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Carbenoxolone ameliorates hepatic lipid metabolism and inflammation in obese mice induced by high fat diet via regulating the JAK2/STAT3 signaling pathway

Yuning Chen[a,b,1], Wen Lu[a,1], Zhengyu Jin[c,1], Jian Yu[b,*], Bimin Shi[a,*]

[a] Department of Endocrinology and Metabolism, The First Affiliated Hospital of Soochow University, Suzhou 215000, China
[b] Department of Geriatrics, The Third Affiliated Hospital of Soochow University, Changzhou 213000, China
[c] Department of General Surgery, The First Affiliated Hospital of Soochow University, Suzhou 215003, China

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ABSTRACT

Carbenoxolone (CBX) is the active principle of licorice, which is used to treat psoriasis, peptic ulcers, and wound healing. However, there is no report on how CBX ameliorates hepatic lipid metabolism and inflammation in obese mice. In this study, our aim is to explore the mechanism by which CBX regulates lipid metabolism in the liver of obese mice. C57BL/6J mice were divided into three groups and were fed with normal chow diet (NC group) or High-fat diet (HFD and CBX group) for eight weeks. Then mice in CBX group were given CBX every day by gavage for twelve weeks (15 mg/kg). Blood was collected for detection of triglycerides (TG), total cholesterol (TC), density lipoprotein (LDL), high-density lipoprotein (HDL), Alanine aminotransferase (ALT), and Aspartate aminotransferase (AST). Liver tissues were stained with hematoxylin-eosin for histological examination. Immunohistochemical staining was performed for detection of SOCS-3 (Suppressor of cytokine signaling 3), SREBP-1 (Sterol regulatory element-binding protein 1), and FAS (Fatty acid synthase) protein. The genes of SOCS-3, SREBP-1, and FAS in liver were assessed by real-time PCR. Western blotting was applied to detect the protein expressions of the phosphorylated JAK2 (Janus kinase 2) and phosphorylated STAT3 (Signal transducer and activator of transcription 3). Our results showed that compared with the HFD group, serum concentrations of TG, TC and LDL were decreased significantly, while the concentration of HDL was increased in the CBX group. CBX could attenuate intracellular lipid accumulation in the liver. Besides, treatment with CBX could significantly decrease levels of inflammatory factors such as IL-6 (Interleukin 6) and TNF-a (Tumor necrosis factor-a), increase expressions of phosphorylated JAK2 and phosphorylated STAT3, decrease the expressions of SOCS-3, SREBP-1 and FAS in the liver. In conclusion, through activating the JAK2/STAT3 signaling pathway in liver and reducing the expression of SCOCS-3, CBX could further decrease the expressions of SREBP-1c, FAS and ameliorate the inflammatory state of liver, so as to protecting the liver from lipid metabolism damage induced by high-fat diet. Therefore, CBX has the possibility for the treatment of obesity, hyperlipidemia, and inflammation.

1. Introduction

In recent years, the prevalence of obesity has increased substantially, resulting in a global burden [1]. Due to excess nutrition or a sedentary lifestyle, excessive lipid accumulation in peripheral tissues or adipose resulting in obesity [2]. Nowadays, obesity has been growing worldwide and raising widespread concerns about health.

Obese individuals have been proposed to be in a state of low-grade inflammation, causing an alteration of inflammatory cytokines production and thereby linking obesity, insulin resistance and inflammation [3]. An important feature of obesity is the accumulation of liver lipid. This process is in part regulated by cytokines activated by the JAK/STAT signaling pathway [4].

Under physiological conditions, leptin binds to its receptor, forming a temporary dimeric structure. It can induce the catalytic activity of the JAK2 enzyme and recruit STAT3 [5]. STAT3 guide the leptin signal to the nucleus, where they coordinate transcription of neurotransmitters responsive to hormonal signals [6]. JAK2 and STAT3 may be inactivated by SOCS-3 proteins [7]. SOCS-3 acts as a negative feedback loop to switch off the pathway, however, the process of transcription is

* Corresponding authors.
E-mail addresses: Yujian802@163.com (J. Yu), bimin_shi@126.com (B. Shi).
[1] These authors contributed equally to this work.

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induced by STAT3 [8]. In obese individuals, the over expression of the inflammatory markers is linked to elevated SOCS-3 expression, characterizing a relationship between inflammation and impaired leptin signaling, as SOCS-3 can inhibit JAK2 and STAT3, contributing to the resistance to leptin [9].

