction of hepatic lipid synthesis in a waterfowl model of steatosis.

Abstract

This experiment was conducted to explore the effects of betaine on the DNA methylation level, expression level, and fat synthesis of *VNN1* in goose hepatocytes by isolating the primary hepatocytes of goose at the cellular level and constructing a fatty degeneration model of goose hepatocytes. In the study, 23-day-old Taizhou goose embryos were used as the research object, and free fatty acid (PA:OA = 2:1) was used to induce steatosis of goose primary hepatocytes. The experiment was randomly divided into seven groups: control group, steatosis model group, and betaine (2, 10, 25, 50, 100mM) group. After 24 h of cell culture, cell viability, oil red O staining, and lipid metabolism-related indicators in cell supernatant were measured, and cells were collected to determine *VNN1*, *FAS*, *ACC*, *SCD*, *SREBPQ* gene expression and *VNN1* promoter region DNA methylation level. (1) The addition of 0.5 mM fatty acids successfully determined the degeneration of goose hepatocytes. The levels of TG and LDL were significantly increased ($p < 0.05$), and the level of HDL was significantly decreased. (2) The addition of 100 mM betaine significantly reduced TG levels, and 10, 25, 50, and 100 mM betaine significantly reduced LDL levels. The addition of betaine had no significant effect on HDL level compared to the FFA group ($p > 0.05$), although a significant overall model effect was observed. Oil red O staining showed that the area of lipid droplets in cells with 50 mM betaine decreased most significantly. (3) The expression levels of *VNN1*, *FAS*, *ACC*, *SCD*, and *SREBPQ* genes in the fat model group were significantly higher compared to the control group ($p < 0.05$), and the DNA methylation level in the promoter region of the *VNN1* gene decreased. (4) The addition of 2, 10, 25, 50, and 100 mM betaine significantly reduced the expression of *VNN1*. The expression of *FAS* in 2, 10, and 25 mM betaine groups significantly decreased. Betaine at 10 and 100 mM significantly reduced the expression of *SREBPQ*, but it showed no significant effect on *ACC* expression. Addition of 2, 50, and 100 mM betaine led to an increased DNA methylation status at the *VNN1* gene promoter region. In summary, the



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addition of betaine can reduce the expression of fatty acid synthesizing genes such as *FAS*, *SCD*, and *SREBPQ*, down-regulate the expression level of the *VNN1* gene, increase the DNA methylation level of the *VNN1* promoter region, and reduce lipid deposition in goose liver steatosis cells.

Keywords: betaine; goose primary hepatocytes; *VNN1*; DNA methylation

1. Introduction

Excessive deposition of abdominal fat is a serious problem faced by the goose industry in recent years. Excessive abdominal fat not only significantly reduces the slaughter rate and carcass quality of goose meat but also causes other problems, such as feed waste and environmental pollution [1]. The addition of essential nutrients is an important measure to control fat deposition [2]. Betaine, also known as tri-methyl glycine, has three active methyl groups in its molecule. Compared with choline and methionine, betaine is a more effective methyl donor [3]. Therefore, the role of betaine in goose liver fat synthesis is of great significance. Goose liver is an important organ for the de novo synthesis of fatty acids in the body and has a strong ability to accumulate lipids. Therefore, the synthesis of fatty acids in the liver directly affects the amount of abdominal fat deposited in geese [3,4]. Previous research shows that betaine reduces fat accumulation and reduces abdominal fat rate by reducing feed intake and increasing lipolysis [5]. Transcriptome sequencing found that *VNN1* is a key candidate gene that is downregulated in response to a lower betaine rate [6]. Furthermore, at the cellular level, it was found that betaine inhibited lipid accumulation in goose liver cells and that methylation in the *VNN1* promoter region negatively regulated its expression [7]. Although previous studies have shown that betaine has a certain regulatory effect on goose liver lipid metabolism, they mostly focused on a single concentration or under specific conditions [5]. Based on the above background, in the current study, we used primary hepatocytes of Taizhou goose embryos as the research object, and constructed a goose hepatocyte fatty degeneration model by adding free fatty acids to the culture medium. On this basis, we further studied the effect of betaine on goose hepatocytes to better demonstrate the regulatory effect of betaine on fat metabolism and *VNN1* expression under different conditions.

2. Materials and Methods

2.1. Test Materials

2.1.1. Obtaining Primary Goose Hepatocytes

The eggs in this experiment were obtained from the National Waterfowl Gene Bank (Taizhou, China). The incubation temperature of the eggs was 37.5 °C–38.5 °C, the humidity was 65–70%, the turning cycle was 2 h/time, and the eggs were candled every 7 days during the incubation process to remove unfertilized eggs. On 22 days of incubation, normal embryo eggs were taken for testing.

2.1.2. Main Test Reagents

The betaine used in this study was obtained from Beijing Xin Dayang Technology Development Co., Ltd., Beijing, China, in the form of white powder or microgranules, good flowability, and a purity of $\geq 98\%$; sodium oleate was purchased from MacLean Reagent Co., Ltd., Shanghai, China; sodium palmitate was purchased from Aladdin Shanghai China; fetal bovine serum, red blood cell lysis buffer, Oil Red O staining kit, and PBS buffer were purchased from Beijing Solebow Co., Ltd., Beijing, China; type IV collagenase

digestion solution was obtained from biosharp; DMEM high glucose medium and penicillin/streptomycin double antibody mixture were purchased from GIBCO, Thermo Fisher Scientific, San Diego, CA, USA; primers were purchased from Jinweizhi Biotechnology Co., Ltd., Suzhou, China; FastPure comPlex Cell/Tissue total RNA extraction reagent, HiScriPtIII RT SuPerMix for QPcr(+gDNAwiPer) reverse transcription kit, and SYBR Master Mix kit were purchased from Novozyme Biotech Co., Ltd., Bagsvaerd, Denmark.

