# Scutellarein regulates the PTEN/AKT/NFκB signaling pathway to reduce pirarubicin related liver inflammation

YING LAN[1], LVLIN CHEN[1], JUNCHENG ZHU[1], HUI LI[1] and ZHIMEI LIN[2]

Departments of [1]Critical Care Medicine and [2]Hematology, The Affiliated Hospital of Chengdu University, Chengdu, Sichuan 610081, P.R. China

Received December 21, 2022; Accepted April 24, 2023

DOI: 10.3892/ijmm.2023.5258

**Abstract.** The side effects of chemotherapy drugs have been hindering the progress of tumor treatment. The liver is the metabolic site of most drugs, which leads to the frequent occurrence of liver injury. Classical chemotherapy drugs such as pirarubicin (THP) can also cause dose-dependent hepatotoxicity, and the related mechanism is closely related to liver inflammation. Scutellarein (Sc) is a potential Chinese herbal monomer exhibiting liver protection activity, which can effectively alleviate the liver inflammation caused by obesity. In the present study, THP was used to establish a rat model of hepatotoxicity, and Sc was used for treatment. The experimental methods used included measuring body weight, detecting serum biomarkers, observing liver morphology with H&E staining, observing cell apoptosis with TUNEL staining, and detecting the expression of PTEN/AKT/NFκB signaling pathways and inflammatory genes with PCR and western blotting. However, whether Sc can inhibit the liver inflammation induced by THP has not been reported. The experimental results showed that THP led to the upregulation of PTEN and the increase of inflammatory factors in rat liver, while Sc effectively alleviated the aforementioned changes. It was further identified in primary hepatocytes that Sc can effectively inhabited PTEN, regulate AKT/NFκB signaling pathway, inhibit liver inflammation and ultimately protect the liver.

## Introduction

The liver is the metabolic organ of the majority of substances in the human organism, particularly various drugs (1,2). Drug-induced liver disease (DLD) is a kind of disease that causes liver damage when patients receive conventional drugs, Chinese herbal medicine, and health care products (3,4). With the advent of various new drugs, the occurrence of DLD is also showing an increasing trend (3-5). The proportion of DLD in acute hepatitis and liver failure, is gradually increasing, which has aroused widespread concern of people and clinicians (3,4). The main clinical manifestations of DLD include fever, fatigue, anorexia and jaundice (3,4,6,7). Laboratory examinations mainly focus on abnormal elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (8). In severe cases, cirrhosis, hypoproteinemia, coagulation dysfunction and even liver failure may occur, threatening life (6,7). Pirarubicin (THP) is a common anthracycline antineoplastic drug, but its clinical application is often affected by its toxic and side effects, mainly including hepatotoxicity, cardiotoxicity and myelotoxicity (9-11). A recent study reported that THP can induce accumulation of reactive oxygen species in hepatocytes, induce apoptosis and ferroptosis of hepatocytes, and finally cause liver damage (9). On the other hand, in the process of liver injury, inflammatory reaction also plays an important role (12,13). How to prevent and treat DLD and broaden the clinical application of corresponding drugs has become a thorny problem.

With the rapid development of the modernization of traditional Chinese medicine, finding effective ingredients from natural plants and developing new drugs from natural sources have become an effective means to protect the liver (14,15). Flavonoids are one of the important categories of secondary metabolites of plants, which are not only widely found in numerous medicinal plants, but also in almost all vegetables and fruits (16,17). They have excellent biological activities including anti-oxidation, anti-inflammatory, anticancer and organ protection, which are of great benefit to human health (16,18-20). Scutellarein (Sc), (chemical name 4', 5,6,7-tetrahydroxyflavone) is the aglycone and active metabolite of scutellarin, which is widely distributed in *Scutellaria* genus of *Labiatae* family and *Erigeron* genus of *Compositae* family, particularly in the stems and leaves of *Erigeron breviscapus* (Vaniot) and *Scutellaria baicalensis* Georgi (21). It is a natural flavonoid compound with stronger biological activity, particularly anti-inflammatory effect (21,22). Previous studies have found that Sc has strong anti-inflammatory, antioxidant, anti-insulin resistance and regulatory effects on nonalcoholic fatty liver disease caused by obesity (23,24). However, it is unknown whether Sc has potential protective effect on DLDs, particularly those induced by THP.

*Correspondence to:* Dr Zhimei Lin, Department of Hematology, The Affiliated Hospital of Chengdu University, North Section 2, 82 2nd Ring Road, Jinniu, Chengdu, Sichuan 610081, P.R. China
E-mail: linzhimei2022@163.com

*Key words:* scutellarein, pirarubicin, hepatotoxicity, PTEN, inflammation

PTEN is a key molecule in the development of certain inflammatory diseases, which is widely expressed in the liver and mediates its physiological activities (25-27). For example, PTEN can promote the transformation of PIP3 to PIP2, weaken the phosphorylation process of AKT promoted by PIP3, and then indirectly activate the expression of NFκB, affect the secretion of inflammatory factors in hepatocytes, and finally regulate liver inflammation (28-30). However, it is unknown whether the anti-inflammatory effect of Sc can be achieved by regulating PTEN.

The purpose of the present study was to investigate the effect of Sc on the expression of PTEN and inflammatory response in THP-induced rat liver and its possible mechanism, so as to provide experimental basis for exploring the specific mechanism of Sc in improving THP induced hepatotoxicity and screening therapeutic targets. It provides a theoretical basis for the development and application of Sc as a potential PTEN natural agonist to treat liver diseases.

## Materials and methods

*Reagents*. THP (cat. no. HY-13725) and Sc (cat. no. HY-N0752) were purchased from MedChemExpress. Hematoxylin-eosin staining kit (cat. no. C0105M) and Cell Counting Kit-8 (CCK-8; cat. no. C0039) were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. ALT (cat. no. C009-2-1), AST (cat. no. C010-2-1), CRP (cat. no. H126-1-2), monocyte chemoattractant protein-1 (MCP-1; cat. no. H115), IL-1β (cat. no. H002-1-2) and IL-6 (cat. no. H007-1-2) assay kits were purchased from Nanjing Jiancheng Bioengineering Research Institute. Lentiviral particles carrying PTEN (LvPTEN) and empty vector (LvControl) were constructed by Biotechnology Co., Ltd. The primary antibodies of PTEN (cat. no. 22034-1-AP), AKT (cat. no. 10176-2-AP), phosphorylated (p)-AKT (cat. no. 80455-1-RR) and GAPDH (cat. no. 10494-1-AP) were purchased from Wuhan Protein Technology Biotechnology Co., Ltd. The primary antibodies of IκBα (cat. no. 4812S), p-p65 (cat. no. 3033T) and t-p65 (cat. no. 8242T) were purchased from Cell Signaling Technology, Inc. Secondary antibody [HRP-conjugated goat anti rabbit IgG H + L (cat. no. 31460)] was purchased from Thermo Fisher Scientific, Inc. All reagents were of analytical grade.

*Animals*. The present study was approved by the Ethics Committee for Experimental Animals of The Affiliated Hospital of Chengdu University (Chengdu, China). The IACUC number of animal experiment is CDFS12020220056. A total of 20 male SD rats (8 weeks-old, 180-200 g) were purchased and raised in the Experimental Animal Center of Chengdu University. Rat grouping was as follows: i) Control group, normal saline; ii) Sc group, 100 mg/kg Sc + normal saline; iii) THP group, normal saline + 3 mg/kg THP; and iv) Sc + THP group, 100 mg/kg Sc + 3 mg/kg THP. Sc was administered by gavage and THP was administered by intravenous injection. Sc was dissolved in a very small amount of DMSO before being dissolved in normal saline (DMSO: normal saline ≈1:1,000). The animal model was established for 6 weeks, venous blood was received once a week, and body weight and food intake were measured once a week. The rats were housed under normal laboratory conditions (21±2˚C, 12/12-h light/dark cycle, humidity 50-60%) with free access to standard pellet diet and water.