SREBP-1c is a major transcription factor involved in the control of cholesterol and fatty acid synthesis [10]. FAS is also a key enzyme involved in fat production [11]. The anti-steatogenic effect of hepatic STAT3 is mediated, at least in part, by inhibition of SREBP-1c [12]. However, mice lacking the negative regulator of liver STAT3 activity showed increased liver lipid accumulation and up-regulated expression of SREBP-1c after fed with the high-fat diet [13].

Carbenoxolone is a semisynthetic derivate of glycyrrhizic acid, which is the active principle of licorice [14]. Carbenoxolone is widely used to treat psoriasis, peptic ulcers, and wound healing [15]. Previous studies showed that CBX has the capability of reducing plasma triglyceride and cholesterol in obese mice [16]. However, the molecular mechanism by which CBX inhibits the liver lipid synthesis and inflammation in obese mice has not been reported. Consequently, we further study on the mechanism of CBX ameliorates hepatic lipid metabolism and inflammation.

2. Materials and methods

2.1. Animals and treatment

Six-weeks-old male C57BL/6J mice were obtained from the Laboratory Animal Center of Soochow University. They were given free food and water under controlled temperature conditions (23 °C) and the 12-h light/dark cycle. The animal experimental procedure was approved by the Animal Ethics Committee of Soochow University. Room temperature and humidity were controlled.

After fed the normal chow diet for acclimation for one week, one group of the mice (NC group, n = 8) were fed normal chow diet for eight weeks, and other mice were fed high-fat diets (60% of calories from fat, 20% from carbohydrates, 20% from protein, Beijing KeAo Feed Co. Ltd., Beijing, China) for eight weeks. Then, mice fed normal control diets were continuously maintained for twelve weeks, however mice fed high-fat diets were randomly classified into two groups and maintained for twelve weeks: (1) mice fed high-fat diets only (HFD group, n = 8); (2) mice fed high-fat diets and CBX treatment group (CBX group, n = 8). Mice in CBX group were given CBX (Sigma-Aldrich, St. Louis, USA) dissolved in sterile water every day by gavage (15 mg/kg), besides mice in HFD and NC group were given the same volume of sterile water by gavage daily. Food intake and body weight of mice were measured daily.

2.2. Analysis of clinical parameters

After twelve weeks of CBX treatment, all mice were sacrificed. As described previously [17], immediately the blood of mice was centrifuged at 2500 rpm for 20 min to collect the serum. The serum was processed for detection of triglycerides (TG), total cholesterol (TC), density lipoprotein (LDL), and high-density lipoprotein (HDL) using commercial kits (Wako Pure Chemical Industries, Japan). The levels of serum Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were identified using commercial kits (Jiancheng biological company, Nanjing, China).

2.3. Histology staining

After mice were sacrificed, a small amount of liver tissue was removed from mice and fixed in 10% neutral formalin immediately. They were dehydrated, embedded in paraffin, cut into 4 μm sections and stained with hematoxylin-eosin (H&E) for histological examination, as previously described [18]. Histologic images of H&E stained were captured by the Leica microscope (Leica Microsystems, Germany). Based on the agreement data, the activity score of non-alcoholic fatty liver disease(NAFLD) is defined as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2); thus ranging from 0 to 8 [19].

2.4. Immunohistochemistry

After mice were sacrificed, liver tissues of mice were fixed in 10% neutral formalin, embedded in paraffin, cut into 4 μm sections. As previously described [20], sections were dewaxed and quenched endogenous peroxidase for 10 min using 3% hydrogen peroxides. Immunohistochemical staining was performed for FAS protein using mouse anti-mouse monoclonal antibody at the dilution of 1:200 (sc-21730, Santa Cruz Biotechnology, USA) overnight at 4 °C. The staining was performed for SREBP-1c protein using mouse anti-mouse antibody at the dilution of 1:200 (sc-13551, Santa Cruz Biotechnology, USA) overnight at 4 °C. The staining was performed for SOCS-3 protein using mouse anti-mouse monoclonal antibody at the dilution of 1:100 (sc-518020, Santa Cruz Biotechnology, USA). Then, the sections were incubated with HRP-connected secondary antibodies for 30 min. Sections were stained with the DAB kit, as previously described [21]. Finally, stained images were captured with optics microscope, and all images were acquired on the Leica microscope. The brown diaminobenzidine precipitates were considered positive. Viewed at 400× magnification, results were evaluated semi-quantitatively according to the percentages of positive cells in eight random images [22].

