Scutellarin Prevents Nonalcoholic Fatty Liver Disease (NAFLD) and Hyperlipidemia via PI3K/AKT-Dependent Activation of Nuclear Factor (Erythroid-Derived 2)-Like 2 (Nrf2) in Rats

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Data Collection B
Statistical Analysis C
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Source of support: This work was supported by the Research Fund for Scientific Undertakings of Liaoning Province in China (No. 2015005006

Background: Nonalcoholic fatty liver disease (NAFLD) is a condition characterized by excessive fat accumulation in the form of triglycerides. The incidence of NAFLD and hyperlipidemia, with their associated risks of end-stage liver and cardiovascular diseases, is increasing rapidly. This study aimed to investigate the effects of scutellarin on the experimental NAFLD in high-fat diet fed and chronic stress rats, and its possible mechanism.

Material/Methods: Sprague-Dawley rats were fed with high-fat diet and subjected to chronic stress for 12 weeks, and administered orally with scutellarin for 4 weeks (n=8), and then blood and livers were harvested for analyzing. Enzyme activity assay, immunofluorescence, Western blot, and quantitative RT-PCR were performed to analyze the factors of the oxidant/antioxidant system and pathway.

Results: After the high-fat diet and chronic stress administration for 12 weeks, serum and liver lipid metabolism of treatment groups with the different doses of SCU effectively improved and the degree of oxidative damage reduced. Using Western blot assay and immunofluorescence (IF) staining assay, Nrf2, HO-1, and PI3K, and AKT proteins significantly increased after SCU treatment for 4 weeks (P<0.01). The hepatic mRNA expression of HO-1, NQO1, and Nrf2 in SCU treatment groups was upregulated significantly through quantitative RT-PCR assay (P<0.05). However, compared to the positive control group, no difference was detected in the SCU (100 or 300 mg/kg) groups (P>0.05). These results indicate that SCU protects against NAFLD in rats via attenuation of oxidative stress.

Conclusions: The antioxidant effects of SCU on NAFLD are possibly dependent on PI3K/AKT activation with subsequent Nrf2 nuclear translocation, which increases expression of HO-1 and NQO1. We therefore suggest that breviscapine may be a potentially useful therapeutic strategy for NAFLD and hyperlipidemia.

MeSH Keywords: Fatty Liver • Hyperlipidemias • NF-E2-Related Factor 1

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/907530

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Background

Hyperlipemia is risk factors for various cardiovascular and metabolic disorders such as atherosclerosis, obesity, and metabolic syndrome. Cardiovascular disease is a major factor contributing to high rates of mortality and morbidity, particularly in the elderly, which has become a serious global health problem [1–3]. Dietary and endogenous lipids are transported in plasma by lipoproteins made mainly by enterocytes and hepatocytes. Once fat metabolism disorder occurs, lesion appear on the liver or intestinal organs.

Nonalcoholic fatty liver disease (NAFLD) is a condition characterized by excessive fat accumulation in the form of triglycerides. NAFLD is the hepatic manifestation of metabolic syndrome, which is highly associated with several metabolic disorders, such as hyperlipidemia, insulin resistance (IR), hypertension, and type 2 diabetes mellitus (T2DM) [4,5]. It causes various types of liver injuries, including simple hepatic liquid accumulation, which may appear alone or associated with inflammation, with or without fibrosis [6,7]. The incidence of NAFLD and hyperlipidemia, with their associated risks of end-stage liver and cardiovascular diseases, is increasing rapidly [8]. Statins inhibit the synthesis and increase of low-density lipoprotein (LDL) receptors in the liver, thus reducing blood cholesterol levels in hyperlipemia patients [3]. However, it reduces plasma lipids and lower incidence for some of these disorders in 20–40% of individuals, and statins can cause myopathy, liver disorder, Type 2 diabetes, and other adverse reactions when applied by high doses and long-term [9]. These indicate a need for new approaches to lower plasma lipids.

Scutellarin (SCU, 4,5,6-trihydroxyflavone-7-glucuronide), which is the major active component (>85%) of breviscapine, is a natural drug consisting of total flavonoids of the plant Erigeron breviscapus (Vant.) Hand-Mazz. (Compositae) has been used for the treatment of angina pectoris, cerebral infarction, atherosclerosis, and coronary heart disease, and has a large market share in China [10]. SCU displays protective effects in cardiovascular and cerebrovascular diseases [10–13]. SCU treatment significantly increases the phosphorylation of PKG-Iα at Ser50, Ser72, and Ser89 in hypoxia reoxygenation (HR) injury in HCMECs [14]. In addition, it is an effective antioxidant, and its antioxidant effect is associated with its structure of free phenol hydroxyl number and location [15]. Hong et al. [15] found that scutellarin attenuated $H_2O_2^-$ induced cytotoxicity, intracellular accumulation of ROS and Ca^{2+}, and lipid peroxidation in PC12 cells with oxidative damage. Further studies showed that breviscapine injection could reduce the oxidative damage of cerebral ischemia by increasing nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and heme oxygenase 1 (HO-1) expression [16]. Recent studies have highlighted that scutellarin can regulate lipid metabolism and reduce plasma

lipid in C57BL/6J mice [17], and Erigerontis injection, in which scutellarin is the main effective constituent, can reduce cholesterin and triglyceride and improve blood viscosity in ischemic stroke patients [18]. However, the specific mechanism behind the antioxidant and antilipemic effects is still unclear.

The Nrf2 is an emerging regulator of cellular resistance to oxidants, and the pathway is crucial in determining the sensitivity of mammalian cells to oxidative insults through the basal and inducible expression of an abundance of detoxification enzymes, antioxidant proteins, xenobiotic transporters, and other stress response proteins [19]. The beneficial effects of Nrf2 activation have been reported by numerous studies in a variety of experimental and clinical settings [20,21]. Nrf2 arises as a critical regulator of liver metabolic homeostasis, regulating fatty acid synthesis and the progression from steatosis to more-severe liver pathologies [22,23]. Therefore, we aimed to investigate whether scutellarin could be a useful tool to improve LDL overexpression in the context of hepatic lipid accumulation and hyperlipemia, and which activates oxidative the stress-induced Nrf2 pathway.

Material and Methods

Drug and reagents

SCU (C21H18O12, purity >90%) was purchased from Shanghai Rong He Bioengineering Company (Shanghai, China). Atorvastatin calcium tablets (Lipitor) were procured from Pfizer Inc. Total Cholesterol (TC) kits, Triglyceride (TG) kits, high-density lipoprotein cholesterol (HDL-C) kits, and low-density lipoprotein cholesterol (LDL-C) kits were purchased from Shanghai Fosun Long March Medical Science Co., Ltd, and lipid control was purchased from Randox laboratories Ltd. Glutathione kits (GSH), total superoxide dismutase kits (T-SOD), malonaldehyde kits (MDA), and total protein quantitative kits were acquired from Jian Cheng Bioengineering Institute (Nanjing, China). BCA Protein Assay Kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Anti-PI3K and anti-AKT (phospho Ser473) were acquired from Immunoway Biotechnology Company (USA), anti-Nrf2 antibody (AP52270) was purchased from Abgent (USA), NQO1 (ab28947) and anti-HO-1 antibody (ab69544) were acquired from ABcam Co. LTD (Shanghai). Realtime PCR kits and Trizol were acquired from TaKaRa Co. LTD (Dalian). Primers for genotypes were:
NRF2, 5'-TGTAGATGACCATGAGTCGC-3'(forward);
NRF2, 5'-TCCTGCCAAACTTGCTCCAT-3'(reverse);
HO-1, 5'-CTCACAGTCGCCAGTCGC-3'(forward);
HO-1, 5'-AAGGCCTCGGACAAATCCTG-3'(reverse);
NQO-1, 5'-AGTACAGCTCGCACTAGCCT-3'(forward);
NQO-1, 5'-GGGCCAATACAATCAGGGCT-3'(reverse);
AKT, 5'-ATGCGGGGCACTATCATCTC-3'(forward) and

AKT, 5'-TAACACCAACTCCGTGCCAG-3'(reverse).

