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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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

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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 lipid 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 brevisscapus (Vant.) Hand-Mazz. (Compositae) has been used as a traditional drug 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-I in the liver, which may appear alone or associated with inflammation or 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.

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-AKT (phospho Ser473) were acquired from Immunoway Institute of Biotechnology (Shanghai, China). Anti-PI3K and anti-NF-kB (IkB, Ser32) were purchased from Abgent (USA), NQO1 (ab28947) and anti-AKT (phospho Ser473) were acquired from Immunoway Biotechnology Company (USA), anti-Nrf2 antibody (AP52270) 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-AKT (phospho Ser473) were acquired from Immunoway Institute of Biotechnology (Shanghai, China). Anti-PI3K and anti-NF-kB (IkB, Ser32) were 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 purchased from TaKaRa Co. LTD (Dalian). Primers for genotypes were: NRF2, 5'-TGTAGATGACCATAGTCCCGC-3'(forward); NRF2, 5'-TCCTGCCAAACTGTCCTCAT-3'(reverse); HO-1, 5'-CTCACAGTCGCAAGTCGC-3'(forward); HO-1, 5'-AGGGCTCCGCAAATCTGCC-3'(reverse); NQO1, 5'-AGTACAGTCGCAACTAGCCT-3'(forward); NQO1, 5'-GGCGTAACATCACAGGCT-3'(reverse); AKT, 5'-ATGCCGCGCCACTATCATCTC-3'(forward) and
AKT, 5’-TAAACCAACTCCGTGCCAG-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 two groups. The treatment groups had 42 rats, and the others were blank control group (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 (Veh) were fed with 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, 2) Scutellarin (50 mg/kg, po) (SCU50), 3) Scutellarin (100 mg/kg, po) (SCU100), 4) Lipitor (5 mg/kg, po) (LIP5); and the rats in the Veh-h-S group and Veh group received gavage of 10 ml/kg basal diet. 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 of the levels of TC, TG, HDL-C, LDL-C and ALT, AST, then the remain was stored at –80°C. 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% formal), 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 \(\mu\)M. The sections were deparaffinized 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% \(\text{H}_2\text{O}_2\) 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 (Y0006, 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 non-specific 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\(^3\)) (Beyotime, China) supplemented with protease and phosphatase inhibitors (1: 100), and then liver protein lysate was centrifuged at 14,000 \(\times\) 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 proteins 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), \(\beta\)-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/\(\beta\)-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). The mRNA expression of Nrf2, HO-1, NQO-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...
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 stress 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).

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).
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) (Figure 2B); 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 2. The effect of hepatic tissue blood flow (HTBF) after SCU treatment. (A) Laser speckle images of hepatic tissue on 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 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).
ANIMAL STUDY

To more deeply assess our established rodent models using histological examinations, the results showed that the 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 NAFLD 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<0.05) (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 LIPS 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 qRT-PCR, 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 the LIPS 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 data (⁎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).
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. We observed that serum TC and LDL of HO-1/β-actin

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SCU50 SCU100 SCU300V eh-h-S

0.6 0.4 0.2 0.0

NQO1/β-actin

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&&

SCU50 SCU100 SCU300V eh-h-S

0.8 0.6 0.4 0.2 0.0

Nrf2/β-actin

**##

&&

SCU50 SCU100 SCU300V eh-h-S

0.8 0.6 0.4 0.2 0.0

PI3K/β-actin

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&&

SCU50 SCU100 SCU300V eh-h-S

0.8 0.6 0.4 0.2 0.0

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 data. (* 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.
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,
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 bloodstream; 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 (Nrf2), 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

**Figure 8.** The gene expression of cytokines on Nrf2 pathway in livers from Sprague Dawley rats after SCU treatment by quantitative RT-PCR. Quantitative values were obtained from the threshold cycle value (2-△△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 data (* 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 LIP groups). (F) The immunoblotting results of these proteins.
disease, and impairment of Nrf2 activity represents a major risk factor for the evolution of NAFLD to nonalcoholic steato-hepatitis (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.

