

# Sodium butyrate alleviates free fatty acid-induced steatosis in primary chicken hepatocytes via the AMPK/PPAR $\alpha$ pathway

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**ABSTRACT** Fatty liver hemorrhagic syndrome (FLHS) is a prevalent metabolic disorder observed in egg-laying hens, characterized by fatty deposits and cellular steatosis in the liver. Our preliminary investigations have revealed a marked decrease in the concentration of butyric acid in the FLHS strain of laying hens. It has been established that sodium butyrate (**NaB**) protects against metabolic disorders. However, the underlying mechanism by which butyrate modulates hepato-lipid metabolism to a great extent remains unexplored. In this study, we constructed an isolated in vitro model of chicken primary hepatocytes to induce hepatic steatosis by free fatty acids (**FFA**). Our results demonstrate that treatment with NaB effectively mitigated FFA-induced hepatic steatosis in chicken hepatocytes by inhibiting lipid accumulation, downregulating the mRNA expression of lipo-synthesis-related genes (sterol regulatory element binding transcription factor 1 (**SREBF1**), acetyl-CoA carboxylase 1

(**ACC1**), fatty acid synthase (**FASN**), stearoyl-CoA desaturase 1 (**SCD1**), liver X receptor  $\alpha$  (**LXR $\alpha$** ), 3-hydroxy-3-methylglutaryl-CoA reductase (**HMGCR**)) ( $P < 0.05$ ), and upregulating the mRNA and protein expression of AMP-activated protein kinase  $\alpha 1$  (**AMPK $\alpha 1$** ), peroxisome proliferator-activated receptor  $\alpha$  (**PPAR $\alpha$** ), and carnitine palmitoyl-transferase 1A (**CPT1A**) ( $P < 0.05$ ). Moreover, AMPK and PPAR $\alpha$  inhibitors (Compound C (**Comp C**) and GW6471, respectively) reversed the protective effects of NaB against FFA-induced hepatic steatosis by blocking the AMPK/PPAR $\alpha$  pathway, leading to lipid droplet accumulation and triglyceride (**TG**) contents in chicken primary hepatocytes. With these findings, NaB can alleviate hepatocyte lipoatrophy injury by activating the AMPK/PPAR $\alpha$  pathway, promoting fatty acid oxidation, and reducing lipid synthesis in chicken hepatocytes, potentially being able to provide new ideas for the treatment of FLHS.

**Key words:** sodium butyrate, free fatty acid, primary chicken hepatocyte, steatosis

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## INTRODUCTION

Fatty liver hemorrhagic syndrome (**FLHS**) is a frequent metabolic disorder in high-producing laying hens featuring hepatic fat accumulation and abnormal lipid metabolism, which can lead to acute mortality and a rapid decline in egg production, causing significant economic losses for the poultry industry. The development of FLHS is influenced by various factors, including environmental conditions, genetic predisposition, hormonal imbalances, nutritional factors, gut microbiota composition, and metabolic status (Wang et al., 2020). Currently, considerable progress has been made in

comprehending FLHS, but the development of robust diagnostic and therapeutic strategies is still at an early stage (Guo et al., 2021; Meng et al., 2021; Liu et al., 2022). Further research and clinical trials are needed to establish more effective approaches for diagnosing and treating FLHS.

Recently, evidence has revealed the vital role of the gut microbiota and associated bacterial metabolites in governing and regulating essential physiological processes and metabolic pathways in the human body (Fang et al., 2022; Palmnäs-Bédard et al., 2022). Gut symbiotic bacteria generate short-chain fatty acids (**SCFA**) via the fermentation of indigestible dietary fibers, among which butyric acid emerges as a principal constituent in this metabolic pathway. Prior studies have shown that butyrate could be utilized as an energy source by colonocytes (Donohoe et al., 2011). Meanwhile, butyrate exerts multiple beneficial effects in mammals. These include the regulation of gut hormone secretion and the inhibition of pro-inflammatory factor

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production (Lin et al., 2012; Matt et al., 2018). These effects are closely associated with the pathogenetic mechanisms involved in developing fatty liver disease. Recently, butyrate has been shown to activate the G protein-coupled receptors, GPR41 and GPR43, which have been implicated in regulating energy metabolism and immune function (Kimura et al., 2011; Chang et al., 2014; Byrne et al., 2015). In addition, sodium butyrate (**NaB**) can enhance peroxisome proliferator-activated receptor  $\alpha$  (**PPAR $\alpha$** ) mRNA transcription level and restore  $\beta$ -oxidation of fatty acids (**FA**) in the mice liver on a high-fat diet, thus reducing liver fat deposition (Sun, 2018). Numerous seemingly contradictory findings show sophisticated interactions between gut microbiota, butyrate content, and host immunity and metabolism (Qin et al., 2012; Zhang et al., 2016; Chen et al., 2020). Nevertheless, the intrinsic mechanisms by which butyrate regulates lipid metabolism in the liver have not been more thoroughly investigated.

AMPK functions as an intracellular receptor and regulator of cellular energy, playing a critical role in maintaining cellular homeostasis. Liver kinase B1 (**LKB1**) regulates the activation of AMPK, which in turn influences the synthesis and breakdown of FA (Shaw et al., 2005). Activation of AMPK inhibits the dimerization of acetyl-CoA carboxylase (**ACC**), subsequently reducing its activity and impeding FA synthesis. Simultaneously, a decrease in malonyl coenzyme A (**malonyl-CoA**) values accelerates FA oxidation within the mitochondria (Munday et al., 1988; Cho et al., 2010). This process is controlled by PPAR $\alpha$ , which detects fluctuations in the body's energy state and regulates FA utilization, ketone synthesis rates, and fat storage under low and high energy conditions. Additionally, PPAR $\alpha$  is a factor in mitochondrial FA transport and regulation of  $\beta$ -oxidation (Hashimoto et al., 2000; Xu et al., 2002). Research has shown that PPAR $\alpha$  can upregulate the expression of CPT1, CPT2, and carnitine acylcarnitine translocase (**CACT**), thereby enhancing lipolysis mediated by lipoprotein lipase (**LPL**) to reduce triglyceride (**TG**) levels, and reducing the secretion of hepatic LPL inhibitor apolipoprotein C-III to improve LPL activity indirectly (Hertz et al., 1995; Schoonjans et al., 1996; Brandt et al., 1998). Consequently, AMPK and PPAR $\alpha$  play crucial roles in sustaining the equilibrium of lipid metabolism. Activation of AMPK and PPAR $\alpha$  using active substances may hold potential as targets for allied diseases.

We previously reported that aberrant activation of the AMPK/PPAR $\alpha$  signaling channel, dysbiosis of the gut microbiota, and reduced production of SCFA, particularly butyric acid, are associated with the development of FLHS (Gao et al., 2019; Huang et al., 2022; Zhou et al., 2022). Therefore, we hypothesize that supplementation of NaB alone could ameliorate lipid accumulation in the livers of FLHS. We further postulated that this amelioration could be linked to regulating crucial genes involved in FA metabolism, such as AMPK/PPAR $\alpha$ . Our results showed that butyrate effectively alleviates lipid accumulation and liver damage induced

by FFA in primary chicken hepatocytes through modulation of the AMPK/PPAR $\alpha$  signaling channel. Based on these findings, the prospective future of NaB is in mitigating steatohepatitis and positioning it as a promising therapeutic intervention for managing FLHS.