*Sample collection and stain*. The rats were anesthetized with pentobarbital sodium (40 mg/kg intraperitoneally) and then sacrificed by cervical dislocation. Blood and liver tissue were collected from aorta rapidly, and the bleeding serum was centrifuged (22˚C, 1,000 x g, 15 min). The venous blood obtained every week was also separated by this method. The liver tissue was cleaned in normal saline, partially fixed in paraformaldehyde (4%) solution (22˚C, 48 h), followed by dehydration, transparency, waxing, and embedding. The tissue was then sliced into sections of ~5 $\mu$m using a slicing machine. The other parts of the liver were frozen at -80˚C for subsequent molecular research. TUNEL staining was performed according to the kit instructions. The paraffin sections of the liver were dewaxed, rinsed and soaked with protease K dropwise (37˚C, 30 min), followed by DNase I reaction solution dropwise (37˚C, 30 min). Subsequently, after cleaning, TdT enzyme reaction solution was added dropwise to the sample (37˚C, 60 min, away from light). Then, after cleaning, streptavidin TRITC working solution was added (37˚C, 30 min, away from light) dropwise to the sample. Finally, DAPI staining solution was used to stain the nucleus (37˚C, 10 min, away from light). The sample was then cleaned, an appropriate amount of sealing agent (glycerol: PBS=6:4) was added dropwise, sealed and observed under an optical microscope.

*Serum biomarkers of liver function and inflammatory factor.* The levels of ALT, AST, CRP, MCP-1, IL-1β and IL-6 in serum were determined according to the corresponding kit protocol.

*Histological analysis*. The liver tissue was paraffin-embedded through fixation, dehydration, transparency, wax penetration and embedding, and finally sectioned to become 4-5-$\mu$m thick paraffin sections. Then, paraffin sections were used for hematoxylin and eosin (H&E) staining for histopathology. Staining images were viewed using a Nikon eclipse 80i microscope (Nikon Corporation) at a magnification of x200.

*Cell extraction of primary rat hepatocytes*. A male SD rat (4 week) was purchased from the Animal Experiment Center of Chengdu University and fixed after disinfection. The abdominal cavity was opened to expose the hepatic portal vein and inferior vena cava. The hepatic portal vein was perfused (5 ml/min), and the inferior vena cava was opened to clear the blood. Then collagenase IV was replaced and perfusion was continued until the liver became soft. After removing the blood vessels and capsule in the liver tissue, the liver tissue was separated, filtered with 100 $\mu$m sieve, and then washed. After centrifugation at 1,800 x g for 5 min (22˚C), it was resuspended in DMEM medium (containing 10% FBS). Cells were counted (primary hepatocytes were magnified at x100 under a light microscope for observation), and the mixed cell suspension was transferred into a cell culture dish for 48 h. Later, it was found that the morphology of primary hepatocytes was consistent, and island like connections were formed between the cells. The extraction scheme of primary hepatocytes in the present study was derived from a previous study (31).

*CCK-8 method to explore the optimal concentration of THP and Sc and the viability of hepatocytes*. Hepatocytes were treated with increasing concentrations of THP (0, 1, 5, 10 and 20 $\mu$mol/l) for the following time intervals: 0, 6, 12, 24 and 48 h. Under the optimal THP treatment concentration and time, the Sc treatment concentration was set to 0, 20, 40, 80, 160 and 320 $\mu$mol/l. According to the instructions of CCK-8 test kit, the cell viability of primary hepatocytes in each group was detected. Briefly, hepatocytes suspension was inoculated into a 96-well plate (~5,000 cells per well), and after drug stimulation, 10 $\mu$l of CCK-8 solution was added to each well. After further incubation for 1 h in the cell culture chamber (pH, 7.2-7.4; temperature, 37˚C; humidity, 95%; $CO_2$, 5%), the absorbance value at 450 nm was measured using an enzyme-linked immunosorbent assay.

*Lentiviral particles processing*. In the present study, PTEN gene was overexpressed by lentiviral particles (Lv). PTEN specific lentivirus (shRNA sequence: 5'GCTAGAACT TATCAAACCCTT-3') and non-targeted control lentivirus (shRNA sequence: 5'CAACAAGATGAAGAGCACCAA-3') were purchased from Qingke Biotechnology Co., Ltd. The packaging and transfection of lentivirus and the screening of cells were all completed by Qingke Biotechnology Co., Ltd. Before transfection, 293T cells (Qingke Biotechnology Co., Ltd.) were cultured to 80-90% degree of polymerization, PEI (1 $\mu$g/$\mu$l) and plasmid were dissolved in Optimem respectively, and then mixed. After 48 h, centrifugation was performed (4˚C, 18,00 x g, 10 min), and the supernatant was received and mixed with 5X PEG8000. Then centrifugation (4˚C, 2,500 x g, 20 min) was performed to obtain the virus. According to the manufacturer's instructions, lentiviral vector was transferred into primary hepatocytes under the 15 $\mu$g/ml Polybrene with a complex multiplicity of infection (MOI) of 10. The DMEM was changed 24 h after infection. After 72 h, primary hepatocytes were screened using 2.0 $\mu$g/ml puromycin and cultured at 37˚C in an incubator containing 95% air and 5% carbon dioxide. The generation system used was 3rd. All the aforementioned reagents were obtained from Qingke Biotechnology Co., Ltd. The corresponding operation scheme can be observed at the following link: https://tsingke.com.cn/equipment/Modified_synthesis.

*Primary hepatocytes grouping and treatment*. Briefly, primary hepatocytes were divided into 7 groups and treated for 24 h: i) Control group, ii) THP group (5 $\mu$mol/l THP), iii) Sc + THP group (80 $\mu$mol/l Sc + 5 $\mu$mol/l THP), iv) LvControl group (non-targeted Control lentivirus vector), v) LvControl + THP group (non-targeted Control lentivirus vector + 5 $\mu$mol/l THP), vi) LvPTEN group (lentiviral vector of PTEN), and vii) LvPTEN + Sc group (lentivirus vector of PTEN + 80 $\mu$mol/l Sc).

*Reverse transcription-quantitative PCR (RT-qPCR)*. Total RNA was extracted from frozen pulverized rat liver and primary hepatocytes using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), then was transcribed by two-step method using Super script First-Strand Synthesis System. The RT-qPCR thermocycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 64˚C for 30 sec. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative number of tested genes (32). The PCR products were quantified with the SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), and the results were normalized to β-actin gene expression. The primer sequences were as follows: PTEN forward, 5'-CAA TGACAGCCATCATCAAAGAG-3' and reverse, 5'-GCT CAGACTTTTGTAATTTGTG-3'; NFκB forward, 5'-AGA GGATTTCGATTCCGCTA-3' and reverse, 5'-CGTGAA GTATTCCCAGGTTTG-3'; IL-1β forward, 5'-GACCTGTTC TTTGAGGCTGAC-3' and reverse, 5'-TTCATCTCGAAG CCTGCAGTG-3'; IL-6 forward, 5'-AACCACGGCCTTCCC TACTTC-3' and reverse, 5'-GATGAATTGGATGGTCTT GGTC-3'; TNF-α forward, 5'-GCCTCTTCTCATTCCTGC TT-3' and reverse, 5'-TGGGAACTTCTCATCCCTTTG-3'; VCAM-1 forward, 5'-AAGTGGAGGTCTACTCATTCC-3' and reverse, 5'-GGTCAAAGGGGTACACATTAG-3'; and β-actin forward, 5'-AGCTGAGAGGGAAATCGTGC-3' and reverse 5'-ACCAGACAGCACTGTGTTGG-3'.