Animals and experimental design

The experimental procedures were performed in accordance with animal testing regulations in China for the care and use of laboratory animals. All animal protocols were approved by the Animal Care Committee in Liaoning University of Traditional Chinese Medicine (Shenyang, China) and performed according to institutional guidelines. The mean weight of Sprague-Dawley rats (n =50) used was 170–190 g and they were provided by the HFK Bioscience Co., Ltd. (Beijing, China) (No. 11401300038822-23). Rats were maintained at an environmental temperature of 23±2°C and relative humidity (40–70%) conditions with a 12-h light-dark cycle-controlled room. All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals. All efforts were made to minimize animal suffering. After one-week acclimatization, the rats were randomly divided into 2 groups. The treatment groups had 42 rats, and the others were blank control groups (n=8). During 12 weeks, the treatment groups were fed with high-fat diet (4% cholesterol, 10% lard, 5% white sugar, 0.5% sodium cholate, 0.2% propylthiouracil and 80.3% basal feed) and subjected to a combination of various stressors, such as irregular noise, hard light, and electric shock. The high-fat diet was provided by the HFK Bioscience Co., Ltd. (Beijing, China) (No. 11003800008012). The vehicle groups (Vehicle, Veh) were fed a basal diet. After feeding for 8 weeks, venous blood was collected for blood lipid detection, and 2 rats were taken from the treatment group for detection of liver pathology to confirm whether the modeling was successful. After the model was successfully built, the rats in the treatment group were randomly divided into the 5 experimental groups (8 rats per group) as follows: 1) Vehicle +high-fat diet+ stress (Veh-h-S); 2) Scutellarin (50 mg/kg.d, po) (SCU50), 3) Scutellarin (100 mg/kg.d, po) (SCU100), 4) Scutellarin (300 mg/kg.d, po) (SCU300); 5) Lipitor (5 mg/kg.d, po) (LIP5); and the rats in the Veh-h-S group and Veh group received gavage of 10 ml/(kg·d) distilled water. The intervention time of drug was 4 weeks [24]. Body weight and physical condition were monitored every week.

Blood and tissue collection and homogenate preparation

After 12-week treatment, the rats were anesthetized with pentobarbital (0.06 g/kg; Euthanyle). Blood was collected from the abdominal aorta, and separated at 3000 rpm for 10 min to obtain serum. Serum was used for the analysis the levels of TC, TG, HDL-C, LDL-C and ALT, AST, then the remain was stored at –80°C. The livers were immediately removed and weighed, and then one-half of the tissue sample was stored at –80°C and the other half was stored in 10% formol. Part of the liver was homogenized in sterile saline using an electric homogenizer (T25, IKA, Germany), then centrifuged at 3500 rpm for

15 min. The supernatants were stored at –80°C for analysis of antioxidant enzymes activity (see below).

Serum biochemistry

To assess the clinical biochemical changes in the rats, the serum concentrations of TC, TG, HDL-C, LDL-C and ALT, and AST were measured using an fully automatic biochemical analyzer (XL-600, Erba, Germany).

Antioxidant enzymes activity assay

The activities of GSH, MDA, and T-SOD in serum and liver were determined by a commercial assay kit according to the manufacturer's instructions.

Hepatic blood flow measurement by laser speckle contrast analysis

Hepatic blood flow measurements were obtained using a commercially available LSCI instrument (moorFLPI Speckle Contrast Imager, Moor Instruments, UK), which was connected to a standard computer equipped with purpose-designed data acquisition software (MoorFLPI Measurement Software Version 4.0; Moor Instruments). The LSCI instrument was mounted on a fully adjustable tabletop stand and positioned 30 cm above the exposed liver surface. Blood flow within the imaging field (15×20 mm) was visualized as a 2-dimensional color-coded map of perfusion and recorded with purpose-designed data acquisition software. Image resolution was 752×580 pixels (exposure time 20 ms) and light was collected through a zoom lens, which provided adjustable magnification. Identical focal lens settings and exposure times were maintained during all measurements. All LSCI blood perfusion measurements are presented in Liaoning University of Traditional Chinese Medicine.

Histopathology

Following standard procedures, pathological analyses were performed on liver tissue sections. Briefly, small pieces of liver were fixed with formalin (10% formol), dehydrated in ethanol-xylene, and embedded in paraffin. Sections of 5-μm thicknesses were obtained on a sliding microtome, mounted on slides, dewaxed with xylene, and stained with hematoxylin-eosin. Images were captured using an Leica DM2000 microscope from 10 randomly selected fields (200×) to assess NAFLD using established histologic criteria that score steatosis, hepatocellular ballooning, and hepatic inflammatory infiltrates. In brief, steatosis was scored as: grade 0 for no fatty hepatocytes, grade 1 for fatty hepatocytes occupying <33% of the hepatic parenchyma, grade 2 for fatty hepatocytes occupying 33–66% of the hepatic parenchyma, or grade 3 for fatty hepatocytes occupying >66% of the hepatic parenchyma.

Hepatocellular ballooning was scored as grade 0 for none, grade 1 for few ballooned cells, and grade 2 for predominant ballooning. Hepatic inflammatory infiltrates were scored as grade 0 for none, grade 1 for <2 foci/field, grade 2 for 2–4 foci/field, or grade 3 for >4 foci/field. An overall score, namely the NAFLD activity score (NAS), was generated based on the individual scores of all the histological features [25].

Immunofluorescence (IF) staining

Paraffin-embedded and formalin-fixed liver tissue was sectioned at a thickness of 4 µM. The sections were deparaffinated in xylene-ethanol, washed 3 times in PBS for 5 min, antigen retrieval in sodium citrate buffer (0.01 mol/l, pH 6.8) for 20 min, washed 3 times in PBS for 5 min, placed in 0.3% H_2O_2 for 20 min, washed 3 times in PBS for 5 min, and incubated with 5% bull serum albumin in PBS/0.1% Triton X-100 for 60 min at 37°C. After draining the solution from the tissue section, the tissue was incubated with AKT (phospho Ser473) antibody (YP0006, Immunoway, USA) and Nrf2 antibody (AP52270, Abgent, USA), and then placed in a 4°C refrigerator overnight. After rinsing with PBS, the sections were incubated with FITC-conjugated secondary antibody for 120 min in 5% bull serum albumin. Nuclear staining was performed with 4,6-diamidino-2-phenylindole (DAPI, Invitrogen), then mounted and photographed using a confocal laser scanning microscope (Axio Scope A1, Zeiss, Germany). The negative control experiments were also performed, in which the secondary antibodies or primary antibodies were omitted to exclude the potential nonspecific binding to the tissue specimen.

Western blot analysis

One part of the liver sample was homogenized in cell lysis buffer (tissue to buffer ratio: 0.1 g·ml^{-1}) (Beyotime, China) supplemented with protease and phosphatase inhibitors (1: 100), and then liver protein lysate was centrifuged at 14,000×g for 15 min and the supernatant stored at –80°C. Protein concentration was determined by BCA Protein Assay Kit (Beyotime, China). Equal quantities of protein were resolved by 10% SDS-PAGE. After electrophoresis, proteins were blotted onto a PVDF membrane (Millipore), blocked for 1 h in 5% dry milk-PBS-0.1% Tween 20 (blocking buffer), and incubated with the respective primary antibodies overnight at 4°C in blocking buffer: Anti-PI3K (dilution 1: 400), anti-AKT (phospho Ser473) (dilution 1: 400), NQO1 (dilution 1: 600), anti-Nrf2 antibody (dilution 1: 300) and Anti-Heme Oxygenase 1 antibody (HO-1) (dilution 1: 600), β-actin (dilution 1: 1000). Secondary HRP-conjugated antibodies were applied for 2 h at room temperature in blocking buffer. The blots were developed with ECL reagent on automatic digital gel image analysis system (4200SF, Tanon, Shanghai). After gathering images, grey value was measured by gel imaging analysis system software, and we put the

grey value target protein/β-actin grey value ratio as the protein relative expression levels, and made statistical analysis.