## MATERIALS AND METHODS

### **Cell Isolation, Culture, and Treatment**

Referring to the previous culture method for isolation and bio-culture of primary chicken liver cells (Huang, 2019), the 12-day-old chicken embryo of Hy-Line Variety Brown layer chickens were taken for cell experiment in vitro. The livers were chopped into small portions (approximately 1 mm<sup>3</sup>), washed with phosphate buffered saline (**PBS**) 3 to 5 times, then digested with 0.1% collagenase at 37°C for 10 to 15 min. Digestion was terminated by additional Dulbecco's modified Eagle's medium (**DMEM**) (Gibco, CA) containing 10% fetal bovine serum (**FBS**) (Bioind, Israeli). Hepatocytes were collected by filtration through 200-mesh and 500-mesh cell sieves and centrifugation at 4°C, 1,200 rpm for 5 min (Centrifuge 5810 R, Eppendorf, Germany). Hepatocytes were resuspended and diluted in serum-free medium (1 × 10<sup>6</sup> cells/mL), plated into tissue culture plates after cell counting, and then maintained at 37°C and 5% CO<sub>2</sub>. All hepatocytes were gathered for subsequent experiments.

The first experiment (Exp1) examined the toxicity of NaB (Selleck, Houston, TX) in chicken hepatocytes. Cells were taken after 48 h of incubation in 96-well cell culture plates, washed twice, and added to serum-free medium pre-warmed to 37°C respectively containing 0, 0.5, 1, 1.5, and 2 mmol/L NaB. Six replicates were set up for each concentration, and the cells were treated for 24h. Our preliminary data supported the choice of 0.25, 0.5, and 1 mmol/L doses for 24 h, showing remarkable efficacy while preserving adequate cell viability. Based on the previous study, a high-fat cell additive (containing 12 mmol/L sodium oleate (**OA**) and 6 mmol/L sodium palmitate (**PA**); Kunchuang, Xi'an, China) formulated as 1 mmol/L free fatty acids (**FFA**) (keeping the ratio of OA and PA concentrations at 2:1) was selected for the second experiment (Exp2) to establish hepatic steatosis model in primary hepatocytes.

To investigate the roles of AMPK and PPAR $\alpha$  in NaB preventing steatosis of chicken hepatocytes, Comp C (Sigma-Aldrich, Shanghai, China) and GW6471 (Selleck), an ATP-competitive AMPK inhibitor and a selective PPAR $\alpha$  antagonist, were separately used to inhibit AMPK and PPAR $\alpha$  in chicken hepatocellular adipose degeneration model. Twenty-five milligram Comp C powder dissolved in 1.2516 mL DMSO to make a 50 mmol/L masterbatch; 10 mg GW6471 powder dissolved in 1.6138 mL DMSO to make a 10 mmol/L masterbatch. In the third experiment (Exp3), hepatocytes were cultured with/without 0.5 mol/L NaB, 1 mmol/L FFA, and 15  $\mu$ mol/L Comp C, while others were cultured with/without 0.5 mol/L NaB, 1 mmol/L FFA,

and 4  $\mu\text{mol/L}$  GW6471. All hepatocytes were collected for subsequent experiments.

### Calculating Cell Damage

The Cell Counting Kit-8 (CCK-8) (Bimake, Charlotte, NC) served to determine the cell viability. The CCK-8 working solution was formulated at a ratio of 100:10, and 100  $\mu\text{L}$  of the mixture was added to each well and incubated at 37°C for 1 h under dark conditions. The optical density (OD) was determined using a multimode reader (Molecular Devices, Shanghai, China), which was adjusted to 450 nm. Three independent runs of each experiment were performed.

### Determination of Biochemical Indexes Related to Liver Fat Metabolism

After washing twice, the treated cells were scraped off and resuspended in 1 mL PBS. Following centrifugation of the cell suspension at 1,000 rpm for 10 min, the precipitate was taken and added to 300  $\mu\text{L}$  of physiological saline, and the hepatocytes were completely fragmented using a homogenizer (Servicebio, Wuhan, China). Hepatocyte homogenates were measured for TG, total cholesterol (T-CHO), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) using commercially available assay kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China) according to the manufacturer's recommendations.

### Chicken Hepatocytes Oil Red O Staining

Chicken hepatocytes were fixed and stained according to the requirements of the oil red O staining kit (Solarbio, Beijing, China). After the operation, the picture results were used in a microscope to observe and obtain. Microscopic examination showed that the red or orange-red substances were lipid droplets, and the nuclei were blue.

**Table 1.** Sequence of target genes primer.

Gene names	GenBank accession no.	Sequence of primer (5'-3')
<b>AMPK<math>\alpha</math>1</b>	NM_001039603.1	F:CAACTACCTGGCTCCGAGA R:CGAAGTCATCCCGATTATAGCTC
<b>PPAR<math>\alpha</math></b>	NM_001001464	F:ACGGAGTTCCAATCGC R:AAACCTTACAACCTTCACAA
<b>CPT1</b>	AY675193	F:GGAGAACCAAGTGAAGTAATGAA R:TGGAAACGCCACATAAAGGCAGAAA
<b>SREBF1</b>	NM_204126.2	F:CATTGGGTCAACGCTTCTTCGTG R:CGTTGAGCAGCTGAAGGTACTCC
<b>ACC</b>	NM_205505.1	F:TGGAACGGAAACGTCTCGG R:CACAGGTACGCCTTACCGT
<b>FASN</b>	NM_205155	F:AAAGCAATTCTGTCACGGACA R:GGCACCATCAGGACTAAGCA
<b>SCD1</b>	NM_204890.1	F:TAACAGCTGGATCTCACCGC R:CGGAGAACTTGTGGTGGACAA
<b>LXR<math>\alpha</math></b>	NM_204542.2	F:CAACTACCTGGCTCCGAGA R:CGAAGTCATCCCGATTATAGCTC
<b>HMGR</b>	NM_204485.2	F:CTGGGTTGGTTCTGCTTGTCA R:ATTGGTCTCTGCTTGTCA
<b><math>\beta</math>-Actin</b>	NM_205518.1	F:CTGACCCCTGAAGTACCCCAT R:TGTCACTCTCTCTGTGGCTT

### RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

Following the manufacturer's instructions, total RNA was extracted using the TransZol Up reagent (TransGen, Beijing, China), and measured with a GeneQuant 1300 spectrophotometer. Then the mRNA therein was reverse transcribed into cDNA using TransScript Uni All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) (TransGen). Primers for the amplification of *AMPK $\alpha$ 1*, *PPAR $\alpha$* , *CPT1*, sterol regulatory element binding transcription factor 1 (*SREBF1*), *ACC*, fatty acid synthase (*FASN*), stearoyl-CoA desaturase 1 (*SCD1*), liver X receptor  $\alpha$  (*LXR $\alpha$* ), 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGR*) and  $\beta$ -Actin were designed by NCBI and synthesized by Tsingke Biotechnology Company (Beijing, China) (Table 1). The Real-time PC Detection System (Bio-Rad CFX384 Touch, Foster City, CA) was used to conduct real-time quantitative polymerase chain reaction using ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China). A hot start at 95°C for 30 s, denaturation at 95°C for 5 s, and annealing/extension at 60°C for 30 s made up the PCR protocol. Forty cycles were performed in total.  $\beta$ -Actin was used as a housekeeping gene, and the mRNA expression levels of related genes were analyzed using the  $2^{-\Delta\Delta CT}$  approach.