2.5. RNA extraction and quantitative real-time PCR

After mice were sacrificed, RNA in liver tissues of mice in each group was isolated using Trizol (Thermo Fisher Scientific, USA). According to the suppliers' protocol, RNA was reverse transcribed using the GoScript Reverse Transcription kit (Promega Corporation, USA). The mRNA levels were measured by RT-qPCR using SYBR-green (Takara, Japan) and normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as described [23]. Relative quantification of gene expression was calculated via the $2^{-\Delta\Delta CT}$ method [24]. The primer sequences used to amplify mRNA were presented in Table 1.

2.6. Protein extraction and western blot analysis

After mice in each group were sacrificed, a small amount of liver tissue was homogenized in order to extract the protein. Homogenates were centrifuged at 15,000*g* for 15 min at 4 °C, and supernatants were

Table 1
Primer sequences designed for mice genes.

Gene name	Primer forward (5′-3′)	Primer reverse (5′–3′)
SREBP-1c	CCCTGCGAAGTGCTCACAA	GCGTTTCTACCACTTCAGGTTTCA
FAS	TGGTCACAGACGATGACAGGA	AGGCGTCGAACTTGGACAGA
SOCS-3	GACCAAGAACCTACGCATCCAGTG	AGGCGTCGAACTTGGACAGA
GAPDH	CAGAACATCATCCCCTGCATC	CTGCTTCACCACCTTCTTGA