*Western blotting*. Firstly, radioimmunoprecipitation assay buffer (cat. no. P0013B; Shanghai Biyuntian Biotechnology Co., Ltd.) was added to the tissue or cells to extract proteins, and the protein concentration was measured using a BCA protein concentration detection kit (cat. no. P0010; Shanghai Biyuntian Biotechnology Co., Ltd.). Subsequently, protein loading buffer was added (cat. no. P0015; Shanghai Biyuntian Biotechnology Co., Ltd.) and lysates were heated at 95˚C for 10 min to denature the protein. In turn, liver tissue lysates or cell lysates (~20 $\mu$g) were subjected to SDS-PAGE (10%). Subsequently, the protein was transferred to the PVDF membrane at 4˚C and PVDF membrane was soaked in QuickBlock™ Blocking Buffer (cat. no. P0220; Shanghai Biyuntian Biotechnology Co., Ltd.) at room temperature for 15 min. The membrane was incubated at 4˚C for 14 h with the following primary antibodies against: PTEN (1:1,000), AKT (1:1,000), p-AKT (1:1,000), IκBα (1:1,000), p-p65 (1:1,000), t-p65 (1:1,000) and GAPDH (1:5,000). Following the primary incubation, the membrane was incubated with secondary antibody (1:10,000) at room temperature for 1 h. Subsequently, protein visualization was performed using BeyoECL Plus (Beyotime Institute of Biotechnology) and Image Lab 2.5.2 software (Bio Rad Laboratories, Inc.). GAPDH was used as an internal reference protein.

*Statistical analysis*. The SPSS software (version 18.0; SPSS, Inc.) was used for statistical analysis. Data are expressed as the mean ± SEM. The normal distribution and homogeneity of variance of the data was detected using one-way or two-way ANOVA. Tukey's multiple comparison post hoc test was used to analyze the significant differences between the groups. $P \leq 0.05$ was considered to indicate a statistically significant difference.

## Results

*Effects of Sc and THP on body weight and feed intake of rats*. During the 6-week period, in THP group rats, the body weight decreased significantly from the third week (vs. Control, Fig. 1A), and the feed intake decreased significantly from the second week (vs. Control, Fig. 1B). The body weight and

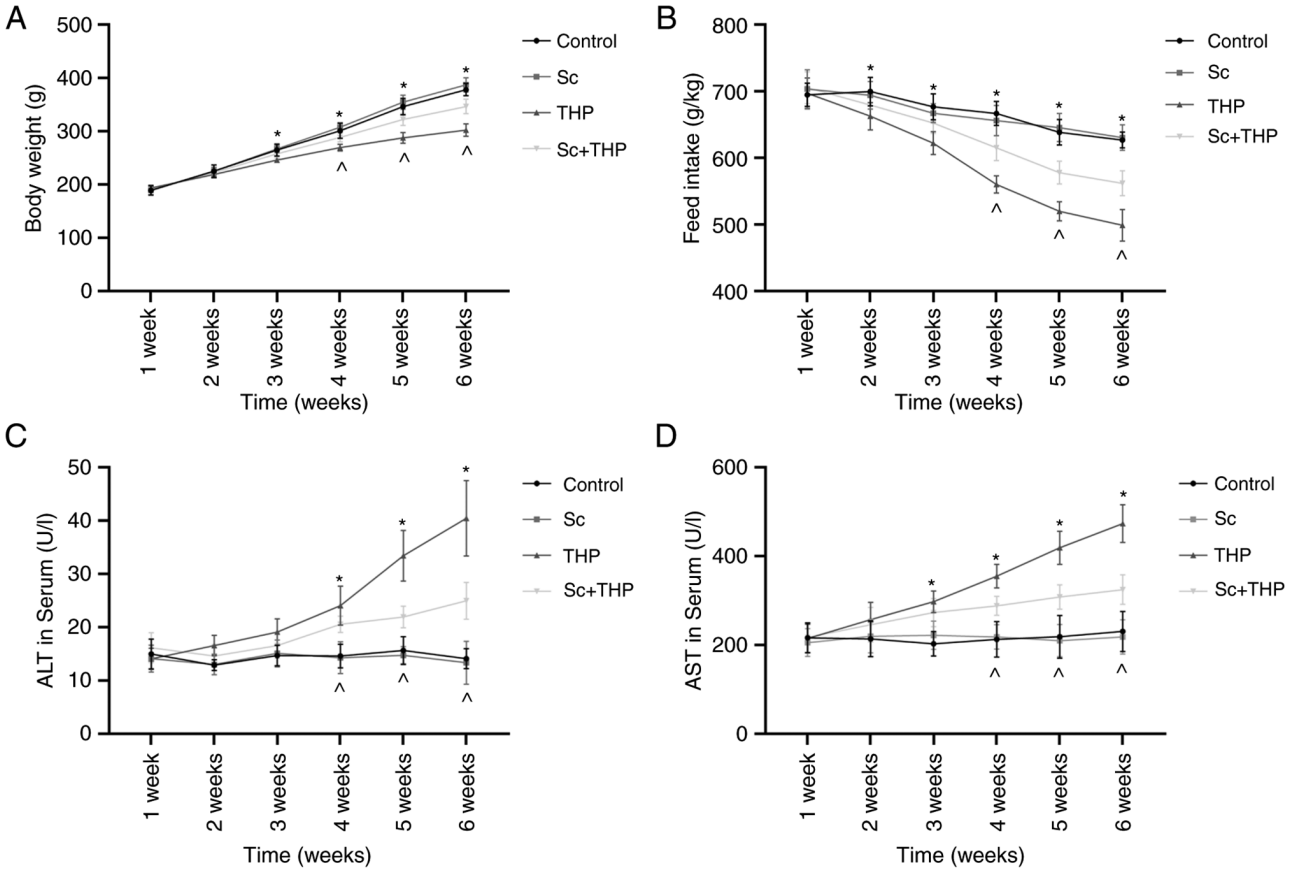


Figure 1. Dynamic changes of body weight, food intake and liver function in rats during 6 weeks. (A) The effect of THP and Sc on the Body weight of rats during 6 weeks. (B) The effect of THP and Sc on the food intake of rats during 6 weeks. (C) The effect of THP and Sc on the ALT in serum of rats during 6 weeks. (D) The effect of THP and Sc on the AST in serum of rats during 6 weeks. Values are expressed as Mean ± SD. *P<0.05 (THP vs. Control) and ^P<0.05 (Sc + THP vs. THP). THP, pirarubicin; Sc, scutellarein; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

food intake of rats in the Sc + THP group began to increase at week 4 (vs. THP, Fig. 1A and B). Sc alone had no statistically significant effect on the weight and feed intake of rats (vs. Control, Fig. 1A and B).

*Effects of Sc and THP on ALT and AST in serum and holism of rats*. During the 6-week period, in THP group rats, the ALT increased significantly from the fourth week (vs. Control, Fig. 1C), and the AST increased significantly from the third week (vs. Control, Fig. 1D). The ALT and AST in serum of rats in the Sc + THP group began to decrease at week 4 (vs. THP, Fig. 1A and B). Sc alone had no statistically significant effect on the ALT and AST in serum of rats (vs. Control, Fig. 1A and B).

*Effects of Sc and THP on serum inflammatory factor and liver histomorphology of rats*. After 6 weeks, the serum CRP (Fig. 2A), IL-1β (Fig. 2B), IL-6 (Fig. 2C) and MCP-1 (Fig. 2D) of THP group rats were significantly increased (vs. Control). Serum CRP (Fig. 2A), IL-1β (Fig. 2B), IL-6 (Fig. 2C) and MCP-1 (Fig. 2D) of Sc + THP group rats were significantly decreased compared with the THP group. Sc alone has no statistically significant effect on inflammatory factors in rat serum (vs. Control, Fig. 2A-D).

As revealed in Fig. 2E, the liver tissue morphology of rats in the control group was normal, while that of rats in the THP group exhibited disorder of hepatocyte fusion and arrangement, increased cell gap, abnormal nucleus and blood cell infiltration, while that of rats in the Sc + THP group was relatively light. Sc alone had no significant effect on the liver histomorphology of rats.

The results demonstrated also that there were almost no apoptotic cells in the liver tissues of rats in the control group and Sc group, while some apoptotic cells appeared in the liver tissues of rats in the THP group, while those in the Sc + THP group were relatively light (Fig. 2F).

*Effects of Sc and THP on liver PTEN/AKT/NFκB signal pathway in rats*. The expression of p-AKT/t-AKT and IκBα protein in the liver of THP group rats decreased significantly, and the expression of p-p65/t-p65 and PTEN protein increased significantly (vs. Control, Fig. 3A-E). The corresponding protein expression in Sc + THP group was significantly reversed (vs. THP, Fig. 3A-E). The liver protein expression in Sc alone group was not significantly abnormal (vs. Control). Semi-quantitative analysis of PTEN (Fig. 3B), p-AKT/t-AKT (Fig. 3C), IκBα (Fig. 3D) and p-p65/t-p65 (Fig. 3E) provided more evidence. Sc alone had no significant effect on PTEN/AKT/NFκB signal pathway in rat liver (vs. Control).