### Protein Extraction and Western Blot

Proteins were detached from hepatocytes using a lysate (RIPA lysate: phenylmethylsulfonyl fluoride: phosphatase inhibitor = 100:1:1) (Solarbio), and protein consistency was assayed with a BCA Protein Assay Kit (Beyotime, Beijing, China). Western Blot was carried out as the method described previously (Zhuang et al., 2019), equal amounts of total cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (**SDS-PAGE**) and analyzed by using primary antibodies as indicated, such as anti-AMP-activated protein kinase  $\alpha 1$  (**AMPK $\alpha 1$** ) (Bioss, Beijing, China), anti-p-AMPK $\alpha 1$  (Bioss), anti-PPAR $\alpha$  (Proteintech, Wuhan, China), anti-carnitine palmitoyl-transferase 1A (**CPT1A**) (Bioss), anti-FASN (Proteintech), anti-SREBF1 (Proteintech). As a reference, glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) (Servicebio) was applied. Corresponding secondary antibodies were also used, such as HRP-conjugated Affinipure Goat Anti-Mouse IgG (H+L) (Proteintech) and HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) (Proteintech). Detection was performed using an ECL chemiluminescent substrate (Abbkine, Wuhan, China). The strips were imaged on a Gel Imaging System (Bio-Rad) and ImageJ software was used to quantify the relative optical density of the bands.

## Statistical Analyses

Statistical comparisons among groups were made by 1-way ANOVA with SPSS 25.0 and presented as mean  $\pm$  standard deviation (mean  $\pm$  SD). At last, data graphs were generated by GraphPad Prism 9 software. Differences were considered significant at  $P < 0.05$  and indicated as follows: ns  $P > 0.05$ ; compared with the Con group, \*  $P < 0.05$ , \*\*  $P < 0.01$ ; compared with the FFA group, #  $P < 0.05$ , ##  $P < 0.01$ .

## RESULTS

### Dysfunction of Lipid Metabolism Brought on by FFA Stimulation in Hepatocytes Can be Mitigated by NaB

Hepatocyte cell viability was not dramatically altered following treatment with 0.5 to 1 mmol/L NaB ( $P > 0.05$ ), whereas it significantly decreased hepatocyte activity at the concentration of 1.5 to 2 mmol/L ( $P < 0.01$ ; **Figure 1A**). Treatment of hepatocytes with low, medium, and high concentrations of NaB, respectively, greatly reduced the large number of lipid droplets produced by FFA stimulation ( $P < 0.01$ ; **Figures 1B** and **1C**). Meanwhile, FFA stimulation markedly enhanced the TG, T-CHO, HDL-C, and LDL-C contents among hepatocytes by comparison with the Control group (**Con**) ( $P < 0.01$ ), and 0.25 to 1 mmol/L NaB treatment significantly inhibited the increasing of TG and LDL-C contents caused by FFA stimulation among hepatocytes ( $P < 0.01$ ; **Figure 1D**). These discoveries demonstrated that NaB suppresses the excessive fat buildup among hepatocytes brought on by FFA activation.

Sodium Butyrate (NaB) Alters Elements involved in Lipid Metabolism and AMPK/PPAR $\alpha$  Correlated Factors Manifestation within Hepatocytes Activated by FFA. Free fatty acids substantially enhanced the quantities of SREBF1, ACC1, LXR $\alpha$  and HMGR mRNA ( $P < 0.01$ ; **Figures 2A**, **2B**, **2E**, and **2F**), inhibited the quantities of AMPK $\alpha 1$  mRNA ( $P < 0.01$ ; **Figure 2G**) and PPAR $\alpha$  and

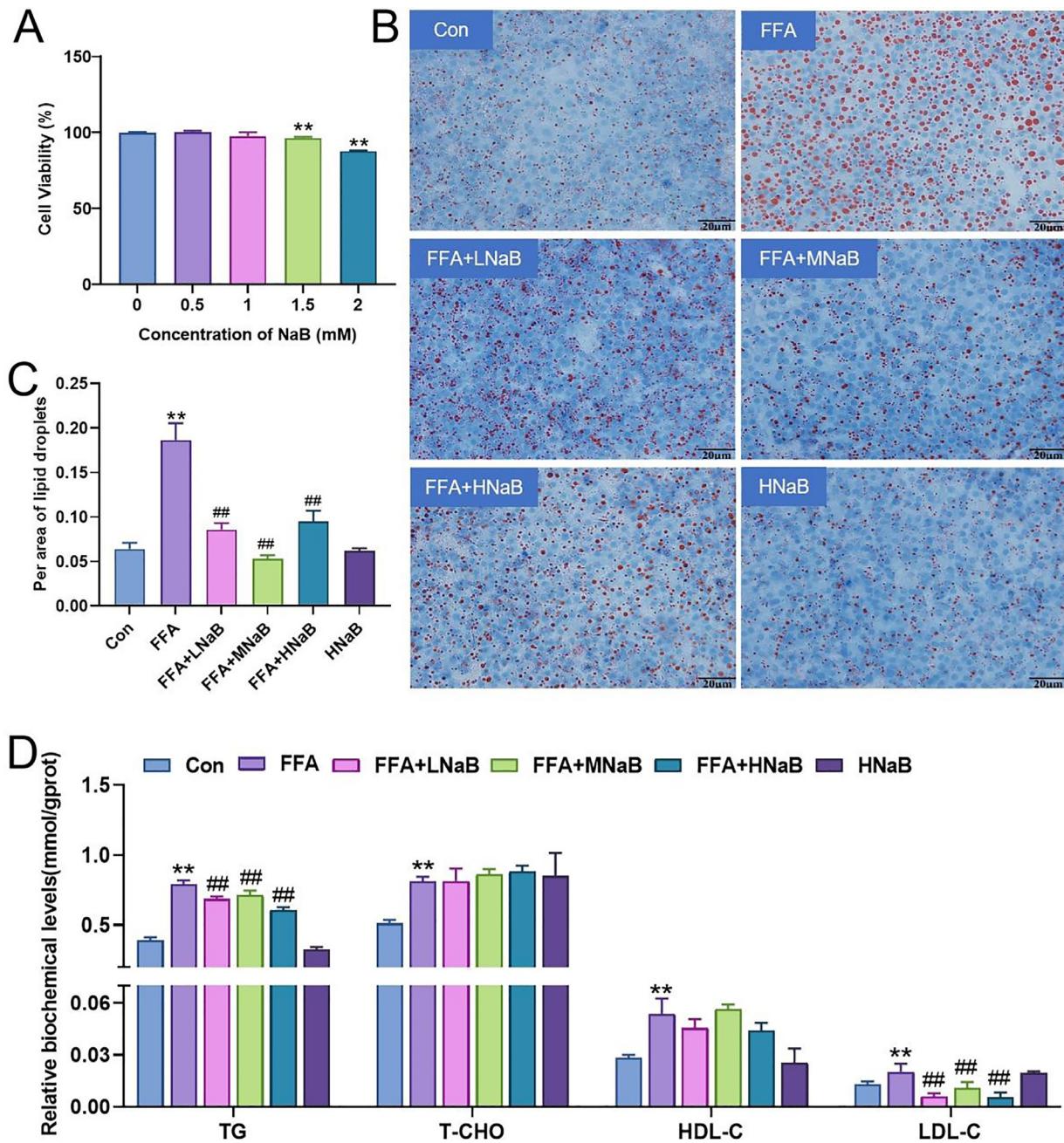
CPT1 mRNA ( $P < 0.01$ ; **Figures 2H** and **2I**) with respect to Con group. However, different concentrations of NaB all blocked the FFA-stimulated hepatocyte-induced increase in adipogenesis-related factors to varying degrees, and the effect was particularly significant in the mRNA expression of ACC1, FASN, and LXR $\alpha$  ( $P < 0.01$ ; **Figure 2A**). Sodium butyrate addition increased the expression of AMPK $\alpha 1$  mRNA at least 1.8 folds in FFA-treated hepatocytes ( $P < 0.01$ ; **Figure 2B**). In addition, the results showed that FFA activation of hepatocytes led to a decrease in the concentrations of p-AMPK $\alpha 1$ , PPAR $\alpha$ , and CPT1A proteins ( $P < 0.01$ ; **Figures 3A–3D**) and an increase in the concentrations of SREBF1 and FASN proteins ( $P < 0.01$ ; **Figures 3A**, **3E**, and **3F**). Treatment with medium concentration of NaB resulted in significantly higher levels of p-AMPK $\alpha 1$ , PPAR $\alpha$ , and CPT1A proteins in FFA-treated hepatocytes ( $P < 0.01$ ; **Figures 3A–3D**). But all 3 concentrations of NaB treatment resulted in a significant decrease in SREBF1 and FASN protein contents in FFA-treated hepatocytes ( $P < 0.01$ ; **Figures 3A**, **3E**, and **3F**).

