

Polygonatum Sibiricum Polysaccharides Alleviate CCl₄-Induced Acute Liver Injury in Mice Via Reducing Inflammation and Oxidative Stress

Jiujie Li^{1,2}, Ting Wang¹, Fuding Zhou¹, Changchun Xiao², Zhengxiang Liu¹, Jinchuan Yu¹,
Di Liang³, Guangjun Wang⁴, Changsheng Shao⁵, Peng Wang^{5,*}, Wenjun Chen^{1,*}

¹Department of Nutrition and Food Hygiene, School of Public Health, Anhui Medical University, Hefei 230032, Anhui, China

²Hefei Center for Disease Control and Prevention, Hefei, 230041, Anhui, China

³Department of Gastroenterology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, Hefei 230001, Anhui, China

⁴School of Public Health, Anhui Medical University, Hefei 230032, Anhui, China

⁵CAS Key Laboratory of High Magnetic Field and Ion Beam Physical Biology, Hefei Institutes of Physical Science, Chinese Academy of Sciences (CAS), Hefei 230031, China

*Corresponding author: chenwj71024@163.com, pengwang@ipp.ac.cn

Co-first authors: Jiujie Li and Ting Wang have contributed equally to this work

Received March 09, 2024; Revised April 10, 2024; Accepted April 17, 2024

Abstract From liver injury to hepatocirrhosis and hepatocarcinoma, irreversible progression correlates closely with inflammation. *Polygonatum sibiricum* has been reported to be beneficial to the liver, but its regulation of inflammation in liver injury has not been determined. Thus, we hypothesize that *Polygonatum sibiricum* polysaccharides (PSP) play a protective role against acute liver injury by inhibiting inflammation and explore the underlying mechanism. The concentration and constituents of polysaccharides in PSP were identified first. We found that, like bifendate, PSP pretreatment significantly ameliorated acute CCl₄ exposure-induced liver injury and associated biomarkers. Meanwhile, PSP markedly attenuated inflammation and macrophage proinflammatory polarization both in the livers of CCl₄-treated mice and LPS-treated Raw264.7 cells. Mechanistic investigation showed that PSP pretreatment markedly attenuated the activation of JAK₂/STAT₃/NF-κB signaling pathway in liver injury. Our findings demonstrated a novel understanding of PSP-mediated macrophage proinflammatory polarization in acute injury, which provides new knowledge regarding its application in acute liver injury treatment.

Keywords: *Polygonatum sibiricum*, macrophage M1 polarization, acute liver injury, inflammation, JAK₂/STAT₃

Cite This Article: Jiujie Li, Ting Wang, Fuding Zhou, Changchun Xiao, Zhengxiang Liu, Jinchuan Yu, Di Liang, Guangjun Wang, Changsheng Shao, Peng Wang, and Wenjun Chen, "Polygonatum Sibiricum Polysaccharides Alleviate CCl₄-Induced Acute Liver Injury in Mice Via Reducing Inflammation and Oxidative Stress." Journal of Food and Nutrition Research, vol. 12, no. 4 (2024): 182-195. doi: 10.12691/jfmr-12-4-3.

1. Introduction

As the main organ of catabolism, liver is highly susceptible to invasion of various pathogens or stimulation of toxins, causing liver damage [1,2]. According to the latest research, liver disease is the 11th leading cause of death worldwide, with more than 2 million people dying from various liver diseases every year, including viral hepatitis, cirrhosis and liver cancer, accounting for 4% of all deaths worldwide [3]. More than a fifth of the population in China is affected by liver disease, particularly hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, cirrhosis, liver cancer, non-alcoholic fatty liver disease (NAFLD), alcohol-related liver disease (ALD) and drug-induced liver injury (DILI). Liver disease has clearly become one of the major factors of morbidity

and mortality in China [4].