2.1.3. Reagent Preparation Method

- (1) Digestion solution of 0.2% collagenase: We centrifuged 100 mg of type IV collagenase powder and added 1 mL of DMEM medium to fully dissolve it. Then we transferred it to a 50 mL centrifuge tube and made the volume up to 50 mL. We washed the tube repeatedly, pipetted and dissolved it, and finally filtered it with a 0.22 μ m filter membrane to sterilize it, and then stored it at -20°C .
- (2) DMEM complete medium: We added 5 mL of fetal bovine serum (FBS) to 45 mL of DMEM medium (FBS: DMEM = 1:9), then added penicillin/streptomycin (double antibody) mixture (added 10 μ L of double antibody to every 1 mL of medium), mixed thoroughly, filtered and sterilized with a 0.22 μ m filter membrane, and stored at 4°C .
- (3) 0.1 mol/L NaOH solution: We dissolved 0.04 g NaOH in 10 mL double distilled water and mixed well for later use.
- (4) 10% BSA stock solution: 1 g BSA was dissolved in 10 mL PBS buffer and dissolved in a 70°C water bath.
- (5) Fatty acid formulation: Fatty acid stock solution, 100 mM: ① Weigh 189.25 mg sodium oleate (OA) and 85.47 mg sodium palmitate (PA) in a 15 mL centrifuge tube, add 10 mL, 0.1 mol/L NaOH solution and dissolve in a 70°C water bath (store at -20°C); ② fatty acid, 10 mM: Take 1 mL of 100 mM fatty acid stock solution and add it to 9 mL BSA mother solution and mix well for later use. ③ solutions of 2 mM, 1 mM, 0.5 mM, 0.25 mM of fatty acid: After using up the 10 mM fatty acid solution, dilute it at 1:4, 1:9, 1:19, 1:39, and filter it at 0.22 μ m for sterilization.

2.1.4. Primer Design

NCBI (<https://www.ncbi.nlm.nih.gov/>) was used to query gene information for primer design. See Table 1 for fluorescent quantitative PCR primers.

Table 1. qPCR primers.

Gene	Primer Sequence (5'-3')	Product Size (bp)	Reference
<i>β-actin</i>	F: GAAATCGTGCGTGACATCAA R: GCAGGACTCCATACCCAAGA	198	XM_066977989.1
<i>VNN1</i>	F: GAACTCAAGCACGAAGATGGAA R: CAGGTCTGTGCTCCTACACTTGA	189	XM-048075766.1
<i>FAS</i>	F: ATGCTTCAGGAGATGGGTATTG R: CCATCAGTGTTACTCCCAGCA	118	XM-048050305.1
<i>ACC</i>	F: TCCAGCAGAACCGCATTGACAC R: GTATGAGCAGGCAGGACTTGGC	187	XM-048074039.1
<i>SCD</i>	F: TAACGGCTGGATCTCATCGC R: AGAGAACTTGTGGTGGACGC	149	XM-048069234.1
<i>SREBPQ</i>	F: GGTCCGGGCCATGTTGA R: CAGGTTGGTGCGGGTGA	175	XM-048066212.1

2.2. Test Method

2.2.1. Isolation and Culture of Primary Goose Hepatocytes

- (1) We took out 22-day-old Taizhou goose embryos from the incubator, soaked the eggshells in Sanisol, took out the goose embryos aseptically and put them in a tray, and rinsed them twice with PBS. Primary hepatocytes were isolated and pooled from the livers of 8–10 embryos per experimental replicate to ensure sufficient cell yield. All eggs were from the same closed Taizhou goose breeding flock within the National Waterfowl Gene Bank, ensuring a consistent genetic background.
- (2) We removed the abdominal feathers of the goose embryo, cut and opened the abdomen with ophthalmic scissors, took 3/4 of the main body of the left and right liver lobes, and rinsed with PBS several times until there was no obvious blood on the surface of the liver.
- (3) We transferred the liver to a new culture dish and minced it with ophthalmic scissors until it became a paste.
- (4) We added about 4 times the volume of 0.1% collagenase digestion solution to the chopped liver and place in a 37 °C water bath for 20 min, and gently shook every 5 min to decompose the tissue blocks for full digestion.
- (5) We added an equal volume of complete medium to the digested liver to terminate digestion, then gently pipetted with a cut pipette tip to ensure that no large pieces of tissue remained.
- (6) We filtered the cells twice with a 220-mesh filter, transferred them to a 15 mL centrifuge tube, centrifuged at 800 rpm for 8 min, and discarded the supernatant.
- (7) Added red blood cell lysis buffer at a volume ratio of 1:3, pipette and mixed, placed on ice for lysis, shaken once every 3 min, repeated 3 times, centrifuged at 700 rpm for 9 min, and discarded the supernatant.
- (8) We added preheated DMEM medium at a volume ratio of 1:3, mixed well by pipetting, centrifuged at 700 rpm for 5 min, and discarded the supernatant.
- (9) Repeat (8).
- (10) We added complete medium to dilute and count.
- (11) After culturing for a certain period of time, various relevant indicators were measured.

2.2.2. Screening of Fatty Acid Concentration

Cell Plating

Concentrations of 1.3×10^5 cells/well were added to a 96-well plate and cultured in a CO₂ incubator (37 °C, 5% CO₂) for 24 h. The non-adherent cells were washed with PBS, replaced with serum-free medium, and returned to the incubator for starvation culture for 12 h to homogenize the cells. The plates were then treated with medium containing different concentrations (0.25, 0.5, 1, 2 mM) of fatty acids and normal medium. All fatty acid concentrations and the control were tested on the same 96-well plate, with each treatment condition replicated in 6 wells ($n = 6$).