### Sodium Butyrate Ameliorates FFA-Induced Lipid Metabolism Disorders by AMPK Signaling

As a critical regulator of maintaining cellular homeostasis and energy, the buildup of intracellular lipid droplets can be reduced by AMPK. Thus, hepatocytes were treated with the NaB, FFA, and AMPK inhibitor Comp C to examine the role of AMPK in alleviating lipid metabolism problems by NaB treatment. Oil red staining analysis showed that NaB treatment resulted in a significant reduction in the number of lipid droplets in FFA-stimulated hepatocytes ( $P < 0.01$ ), whereas the addition of the AMPK inhibitor Comp C twice increased the number of lipid droplets when compared with that in the FFA + NaB group ( $P < 0.01$ ; **Figures 4A** and **4B**); the similar results were seen in the TG content assay ( $P < 0.01$ ; **Figure 4C**). Compound C (**Comp C**) further effectively downregulated p-AMPK $\alpha 1$  protein expression in FFA-stimulated hepatocytes ( $P < 0.01$ ), but it did not lead to a decrease in p-AMPK $\alpha 1$  protein expression and an increase in SREBF1 protein expression in hepatocytes co-treated with NaB and FFA ( $P < 0.01$ ; **Figures 5A**, **5B**, and **5E**). However, the levels of PPAR $\alpha$  and CPT1A proteins were significantly increased in hepatocytes induced by NaB with FFA ( $P < 0.01$ ), whereas they were significantly decreased under Comp C treatment ( $P < 0.01$ ; **Figures 5A**, **5C**, and **5D**).

### Sodium Butyrate Ameliorates FFA-Induced Lipid Metabolism-Related Problems by Activation of AMPK/PPAR $\alpha$ Signaling

To explore the role of PPAR $\alpha$  in mitigating lipid metabolism disorders in NaB therapy, the hepatocytes were stimulated with NaB, FFA, and PPAR $\alpha$  inhibitor GW6471. When contrasted to the Con group, FFA stimulation raised lipid droplet quantities ( $P < 0.01$ ); NaB