*Effects of Sc and THP on liver PTEN gene and inflammatory gene in rats*. PCR results of rat liver demonstrated that PTEN

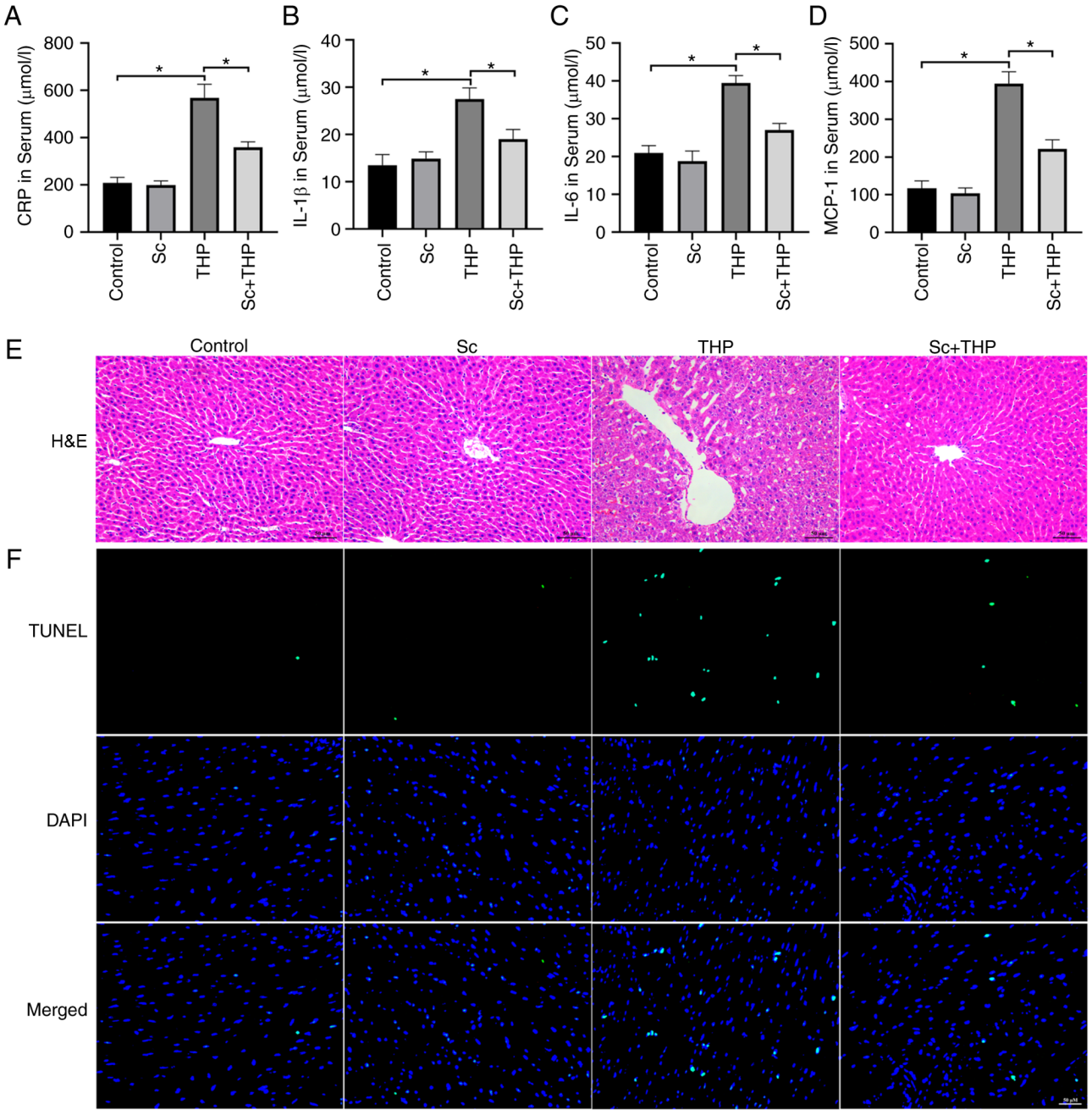


Figure 2. Changes of serum inflammatory factors and liver tissue morphology in rats. (A-D) The effect of THP and Sc on the (A) CRP (B) IL-1β (C) IL-6 and (D) MCP-1 in serum of rats. (E) The effect of THP and Sc on the liver tissue morphology in rats. (F) TUNEL stain. Values are expressed as the mean ± SD. *P<0.05. THP, pirarubicin; Sc, scutellarein; MCP-1, monocyte chemoattractant protein-1.

gene (Fig. 3F) and inflammatory gene NFκB (Fig. 3G), TNF-α (Fig. 3H), IL-1β (Fig. 3I), IL-6 (Fig. 3J) and VCAM-1 (Fig. 3K) were significantly increased and IL-10 (Fig. 3L) gene was significantly decreased in the liver of rats in THP group (vs. Control), while the level of Sc + THP group was significantly reversed (vs. THP, Fig. 3F-L). Sc alone had no significant effect on PTEN gene and inflammatory gene in rat liver (vs. Control).

*Optimum concentrations of THP and Sc in primary hepatocytes*. As revealed in Fig. 4A, in the time-concentration gradient relationship of THP treatment of primary hepatocytes, 1 μmol/l THP had little effect on the viability of hepatocytes within 48 h. However, 10 and 20 μmol/l THP had significant effect on the viability of hepatocytes within 48 h; Therefore, 5 μmol/l THP was selected as the treatment concentration. At 24 h, 5 μmol/l THP inhibited the viability of hepatocytes to ~50%, which was suitable for the experiment. Therefore, 5 μmol/l THP and 24 h were selected as the final treatment concentration and time.

It was identified that in the concentration gradient relationship of primary hepatocytes treated with Sc, 20 and 40 μmol/l Sc have little effect on the viability of THP-treated hepatocytes (vs. THP, Fig. 4B). A total of 80 and 160 μmol/l Sc significantly increased the viability of hepatocytes (vs. THP), thus 80 μmol/l Sc was selected as the treatment concentration.