CCK8 Test Cell Viability

After 24 h of fatty acid treatment, 10 µL of CCK8 solution was added to each well along the cell plate wall and gently shaken to avoid bubble formation. The cells were then incubated in an incubator for 1–4 h, and the absorbance was measured at a wavelength of 450 nm.

2.2.3. Establishment of Fatty Degeneration Model and Experimental Grouping

The construction of the goose liver cell fatty degeneration model was prepared according to the method in Table 2. The experimental groups are as follows:

Table 2. Experimental grouping for the establishment of the fatty degeneration model.

Reagent Name	Dosage
RNase-free ddH ₂ O	to 16 µL
4 × gDNA wiper Mix	4 µL
RNA	Total RNA: 1 pg ^{−1} µg
5 × HiScript IIIqRT SuperMix	4 µL

Note: Before adding 5 × HiScript IIIqRT SuperMix, use a pipette to gently mix the enzyme-free water, 4 × gDNA wiper Mix, and RNA. Utilize a 42 °C metal bath for 2 min.

2.2.4. Determination of Lipid Metabolism-Related Indices in Cell Supernatant

After 24 h of cell culture, the culture medium was aspirated, centrifuged at 1000 rpm/min for 10 min, and the supernatant was taken for determination. There were 4 replicates in each group, with a total of 28 samples. The operation was strictly in accordance with the instructions of the kit, and then the corresponding OD value was measured at a specific wavelength on the microplate reader, and the results were calculated using MS Excel.

2.2.5. Oil Red O Staining

The cell culture medium was removed, the cells were washed twice with PBS, and then processed according to the instructions of the Oil Red O staining kit.

2.2.6. Total RNA Extraction, Reverse Transcription, and Real-Time Fluorescence Quantification

(1) Extraction of total cellular RNA

After discarding the cell culture supernatant, wash twice with pre-cooled PBS, add 500 µL Buffer CRL to each well of the 12-well plate to fully cover the cell surface, and then use a pipette to repeatedly blow the cells to make them fall off.

All the above lysate products were transferred to RNA Columns I, centrifuged at 12,000 rpm (13,400 × g) for 30 s, and the extra liquid was discarded.

We added 500 µL Buffer RWA (with anhydrous ethanol) to RNA Columns I, centrifuged at 12,000 rpm (13,400 × g) for 30 s, and discarded the extra liquid.

We added 500 µL Buffer RWB (with anhydrous ethanol) to RNA Columns I, centrifuged at 12,000 rpm (13,400 × g) for 1 min, and discarded the waste liquid.

We placed RNA Columns I back into the collection tube and centrifuged at 12,000 rpm (13,400 × g) for 1 min to prevent alcohol contamination.

We carefully transferred RNA Columns I to a new 1.5 mL enzyme-free tube, used a pipette to pipette 20 µL of enzyme-free water vertically into the center of the adsorption column membrane in the 1.5 mL tube, incubated at normal temperature for 1 min, then centrifuged at 12,000 rpm (13,400 × g) for 1 min to collect RNA. The extracted RNA concentration was tested using a nucleic acid concentration detector. The OD₂₆₀/OD₂₈₀ of 1.8~2.0 indicates that the RNA purity is good and can be directly used in downstream reverse transcription experiments.

(2) Cellular reverse transcription

HiScript IIIqRT SuperMix for QPCR(+gDNAwiPer) reverse transcription kit was used to reverse transcribe RNA, using 20 µL of reverse transcription system:

- Genomic DNA removal, reaction system, see Table 2.
- RNA reverse transcription to cDNA: 37 °C, 15 min; 85 °C, 5 s. After reverse transcription, it can be used directly in downstream experiments or stored at −20 °C.

(3) Real-time fluorescence quantification

According to the Novozyme SYBR Master Mix kit, the expression of *VNN1*, *FAS*, *ACC*, and other gene mRNAs was detected, with β -actin as the reference gene. The primer sequences are shown in Table 1. Quantitative operations must be performed on a clean bench and ice box. Prepared the following mixed solution for each reaction: 2 $\times$ ChamQ SYBR QPCR Master Mix 10 μ L, 0.4 μ L each of forward and reverse primers, 2 μ L of cDNA template, 7.2 μ L of ddH₂O. The reaction mixtures were aliquoted into a 96-well qPCR plate. The plate was sealed with a sealing all around, gently pop out the small bubbles in the well, then centrifuged briefly, and put it in the machine. Performed qPCR reaction under the following conditions: 95 $^{\circ}$ C 30 s, 40 cycles (95 $^{\circ}$ C 10 s, 60 $^{\circ}$ C 30 s). Made 3 parallel wells for each sample, took the average CT value for experimental data analysis, and used the $2^{-\Delta\Delta CT}$ method to calculate the relative expression of each gene mRNA.

2.2.7. BSP Detection of DNA Methylation Level in the VNN1 Promoter Region

After testing the quality and concentration of the DNA samples, sodium bisulfite was used for methylation detection, a PCR reaction solution was prepared, and amplification was performed using the amplification PCR reaction program (PCR amplification primers are shown in Table 3); the PCR purified product was connected to the Puc18-T vector to construct 10 clones. The connection condition was retained overnight at 18 $^{\circ}$ C. After the connection product transformation, it was prepared into competent cells and spread on an ampicillin plate that had been previously coated with 20 μ L 100 mM IPTG and 100 μ L 20 mg/mL X-gal, and then inverted and cultured until the next day. When performing blue-white screening, pick out the white colonies growing on the IPTG/X-gal plate, transfer them to a liquid culture medium containing ampicillin with a toothpick, and culture them overnight at 37 $^{\circ}$ C. After the target DNA fragment was cloned, 1% agarose gel electrophoresis was used to detect and identify the colony PCR. Plasmid extraction and plasmid sequencing M13+/- primer sequencing. The obtained gene sequence was analyzed using the software DNASTar v.18.0.1, the sequence was aligned using the SeqMan software, and the methylation dot plot was made using QUMA. The detected VNN-1 gene fragment contains 12 CPG sites. The positions of these sites in the sequence were as follows: 7, 41, 70, 89, 132, 173, 187, 200, 208, 233, 245, 257.