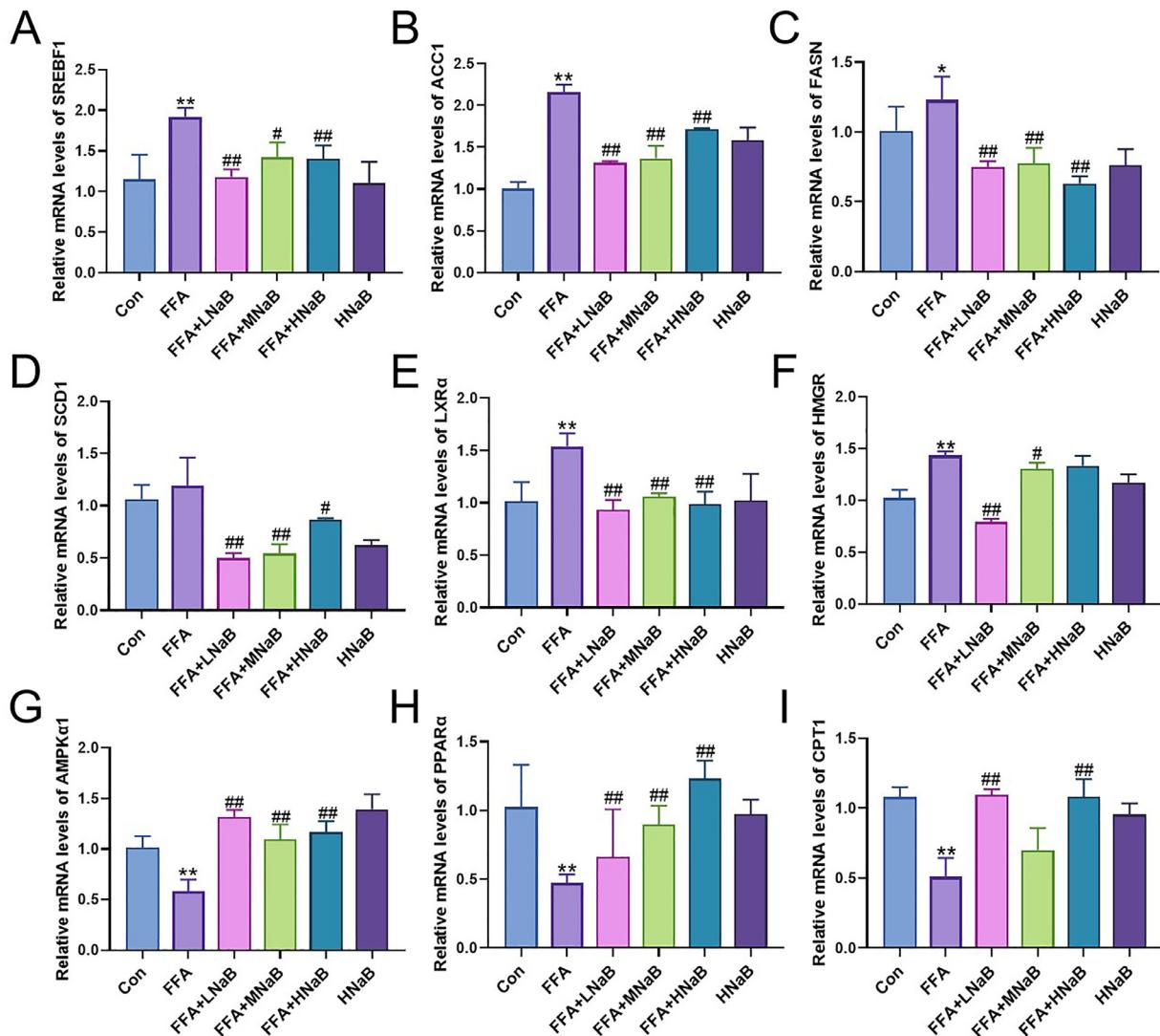


**Figure 1.** Sodium butyrate (NaB) concentration exploration, pathological section and biochemical indicators of chicken hepatocellular adipose degeneration model. Low, medium, and high NaB concentrations represent 0.25, 0.5, and 1 mmol/L concentrations of NaB, respectively. (A) Effect of NaB on the viability of primary chicken embryo hepatocytes. (B) Hepatocyte oil red O stain ( $400\times$ ). (C) Hepatocyte lipid droplet area ratio. (D) The TG, T-CHO, HDL-C, and LDL-C levels of liver cells homogenate. Data are displayed as the mean and standard deviation of at least 3 separate studies. “\*\*” denotes a change that is significantly different from the Con group ( $*P < 0.05$  and  $**P < 0.01$ ). “#” denotes a change that is significantly different from the FFA group ( $\#P < 0.05$  and  $\#\#P < 0.01$ ). It is the same below.

exposure effectively inhibited the FFA-induced increase in the number of lipid droplets in hepatocytes ( $P < 0.01$ ), whereas the addition of GW6471 in turn increased the number of lipid droplets under both NaB and FFA treatments ( $P < 0.01$ ; Figures 6A and 6B). Similar outcomes were also observed when TG content was determined ( $P < 0.01$ ; Figure 6C).

Sodium butyrate treatment increased the expression of PPAR $\alpha$  and CPT1A proteins in FFA-stimulated hepatocytes ( $P < 0.01$ ), whereas the expression of PPAR $\alpha$  and CPT1A proteins decreased dramatically in

hepatocytes after the addition of FFA, NaB, and GW6471 ( $P < 0.01$ ; Figures 7A, 7C, and 7D). Under treatment with GW6471, NaB treatment still led to an upregulation of p-AMPK $\alpha 1$  protein expression among FFA-induced hepatocytes ( $P < 0.01$ ; Figures 7A and 7B). Consistent with prediction, GW6471 inhibited the down-regulation of SREBF1 protein expression under NaB and FFA treatment ( $P < 0.01$ ; Figures 7A and 7E). Combining the previous results, NaB might ameliorate chicken hepatocyte steatosis through the AMPK/PPAR $\alpha$  signaling pathway.



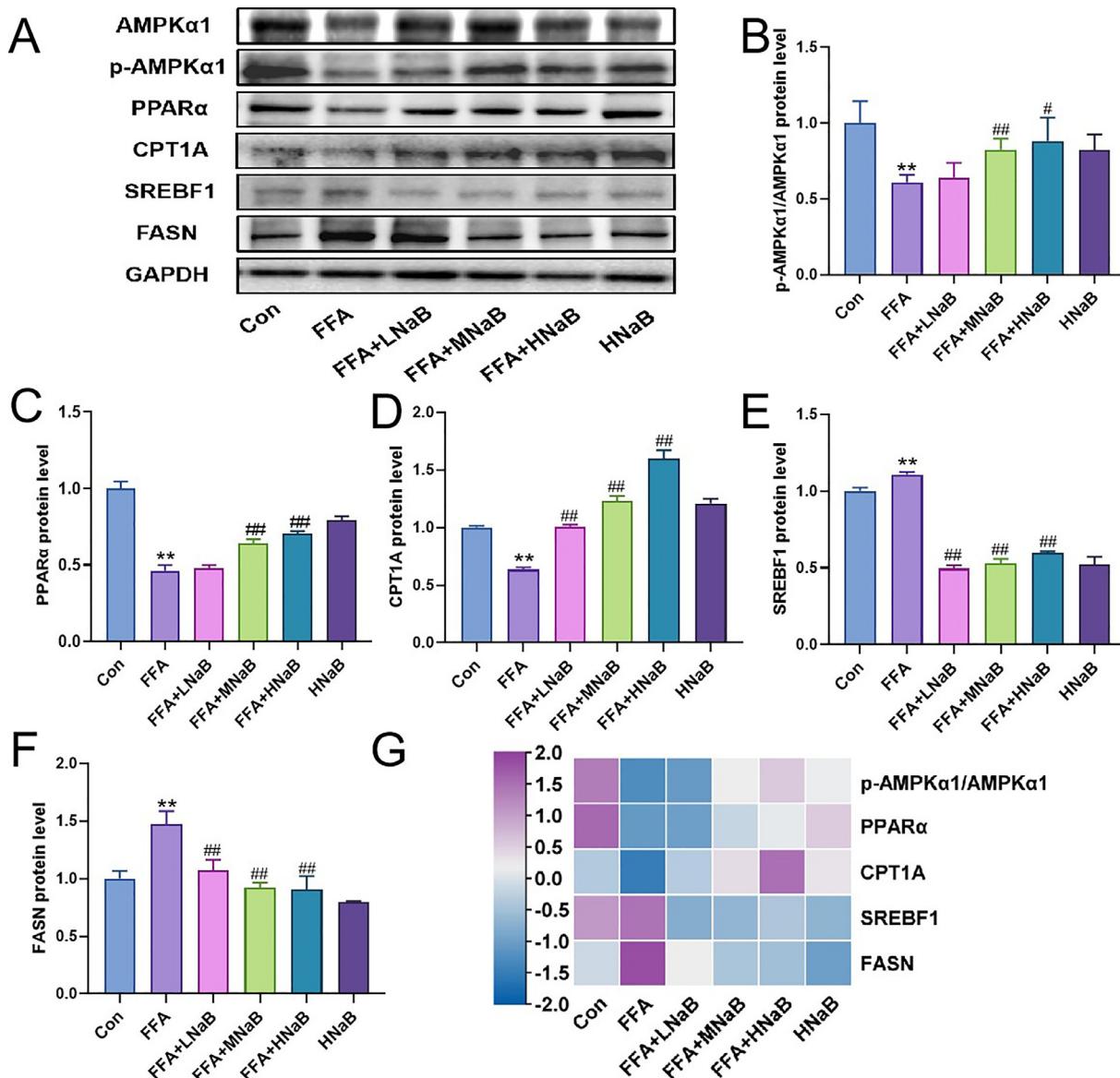
**Figure 2.** Effect of NaB on relative genes in FFA induced chicken hepatocellular adipose degeneration model. Transcript levels of mRNA of lipid synthesis-related genes *SREBF1* (A), *ACC1* (B), *FASN* (C), *SCD1* (D), *LXRα* (E), and *HMGR* (F) mRNAs were detected by RT-qPCR in hepatocytes of each group. The mRNA transcript levels of *AMPKα1* (G), a key factor gene for energy regulation, *PPARα* (H), a key factor gene for lipolysis, and *CPT1* (I), a rate-limiting enzyme gene for fatty acid oxidation, were detected by RT-qPCR in hepatocytes of each group. RT-qPCR, real-time quantitative polymerase chain reaction.