Quantitative RT-PCR

Extraction and reverse transcription-quantitative PCR (qRT-PCR) analysis of RNAs from the liver were performed as described previously [26]. The tissue samples were frozen at –80°C. Total RNA was extracted using Trizol reagent (TaKaRa, Dalian, China). Total RNA was quantified using a Nano2000 ultraviolet spectrophotometer (Thermo Fisher Scientific Inc., USA). The mRNA expression of NRF2, HO-1, NOQ-1, and AKT in the livers were examined via qRT-PCR using the ABI 7500 Real-Time PCR system (Applied Biosystems, USA) according to the manufacturer's instructions. Quantitative values were obtained from the threshold cycle value (Ct). An internal control, GAPDH, was used to calculate the relative quantitative values of the target genes of each sample.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (Version 6.0, GraphPad Software) and SPSS 19.0 statistical software. The results are expressed as means and standard deviation of the mean (SD), and P values of 0.05 were considered statistically significant. Differences between the body weight and biochemical and molecular biology results from the 6 experimental groups were evaluated by one-way ANOVA, followed by LSD Fisher test. Comparisons between the histology NAS and hepatosomatic index results were made using the Kruskal-Wallis H test.

Results

Scutellarin effects the body weight of animals

To determine whether scutellarin affected the body weight in NAFLD rats, we weighed the animals and recorded the result every 4 weeks. The results showed that the body weights of animals in the treatment groups with high-fat diet and stress did not significantly increase compared to Veh groups (P<0.05) (Table 1), especially in the first 4 weeks. The body weight significantly increased after SCU (100 or 300 mg/kg) treatment for 4 weeks (P<0.05) (Table 1). Furthermore, the increase in body weight observed in the Lipitor group was also significant (P<0.01), and had no statistical difference from SCU treatment groups (Table 1).

Scutellarin improves serum TC, TG, HDL-C, and LDL-C concentrations

To determine whether scutellarin increased the circulating cholesterol and lipid efflux in NAFLD rats, we measured serum TC, TG, HDL-C, and LDL-C levels. The results showed that

Table 1. The effect of the body weight of animals every 4 week on this test.

Groups	N	0 w	4 w	8 w	12 w
Veh	8	191.50±7.17	333.88±6.03	421.00±14.47	489.88±11.90
Veh-h-S	8	191.75±4.65	192.12±10.16**	195.25±11.04**	196.50±13.91**
LIP5	8	186.75±4.77	187.25±7.48**	194.38±12.22**	226.50±18.09**##
SCU50	8	187.38±7.76	189.75±12.33**	196.50±14.36**	209.25±17.27**
SCU100	8	192.88±6.56	193.12±16.90**	199.75±12.54**	213.00±12.92**#
SCU300	8	186.25±3.45	186.50±15.92**	195.62±15.30**	212.00±14.23**#

Under high-fat diet and chronic stress, rats were treated with different concentrations of scutellarin (SCU, 50, 100, 300 mg/kg) for 4 weeks, and the body weight of animals was recorded and analyzed. All data were represented as means±standard deviation (n=8). Statistical analysis of the body weight (* $P<0.05$, ** $P<0.01$, Veh-h-S group versus Veh group; # $P<0.05$, ## $P<0.01$, treatment groups versus Veh-h-S group).
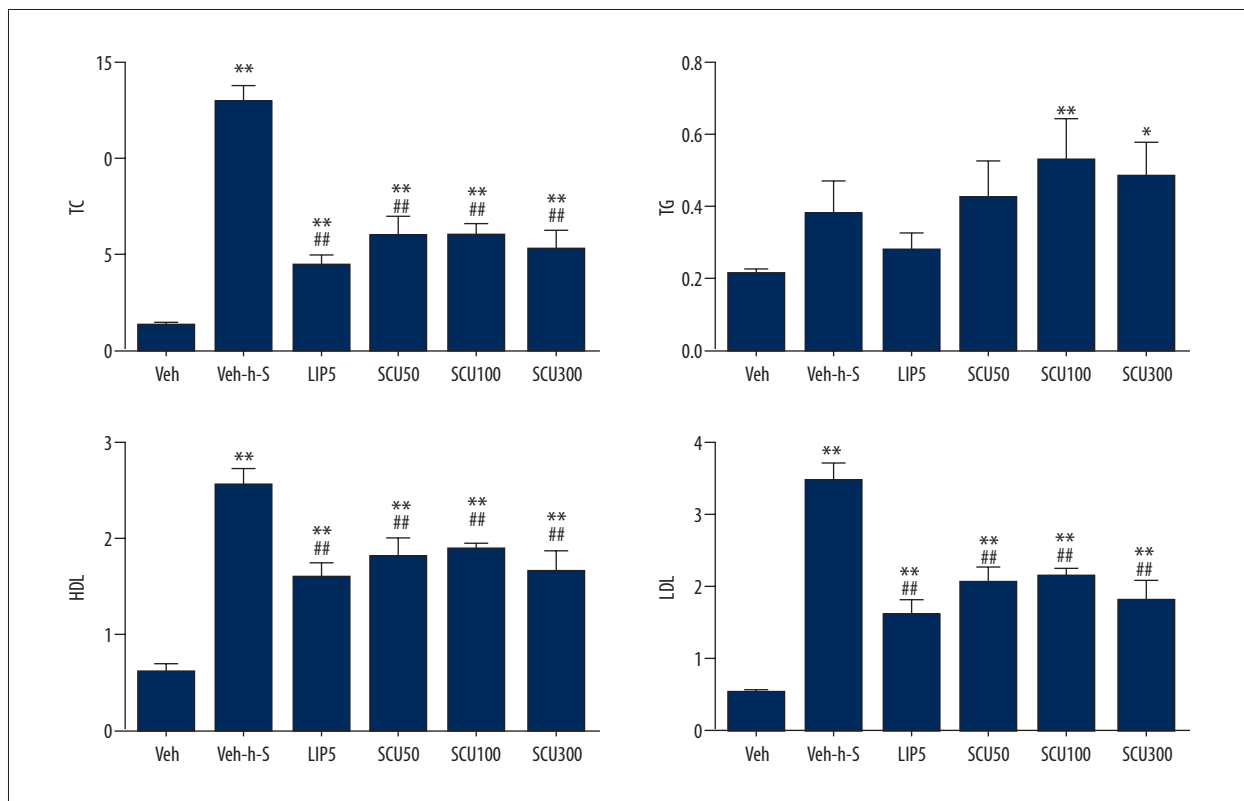

Figure 1. The effect of serum TC, TG, HDL-C and LDL-C concentrations after SCU treatment. Under high-fat diet and chronic stress, rats were treated with different concentrations of SCU (50, 100, 300 mg/kg) for 4 weeks, and serum biochemistry was determined by clinical chemistry analyzer. All data were represented as means±standard deviation (n=8). Statistical analysis of TC, TG, HDL-C and LDL-C (* $P<0.05$, ** $P<0.01$, Veh-h-S group versus Veh group; # $P<0.05$, ## $P<0.01$, treatment groups versus Veh-h-S group).