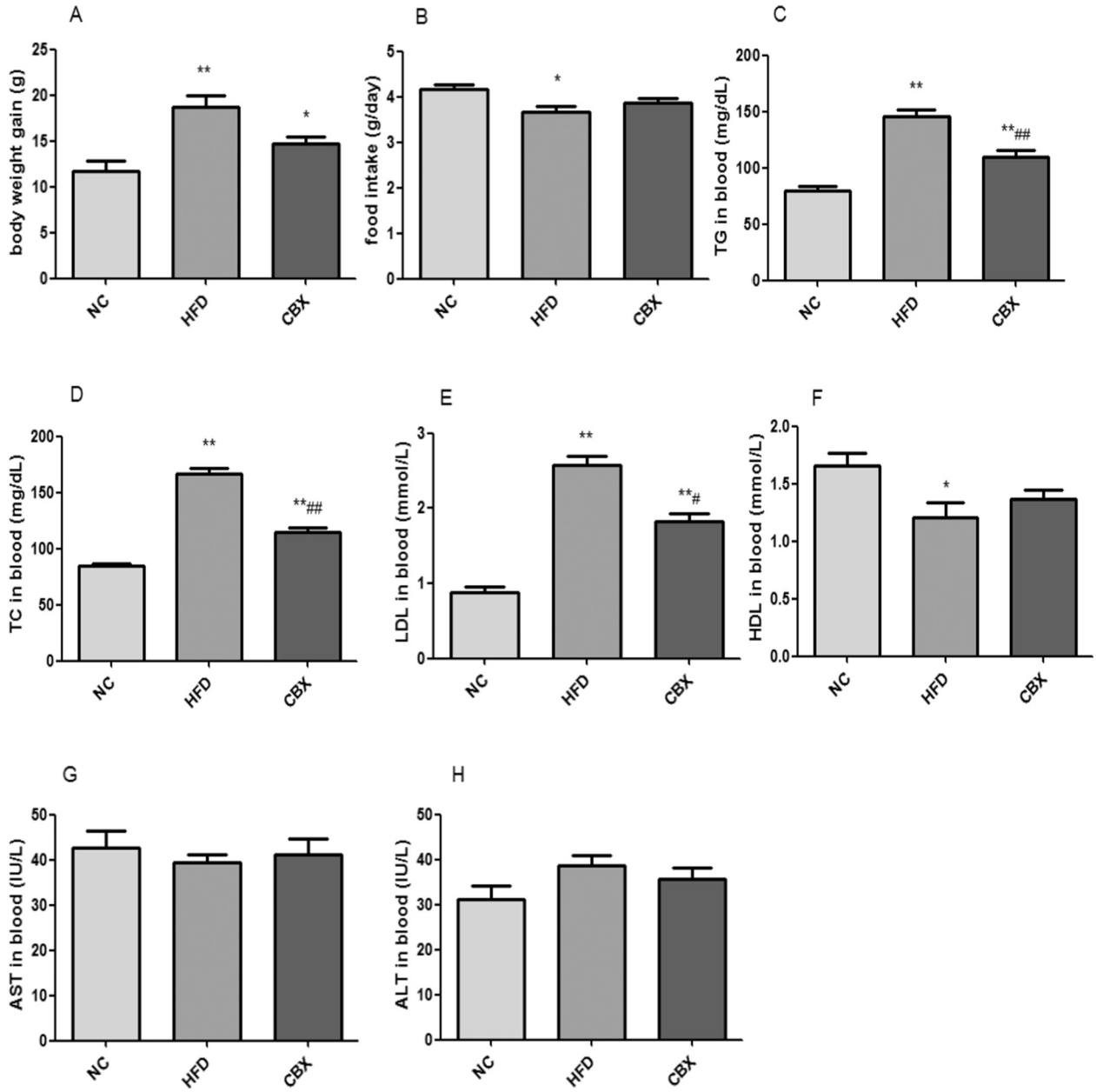

Fig. 1. Changes in the body weight gain, food intake, and clinical parameters. (A). The change of body weight gain in each group. (B). The change of food intake in each group. (C). The change of TG in blood. (D). The change of TC in blood. (E). The change of LDL in blood. (F). The change of HDL in blood. (G). The change of AST in blood. (H). The change of ALT in each group. *$p < 0.05$ and **$p < 0.01$ vs. NC group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. DM group. Differences in multiple groups were analyzed by one-way ANOVA with Tukey multiple comparison post hoc.

collected [25]. Detect the protein concentration by the BCA assay (Beyotime Biotechnology, China). Samples were separated by SDS-PAGE, proteins were transferred from gel to PVDF membrane. Then membranes were incubated with primary antibodies at 4 °C overnight. Primary antibodies used include Phospho-JAK2 (#3771, CST, USA, 1:1000 dilution), JAK2 (#3230,CST, USA, 1:1000 dilution), Phospho-STAT3 (#9145, CST, USA, 1:2000 dilution), STAT3 (#9139, CST, USA,1:1000 dilution), SOCS-3 (sc-518020, Santa Cruz, USA, 1:500 dilution), TNF-a (sc-52746, Santa Cruz, USA, 1:500 dilution), IL-6 (sc-57315, Santa Cruz, USA, 1:500 dilution). As previously described [26], at room temperature, membranes were incubated with the HRP-conjugated secondary antibody (Santa Cruz, USA, 1:5000 dilution) for 1 h. Then target bands of membranes were exposed to ECL kit (Beyotime Institute of Biotechnology, China). The intensity of band was quantified by Image J software and normalized to the control group. β-tubulin (#2128, CST, USA, 1:1000 dilution) was used as a loading control.

2.7. Statistical analysis

Statistical analyses were performed by SPSS 20.0. Experiments were done at least three times. All data were presented as mean ± standard deviation. Differences in multiple groups were analyzed by one-way ANOVA with Tukey multiple comparison post hoc. Double-tailed $p < 0.05$ was considered statistically significant. All figures were performed using Graph Pad Prism 5.0.

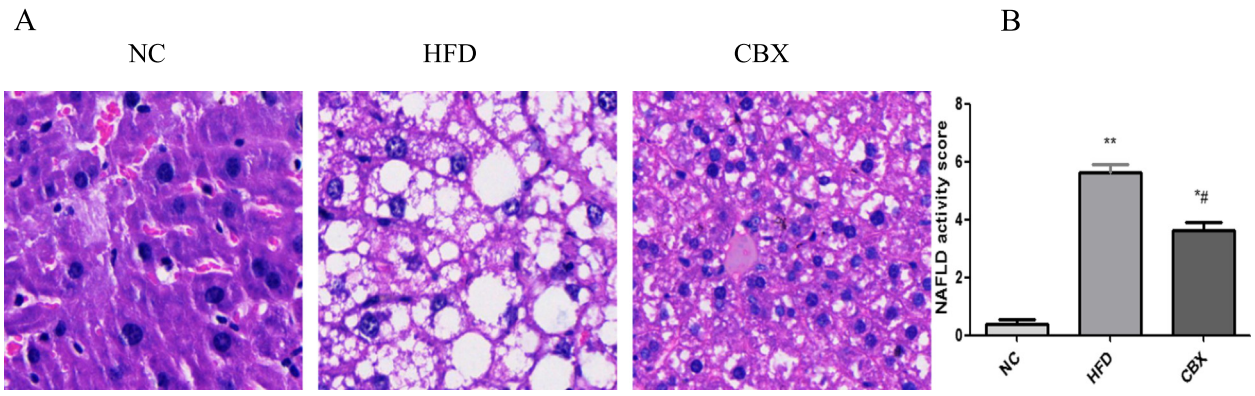

Fig. 2. Changes of liver histopathology. (A). Changes of liver histopathology under optical microscope (Magnifications × 400). HE staining was utilized to analyze histological abnormalities. (B). The NAFLD activity score is defined as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2). Data are presented as mean ± SD of 8 mice per group. *$p < 0.05$ and **$p < 0.01$ vs. NC group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. DM group.