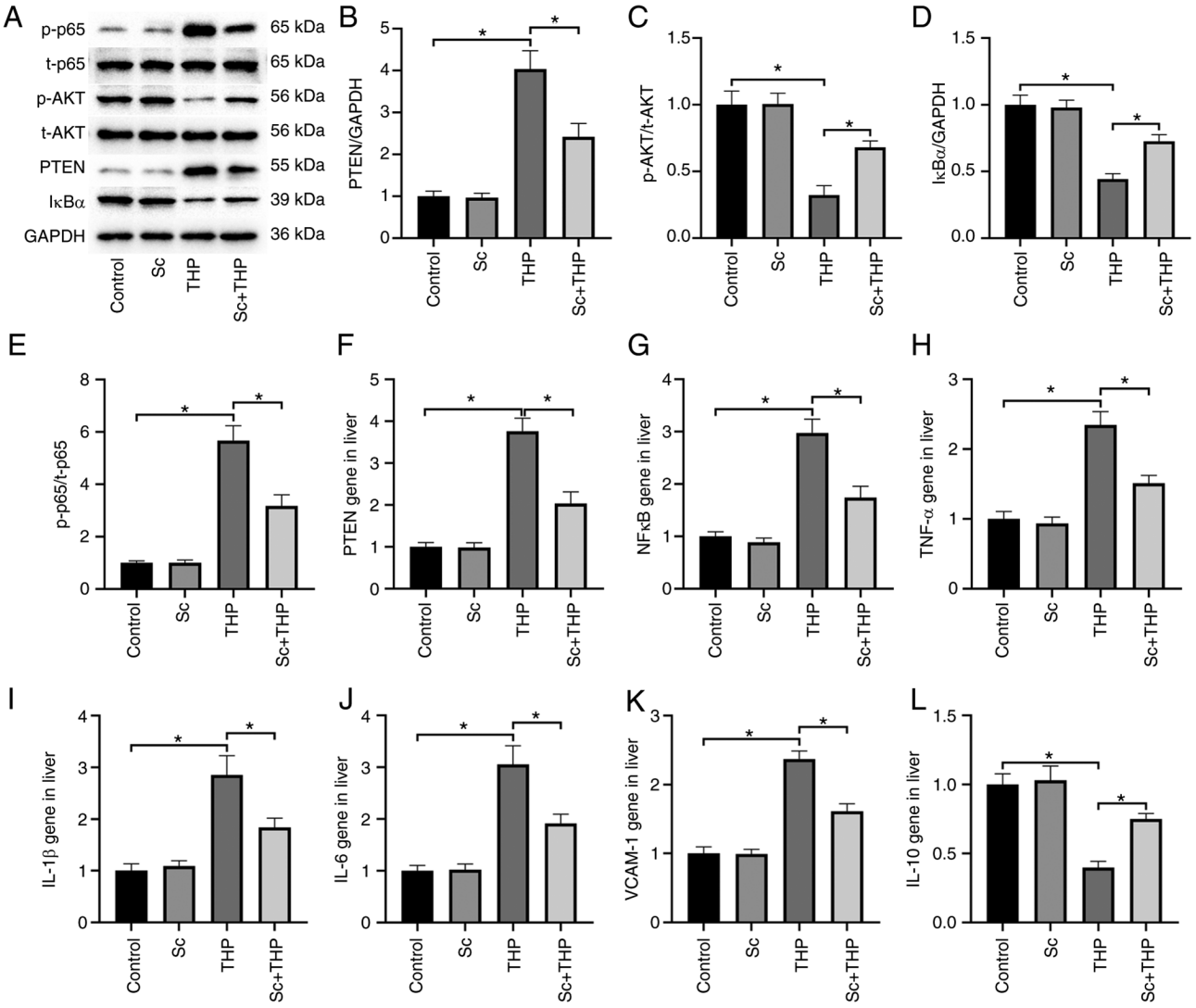


Figure 3. Changes of the hepatic PTEN/AKT/NFκB signaling pathway and inflammation-related genes in rats. (A) The effect of THP and Sc on the p65, AKT, PTEN and IκBα protein expression in liver of rats. (B-E) Semi-quantitative analysis of (B) PTEN, (C) AKT, (D) IκBα and (E) p65 protein expression. (F-L) The effect of THP and Sc on (F) PTEN, (G) NFκB, (H) TNF-α, (I) IL-1β, (J) IL-6, (K) VCAM-1 and (L) IL-10 gene expression in liver of rats. Values are expressed as the mean ± SEM. *P<0.05. THP, pirarubicin; Sc, scutellarein; p-, phosphorylated.

Similarly, 80 $\mu$mol/l Sc alone did not significantly affect the viability of hepatocytes (vs. Control).

*Effects of Sc and THP on hepatocyte viability*. As shown in Fig. 4C, both THP (vs. Control) and LvPTEN (vs. LvControl) significantly decreased the viability of hepatocytes, while the viability of hepatocytes in the Sc + THP (vs. THP) and LvPTEN + Sc groups (vs. LvControl + THP) increased significantly. LvControl alone did not significantly affect the viability of hepatocytes (vs. Control).

*Effects of Sc and THP on PTEN/AKT/NFκB signal pathway in hepatocytes*. The expression of p-AKT/t-AKT and IκBα protein in the THP (vs. Control) and LvPTEN groups (vs. LvControl) decreased significantly, and the expression of p-p65/t-p65 and PTEN protein in the THP (vs. Control) and LvPTEN groups (vs. LvControl) increased significantly (Fig. 4D-H). The corresponding protein expression in the Sc + THP (vs. THP) and LvPTEN + Sc (vs. LvControl + THP) groups was significantly reversed (Fig. 4D-H). The liver protein expression in LvControl alone group was not significantly aberrant (vs. Control). Semi-quantitative analysis of PTEN (Fig. 4E), p-AKT/t-AKT (Fig. 4F), p-p65/t-p65 (Fig. 4G) and IκBα (Fig. 4I) provided more evidence.

The results also revealed that the expression of nuclear p-p65/t-p65 protein was significantly increased in the THP (vs. control group) and LvPTEN groups (vs. LvControl), while it was significantly reversed in the Sc + THP group (vs. THP) and LvPTEN + Sc group (vs. LvControl + THP, Fig. 4I). There was no significant abnormality in nuclear protein expression in the single LvControl group (compared with the control group). Semi-quantitative analysis provides additional evidence for nuclear p-p65/t-p65 (Fig. 4J).

*Effects of Sc and THP on PTEN gene and inflammatory gene in hepatocytes*. PCR results of hepatocytes showed that PTEN gene (Fig. 5A) and inflammatory gene NFκB (Fig. 5B), TNF-α (Fig. 5C), IL-1β (Fig. 5D), IL-6 (Fig. 5E) and VCAM-1 (Fig. 5F) increased significantly and IL-10 (Fig. 5G) decreased significantly in the THP (vs. Control) and LvPTEN groups