There is increasing evidence that inflammatory responses play a key role in liver injury and disease progression [5,6]. Inflammatory response is an immune response caused by macrophages after injury to protect tissues and organs of the body from injury, but the inflammatory factors released by high-level inflammatory response will further damage tissues and organs of the body [7]. Studies have shown that continuous exposure of the liver to exogenous and endogenous harmful substances will release a set of damage-related molecular pattern factors, which are mainly recognized by cell surface pattern recognition receptors, triggering the activation of nuclear factor-κB, acute expression and release of inflammatory factors and chemokines, and participating in the regulation of liver inflammatory signaling pathways and responses [8]. Macrophages in the liver undergo different types of polarization depending on the signal

they receive: M1, M2. After M1-type polarization, the main function of macrophages is to clear foreign pathogens and activate T immune cells to produce immune response. At the same time, M1-type macrophages secrete a variety of inflammatory factors, including TNF- α , IL-1 β , reactive oxygen species and monocyte chemotactic protein-1, etc. Continuous inflammatory response will lead to a large accumulation of inflammatory factors in the body and eventually lead to tissue damage [9]. Therefore, targeting the pathogenesis of hepatic proinflammatory polarization and inhibiting its activation are of great significance for the prevention and treatment of liver-related diseases.

Inflammatory response is an immune response caused by macrophages to protect the tissues and organs of the body from damage after injury. However, inflammatory factors released by high-level inflammatory response will further damage the tissues and organs of the body. Therefore, maintaining the homeostasis of immune response in the body is essential for the normal physiological function of the body [7]. Macrophages in the liver can be divided into two categories: Kupffer cells (KCs) and Monocyte-derived macrophages (MDMs) derived from bone marrow. As resident macrophages in the liver, KCs are located in the hepatic sinusum and mainly play a role in antigen presentation and regulation of immune response. MDMs are differentiated from monocytes circulating in peripheral blood and promote their differentiation and maturation by receiving signals from the local microenvironment. In vivo, macrophages undergo different types of polarization depending on the signals they receive. According to the cell surface markers and the functions they perform, differentiated macrophages can be divided into Classically activated macrophages and alternatively activated macrophages), among which the former is called M1 macrophages, and the latter is called M2 macrophages [10]. The function of M2 macrophages is closely related to the malignant progression of tumors. When activated and polarized, macrophages will secrete cytokines to play anti-inflammatory roles, participate in angiogenesis and extracellular matrix remodeling, and promote the malignant progression of tumors by inhibiting the function of M1 macrophages [11]. The main function of macrophages after M1 polarization is to clear foreign pathogens, activate and cause the body's T immune cells to produce immune responses. M1 macrophages can secrete a variety of inflammatory factors, including TNF- α , IL-1 β , Reactive oxygen species (ROS) and Monocyte chemotactic protein-1, MCP-1, etc. Persistent inflammatory response can lead to a large accumulation of inflammatory factors in the body and eventually lead to tissue damage [9]. Studies have found that the inflammatory response caused by M1-type macrophages plays an important role in the occurrence and development of various liver diseases, including alcoholic and non-alcoholic fatty liver disease, hepatitis and cirrhosis [12,13,14]. Therefore, targeting the mechanism of hepatic M1 macrophages and inhibiting their activation are of great significance for the prevention and treatment of liver diseases.

At present, there are four main types of liver disease drugs on the Chinese market: antiviral drugs, immune modulators, proprietary Chinese medicines, and liver-protecting drugs. From the perspective of the use of

various liver diseases, the most commonly used drugs are antiviral drugs, but in recent years, liver diseases including hepatitis B and cirrhosis have the characteristics of easy recurrence and long course of disease, and treatment is more difficult. In addition, most of the drugs for the treatment of liver diseases tend to be Western medicines, which have the disadvantages of large side effects and high cost. Compared with traditional liver disease treatment drugs, natural herbs have many advantages such as multi-target, low toxicity and side effects, and have been widely concerned and studied by researchers, among which flavonoids is one. A number of studies have proved that flavonoids show great advantages in the prevention and treatment of liver injury. Wang et al. found that Huangjing granules could protect the liver from alcohol-induced injury [15]. Li et al.'s studies have shown that Huangjing has an anti-tumor effect on liver cancer by inducing cell cycle arrest and apoptosis [16].