TC, HDL-C, and LDL-C were upregulated by 10.3-fold, 4.1-fold, and 6.5-fold in the rats under high-fat diet and chronic stresses for 12 weeks, respectively ($P<0.01$) (Figure 1), and these 3 concentrations were all significantly downregulated after SCU treatment for 4 weeks ($P<0.01$). Although TG concentration of the Veh-h-S group was not significantly different from that of the Veh group, TG showed an increasing tendency in Veh-h-S group rats. Furthermore, Lipitor also significantly upregulated TC, HDL-C, and LDL-C in the 12th week ($P<0.01$), and had no statistical difference from SCU treatment groups (Figure 1).
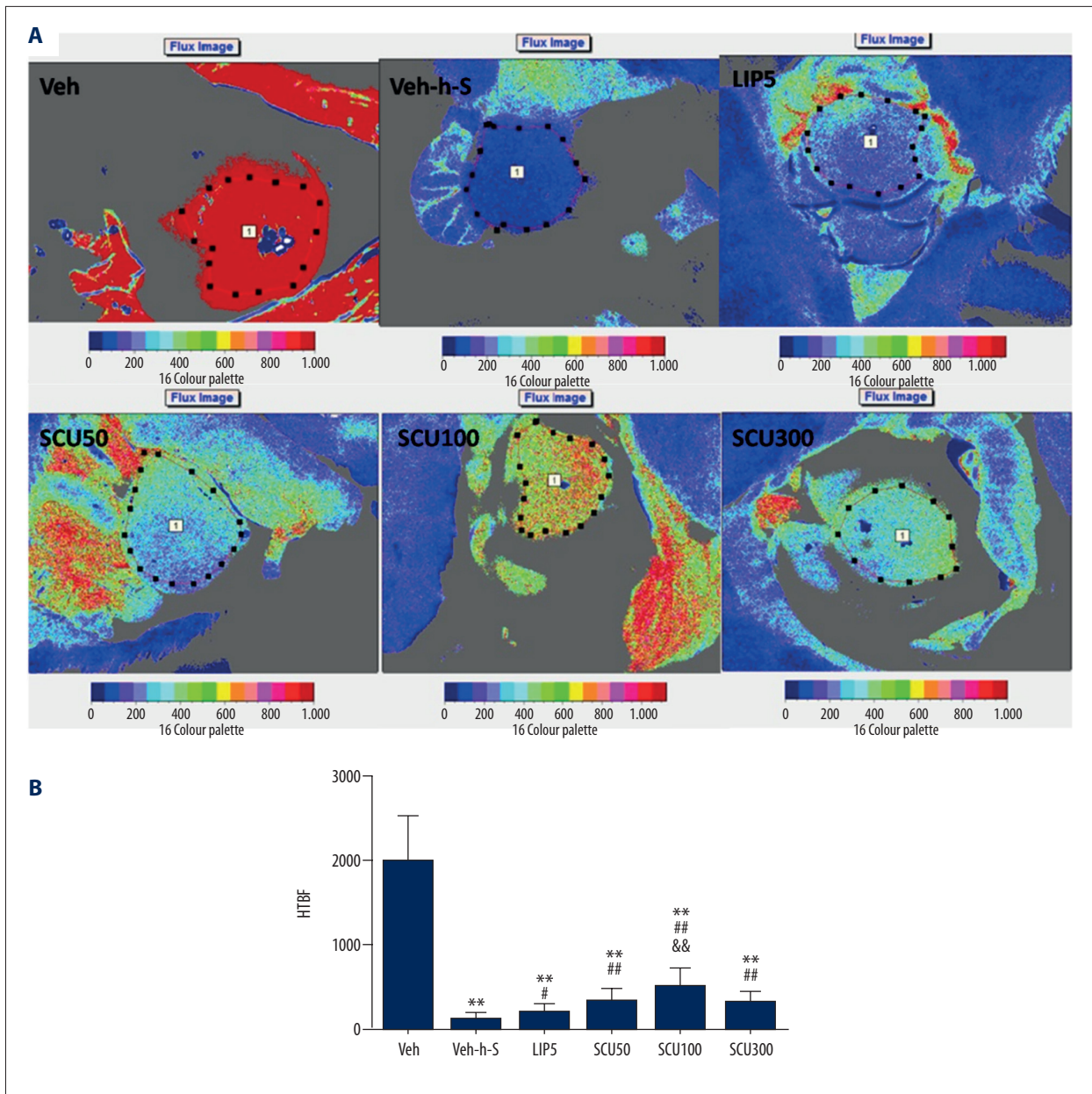
Figure 2. The effect of hepatic tissue blood flow (HTBF) after SCU treatment. (**A**) Laser speckle images of hepatic tissueon the dual intervention of high-fat diet and chronic stress 12 weeks and SCU treatment 4 weeks. The area of the dotted line is the location of collecting hepatic tissue blood flow. Relative perfusion in the arbitrary unit flux was portrayed in a color-coded laser speckle image (red=high flow, blue=low flow). (**B**) Bar graphs showing the HTBF in different groups.All data were represented as means ± standard deviation (n=8). Statistical analysis of HTBF (* P<0.05, ** P<0.01, Veh-h-S group versus Veh group; # P<0.05, ## P<0.01, treatment groups versus Veh-h-S group; & P<0.05, && P<0.01, SCU treatment groups versus LIP5 group).

Scutellarin improves hepatic tissue blood flow (HTBF)

To assess whether SCU could improve hepatic tissue blood flow (HTBF) in NADLF rats, we used laser speckle contrast analysis. The HTBF color image of all groups is showed in Figure 2A. During the 12-week period of induction, the HTBF of animals in the Veh-h-S group (111.77±58.54) with high-fat diet and

chronic stress was less than in the Veh group (1981.39±568.05) (P<0.01) (Figure2B); however, the HTBF recovered gradually after being treated with different doses of SCU (352.31±187.92, 494.39±240.46, 309.46±141.41) (P<0.01) for 4 weeks, and the HTBF of the LIP5 group (208.11±107.62) also increased (P<0.05). Importantly, the effect of HTBF in the LIP5 group was significant less than in the SCU100 group (P<0.01) (Figure 2B).
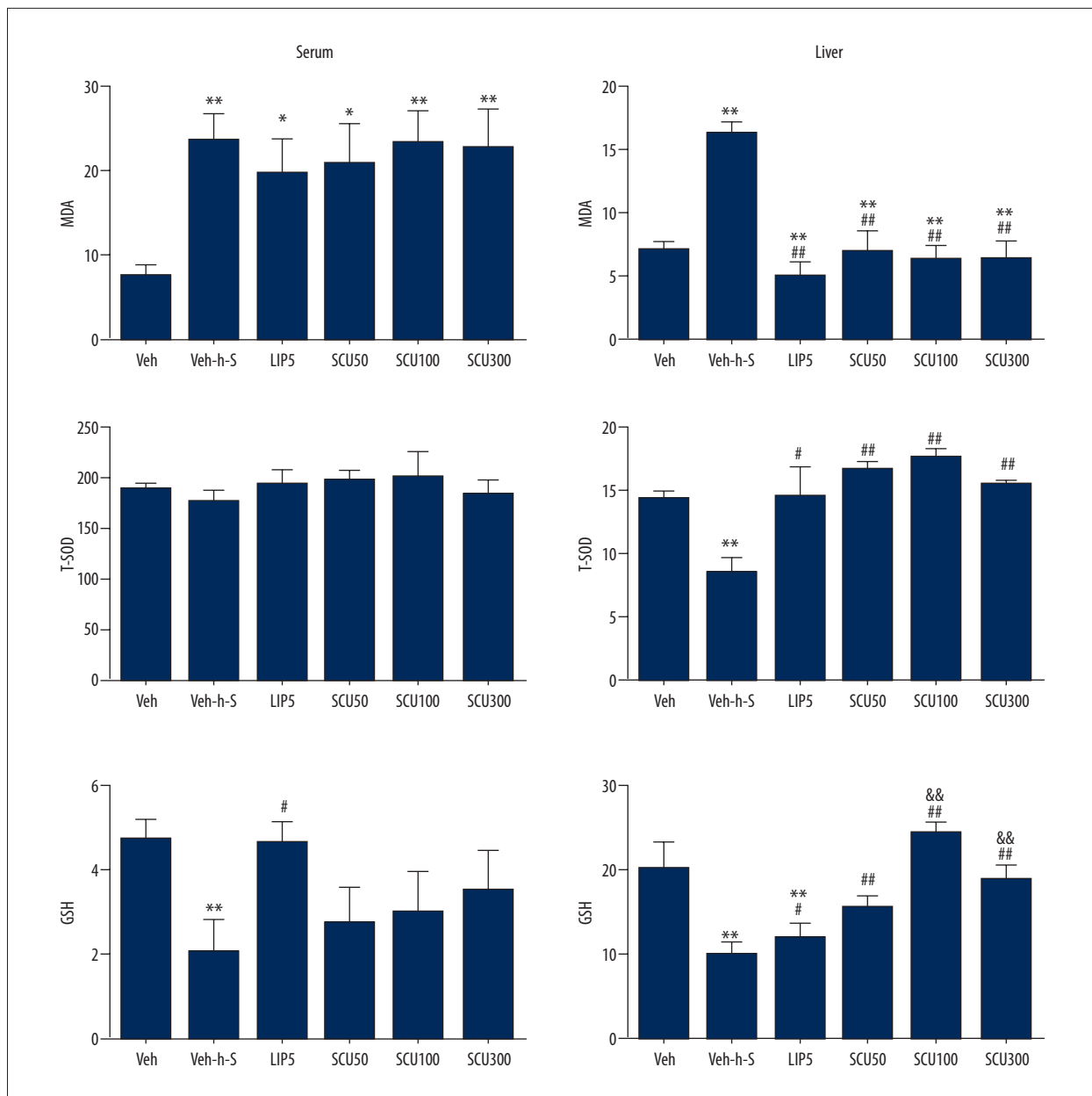
Figure 3. The effects of oxidative damage through MDA, T-SOD and GSH in serum and liver after SCU treatment.Under high-fat diet and chronic stress, rats were treated with different concentrations of scutellarin (SCU, 50, 100, 300 mg/kg) for 4 weeks, and these contents were determined by acommercial assay kits. All data were represented as means ± standard deviation (n=8). Statistical analysis of MDA, T-SOD and GSH (* P<0.05, ** P<0.01, Veh-h-S group versus Veh group; # P<0.05, ## P<0.01, treatment groups versus Veh-h-S group; & P<0.05, && P<0.01, SCU treatment groups versus LIP5 group).