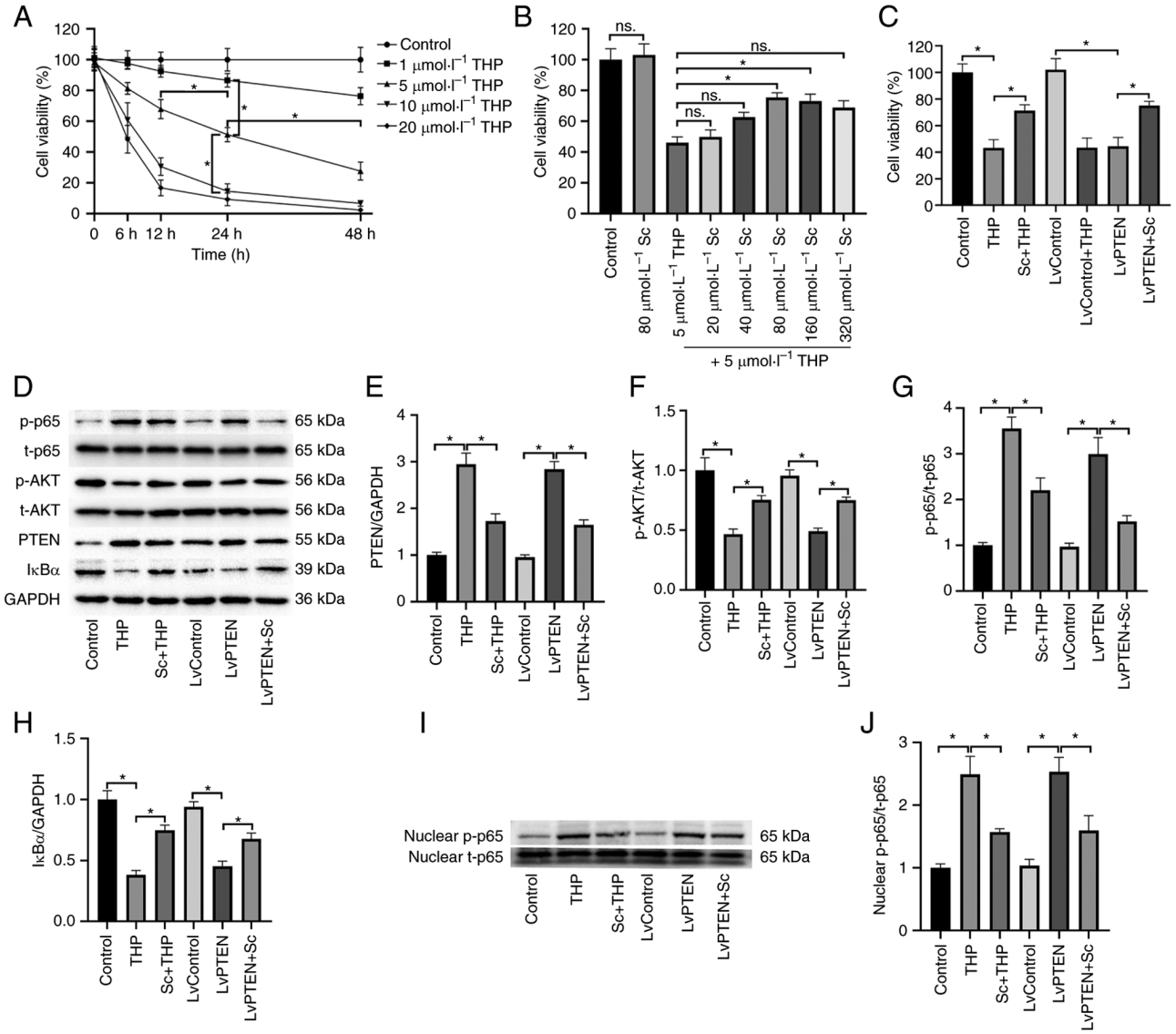


Figure 4. Changes of hepatocyte viability and the PTEN/AKT/NFκB signaling pathway. (A) Selection of the optimal concentration of THP for hepatocyte treatment. (B) Under the condition of 5 $\mu$mol/l THP treatment, the optimal concentration of Sc for hepatocyte treatment was selected. (C) Effects of THP, Sc and Lentivirus on the viability of hepatocytes. (D) The effect of THP, Sc and Lentivirus on the p65, AKT, PTEN and IκBα protein expression in hepatocytes. (E-H) Semi-quantitative analysis of (E) PTEN, (F) AKT, (G) p65 and (H) IκBα protein expression. (I) The effect of THP, Sc and Lentivirus on the nucleus p65 protein expression in hepatocytes. (J) Semi-quantitative analysis of nucleus p65 protein expression. Values are expressed as the mean ± SEM. *P<0.05. THP, pirarubicin; Sc, scutellarein; p-, phosphorylated.

(vs. LvControl), and the corresponding gene expression in the Sc + THP (vs. THP) and LvPTEN + Sc (vs. LvControl + THP) groups was significantly reversed. The hepatocytes gene expression in LvControl alone group was not significantly aberrant (vs. Control).

*Diagram of PTEN/AKT/NFκB signal pathway and inflammation*. As shown in the diagram of Fig. 5H, THP activates the expression of PTEN in hepatocytes, leading to the decrease of PIP3, which leads to the decrease of AKT phosphorylation, and finally activates the expression of NFκB. NFκB binds IκBα and then transfers to the nucleus, eventually causing the explosion of inflammatory factors in the hepatocytes, leading to liver inflammation. Sc effectively inhibits the expression of PTEN activated by THP, and finally alleviates the liver inflammatory reaction.

## Discussion

In the present study, it was found that the hepatotoxicity caused by THP increased in a dose-dependent and time-dependent manner. For example, during the 6-week THP administration period, the body weight and food intake of rats decreased continuously, and the serum ALT and AST increased continuously. When hepatocyte necrosis is caused by various reasons, ALT and AST are released into the blood in large quantities; as a result, they are important indicators and sensitive markers for the diagnosis of viral hepatitis and drug-induced hepatitis (33,34). Drugs and compounds that are toxic to the liver, including chlorpromazine, isoniazid, quinine, salicylic acid preparations, ampicillin, carbon tetrachloride and organic phosphorus, can lead to increased serum ALT and AST activities (3,35).

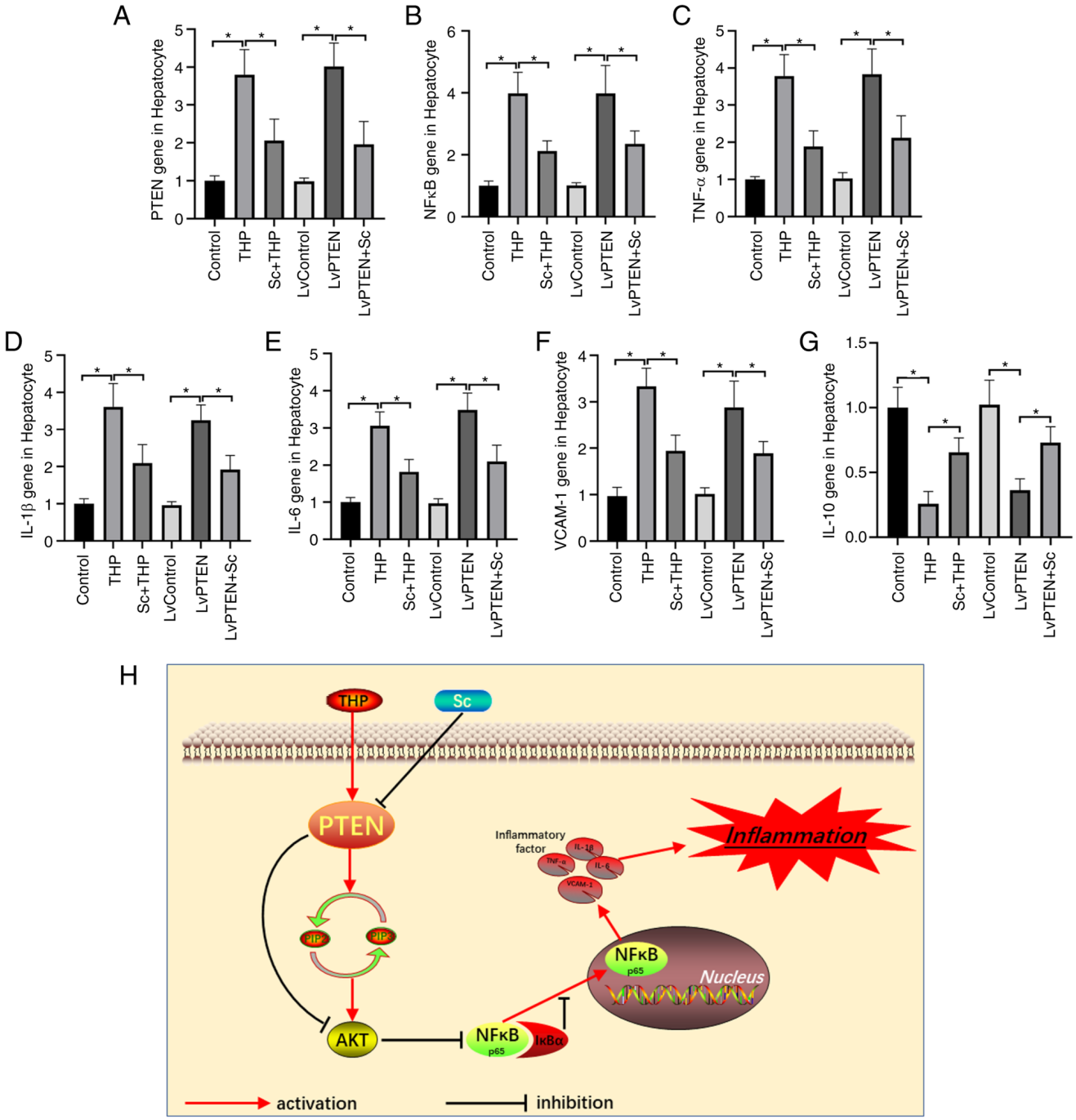


Figure 5. Changes of inflammation gene in hepatocytes. (A-G) The effect of THP, Sc and Lentivirus on the (A) PTEN, (B) NFκB, (C) TNF-α, (D) IL-1β, (E) IL-6, (F) VCAM-1 and (G) IL-10 gene expression in hepatocytes. (H) Diagram of the PTEN/AKT/NFκB signaling pathway and inflammation. Values are expressed as the mean ± SEM. *P<0.05. THP, pirarubicin; Sc, scutellarein.