## DISCUSSION

Fatty liver hemorrhagic syndrome is a common metabolic disorder characterized by hepatic fat accumulation and cellular steatosis, which can lead to sudden death and a dramatic decline in egg production among adult laying hens (Zhuang et al., 2019). Butyrate, a SCFA formed by microbial fermentation, has been shown to positively affect energy metabolism in skeletal muscle, brown adipose tissue, and pancreatic  $\beta$ -cells (Ji et al., 2019). Previous reports have suggested that dysbiosis of the gut microbiota and reduced production of SCFA, especially butyric acid, may be associated with FLHS incidence (Yang et al., 2023). Nevertheless, it remains to be seen whether butyric acid can regulate lipid metabolism in chicken liver and alleviate hepatocellular steatosis, thus serve as a means of preventing or treating FLHS in the future. The current work demonstrated that NaB has a practical protective effect against FFA-

induced hepatic steatosis in primary chicken hepatocytes and identified its mechanism of action in hepatocytes, which is expected to serve as a new approach to alleviate hepatic lipid deposition and prevent FLHS.

Normal hepatic lipid metabolism is particularly important in peak laying hens. While disorders of hepatic lipid metabolism are characterized by elevated TG levels, increased lipoproteins and cellular steatosis. A multitude of investigators have effectively developed in vitro research models to induce hepatic steatosis by applying an excess of FFA (Ricchi et al., 2009; Lin et al., 2020). Our recent study showed that the administration of a blend of FFA, composed of OA and PA in a ratio of 2:1, 1 mmol/L as a concentration, significantly induced steatosis in primary chicken hepatocytes after a 24-h treatment characterized by increased levels of TG, T-CHO, HDL-C, and LDL-C, which consistency with previously studies. A study showed that NaB could alleviate hepatic steatosis, improve lipid profile, and enhance

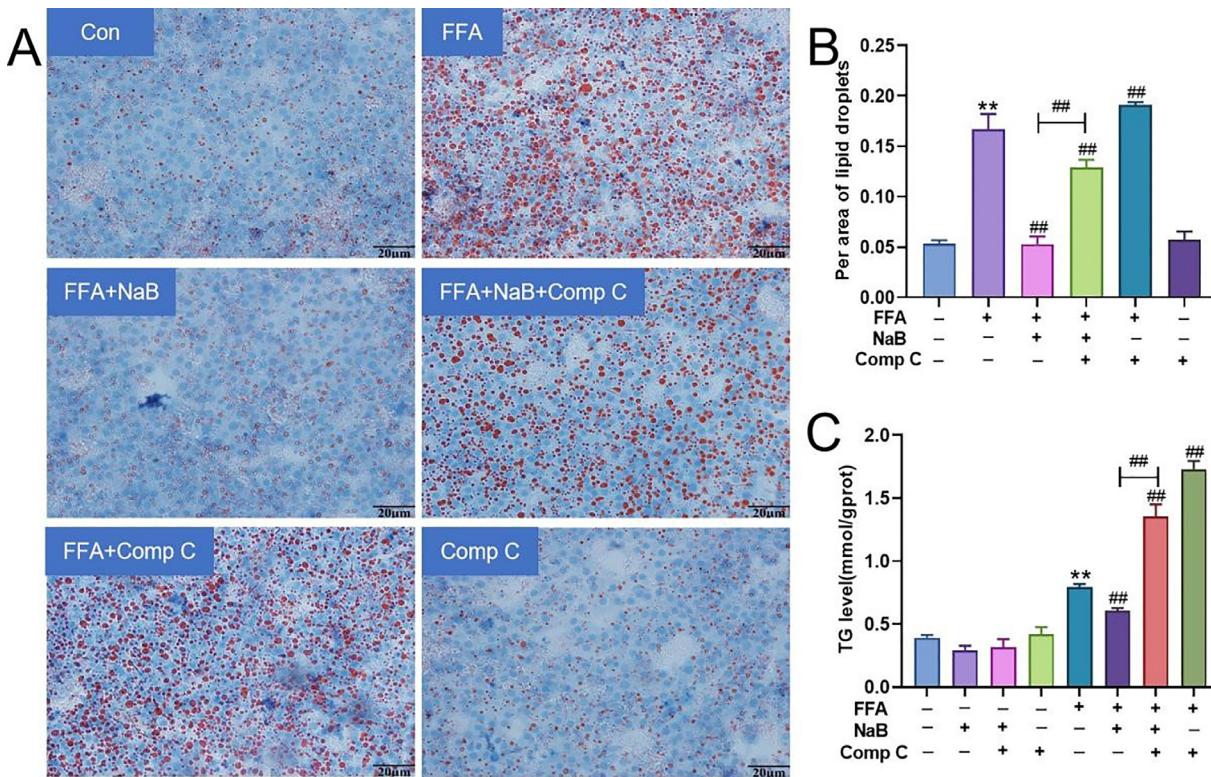


**Figure 3.** Effect of NaB on lipid metabolism relative proteins in FFA induced chicken hepatocellular adipose degeneration model. (A) Hepatocyte-expressed p-AMPK $\alpha$ 1, AMPK $\alpha$ 1, PPAR $\alpha$ , CPT1A, SREBF1, and FASN protein bands under different NaB concentrations with FFA treatment were demonstrated. Protein expression levels of p-AMPK $\alpha$ 1 (B), PPAR $\alpha$  (C), CPT1A (D), SREBF1 (E), and FASN (F) in hepatocytes of each group was obtained from the analysis of gray values of the bands. (G) Heatmap of p-AMPK $\alpha$ 1, PPAR $\alpha$ , CPT1A, SREBF1 and FASN protein expression in hepatocytes under different treatments was shown.

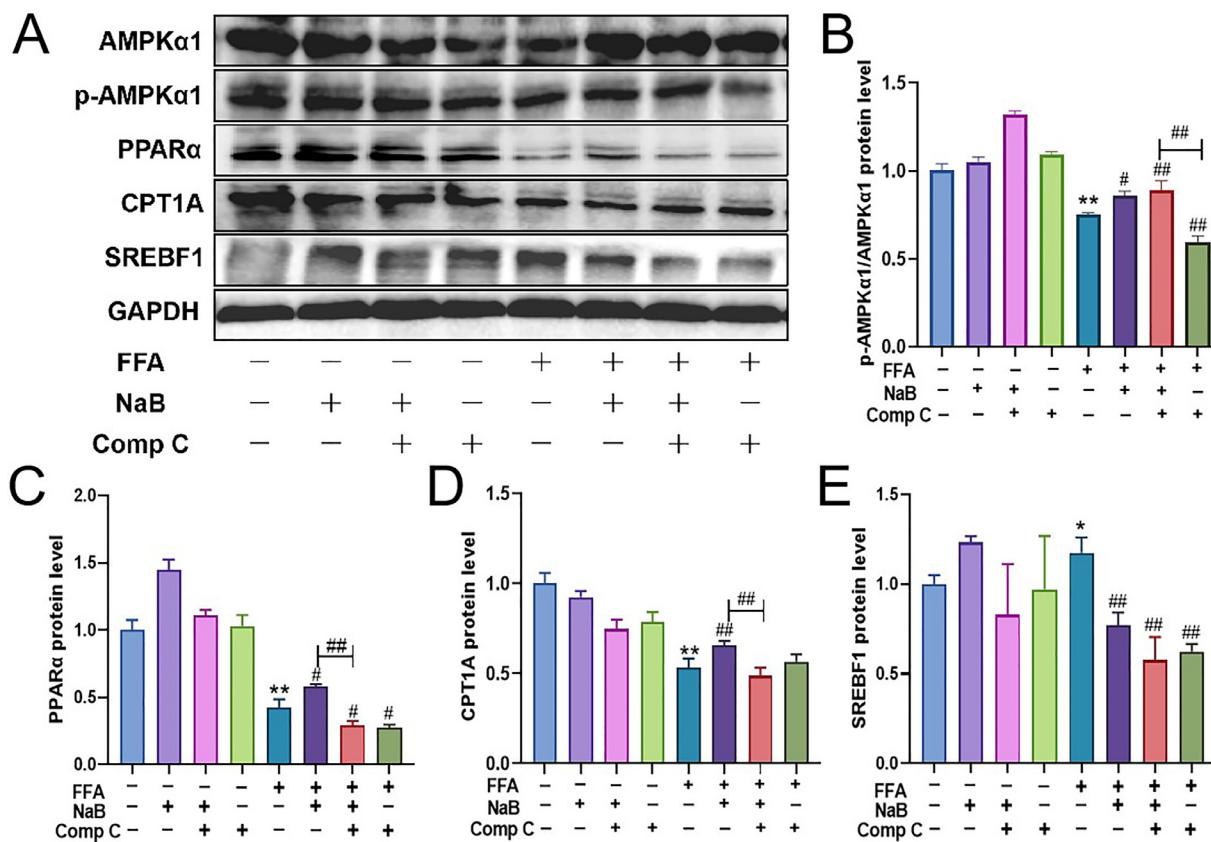
liver function primarily by activating the LKB1-AMPK-INSIG signaling pathway (Zhao et al., 2021). In the current investigation, after FFA induction, the treatment with 0.25 to 1 mmol/L of NaB dramatically boosted cell viability and lowered the increase in TG content.