3. Results

3.1. Changes in the body weight gain and food intake

The body weight gain of the mice in the HFD group was remarkably higher compared with the control group ($p < 0.01$). However, treatment with CBX ameliorated body weight gain induced by high fat diet. Besides, daily food intake per mouse in HFD group was lower than which in control group. After treatment with CBX, the food intake increased (Fig. 1A–B).

3.2. Changes in serum lipid levels and liver function

A significant increase in triglyceride, total cholesterol, and density lipoprotein was observed in the high-fat diet induced obesity mice, besides a significant decrease in high density lipoprotein was observed compared with the NC group ($p < 0.01$). However, treatment with CBX could substantially reduce the concentrations of TG. TC and LDL, increase the concentration of HDL ($p < 0.01$ or $p < 0.05$). As for ALT and AST, there was no statistically significant difference among three groups (Fig. 1C–H).

3.3. Changes in liver histopathology

HE staining of liver sections showed that in the NC group, the morphology and structure were normal under the optical microscope. While in liver tissues of high-fat diet mice, intracellular lipid accumulation and steatosis, the inflammation of liver aggravation, and hepatocellular ballooning was observed (magnification, 400×). However, treatment with CBX could relieve the inflammatory state of liver, ameliorate hepatic steatosis and hepatocellular ballooning, which were consistent with lower levels of triacylglycerols in the CBX group (Fig. 2A). These changes were assessed using the NAFLD activity score (Fig. 2B).

3.4. Detection of liver protein changes by immunohistochemistry

Expressions of SOCS-3, SREBP-1c, and FAS were confirmed by immunohistochemistry. Compared with the NC group, the level of SOCS-3, SREBP-1c, and FAS protein in the liver of the HFD group showed a significant increase ($p < 0.01$). However, after using CBX for a period of time, the expressions of SOCS-3, SREBP-1c, and FAS decreased significantly ($p < 0.05$). This result showed that in obese mice induced by the high-fat diet, the function of hepatic lipid synthesis increased significantly, while CBX could partially inhibit lipid synthesis in liver (Fig. 3).

3.5. Detection gene expressions by qRT-PCR

In the liver of the HFD group, the mRNA expressions of SREBP-1c, FAS and SOCS-3 were significantly higher than the control group ($p < 0.05$ or $p < 0.01$). However, compared with the HFD group, treatment with CBX could reduce the levels of SREBP-1c, FAS and SOCS-3. The results also showed that long-term use of high-fat diet could significantly increase lipid synthesis and accumulation in the liver. However, lipid accumulation in liver will decrease after treatment with CBX. (Fig. 4).

3.6. Detection of liver protein changes by western blot

In the liver of high-fat diet group, the protein expressions of TNF-a and IL-6 were considerably higher than the control group ($p < 0.01$), however the protein expressions of p-JAK2/JAK2 and p-STAT3/STAT3 were decreased significantly ($p < 0.01$). In addition, treatment with CBX could inhibit the protein expressions of TNF-a and IL-6, while increase the expressions of p-JAK2/JAK2 and p-STAT3/STAT3. Our findings showed that in the liver of high-fat diet group, expressions of inflammatory factors increased, besides the JAK2/STAT3 signaling pathway was inhibited. This further aggravated leptin resistance and inhibited leptin to play its role in weight loss and blood lipid lowering, thus further leading to increased the expressions of SREBP-1c, FAS, and lipid accumulation in the liver. However, treatment with CBX could reduce the expression of SOCS-3, promote the activation of JAK2/STAT3 signaling pathway and ameliorate leptin resistance, further decrease expressions of SREBP-1c, FAS, and alleviate the accumulation of lipid in the liver (Fig. 5).

4. Discussion

Recently, obesity has grown up to become a serious threat to public health worldwide [27]. Obesity is causally linked to debilitating conditions such as type 2 diabetes, hyperlipidemia, atherosclerosis, stroke, and cardiovascular disease [28]. Obesity is due to energy imbalance toward overnutrition. Abnormality in the central nervous system involving regulation of energy intake and expenditure and feeding behavior has been recognized as a possible pathogenetic mechanism of obesity [29].