Table 3. Reverse transcription reaction system.

Gene	Primer Sequence (5'-3')	Product Size (bp)
<i>VNN1</i>	TTTATAGTTTTGAGTTAGGGGTGG AACCRAAATCCTCTAAAACTATCC	311 bp

2.2.8. Data Analysis

The experimental data were initially recorded and processed using Excel 2025, and the data were analyzed using SPSS 26.0 software. The fluorescence quantitative data were calculated by the $2^{-\Delta\Delta CT}$ method to calculate the relative expression of each gene mRNA. Image J 1.54 software was used to analyze the gray value of the protein bar, and Prism 10 software was used to complete the drawing. Duncan's one-way ANOVA method was used to analyze and process the biochemical index data, and Tukey's Honest Significant Difference (HSD) was used for multiple comparisons. $p < 0.05$ was used as the criterion for judging the significance of the difference. The whole data was presented as mean and standard error (SEM).

3. Results

3.1. Establishment of a Fatty Degeneration Model of Goose Liver Cells

Four concentrations of fatty acids, 0.25 mM, 0.5 mM, 1 mM, and 2 mM, were used to treat goose primary hepatocytes to induce cell degeneration. Oil red staining showed that all four concentrations could cause cell degeneration, as shown in Figure 1. Cell viability was measured by CCK-8, and the results showed that there was no significant difference between the 0.5 mM fatty acid treatment group and the control group ($p > 0.05$), as shown in Figure 2. Therefore, 0.5 mM was used to establish the fatty acid model.

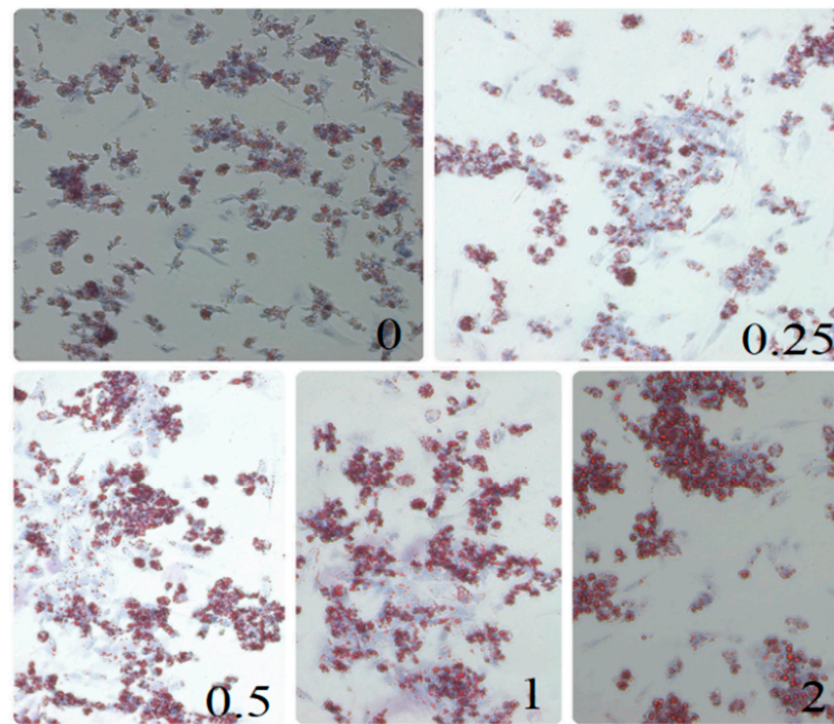


Figure 1. Morphological observation of goose hepatocytes treated with oil red staining for 24 h (0, 20×, control group; 0.25, 20×, 0.25 mmol/L FFA group; 0.5, 20×, 0.5 mmol/L FFA group; 1, 20×, 1 mmol/L FFA group; 2, 20×, 2 mmol/L FFA group; scale = 20 μ m.).

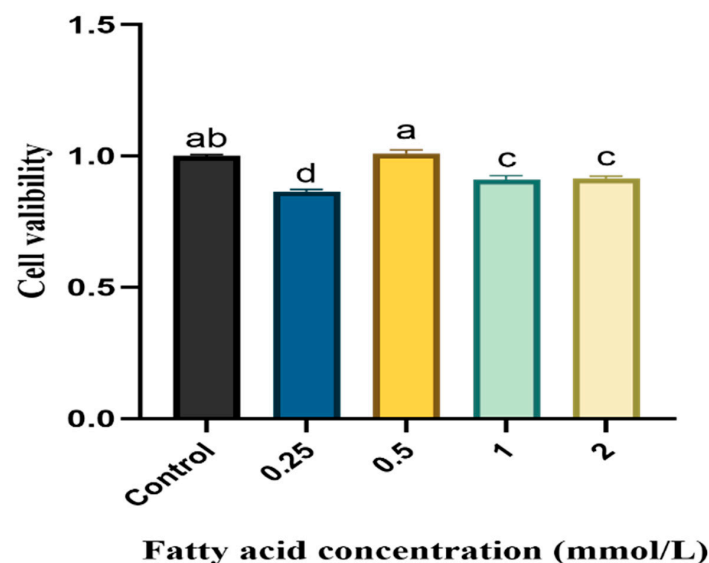


Figure 2. Effects of different concentrations of fatty acids on the activity of goose hepatocytes. Note: Data results were expressed as mean $\pm$ SEM. Different lowercase letters indicate $p < 0.05$.