In order to find out how THP causes liver damage, the relevant inflammatory markers and genes were examined. The results showed that inflammatory markers and inflammatory genes were significantly increased in serum and hepatocytes. The expression of p-AKT and IB decreased, while the expression of PTEN and p-p65 increased. Similarly, the gene expression of PTEN also increased. In further cell research, it was found that overexpression of PTEN gene showed a similar phenomenon to THP treatment, such as downregulation of p-AKT and upregulation of p-p65, and the expression of corresponding inflammatory genes, including NFκB, TNF-α, IL-1β, IL-6 and VCAM-1, were significantly upregulated. PTEN gene exists in almost all tissues in the organism and can modify other proteins and fats by removing phosphate groups (36). Therefore, it is characterized as a phosphatase gene. It is the first tumor suppressor gene with double specific phosphatase activity found so far and plays an important role in cell growth, apoptosis, adhesion, migration and invasion (27,36,37). PIP3 is the main substrate of PTEN, while AKT is the serine and threonine kinase downstream of PI3K/PTEN (29,36). PTEN converts PIP3 into PIP2 through dephosphorylation, maintaining a low level of

PIP3 in cells (38). PIP3 is an important second messenger, which can recruit AKT and PDK to the inner side of the cell membrane and activate PDK to promote the phosphorylation of AKT (30,36). PTEN attenuates this change, thereby negatively regulating the AKT signal.

AKT is an important target to interfere with inflammation (39). When AKT signal is activated, it can inhibit the release of proinflammatory factors including NFκB, IL-1β, IL-6 and TNF-α and promote the expression of anti-inflammatory factors such as IL-10 and TGF (39,40). As a key nuclear transcription factor in inflammatory reaction, NFκB usually binds to the inhibitor proteins of NFκB (IκB) in the form of heterodimer formed by p50 and p65, and exists in the cytoplasm in an inactive form (41,42). When subjected to upstream stimulus signal, IκB is phosphorylated and degraded under IKK induction to activate NFκB (42-44). The activated NFκB enters the nucleus to initiate gene transcription, and produces and releases inflammatory factors including IL-1β, IL-6 and TNF-α, and the released inflammatory factors can in turn act again and activate NFκB, forming positive feedback regulation, thus amplifying the inflammatory reaction cascade (45,46).

Another outstanding finding of the present study was that Sc appears to have the potential to prevent THP-induced liver inflammation. Similar to its pathogenesis, Sc can inhibit the activation of PTEN, in turn activate the phosphorylation of AKT, inhibit the release of numerous proinflammatory factors, and ultimately improve the liver inflammatory state. As one of the representative drugs of natural flavonoids, Sc has great potential in anti-inflammatory efficacy (21,22). In the study of non-alcoholic fatty liver disease, it was found that Sc has significant anti-obesity, anti-insulin resistance, anti-inflammatory and antioxidant effects in the liver (23,24). The specific mechanism is closely related to the inhibition of gene expression of inflammatory cytokines and the fine regulation of genes responsible for energy metabolism (23,24). However, it is worth noting that Sc can inhibit the proliferation and metastasis of HepG2 cells by upregulating PTEN and the PI3K/AKT/NFκB signaling pathway in the study of liver cancer (20). A previous study also found that Sc induced Fas-mediated exogenous apoptosis and G2/M cell cycle arrest in Hep3B hepatoma cells, indicating that Sc may be used as a potential natural drug for the treatment of liver cancer (47). Although this is in contrast to the results of the present study in hepatocytes, it is considered that this may be due to the heterogeneity between liver cancer cells and normal hepatocytes. In short, Sc is beneficial in both anti inflammation and elimination of liver cancer cells in the liver. The therapeutic effect and even adjuvant anti-tumor effects of Sc have been demonstrated in studies targeting other organs (48,49). For example, in the study of bleomycin (BLM)-induced pulmonary fibrosis, it was found that Sc not only alleviated BLM induced pulmonary fibrosis, but also increased tumor cell apoptosis in combination with BLM treatment (49). In another study, Sc was confirmed to play a role in treating atherosclerosis by regulating the Hippo-FOXO3A and PI3K/AKT signaling pathways (48). These studies have proven that Sc is a powerful organ-protective compound, but there remains a long way to go in terms of its specific application in clinical treatment of human diseases.

In conclusion, increasing evidences have demonstrated that inhibiting the activation of PTEN is considered to be an effective therapeutic strategy to alleviate hepatitis, whether viral or pharmaceutical. Chinese traditional herbal extracts have attracted increasing researchers' interest due to their competitive advantages of safety, cheapness and easy access. Although most Chinese herbal extracts have not been fully characterized, the research is also in the preliminary stage, and the clinical efficacy remains to be determined. Nevertheless, a large number of studies have identified that Sc has numerous beneficial effects on various liver diseases, particularly anti-inflammatory effects. It is worth noting that the current study demonstrated that Sc, as a potential natural inhibitor of PTEN, regulates the AKT/NFB signaling pathway, effectively alleviates the upregulation of PTEN and the outbreak of inflammatory factors in the liver caused by THP, and ultimately protects liver function, providing a theoretical and experimental basis for clarifying the pharmacological role of Sc in liver protection.

**Acknowledgements**

Not applicable.

**Funding**

The present study was supported by Chengdu University Clinical Medical College Affiliated Hospital Innovation Team Project (grant no. CDFYCX202202).

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

**Authors' contributions**

ZL was responsible for the conception and design of the research, the key revision of the knowledge content and the approval of the final version of the manuscript to be published, and agrees to be responsible for all aspects of the work, YL made substantial contributions to data acquisition, analysis and interpretation, and participated in the drafting of the manuscript. LC, JZ and HL made substantial contributions to data analysis and interpretation. YL, LC, JZ, HL and ZL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

**Ethics approval and consent to participate**

The present study was approved (approval no. CDFS12020220056) by and followed the guidelines of the Ethics Committee for Experimental Animals of The Affiliated Hospital of Chengdu University (Chengdu, China).