Scutellarin reduces the degree of oxidative damage

To investigate whether scutellarin affected the antioxidant enzymes activity in NAFLD rats, we measured GSH, MDA, and T-SOD in serum and liver, respectively. The results showed that although these antioxidant enzymes levels in serum of SCU groups were not significantly different from in the Veh-h-S group, GSH and T-SOD in livers increased, and MDA was

downregulated (P<0.01) (Figure 3). Meanwhile, LIP5 also regulated GSH, MDA, and T-SOD activity in livers (P<0.01, P<0.05, P<0.05), but the level of upregulating GSH was lower than in SCU100 and SCU300 groups (P<0.01) (Figure 3).
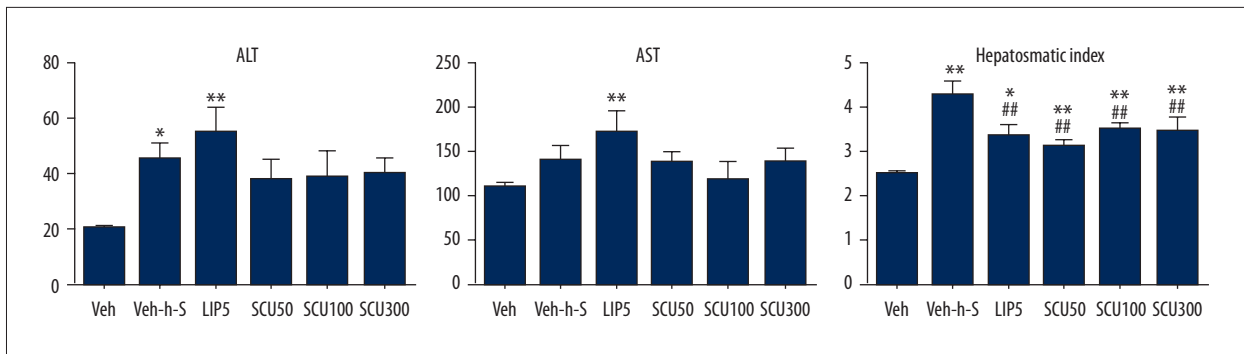
Figure 4. The effect of the liver function indicator through ALT, AST and hepatosmatic index in different groups. Under high-fat diet and chronic stress rats were treated with various concentrations of SCU (50, 100, 300 mg/kg) for 4 weeks, and serum ALT and AST were determined by clinical chemistry analyzer, and hepatosmatic indexwas the ratio of liver to body weight. All data were represented as means ± standard deviation (n=8).Statistical analysis of these date (* P<0.05, ** P<0.01, Veh-h-S group versus Veh group; # P<0.05, ## P<0.01, treatment groups versus Veh-h-S group).

Scutellarin affects the liver function and hepatosomatic index

To determine whether scutellarin protected the liver function in NAFLD rats, we measured serum ALT and AST levels. The results showed that ALT in serum of Veh-h-S group was significantly upregulated in the 12th week, specifically compared to the Veh group (P<0.05) (Figure 4). After SCU treatment, the content of ALT was downregulated, but had no significant difference from the Veh-h-S group (P>0.05). However, AST level in serum of the Veh-h-S group was higher than in the Veh group, but was not significantly different (P>0.05). In addition, we weighed the liver and calculated hepatosomatic index after 12 weeks. Hepatosomatic index showed a significant increase in Veh-h-S group rats (4.55±0.29) (P<0.01), and then the same index (3.16±0.47, 3.60±0.28, 3.50±0.84) decreased in SCU treatment groups (P<0.01, P<0.01, P<0.05) (Figure 4). Furthermore, hepatosomatic index (3.15±0.62) of LIP5 group was also significantly upregulated in the 12th week (P<0.01), and the different SCU groups showed similar efficacy to that of the LIP5 group (P>0.05) (Figure 4).

Scutellarin inhibits hepatic lipid accumulation

To more deeply assess our established rodent models using histological examinations, the results showed that livers of Veh-h-S group rats appeared to specifically be filled with macrovesicular fat within hepatocytes, and specifically compared to Veh group according to histological staining with hematoxylin and eosin (H&E) (P<0.05) (Figure 5), demonstrating that high-fat diet and chronic stress treatment induced lipid accumulation in the liver.

To assess whether SCU could improve hepatic lipid accumulation in NADLF rats, we used histological examinations scored through microscopy. The histological images of all groups are

shown in Figure 5. The degrees of steatosis and hepatocellular ballooning were significantly higher than those of the Veh group (P<0.01). With regard to the pathological changes, the livers in the SCU treatment group were observed to be significantly improved compared with the Veh-h-S group in the same period (P<0.01, P<0.05) (Figure 5); however, hepatic inflammatory infiltrates were not significantly different among SCU treatment groups and the Veh-h-S group. For the overall scores, the NAS of SCU treatment groups (2.78±0.88, 2.59±0.88, 2.81±0.51) were less than the Veh-h-S group (4.54±0.78) (P<0.01). Especially, SCU at 100 mg/kg showed similar efficacy to that of the LIP5 group (5 mg/kg) (P>0.05).