3.2. Effects of Betaine on Lipid Droplet Morphology and Activity of Steatotic Cells in Goose Liver

Different concentrations of betaine (0, 2, 10, 25, 50, 100 mM) were used to treat fatty acid-induced goose liver steatosis cells, and the lipid accumulation of goose liver cells was observed by Oil Red O staining, shown in Figure 3. A small number of red lipid droplets were visible in the control group. Compared with the control group, the lipid droplets in the FFA group increased significantly, the lipid droplet area became larger, and they were relatively aggregated. In Figure 4, compared with the FFA group, the lipid droplets in the betaine group decreased but not significantly ($p > 0.05$). As shown in Figure 4, the addition of betaine had no significant effect on the viability of goose liver steatosis cells.

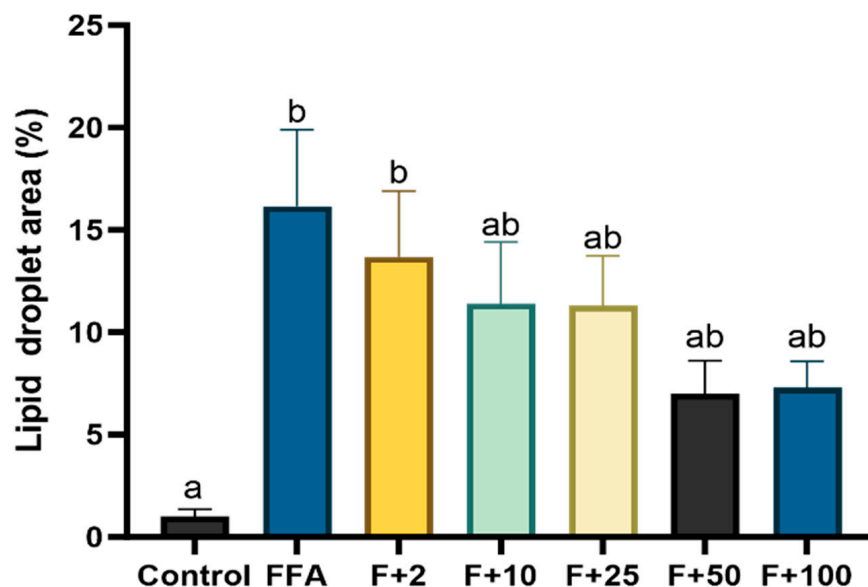


Figure 3. The proportion of lipid droplet area in goose hepatocytes under different concentrations of betaine. Note: Data results were expressed as mean $\pm$ SEM. Different lowercase letters indicate $p < 0.05$.

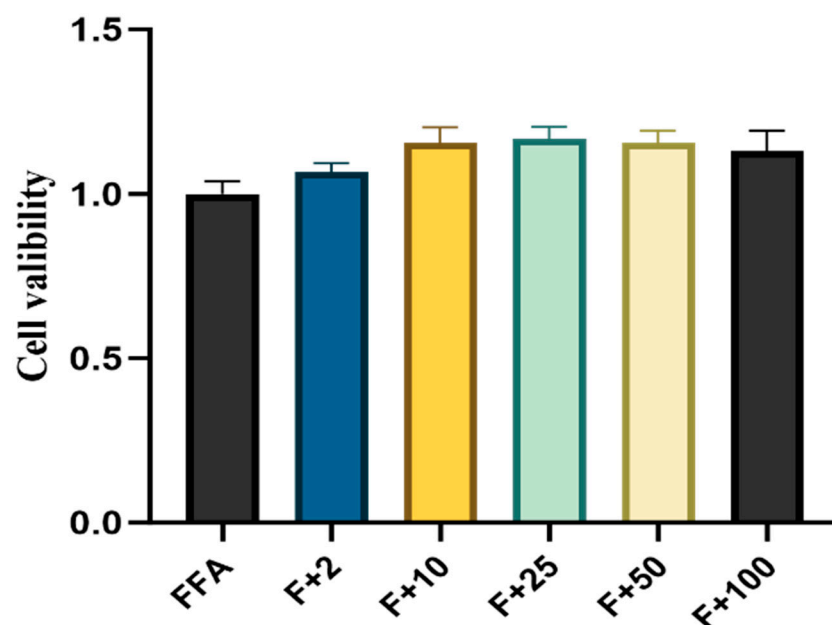


Figure 4. Effects of different concentrations of betaine on the viability of goose liver steatosis cells.

3.3. Effects of Betaine on Lipid Metabolism Indices in the Supernatant of Goose Liver Steatotic Cells

The effects of different concentrations of betaine on TG, HDL, and LDL in the supernatant of goose liver steatosis cells are presented in Table 4. The TG and LDL levels of the FFA group were significantly higher than those of the control group and the HDL level was significantly lower than control group ($p < 0.05$); compared with the FFA group, the TG levels of the 2 mM, 10 mM, 25 mM, and 50 mM betaine groups had no significant effect on the cell supernatant, while the 100 mM betaine group had a significant effect on the supernatant TG level. The LDL levels of the 10 mM, 25 mM, 50 mM, and 100 mM betaine groups were significantly lower than those of the FFA group, and the 2 mM betaine group had no significant effect on the FFA group. The HDL levels of the various betaine treatment groups had no significant effect compared to the FFA group ($p > 0.05$).

Table 4. Effects of different concentrations of betaine on TG, HDL, and LDL in the supernatant of goose liver steatosis cells.