The primary functions of leptin are to be able to control food intake, body thermogenesis and energy consumption [30]. Leptin resistance, defined as an impaired neuronal response to leptin, is engaged in the development of obesity [4]. Most obese individuals have very high plasma leptin concentrations [31]. However, because of leptin resistance this hyper endogenous plasma leptin does not reduce appetite

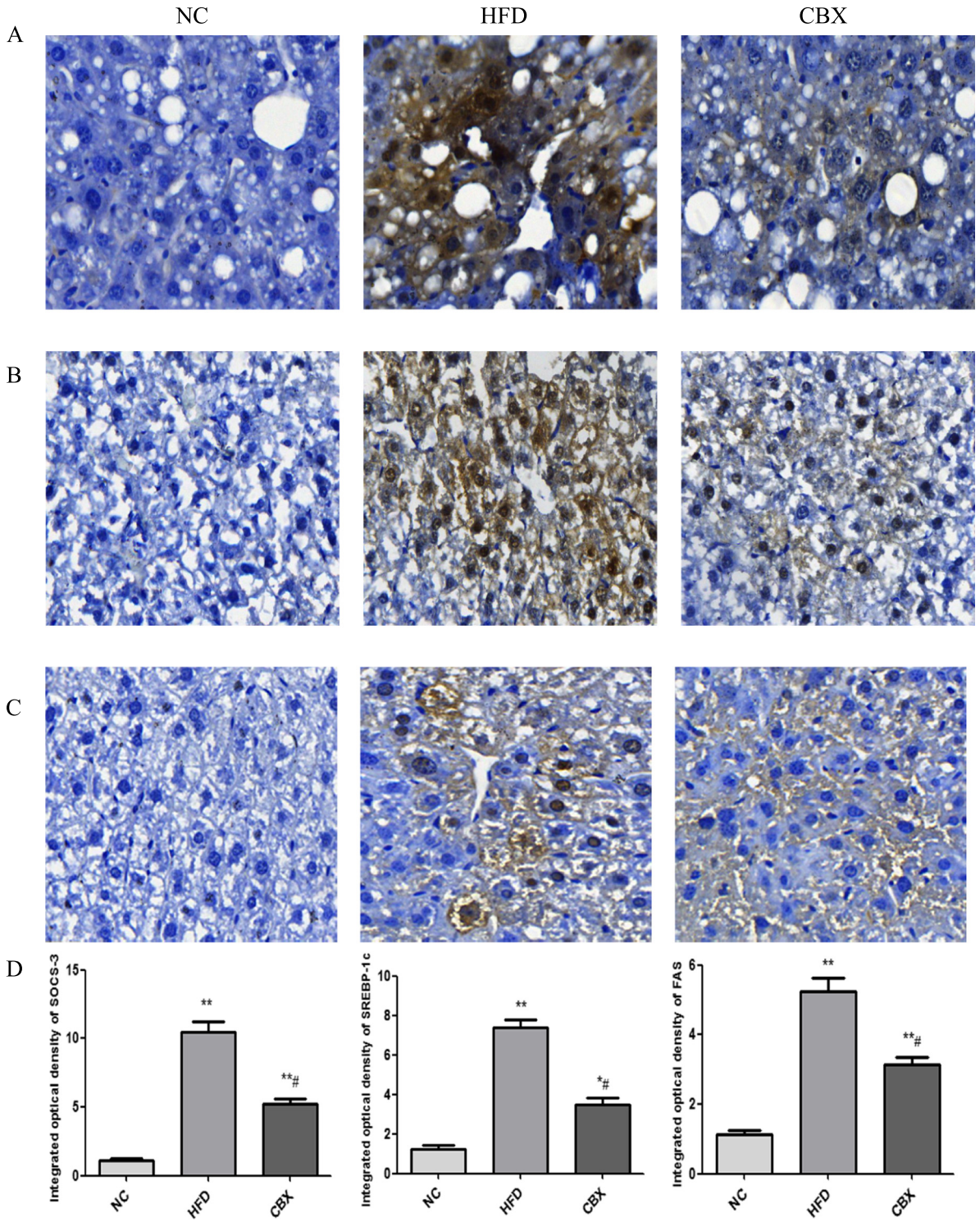

Fig. 3. Representative photomicrographs of liver section by immunohistochemistry (Magnifications × 400). (A). The changes in expressions of SOCS-3 protein. (B). The changes in expressions of SREBP-1c protein. (C). The changes in expressions of FAS protein. (D). Integrated optical density of SOCS-3, SREBP-1c and FAS. Data are presented as mean ± SD of 8 mice per group. *$p < 0.05$ and **$p < 0.01$ vs. NC group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. DM group.