References:

1. Kones R: Primary prevention of coronary heart disease: integration of new data, evolving views, revised goals, and role of rosuvastatin in management. A comprehensive survey. Drug Des Devel Ther, 2011; 5: 325–80
2. Ye JX, Chen DZ: Novel cardio protective strategy combining three different preconditioning methods to prevent ischemia/reperfusion injury in aged hearts in an improved rabbit model. Exp Ther Med, 2015; 10: 1339–47
3. Soh J, Iqbal J, Queiroz J et al: MicroRNA-30c reduces hyperlipidemia and atherosclerosis by decreasing lipid synthesis and lipoprotein secretion. Nat Med, 2013; 19: 892–900
4. Abenavoli L, Milic N, Renzo LD et al: Metabolic aspects of adult patients with nonalcoholic fatty liver disease. World J Gastroenterol, 2016; 22:7008
5. Greenfield V, Cheung O, Sanyal A: Recent advances in nonalcoholic fatty liver disease. Curr Opin Gastroenterol, 2008; 24:320–27
6. Stagrans I, Pan XM, Rapp JH, Feingold KR: The role of dietary oxidized cholesterol and oxidized fatty acids in the development of atherosclerosis. Mol Nutr Food Res, 2005; 49: 1075–82
7. Angelo P: Nonalcoholic fatty liver disease. N Engl J Med, 2002; 346: 1221–31
8. Zarzour RHA, Ahmad M, Asmawi MZ et al: Phyllanthus niruri standardized extract alleviates the progression of non-alcoholic fatty liver disease and decreases atherosclerotic risk in Sprague-Dawley rats. Nutrients, 2017;9: pii: E766
9. Macedo AF, Taylor FC, Casas JP et al: Unintended effects of statins from observational studies in the general population: Systematic review and meta-analysis. BMC Med, 2014; 12: 51
10. Lin H, Zhang W, Dong ZX: A new and practical synthetic method for the synthesis of 6-methyl-scutellarein: One metabolite of scutellarein in vivo. Int J Mol Sci, 2015;16: 7587–94
11. Fang M, Yuan Y, Rangarajan P et al: Scutellariin regulates microglia mediated TNC1 astrocytic reaction and astroglisis in cerebral ischemia in the adult rats. BMC Neuroscience, 2015; 16: 84
12. Wang W, Ma X, Han J et al: Neuroprotective effect of scutellariin on ischemic cerebral injury by down-regulating the expression of angiotensin-converting enzyme and AT1 receptor. Plos One, 2016; 11: e0146197
13. Gao J, Chen G, He H et al: Therapeutic effects of breviscapine in cardiovascular diseases: A review. Front Pharmacol, 2017; 8: 289
14. Li L, Li L, Chen C et al: Scutellcin’s cardiovascular endothelium protective mechanism: Important role of PKG-Ix. PLoS One, 2015; 10: e0139570
15. Hong H, Liu GQ: Protection against hydrogen-peroxide-induced cytotoxicity in PC12 cells by scutellariin. Life Sci, 2004; 74: 2989–73
16. Guo C, Zhu Y, Weng Y et al: Therapeutic time window and underlying therapeutic mechanism of breviscapine injection against cerebral ischemia/reperfusion injury in rats. J Ethnopharmacol, 2014; 15: 660–66
17. Pu P: Study of the effect of scutellariin on metabolic syndrome. Wu Han University Doctor Thesis, 2013; 4–25
18. Gong Y, Yu X, Guan L et al: [Effects of Breviscapine on memory deficits, liver and kidney abnormal changes induced by D-galactose in mice.] Pharmacology and Clinics of Chinese Materia Medica, 2012; 28: 73–76 [in Chinese]
19. Coppé JM: The Keap1-Nrf2 cell defense pathway – a promising therapeutic target? Adv Pharmacol, 2012; 63: 43–79
20. Hardwick RN, Fisher CD, Canet MJ et al: Diversity in antioxidant response enzymes in progressive stages of human nonalcoholic fatty liver disease. Drug Metab Dispos, 2010; 38: 293–301
21. Chowdhry S, Nazmy MP: Loss of Nrf2 markedly exacerbates nonalcoholic steatohepatitis. Free Radic Biol Med, 2010; 48: 357–71
22. Meakin PJ, Chowdhry S, Sharma RS et al: Susceptibility of Nrf2-null mice to steatohepatitis and cirrhosis upon consumption of a high-fat diet is associated with oxidative stress, perturbation of the unfolded protein response, and disturbance in the expression of metabolic enzymes but not with insulin resistance. Mol Cell Biol, 2014; 34: 3305–20

23. Urano A, Yagiishita Y, Yamamoto M: The Keap1-Nrf2 system and diabetes mellitus. Arch Biochem Biophys, 2015; 566: 76–84

24. Takeshita Y, Watanabe S, Hattori T et al: Blockade of glucocorticoid receptors with RU486 attenuates cardiac damage and adipose tissue inflammation in a rat model of metabolic syndrome. Hypertens Res, 2015; 38: 741–50

25. Tessari P, Coracina A, Cosma A, Tiengo A: Hepatic lipid metabolism and non-alcoholic fatty liver disease. Nutr Metab Cardiovasc Dis, 2009; 19: 291–302