Item	Control	FFA	2	10	25	50	100	SEM	<i>p</i> -Value		
									Between Groups	Linear	Secondary
TG (mmol/L)	0.173 b	0.193 a	0.185 ab	0.184 ab	0.184 ab	0.181 ab	0.180 b	0.014	0.005	0.155	0.952
HDL (mmol/L)	0.050 a	0.041 b	0.042 ab	0.043 ab	0.048 ab	0.046 ab	0.045 ab	0.008	0.023	0.807	0.334
LDL (mmol/L)	0.015 b	0.021 a	0.018 ab	0.016 b	0.017 b	0.016 b	0.016 b	0.005	0.002	0.027	0.232

Note: Control = control group, FFA = fatty acid group, 2 = 2 mM betaine group, 10 = 10 mM betaine group, 25 = 25 mM betaine group, 50 = 50 mM betaine group, 100 = 100 mM betaine; the difference was significant ($p < 0.05$). Data results were expressed as mean $\pm$ SEM. Different lowercase letters indicate $p < 0.05$.

3.4. Effects of Betaine on mRNA Expression of Genes Related to Lipid Metabolism in Steatotic Cells of Goose Liver

The effects of different concentrations of betaine on the mRNA expression of *VNN1*, *FAS*, *ACC*, *SCD*, and *SREBPQ* genes in cells are shown in Figure 5. As shown in, the *VNN1* gene expression in the 2 mM, 10 mM, 25 mM, and 50 mM betaine groups was significantly lower than the control group shown in Figure 5A. However, when betaine was added at 2 mM, 10 mM, and 25 mM, the *FAS* gene was downregulated significantly in the FFA group ($p < 0.05$), see Figure 5B. Whereas the betaine group had no significant effect on the *ACC* gene expression in fatty degeneration cells (Figure 5C). The *SCD* gene expression in the 2 mM, 25 mM, and 50 mM betaine groups was significantly lower than that in the FFA group, as shown in Figure 5D. Compared with the FFA group, the *SREBPQ* gene expression in the 10 mM and 100 mM betaine groups was significantly reduced, as presented in Figure 5E.

3.5. Effects of Betaine on DNA Methylation in the Promoter Region of *VNN1* in Goose Liver Steatotic Cells

The effect of betaine on DNA methylation in the promoter region of *VNN1* in goose liver steatosis cells was assessed. The DNA methylation level in the promoter region of the *VNN1* gene in the FFA group was lower than that of the control group, and the addition of 2 mM, 50 mM, and 100 mM concentrations of betaine can increase the DNA methylation in the promoter region of the *VNN1* gene (Figure 6).

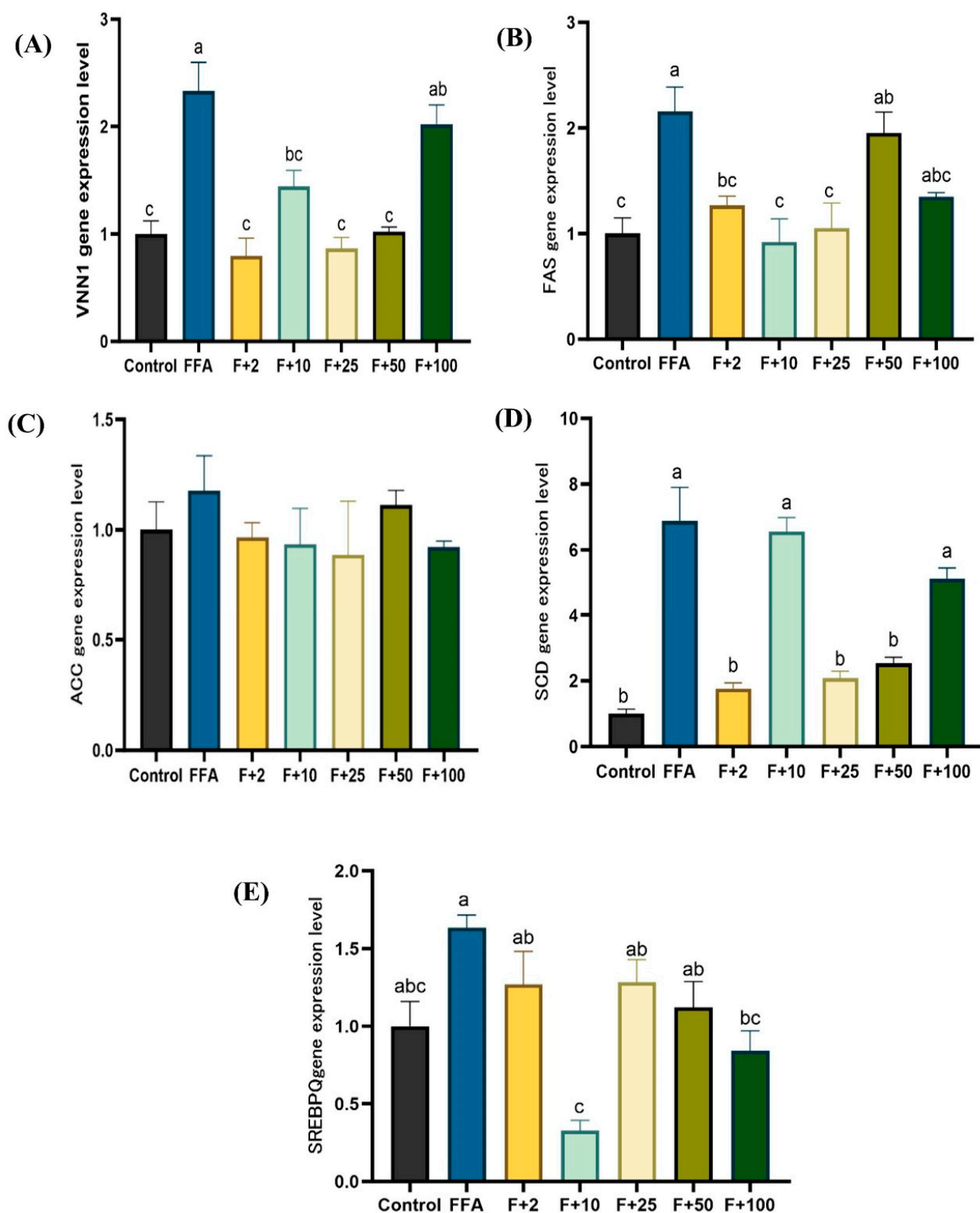


Figure 5. Effects of different concentrations of betaine on the expression of *VNN1*, *FAS*, *ACC*, *SCD*, and *SREBPQ* genes in goose liver steatosis cells. Note: Data results were expressed as mean $\pm$ SEM. Different lowercase letters indicate $p < 0.05$.