Scutellarin enhances the Nrf2-mediated Antioxidant Defense System

To further study the relationship between the anti-hyperlipidemia effect and the antioxidant response of SCU, we examined the effects of the Nrf2-mediated antioxidant defense system, such as HO-1, NQO1, P-AKT, PI3K, Nrf2 protein and HO-1, NQO1, AKT, Nrf2 mRNA expression. All proteins activities were evaluated by Western blot, and the results showed that Nrf2, HO-1 and PI3K proteins increased in the rats under high-fat diet and chronic stresses for 12 weeks (P<0.05), and these proteins significantly increased after SCU treatment for 4 weeks (P<0.01) (Figure 6). In the different doses of SCU groups, the NQO1 and P-AKT proteins also upregulated in comparison with Veh or Veh-h-S groups (P<0.01). We also applied immunofluorescence for detecting P-AKT and Nrf2 protein levels; P-AKT[+] and Nrf2^{+} hepatocyte were increased (green fluorescence intensity) after SCU treatment in comparison with Veh or Veh-h-S group (Figure 7). The mRNA expression of Nrf2, HO-1 and NQO-1 in the livers examined via qRTPCR, the results indicated that the key gene Nrf2 and its downstream targets NQO-1 and HO-1 were upregulated after SCU treatment (P<0.01) (Figure 8). Especially, SCU at 100 mg/kg showed similar efficacy to that of LIP5 group (5 mg/kg) (P>0.05).
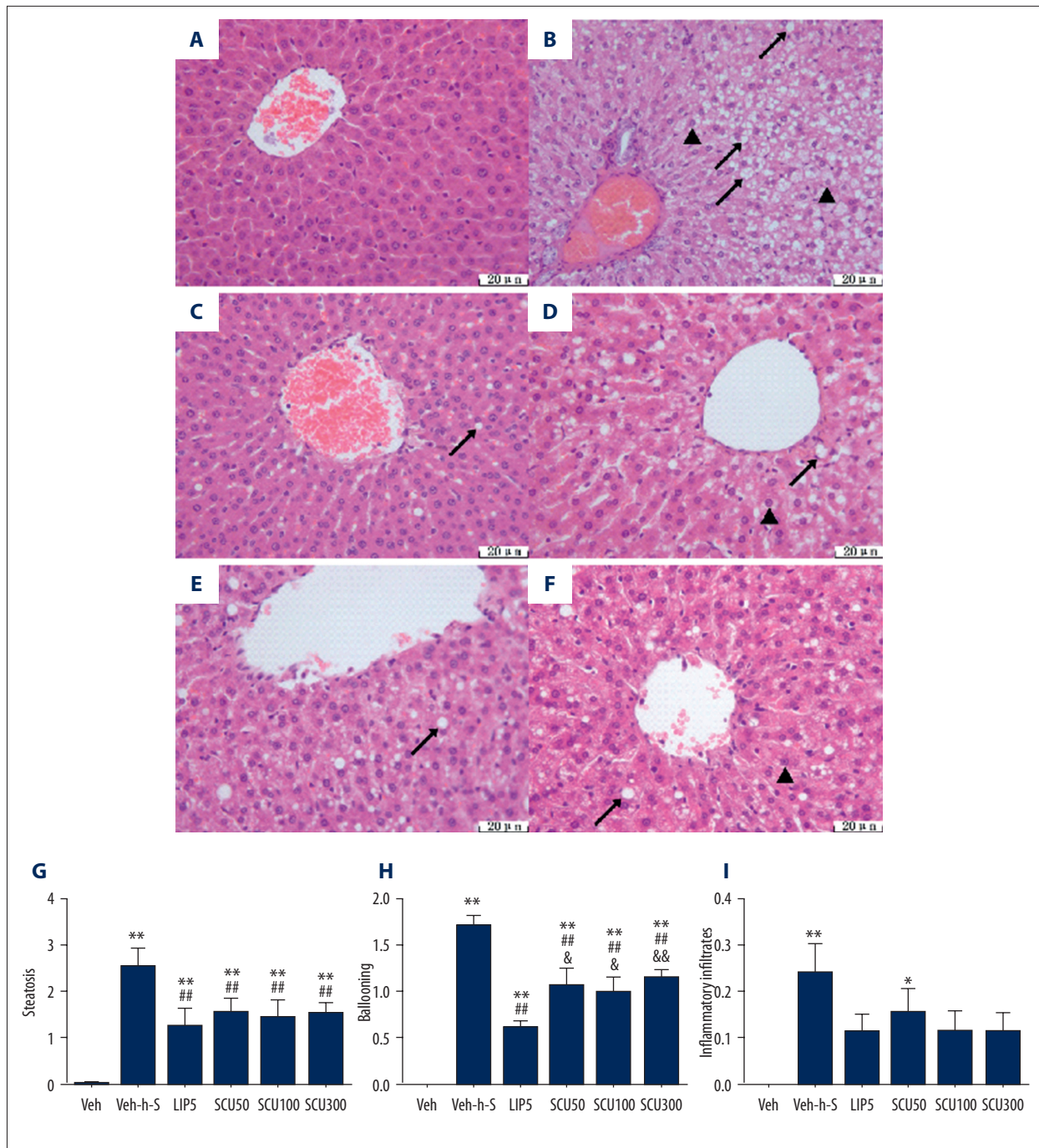
Figure 5. Histologic evaluation of livers from Sprague Dawley rats after SCU treatment. (**A–F**) Representative hematoxylin and eosin-stained liver sections (original magnification 200×) in different groups. (**A**) Veh group, (**B**) Veh-h-S group, (**C**) LIP5 group, (**D**) SCU50 group, (**E**) SCU100 group, (**F**) SCU300 group. Veh-h-S group rats had exacerbated hepatic steatosis (↑) and hepatocyte ballooning (▲) relative to Veh group. SCU reduced these parameters, but the effects had no dose-dependent relationship. Histologic scoring of liver steatosis (**G**), hepatocyte ballooning (**H**) and inflammatory infiltrates (**I**). All data were represented as means±standard deviation (n=8). Statistical analysis of these date (*P<0.05, **P<0.01, Veh-h-S group versus Veh group; # P<0.05, ## P<0.01, treatment groups versus Veh-h-S group; & P<0.05, && P<0.01, SCU treatment groups versus LIP5 group).
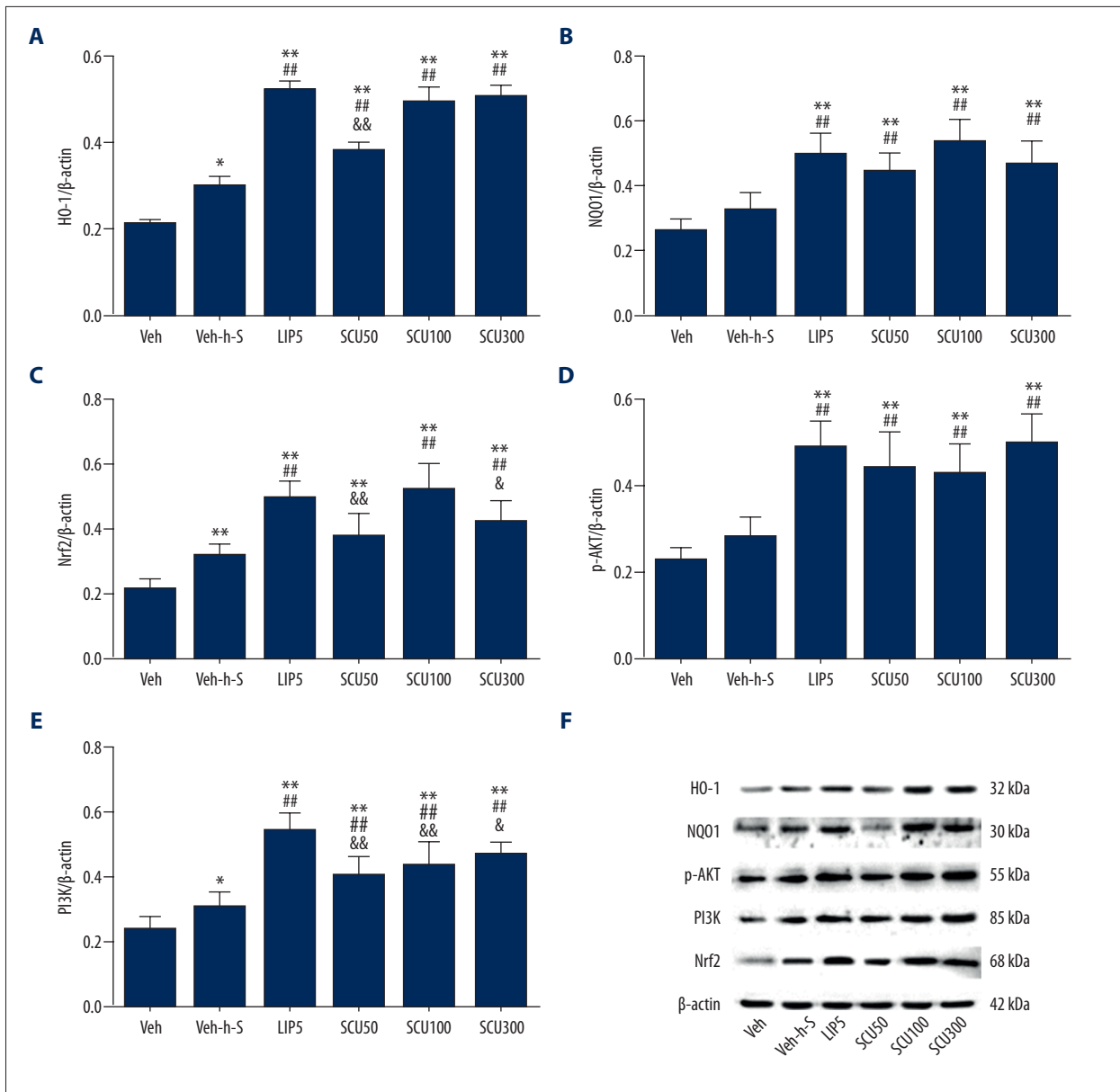
Figure 6. The evaluation on Nrf2-mediated antioxidant defense system of livers from Sprague Dawley rats after SCU treatment by western blotting. The levels of AKT/Nrf2 pathway proteins were evaluated by western blotting. β-actin was used as the internal control, respectively. (**A**) HO-1, (**B**) NQO1, (**C**) Nrf2, (**D**) p-AKT, and (**E**) PI3K proteins in different groups. All data were represented as means ± standard deviation (n=6).Statistical analysis of these date. (* $P<0.05$, ** $P<0.01$, Veh-h-S group versus Veh group; # $P<0.05$, ## $P<0.01$, treatment groups versus Veh-h-S group; & $P<0.05$, && $P<0.01$, SCU treatment groups versus LIP5 group). (**F**) The immunoblotting results of these proteins.