[32]. In contrast, it appears to be a critical factor in obesity-induced pancreatic β cell dysfunction [33]. Appropriate expressions of JAK2, STAT3, and SOCS-3 proteins, are necessary for normal hepatic physiology [4]. Once the expressions of JAK2, STAT3, and SOCS-3 is unusual, it will lead to abnormal accumulation of fat in the liver and even fatty liver.

High-fat diet affects eating behavior through the leptin signaling pathway, namely the JAK2/STAT3 pathway [34]. STAT3 activation in hepatocytes may prevent steatosis. Treatment of obese mice with STAT3 inducing cytokines can ameliorate hepatic fat accumulation induced by high-fat diet [35]. However, deletion of STAT3 in mouse hepatocytes exacerbates steatosis mediated by a high-fat diet [36]. Furthermore, as the major upstream kinases required for STAT3 activity, JAK2 proteins play critical roles in the control of serum lipid [4]. Mice with hepatocyte-specific deletion of JAK2 develop spontaneous steatosis at an early age [37]. Besides, they showed protection against

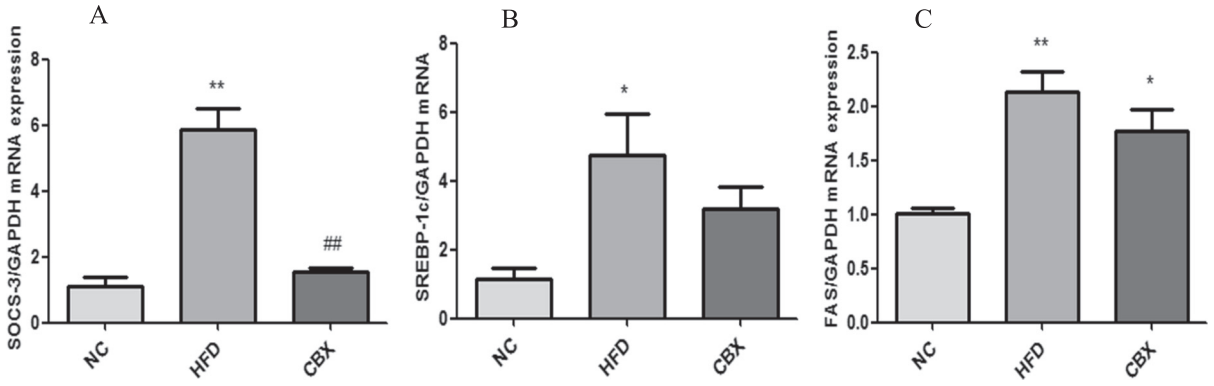

Fig. 4. Detection the changes of gene expressions by qRT-PCR in liver. (A). SOCS-3 mRNA expression. (B). SREBP-1c mRNA expression. (C). FAS mRNA expression. *p < 0.05 and **p < 0.01 vs. NC group; #p < 0.05 and ##p < 0.01 vs. DM group. Differences in multiple groups were analyzed by one-way ANOVA with Tukey multiple comparison post hoc.