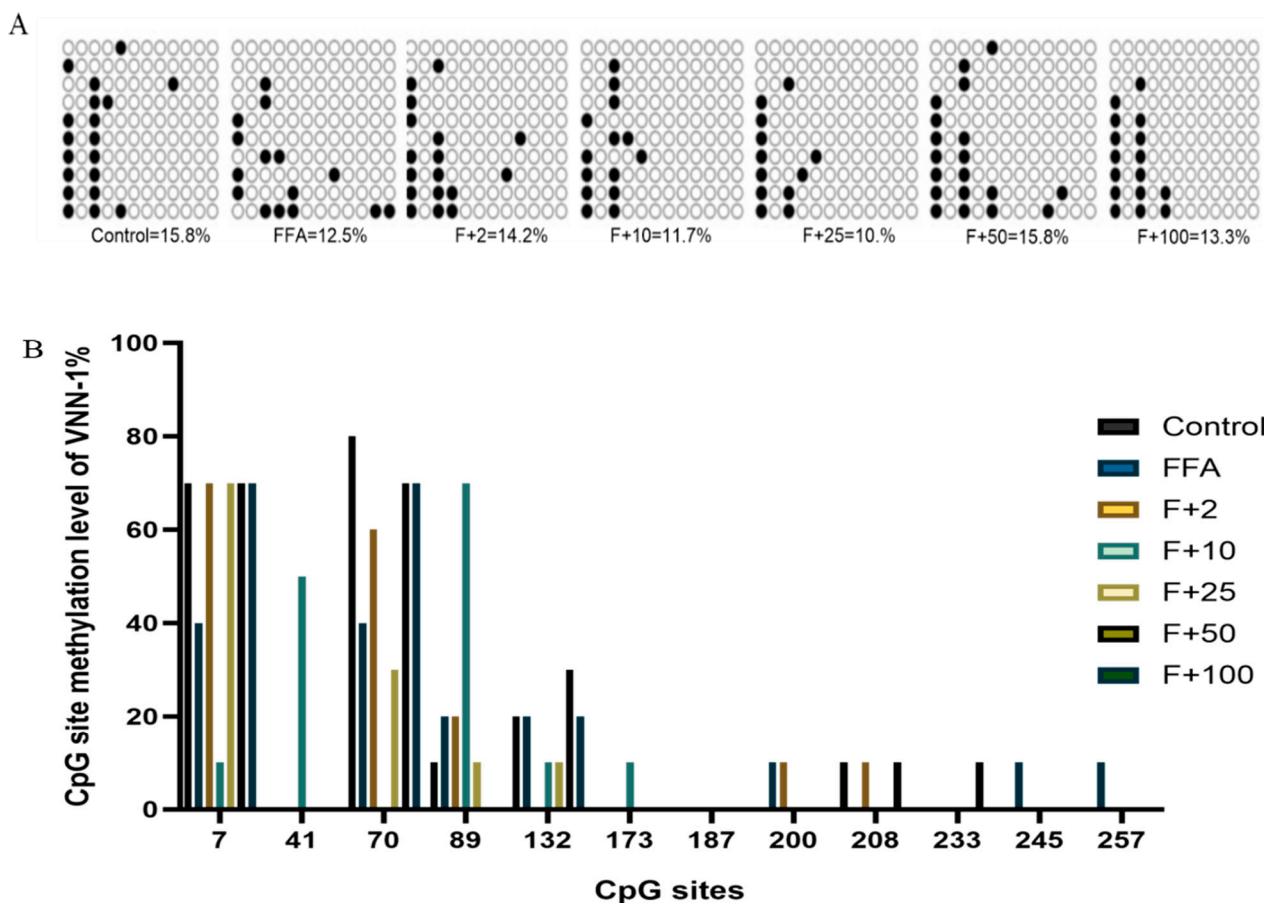


Figure 6. Effects of betaine on DNA methylation of the *VNN1* gene promoter region in goose hepatic steatosis cells. Note: Control = control group, FFA = fatty acid group, F + 2 = 2 mM Betaine, F + 10 = 10 mM Betaine, F + 25 = 25 mM Betaine, F + 50 = 50 mM Betaine, F + 100 = 100 mM Betaine; Figure (A) is the methylation map measured by BSP. The closing of the round hole indicates that the site is methylated, and the opening of the round hole indicates that the site is not methylated. The methylation of each CpG site was calculated based on the sequencing of 10 clones per sample. Figure (B) is the effect of different concentrations of betaine on the methylation level of each CpG site in the *VNN1* promoter region of each group.

4. Discussion

The large amount of fat stored in the liver of geese is due to the imbalance between the storage and secretion of exogenous and endogenous lipids [8]. Exogenous lipids are mainly ingested through the diet, while endogenous lipids are produced through the de novo synthesis pathway in the liver. Under normal circumstances, the liver can effectively regulate the synthesis, storage, and secretion of lipids to maintain the balance of lipid metabolism [9,10]. However, under certain conditions, such as overfeeding or genetic factors, this balance may be broken, leading to excessive accumulation of lipids in the liver [4,11]. This study mainly used goose primary hepatocytes as the research object and constructed a hepatocyte fatty degeneration model by adding free fatty acids (FFA) to the culture medium. FFA is mainly composed of PA and OA (PA: OA = 2:1). Zhao Chenbao and Li Lihong successfully constructed a hepatocyte fatty degeneration model with 1 mmol/L FFA [6,12]. In this experiment, four concentrations of fatty acids, 0.25 mmol/L, 0.5 mmol/L, 1 mmol/L, and 2 mmol/L, were selected. Through Oil Red O staining observation and cell viability determination, it was finally determined that 0.5 mmol/L FFA was used to construct a hepatocyte fatty degeneration model. From the results, compared with the control group, the TG and LDL contents in the degeneration group increased, and

obvious red lipid droplets appeared in the cells [6,12–14]. The lipid droplets were larger and aggregated, indicating that goose primary hepatocytes had undergone fatty degeneration.