Discussion

In modern times, people are experiencing high-intensity learning, working stress, and unhealthy diet and living habits, which are chronic stresses (CS) on our bodies. The long-term high level of corticosteroids caused by CS could affect the lipid metabolism, forming NAFLD and metabolic syndrome in humans [27]. The liver plays a key role in lipid metabolism. The

abnormal hormone, caused by abnormality of the nervous and endocrine system, is the main factor leading to the formation and exacerbating of the " multiple hit" of NAFLD [28–30]. In the present study, we simulated an unhealthy human condition of stress with high-fat diet and unpredictability of chronic stress, establishing the NAFLD rat model. The model is to the normal human condition resulting from unhealthy diet and chronic stress factors. We observed that serum TC and LDL of
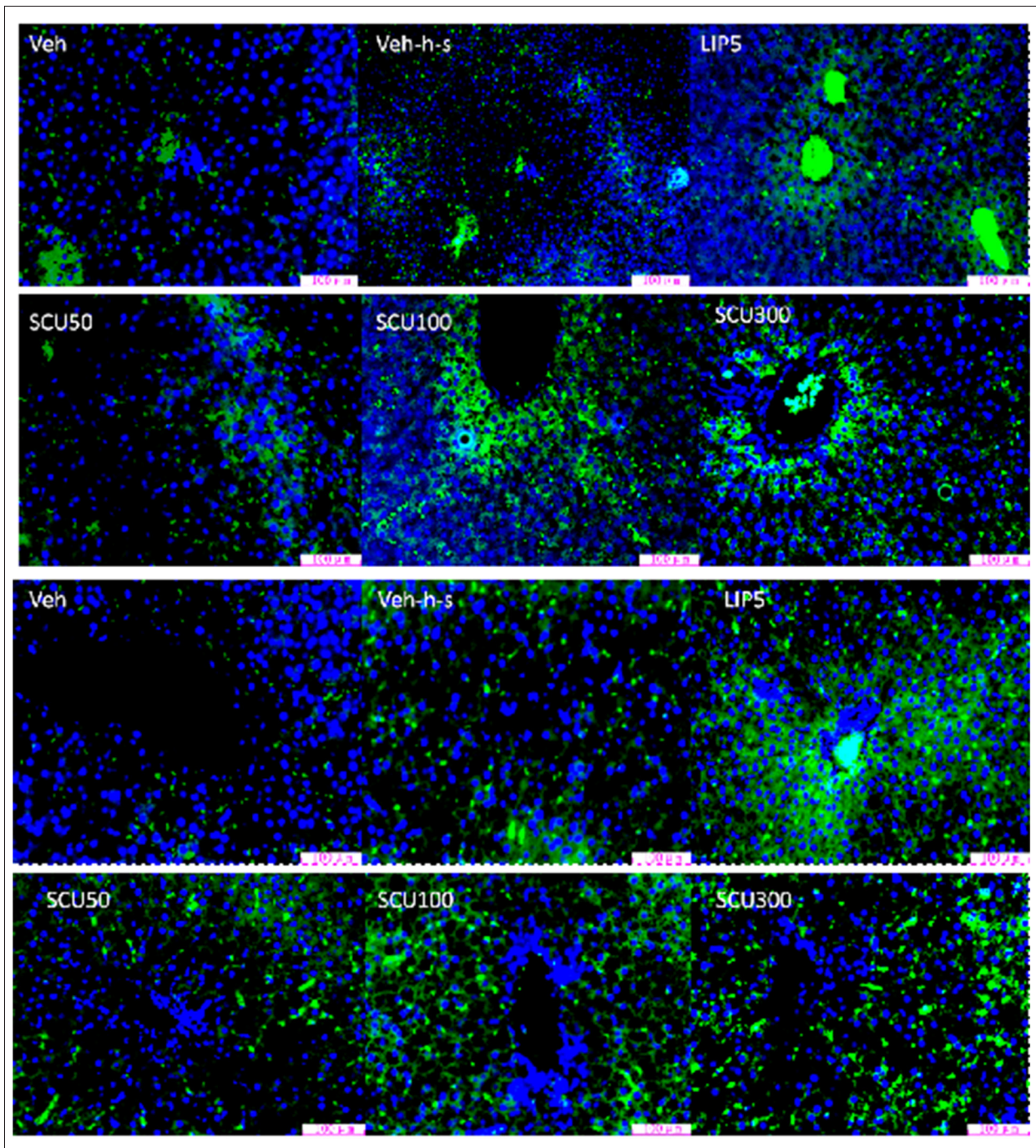
Figure 7. The effect of key protein on Nrf2-mediated antioxidant defense system of livers from Sprague Dawley rats after SCU treatment by immunofluorescence. Anti-NRF2 antibody (up) and anti-p-AKT antibody (under) were measured in livers, respectively. Green fluorescence was positive expression, and nuclei were stained with DAPI. Images of different groups were photographed using confocal fluorescence microscopy (original magnification 200×).