**Patient consent for publication**

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References


1. Trefts E, Gannon M and Wasserman DH: The liver. Curr Biol 27: R1147-R1151, 2017.
2. Jetter A and Kullak-Ublick GA: Drugs and hepatic transporters: A review. Pharmacol Res 154: 104234, 2020.
3. Andrade RJ, Chalasani N, Björnsson ES, Suzuki A, Kullak-Ublick GA, Watkins PB, Devarbhavi H, Merz M, Lucena MI, Kaplowitz N and Aithal GP: Drug-induced liver injury. Nat Rev Dis Primers 5: 58, 2019.
4. European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu; Clinical Practice Guideline Panel: Chair:; Panel members; EASL Governing Board representative: EASL Clinical practice guidelines: Drug-induced liver injury. J Hepatol 70: 1222-1261, 2019.
5. Saithanyamurthi H and Faust AJ: Drug-Induced liver disease: Clinical course. Clin Liver Dis 21: 21-34, 2017.
6. Luedde T, Kaplowitz N and Schwabe RF: Cell death and cell death responses in liver disease: Mechanisms and clinical relevance. Gastroenterology 147: 765-783.e4, 2014.
7. Giordano CM, Zervos XB: Clinical manifestations and treatment of drug-induced hepatotoxicity. Clin Liver Dis 17: 565-573, viii, 2013.
8. Liu Y, Zhao P, Cheng M, Yu L, Cheng Z, Fan L and Chen C: AST to ALT ratio and arterial stiffness in non-fatty liver Japanese population:a secondary analysis based on a cross-sectional study. Lipids Health Dis 17: 275, 2018.
9. Shi H, Yan Y, Yang H, Pu P and Tang H: Schisandrin B diet inhibits oxidative stress to reduce ferroptosis and lipid peroxidation to prevent pirarubicin-induced hepatotoxicity. Biomed Res Int 2022: 5623555, 2022.
10. Zhang Y, Ma XY, Zhang T, Qin M, Sun B, Li Q, Hu DW and Ren LQ: Protective effects of apocynum venetum against pirarubicin-induced cardiotoxicity. Am J Chin Med 47: 1075-1097, 2019.
11. Hori H, Kudoh T, Nishimura S, Oda M, Yoshida M, Hara J, Tawa A, Usami I, Tanizawa A, Yumura-Yagi K, *et al*: Acute and late toxicities of pirarubicin in the treatment of childhood acute lymphoblastic leukemia: Results from a clinical trial by the Japan Association of Childhood Leukemia Study. Int J Clin Oncol 22: 387-396, 2017.
12. Jiménez-Castro MB, Cornide-Petronio ME, Gracia-Sancho J and Peralta C: Inflammasome-mediated inflammation in liver ischemia-reperfusion injury. Cells 8: 1131, 2019.
13. Dai C, Xiao X, Li D, Tun S, Wang Y, Velkov T and Tang S: Chloroquine ameliorates carbon tetrachloride-induced acute liver injury in mice via the concomitant inhibition of inflammation and induction of apoptosis. Cell Death Dis 9: 1164, 2018.
14. Liu YT, Qi SL and Sun KW: Traditional Chinese medicine, liver fibrosis, intestinal flora: Is there any connection?-a narrative review. Ann Palliat Med 10: 4846-4857, 2021.
15. Chen M, Xie Y, Gong S, Wang Y, Yu H, Zhou T, Huang F, Guo X, Zhang H, Huang R, *et al*: Traditional Chinese medicine in the treatment of nonalcoholic steatohepatitis. Pharmacol Res 172: 105849, 2021.
16. Serafini M, Peluso I and Raguzzini A: Flavonoids as anti-inflammatory agents. Proc Nutr Soc 69: 273-278, 2010.
17. Koirala N, Thuan NH, Ghimire GP, Thang DV and Sohng JK: Methylation of flavonoids: Chemical structures, bioactivities, progress and perspectives for biotechnological production. Enzyme Microb Technol 86: 103-116, 2016.
18. Yi YS: Regulatory roles of flavonoids on inflammasome activation during inflammatory responses. Mol Nutr Food Res 62: e1800147, 2018.
19. Krych-Madej J, Stawowska K and Gebicka L: Oxidation of flavonoids by hypochlorous acid: Reaction kinetics and antioxidant activity studies. Free Radic Res 50: 898-908, 2016.
20. Ha SE, Kim SM, Vetrivel P, Kim HH, Bhosale PB, Heo JD, Lee HJ and Kim GS: Inhibition of cell proliferation and metastasis by scutellarein regulating PI3K/Akt/NF-κB signaling through PTEN activation in hepatocellular carcinoma. Int J Mol Sci 22: 8841, 2021.
21. Russo M, Moccia S, Spagnuolo C, Tedesco I and Russo GL: Roles of flavonoids against coronavirus infection. Chem Biol Interact 328: 109211, 2020.
22. Chagas MDSS, Behrens MD, Moragas-Tellis CJ, Penedo GXM, Silva AR and Gonçalves-de-Albuquerque CF: Flavonols and flavones as potential anti-inflammatory, antioxidant, and antibacterial compounds. Oxid Med Cell Longev 2022: 9966750, 2022.
23. Lin Y, Ren N, Li S, Chen M and Pu P: Novel anti-obesity effect of scutellarein and potential underlying mechanism of actions. Biomed Pharmacother 117: 109042, 2019.
24. Gao L, Tang H, Zeng Q, Tang T, Chen M and Pu P: The anti-insulin resistance effect of scutellarin may be related to antioxidant stress and AMPKα activation in diabetic mice. Obes Res Clin Pract 14: 368-374, 2020.
25. Brenner C, Galluzzi L, Kepp O and Kroemer G: Decoding cell death signals in liver inflammation. J Hepatol 59: 583-594, 2013.
26. Cavalli G and Dinarello CA: Suppression of inflammation and acquired immunity by IL-37. Immunol Rev 281: 179-190, 2018.
27. Peyrou M, Bourgoin L and Foti M: PTEN in non-alcoholic fatty liver disease/non-alcoholic steatohepatitis and cancer. Dig Dis 28: 236-246, 2010.
28. Álvarez-Garcia V, Tawil Y, Wise HM and Leslie NR: Mechanisms of PTEN loss in cancer: It's all about diversity. Semin Cancer Biol 59: 66-79, 2019.
29. Piao Y and Yin D: Mechanism underlying treatment of diabetic kidney disease using Traditional Chinese Medicine based on theory of Yin and Yang balance. J Tradit Chin Med 38: 797-802, 2018.
30. Yu G, Yu H, Yang Q, Wang J, Fan H, Liu G, Wang L, Bello BK, Zhao P, Zhang H and Dong J: Vibrio harveyi infections induce production of proinflammatory cytokines in murine peritoneal macrophages via activation of p38 MAPK and NF-κB pathways, but reversed by PI3K/AKT pathways. Dev Comp Immunol 127: 104292, 2022.
31. Pu P, Wang XA, Salim M, Zhu LH, Wang L, Chen KJ, Xiao JF, Deng W, Shi HW, Jiang H and Li HL: Baicalein, a natural product, selectively activating AMPKα(2) and ameliorates metabolic disorder in diet-induced mice. Mol Cell Endocrinol 362: 128-138, 2012.
32. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
33. Sookoian S and Pirola CJ: Liver enzymes, metabolomics and genome-wide association studies: From systems biology to the personalized medicine. World J Gastroenterol 21: 711-725, 2015.
34. Korver S, Bowen J, Pearson K, Gonzalez RJ, French N, Park K, Jenkins R and Goldring C: The application of cytokeratin-18 as a biomarker for drug-induced liver injury. Arch Toxicol 95: 3435-3448, 2021.
35. Stravitz RT and Lee WM: Acute liver failure. Lancet 394: 869-881, 2019.
36. Worby CA and Dixon JE: PTEN. Annu Rev Biochem 83: 641-669, 2014.
37. Ho J, Cruise ES, Dowling RJO and Stambolic V: PTEN nuclear functions. Cold Spring Harb Perspect Med 10: a036079, 2020.
38. Chen J, Zhang XD and Proud C: Dissecting the signaling pathways that mediate cancer in PTEN and LKB1 double-knockout mice. Sci Signal 8: pe1, 2015.
39. Tang F, Wang Y, Hemmings BA, Rüegg C and Xue G: PKB/Akt-dependent regulation of inflammation in cancer. Semin Cancer Biol 48: 62-69, 2018.
40. Sun X, Chen L and He Z: PI3K/Akt-Nrf2 and anti-inflammation effect of macrolides in chronic obstructive pulmonary disease. Curr Drug Metab 20: 301-304, 2019.
41. Mishra V, Banga J and Silveyra P: Oxidative stress and cellular pathways of asthma and inflammation: Therapeutic strategies and pharmacological targets. Pharmacol Ther 181: 169-182, 2018.
42. Mulero MC, Huxford T and Ghosh G: NF-κB, IκB, and IKK: Integral components of immune system signaling. Adv Exp Med Biol 1172: 207-226, 2019.
43. Lin JK: Cancer chemoprevention by tea polyphenols through modulating signal transduction pathways. Arch Pharm Res 25: 561-571, 2002.
44. Kondylis V, Kumari S, Vlantis K and Pasparakis M: The interplay of IKK, NF-κB and RIPK1 signaling in the regulation of cell death, tissue homeostasis and inflammation. Immunol Rev 277: 113-127, 2017.
45. Gasparini C and Feldmann M: NF-κB as a target for modulating inflammatory responses. Curr Pharm Des 18: 5735-5745, 2012.

46. Dumortier C, Danopoulos S, Velard F and Al Alam D: Bone cells differentiation: How CFTR mutations may rule the game of stem cells commitment? Front Cell Dev Biol 9: 611921, 2021.
47. Sang Eun H, Seong Min K, Ho Jeong L, Vetrivel P, Venkatarame Gowda Saralamma V, Jeong Doo H, Eun Hee K, Sang Joon L and Gon Sup K: Scutellarein induces Fas-mediated extrinsic apoptosis and G2/M cell cycle arrest in Hep3B hepatocellular carcinoma cells. Nutrients 11: 263, 2019.
48. Fu Y, Sun S, Sun H, Peng J, Ma X, Bao L, Ji R, Luo C, Gao C, Zhang X and Jin Y: Scutellarin exerts protective effects against atherosclerosis in rats by regulating the Hippo-FOXO3A and PI3K/AKT signaling pathways. J Cell Physiol 234: 18131-18145, 2019.
49. Nie J, Yang HM, Sun CY, Liu YL, Zhuo JY, Zhang ZB, Lai XP, Su ZR and Li YC: Scutellarin enhances antitumor effects and attenuates the toxicity of bleomycin in H22 ascites tumor-bearing mice. Front Pharmacol 9: 615, 2018.