Betaine, as a quaternary ammonium alkaloid, is widely present in animals and plants. It is an intermediate product of choline metabolism in animals and plays an important role in the metabolism of nutrients [3]. Researchers fed mice with betaine and found that it could reduce alcoholic fatty liver in mice [4]. It was established that as the dose of betaine increased, the levels of TG and LDL in broiler serum decreased, and at the same time, the abdominal fat weight of broilers decreased [1,13,15]. They found that the addition of betaine reduced the synthesis of fatty acids, thereby reducing the abdominal fat deposition of broilers [16]. Scientists studied the effect of adding betaine to a low-protein diet on commercial pigs and found that the addition of betaine reduced the levels of LDL and HDL. The team's study found that the addition of betaine to cell culture medium significantly reduced LDL [10]. The results of this experiment showed that compared with the FFA group, the TG and LDL content in the betaine group was significantly reduced, and different concentrations of betaine had no significant effect on cell viability [11,17]. The effect of betaine on lipid accumulation in FFA-induced goose liver cells was further observed by Oil Red O staining, and it was found that betaine at various concentrations could improve lipid accumulation to a certain extent, especially at the 50 μ M concentration of betaine [18,19]. This shows that betaine plays an important role in fat metabolism, and betaine supplementation can inhibit fat deposition [20].

VNN1 is a hydrolase of pantothenic acid, the raw material for fatty acid synthesis, and plays an important role in the synthesis of fatty acids [21]. In the biosynthesis of fatty acids, ACC plays an important catalytic and rate-limiting role [22]. FAS is the last step in the fatty acid biosynthesis pathway that catalyzes fatty acid production and is physiologically regulated by energy balance [23]. Similarly, SCD and SREBPQ are also closely related to fatty acid synthesis. In order to further explore the effect of betaine on steatosis cells in goose liver, this experiment also used qPCR technology to detect the regulatory effect of betaine on genes related to fatty acid synthesis [11]. Wang studied the effect of adding betaine to the diet on the expression of *VNN1* in the liver of geese and found that the expression of *VNN1* and *FAS* genes decreased [7]. It was studied that the effects of betaine on alcoholic fatty liver and liver lipid metabolism disorders in mice [24]. Our results showed that betaine improved liver lipid metabolism by inhibiting the expression levels of *SREBP-1c*, *FAS*, and *SREBP-2* genes. Researchers found in a study on laying hens that adding betaine to the diet of 165-day-old laying hens could significantly reduce the mRNA expression level of *FAS* [9]. He established that the low expression of the *FAS* gene was one of the main reasons for the reduction of fat deposition [25]. The qPCR results of this experiment showed that the addition of betaine could reduce the levels of *VNN1*, *FAS*, *SCD*, and *SREBPQ* genes, which is consistent with the results of previous studies [5,6,15,25]. However, Madeira's research results found that betaine did not significantly change the expression levels of *SCD* and *SREBPQ* in pig livers [10]. This may be due to the different sites of fat deposition in poultry and mammals and the influence of multiple factors [26,27].

In addition to its lipid-lowering effect, betaine is also a highly efficient methyl donor that can replace part of the methionine and choline in the body, thereby improving the efficiency of methyl conversion in the body [17,28]. Therefore, it plays an important nutritional regulatory role in the methyl metabolism process. Our results demonstrated that betaine treatment altered lipid droplet accumulation and downregulated the expression of key lipogenesis-related genes in FFA-induced steatotic goose hepatocytes. The CpG island methylation distribution pattern of *VNN1* in the promoter region was further analyzed by BSP sequencing [2]. The results suggest that betaine can regulate FFA-induced fatty acid degeneration by affecting the transcription of lipogenesis-related genes. DNA methylation

is an epigenetic process affected by the environment. A large number of research results show that there is a negative regulatory relationship between gene expression levels and the degree of methylation in their promoter regions [2,15,16,29,30].

The structure of DNA changes with the methylation of the gene promoter region, hindering the binding between transcription factors and promoters. Transcription factors are key regulatory proteins that initiate gene transcription. They bind to specific sequences on DNA to initiate RNA polymerase binding and transcription. When the promoter is methylated, the accessibility of these binding sites decreases, resulting in the inability of transcription factors to bind effectively, thereby inhibiting gene transcription [31,32]. The results of this experiment also clearly showed at the cellular level that the expression of the *VNN1* gene was reduced and the degree of DNA methylation in its promoter region was increased, indicating that the expression of the *VNN1* gene is regulated by the gene methylation status.

5. Conclusions

In this study, a model of fatty degeneration of goose liver cells was constructed, and it was found that the addition of betaine could alleviate the fatty degeneration of goose liver cells induced by FFA. The expression levels of fatty acid synthesis-related genes *VNN1*, *FAS*, *SCD*, and *SREBPQ* decreased, and the DNA methylation level of the promoter region of the *VNN1* gene increased, indicating that betaine, as a methyl donor, promoted the DNA methylation of *VNN1* and inhibited its expression. This will help to clarify the molecular mechanism of betaine in reducing goose liver fat synthesis and provide new ideas for solving the problem of excessive abdominal fat deposition in meat geese.

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