The dynamic balance between lipogenesis and lipolysis is controlled by fundamental lipid metabolism variables (Saponaro et al., 2015). During the lipo synthesis phase, the transcription factor SREBF1 controls the expression of genes related to FA and cholesterol synthesis in the cell (Ferré and Foufelle, 2010; Saponaro et al., 2015). Previous studies have shown that *SREBP-1(-/-)* (*SREBF1*) mice have reduced mRNA expression levels of both FA and TG synthesis-related enzymes, such as *ACC* and *FASN* (Liang et al., 2002; Stoeckman and Towle, 2002). Recent studies have indicated an elevation in the levels of liver fat synthesis factors (such as *ACC*,

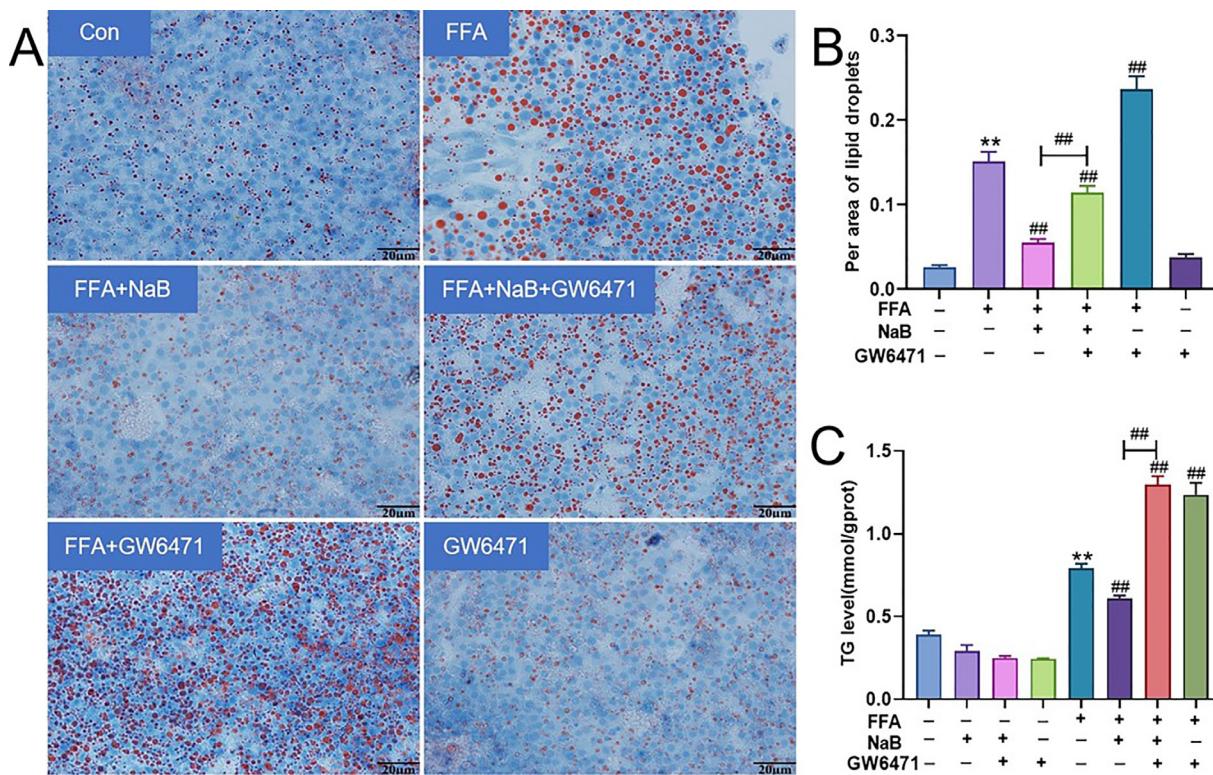
*FASN*, mRNA and proteins expression) in vitro and in vivo models of fatty liver (Wang et al., 2017). Concurrently, in our current study, FFA stimulation showed significant increases in SREBF1 and FASN mRNA and proteins expression levels, whereas the addition of NaB resulted in a decrease in the expression levels of the 2 fat synthesis-related genes and proteins, suggesting that NaB inhibits the FFA-induced increase in fat synthesis. Additionally, we have observed that NaB can promote the expression of FA oxidation genes like *PPAR $\alpha$*  and limit the expression of FA synthesis rate-limiting enzyme genes like *ACC*. Previous research has revealed that inhibiting ACC activity can decrease intracellular levels of malonyl-CoA, thereby activating FA  $\beta$ -oxidation, including increased *PPAR $\alpha$*  and *CPT1* expression (Kim et al., 2017). Therefore, our data demonstrate that NaB alleviates FFA-induced lipid metabolism disorders