26. Ma KL, Ruan XZ, Powis SH et al: Inflammatory stress exacerbates lipid accumulation in hepatic cells and fatty livers of apolipoprotein E knockout mice. Hepatology, 2008; 48: 770–81

27. Lehto SM, Ruusunen A, Niskanen L et al: Elevated depressive symptoms and compositional changes in LDL particles in middle-aged men. Eur J Epidemiol, 2010;25: 403–9

28. Mu J, Wang QG, Wang XQ et al: [Mechanisms of non-alcoholic fatty liver disease and its correlation with chronic stress.] Shi jie Hua ren Xiao hua Za zhi, 2016; 24: 692–98 [in Chinese]

29. Buzzetti E, Pinzani M, Tsochatzis EA: The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). J Hepatol, 2012; 56: 952–64

30. Tian X, Chang L, Ma G et al: Delineation of platelet activation pathway of Scutellarin revealed its intracellular target as Protein Kinase C. Biol Pharm Bull, 2016;39: 181–91

31. Tang H, Tang Y, Li NG et al: Inflammatory stress exacerbates lipid accumulation in hepatic cells and fatty livers of apolipoprotein E knockout mice. J Physiol Gastrointest Liver Physiol, 2006; 290: G852–58

32. Wang Y, Li J, Han M et al: Prevention and treatment effect of total flavonoids in Stellera chamaejasme L. on nonalcoholic fatty liver in rats. Lipids Health and Disease, 2015; 14: 85

33. Bechmann LP, Hannivoort RA, Gerken G et al: The interaction of hepatic lipid and glucose metabolism in liver diseases. J Hepatol, 2012; 56: 952–64

34. Reddy JK, Rao MS: Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. Am J Physiol Gastrointest Liver Physiol, 2006; 290: G852–58

35. Nguyen P, Leray V, Diez M et al: Liver lipid metabolism. J Anim Physiol Anim Nutr, 2008; 92: 272–83

36. Liang S, Wang T, Hu X et al: Administration of lactobacillus helveticus NS8 improves behavioral, cognitive, and biochemical aberrations caused by chronic restraint stress. Neurosciience, 2015; 310: 561–77

37. Vrzhesinskaya OA, Kodentsova VM, Beketova NA et al: Influence of combined vitamin deficiency on unconditioned reflexes and learning in growing rats. Vopr Pitan, 2015; 84: 31–37

38. Wu P, Zhang F, Dai Y et al: Serum TNF-α, GTH and MDA of high-fat diet-induced obesity and obesity resistant rats. Saudi Pharm J, 2016; 24: 333–36

39. Collins AR, Gup te AA, Ji R et al: Myeloid deletion of nuclear factor erythroid 2-related factor 2 increases atherosclerosis and liver injury. Arterioscler Thromb Vasc Biol, 2012; 32: 2839–46

40. Ma Q: Role of nrf2 in oxidative stress and toxicity. Annu Rev Pharmacol Toxicol, 2013; 53: 401–26

41. Lee JM, Johnson JA: An important role of Nrf2ARE pathway in the cellular defense mechanisms. J Biochem Mol Biol, 2004; 37: 139–43

42. Li X, Chen H, Yi Z et al: Imdiazoline 12 receptor inhibitor idazoxan regulates the progression of hepatic fibrosis via AKT-Nrf2-Smad2/3 signaling pathway. Oncotarget, 2017; 8: 21015–30

43. Wu J, Li Q, Wang X et al: Neuroprotection by curcumin in ischemic brain injury involves the AKT/Nrf2 pathway. Plos One, 2013;8: e59843

44. Tsai CY, Wang CC, Lai TY et al: Antioxidant effects of diallyl trisulfide on high glucose-induced apoptosis are mediated by the PI3K/AKT-dependent activation of Nrf2 in cardiomyocytes. Int J Cardiol, 2013; 168: 1286–97

45. Na HK, Kim EH, Jung JH et al: (−)-Epigallocatechingallate induces Nrf2-mediated antioxidant enzyme expression via activation of PI3K and ERK in human mammary epithelial cells Arch Biochem Biophys, 2008;476: 171–77

46. Li Q, Wu JH, Guo DJ et al: Suppression of diet-induced hypercholesterolemia by Scutellarin in rats. Planta Med, 2009; 75: 1203–8

47. Brunt EM, Kleiner DE, Wilson IA et al: The NAS and the histopathologic diagnosis in NAFLD: Distinct Clinico pathologic meanings. Hepatology, 2011; 53: 810–20

48. Xiao W, Jia Z, Zhang Q et al: Inflammation and oxidative stress, rather than hypoxia, are predominant factors promoting angiogenesis in the initial phases of atherosclerosis. Mol Med Rep, 2015; 12: 3315–22