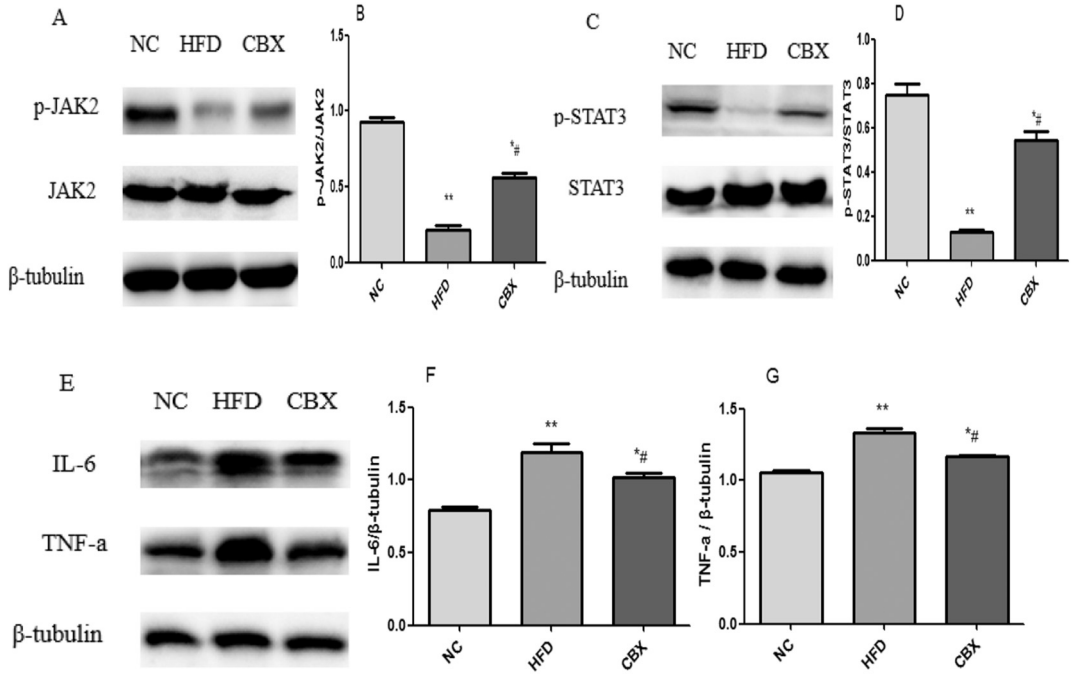

Fig. 5. The changes of JAK2/STAT3 pathway and inflammatory factors in the liver. (A). The changes of expressions of p-JAK2 and JAK2. (B). The quantifications of p-JAK2/JAK2 expressions. (C). The changes of expressions of p-STAT3 and STAT3. (D). The quantifications of p-STAT3/STAT3 expressions. (E). The changes of expressions of IL-6 and TNF-a. (F). The quantifications of IL-6 expressions. (G). The quantifications of TNF-a expressions. Data is presented as the means ± SD. *p < 0.05 and **p < 0.01 vs. NC group; #p < 0.05 and ##p < 0.01 vs. DM group. Differences in multiple groups were analyzed by one-way ANOVA with Tukey multiple comparison post hoc.

high-fat diet-induced increasing blood lipid and leptin resistance [4].

SOCS-3 is the feedback inhibitor of the leptin signaling pathway [38]. Decreased leptin transport to the brain, elevated SOCS-3 activity and inflammatory state have been proposed to be the underlying cause of low leptin signaling [32]. Our research showed that, in the liver of obese mice, the expression of SOCS-3 increased significantly, and the expressions of p-JAK2 and p-STAT3 decreased dramatically. However, CBX could significantly ameliorate this situation. The results illustrated that in the liver of obese mice, leptin resistance was found and the leptin pathway was inhibited, while CBX can partially ameliorate leptin resistance.

In the SREBPs family, SREBP-1c has a significant role in regulating genes associated with fatty acid synthesis [39]. It can up-regulate the function of PPARγ. Besides, the over expression of SREBP-1c has been demonstrated to enhance the adipogenic activity [40]. FAS is a critical enzyme in fatty acid synthesis, and can significantly increase the deposition of triglyceride in the body and lead to obesity [41]. Therefore, both SREBP-1c and FAS are essential genes regulating liver lipid metabolism [42]. The results of Seo, M. S showed that AMPK regulates lipid metabolism through controlling SREBP-1c, which can further increase the transcription of FAS, resulting in an increase in the synthesis of triglyceride [43]. Our results pointed out that the mRNA and protein levels of SREBP-1c and FAS were significantly increased in the liver of obese mice, however, after treatment with CBX, the expressions of SREBP-1c and FAS were decreased. In addition, the HE staining showed that the lipid accumulation in the liver ameliorated considerably after CBX treatment. This further suggests that CBX may inhibit liver lipid accumulation by reducing the expression of SREBP-1c and FAS, thereby decreasing blood lipids.

Inflammation plays a crucial role in the pathogenesis of obesity [44]. In obese individuals, a large number of inflammatory cytokines are produced, besides, ectopic lipid deposits in liver or skeletal muscle [45]. Our results showed that the gene and protein expressions of inflammatory factors, such as IL-6 and TNF-a, were increased in liver of

mice fed with high-fat diet. It indicated that the inflammatory state of the liver was significantly aggravated in mice fed with high-fat diet. However, after treatment with CBX, the expressions of inflammatory factors decreased dramatically, which indicated that CBX could ameliorate the inflammatory state of liver caused by high-fat diet. In addition, results of HE staining also pointed out that there was ectopic lipid deposition in the liver of obese mice. However, after treatment with CBX, there was a major decrease in ectopic lipid deposition in the liver.

In conclusion, we found that CBX could partially improve the activity of JAK2/STAT3 signaling pathway in the liver, reduce the expression of SOCS-3, so as to further reduce the expressions of SREBP-1c, FAS, and inflammatory factors in the liver, resulting in a decrease in lipid accumulation of liver. Therefore, CBX has a protective effect on liver fat accumulation and inflammation induced by obesity. It has potential application in the treatment of fatty liver, hyperlipidemia and obesity.

Acknowledgments

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Conflicts of interest

The authors declare that there is no conflict of interests.

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