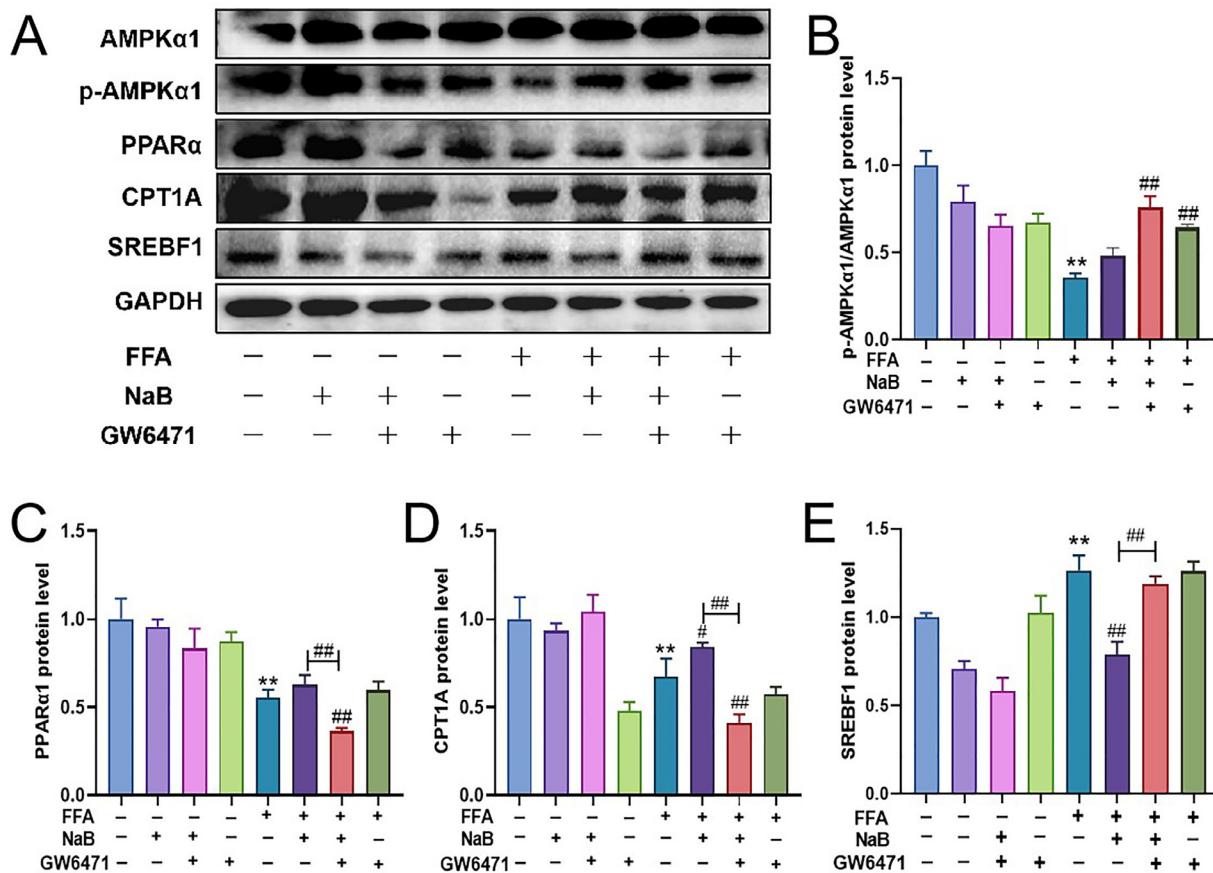
**Figure 4.** Effect of AMPK inhibition on lipid metabolism in chicken hepatocellular adipose degeneration model induced by FFA and NaB. (A) Hepatocyte oil red O stain ( $400\times$ ). (B) Hepatocyte lipid droplet area ratio. (C) TG concentrations in primary hepatocytes of chicken embryos.



**Figure 5.** Effect of AMPK inhibition on proteins related to lipid metabolism in chicken hepatocellular adipose degeneration model induced by FFA and NaB. (A) Hepatocyte-expressed p-AMPK $\alpha$ 1, AMPK $\alpha$ 1, PPAR $\alpha$ , CPT1A, and SREBF1 protein bands under FFA, NaB, and AMPK inhibitor Comp C treatments were demonstrated. Protein expression levels of p-AMPK $\alpha$ 1 (B), PPAR $\alpha$  (C), CPT1A (D), and SREBF1 (E) in hepatocytes of each group was obtained from the analysis of gray values of the bands.



**Figure 6.** Effect of PPAR $\alpha$  inhibition on lipid metabolism in chicken hepatocellular adipose degeneration model induced by FFA and NaB. (A) Hepatocyte oil red O stain (400 $\times$ ). (B) Hepatocyte lipid droplet area ratio. (C) TG concentrations in primary hepatocytes of chicken embryos.



**Figure 7.** Effects of PPAR $\alpha$  inhibition on proteins related to lipid metabolism in chicken hepatocellular adipose degeneration model induced by FFA and NaB. (A) Hepatocyte-expressed p-AMPK $\alpha$ 1, AMPK $\alpha$ 1, PPAR $\alpha$ , CPT1A and SREBF1 protein bands under FFA, NaB, and PPAR $\alpha$  inhibitor GW6471 treatments were demonstrated. Protein expression levels of p-AMPK $\alpha$ 1 (B), PPAR $\alpha$  (C), CPT1A (D), and SREBF1 (E) in hepatocytes of each group was obtained from the analysis of gray values of the bands.

in hepatocytes by inhibiting lipogenesis and promoting lipolysis.

It has been demonstrated that the AMPK and PPAR $\alpha$  signaling pathways can modulate the expression and activity of critical enzymes involved in lipid metabolism, such as ACC, FASN, and CPT1 (Wang et al., 2020). Our experimental results indicate that NaB can enhance the regulation of AMPK and PPAR $\alpha$ , essential factors in FA synthesis and oxidation. As a vital cellular energy sensor, AMPK is necessary for controlling the body's inflammatory reactions, oxidative stress, glucose metabolism, and lipid metabolism. Targeting AMPK has been constituted as a potential therapeutic strategy for treating and preventing non-alcoholic fatty liver disease in mammals. By regulating the expression of genes involved in lipogenesis, triglyceride production, reverse cholesterol transport, lipolysis, and FA oxidation, PPAR also plays a variety of roles in lipid metabolism. Activation of PPAR $\alpha$  can be an essential pharmacological approach to alleviate fatty liver and type 2 diabetes (Okada-Iwabu et al., 2013; Pawlak et al., 2015). Conversely, inhibition of PPAR $\alpha$  exacerbates hepatic lipid synthesis (Zhang et al., 2021). In the present study, our data suggested that inhibiting AMPK and PPAR $\alpha$  can prevent the beneficial effect of NaB on FFA-induced lipid deposition in liver cells. Accumulating evidence has demonstrated that NaB could alleviate hepatic lipid disorders, intestinal inflammation, and other pathologic features by regulating the AMPK and PPAR $\alpha$  signaling pathways (Hong et al., 2016; Sun et al., 2018). However, the relationship between AMPK and PPAR $\alpha$  is complex, particularly in poultry, and has not been extensively studied. Previous studies have shown that AMPK is required to activate PPAR $\alpha$  in mice (Luo et al., 2022). Additionally, studies have found that activated PPAR $\alpha$  can enhance AMPK expression (Bordoloi et al., 2019). In the present study, inhibiting AMPK activation with Comp C effectively counteracts the impact of NaB on PPAR $\alpha$  protein expression. Interestingly, inhibiting PPAR $\alpha$  with GW6471 does not disrupt the activation of the AMPK signaling pathway mediated by NaB. These findings suggest that NaB modulates hepatic lipid metabolism by regulating the interplay between the AMPK and PPAR $\alpha$  signaling pathways.

## CONCLUSION

In summary, NaB promotes fatty acid oxidation and inhibits lipid synthesis in the FFA-induced chicken hepatocellular adipose degeneration model targeting the AMPK/PPAR $\alpha$  pathway. Attempts to generate novel therapies for metabolic diseases like FLHS via AMPK/PPAR $\alpha$  activation by NaB may be worthy of pursuit.

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## DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103482.

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