the treatment group began to rise at the 8th week, at the same time the liver histological examination showed hepatocellular steatosis. In the 12th week, the blood biochemistry, liver tissue blood flow, and oxidation indexes were obviously abnormal (P<0.05), and hepatocyte steatosis was obvious in the Veh-h-S

group, consistent with our hypothesis and the work of others [31]. It was further proved that the dual intervention of high-fat diet and chronic stress could promote lipid disorder and abnormal lipid metabolism in the liver. However, no significant increase in weight was observed in Veh-h-S group rats,
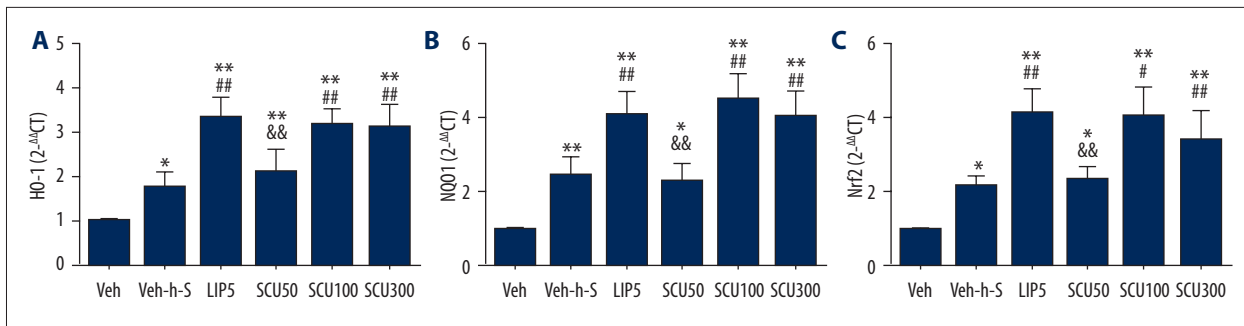
Figure 8. The gene expression of cytokines on Nrf2 pathway in livers from Sprague Dawley rats after SCU treatmentby quantitative RT-PCR.Quantitative values were obtained from the threshold cycle value ($^{\Delta\Delta}$Ct). (**A–C**) The quantitative values of HO-1, NQO1, Nrf2 gene expression in different groups.All data were represented as means±standard deviation (n=3).Statistical analysis of these date (* $P<0.05$, ** $P<0.01$, Veh-h-S group versus Veh group; # $P<0.05$, ## $P<0.01$, treatment groups versus Veh-h-S group; & $P<0.05$, && $P<0.01$, SCU treatment groups versus LIP5 group). (**F**) The immunoblotting results of these proteins.

which may be related to the endocrine abnormality of chronic stress. This result showed that the occurrence of weight and NAFLD may not be positively correlated.

Several plants have been widely used for treating or preventing diseases in various countries. For instance, Herba Erigerontis has been used in folk remedies to treat cerebral ischemia, coronary heart disease, and hypertension [10]. Recently, scientific studies have shown that breviscapine holds pharmacological activity, including anti-inflammatory and vasorelaxant effects and platelet activation, as well as alleviation of cardiac dysfunction and cerebral dysfunction [32–34]. However, the effects and mechanism of extracts from this plant on the NAFLD have not been studied so far. Therefore, we investigated whether SCU treatment could protect against liver damage through activating oxidative stress-induced nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway, used in *in vivo* models.

We designed the protective effect of baicalin on NAFLD and hyperlipidemia from different perspectives such as serum biochemistry, antioxidant enzymes activity assay, hepatic blood flow, and histopathology. Our experiments showed that SCU, the important flavonoids in the Erigeron breviscapus, effectively improved serum lipid metabolism and hepatic lipid accumulation, consistent with our hypothesis and the study of other flavonoids [35]. At the same time, SCU could increase hepatic blood flow, and this may be related to the mechanism of SCU to relax blood vessels and improve vascular stress [32]. The liver, which is the major tissue producing fatty acids in the body, plays a key role in lipid metabolism. Triglycerides (TG) derived from *de novo* lipogenesis are either stored in lipid droplets (LD) or packed into VLDL or exported into the blood stream; when hepatic synthesis of TG could not be transported in time, it causes the liver fat metabolism disorder, and then causes fatty liver [36,37]. After SCU treatment, the blood flow of liver tissue is upregulated, which partially improved the transporting ability of triglyceride transshipment and regulated lipid

metabolism cycle, thereby protecting liver function. It was also observed that the hyperlipidemia regulated activity of SCU may be correlated with the hepatic blood flow and liver function. Nguyen [38] reported that hepatic fatty acids regulated overall lipid metabolism by binding nuclear receptors that modulate gene transcription, consistent with our results.

After 12 weeks of the high-fat diet and chronic stresses, serum levels of lipid peroxidation marker (MDA) went higher, accompanied with the levels of GSH and antioxidant enzymes going down. The increased MDA and decreased antioxidant levels (including GSH, SOD) confirmed the occurrence of oxidative damage in the liver of Veh-h-S group rats; this alteration was also observed in the rodents submitted to chronic stress or high-fat diet by this procedure in several studies [39–41]. These stressors can disturb the oxidant and antioxidant balance, and increase production of free radicals parallel to suppressing antioxidant capacity. The increased ROS lowered the nuclear factor (erythroid-derived-2)-like 2 (Nrf-2), a primary transcriptional regulator of most antioxidants, including SOD and GST [42]. This may be a possible explanation for the decreased SOD and GSH activities and increased MDA contents that were seen in Veh-h-S group rats. The group treated with SCU showed a higher increase in the activities of these enzymes as compared to the Veh-h-S group. Considering that, the liver-protective effect of SCU may be mediated by SOD; however, further studies are needed to determine the direct relationship between the anti-hyperlipidemia effect of SCU and the antioxidant response.

The Nrf2 pathway is crucial in determining the sensitivity of mammalian cells to oxidative insults through the basal and inducible expression of an abundance of detoxification enzymes, antioxidant proteins, xenobiotic transporters, and other stress response proteins [19]. When the body is not under oxidative stress, Keap1 inhibits the activity of Nrf2 and accelerates the degradation of Nrf2. Nrf2 is activated in human fatty liver

disease, and impairment of Nrf2 activity represents a major risk factor for the evolution of NAFLD to nonalcoholic steatohepatitis (NASH) [20,21]. Nrf2 is able to regulate a variety of antioxidants, including phase II detoxifying enzyme, and it affects gene expression throughout the body through the induction of antioxidant response elements [43], which play a central role in the regulation of the oxidative stress response. The NQO-1 and HO-1 genes are the downstream targets of Nrf2 and these are regulated by Nrf2, which is regarded as one of the most important intracellular antioxidant mechanisms [44]. Data from the present study reveal that hyperlipidemia and NAFLD increase the protein levels of Nrf2, NQO-1, and HO-1 and upregulate the expression of NQO-1 and HO-1 mRNA. The increase in the expression of antioxidant factors is due to the body's NRF2 signal, which is reflexively activated when suffering from external oxidative stress. In the present study, our findings revealed that SCU could significantly increase the binding activity of Nrf2 and upregulate the protein expression of antioxidant enzymes HO-1 and NQO1, which consequently enhanced resistance to oxidative stress in the NAFLD rats.

The phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway is considered to be one of the upstream pathways of Nrf2-ARE signaling. In mammalian cells, PI3K/AKT is an important upstream regulator of the Nrf2 and its activation can lead to Nrf2 nuclear translocation [45]. To examine the gene expression alteration regulated by the Nrf2-ARE pathway, we measured the gene expression of AKT and Nrf2. In this study,

we found that SCU induced a significant increase of p-AKT level and upregulate gene expression in the liver of rats. Activation of PI3K results in the synthesis of PIP3, which activates the protein kinase AKT [46]. The high level of phosphorylated AKT indicated that Nrf2 was activated, regulated its nuclear translocation, and then its downstream series of substrate such as NQO-1 and HO-1 was released to regulate the anti-oxidative effect of the cells [47,48]. This result further demonstrated that PI3K/AKT could activate Nrf2 signaling in rats with liver injury. Our results indicate that SCU activates the PI3K/AKT signaling pathway, and promotes Nrf2 regulation, and releases antioxidants, thus leading to improved NAFLD and hyperlipidemia.

Conclusions

In conclusion, our study demonstrates that SCU protects against NAFLD and hyperlipidemia in rats via attenuation of oxidative stress. The antioxidant effects of SCU on NAFLD are possibly dependent on PI3K/AKT activation with subsequent Nrf2 nuclear translocation, which increases expression of HO-1 and NQO1. We suggest that breviscapine may be a potentially useful therapeutic strategy for NAFLD and hyperlipidemia.

Conflict of interest

None.

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