Polygonatum sibiricum polysaccharides (PSP) is the most abundant chemical component of polysaccharides and has important medicinal value. The 2020 edition of Chinese Pharmacopoeia used PSP as the standard to evaluate the quality of polysaccharides. Modern pharmacological studies have shown that PSP has various pharmacological effects such as anti-oxidation, improvement of learning and memory ability, improvement of myocardial cell damage, regulation of hematopoiesis, lowering of blood sugar and blood lipids, regulation of immunity, anti-tumor, protection of liver and kidney, improvement of osteoporosis, anti-inflammation and anti-bacteria [17,18,19,20,21]. Although many studies have confirmed that PSP plays an important role in the prevention and treatment of tissue damage caused by inflammatory response [22,23], the protective effect of PSP on acute liver injury and its anti-inflammatory mechanism are still unclear. Therefore, it can be hypothesized that the hepatoprotective effects of PSP were mediated through anti-inflammatory mechanisms and inhibition of macrophage M1 polarization. In this study, mice and cell models were established to explore the effect of inflammatory process on the hepatoprotective effect of PSP and the possible signaling pathways.

2. Materials and Methods

2.1. Chemicals and Reagents

Polygonatum sibiricum Redouté was purchased from Anhui Jingcheng Kangteng Agricultural Development Limited Company and the plant name was checked with <http://www.worldfloraonline.org> in Oct 11, 2022. Lipopolysaccharide (LPS) was purchased from Sigma. DMEM, antibiotic-antimycotic, and fetal bovine serum were purchased from Gibco. NO, ALT, AST, T-AOC, T-SOD, CAT, GSH-PX, and MDA kits were obtained from Nanjing Jiancheng Bioengineering Institute.

2.2. Preparation of *Polygonatum sibiricum* Aqueous Extract and Quantification

Polygonatum sibiricum were crushed and filtrated through a 120-mesh sieve. After extraction at 75°C by

using an ultrasonic method at 28 kHz, the supernatant was collected, distilled and vacuum dried. Afterwards, PSP was diluted in distilled water and kept at -80°C . The polysaccharide concentration of PSP was measured by using the anthrone-sulfuric acid method.

2.3. Determination of Polysaccharide Molecular Weight

To measure the molecular weight of polysaccharides in PSP, PSP and the standard were diluted in distilled water to a final concentration of 5 mg/ml. After centrifugation, the supernatant was filtered by passing through a $0.22\ \mu\text{m}$ membrane. Then, the molecular weight was measured by using high-performance gel permeation chromatography (HPGPC).

2.4. Identification of Monosaccharides

The standards of 16 monosaccharides (fucose, arabinose, rhamnose, galactose, xylose, glucose, mannose, ribose, fructose, galacturonic acid, galacturonic acid hydrochloride, glucuronic acid, glucosamine hydrochloride, guluronic acid, mannose aldehyde, and N-acetyl-D-glucosamine) were diluted to a final concentration of 10 mg/ml. Then, 10 mg of PSP was hydrolyzed with 3 M TFA at 120°C for 3 h. Then, monosaccharides were identified by using a high-efficiency gel ion exchange method (HPIEC).

2.5. Animal Assay

Five-week-old male C57 mice were purchased from Vital River Laboratory Animal Technology (Beijing, China). Mice were kept in the Animal Laboratory of Anhui Medical University in accordance with Chinese Regulations on the Use and Breeding of Experimental Animals and the guidelines developed by the Experimental Animal Research Institute of Anhui Medical University. This research was approved by the Institutional Animal Care and Use Committee of Anhui Medical University (Number: LLSC20211182).

Sixty mice were randomly divided into six groups: Ctrl group (control), Model group, LPSP (low level of PSP) group, MPSP (medium level of PSP) group, HPSP (high level of PSP) group, and Befindate group. The Ctrl and Model groups were subjected to water gavage for 30 days. The LPSP, MPSP, and HPSP groups were subjected to 0.5 g/kg, 2 g/kg, and 10 g/kg PSP for 30 days, respectively. The Befindate group was subjected to 20 mg/kg of Befindate for 30 days. At the end of gavage, the Model, LPSP, MPSP, HPSP, and Befindate groups were subjected to 0.5% CCl_4 (10 ml/kg) for 24 h. Body weight was measured twice a week. Then, the mice were mercy killed, and samples were collected.

2.6. Organ Index Calculation

After mercy killed, the mouse body, liver and spleen weights were measured. The organ index was calculated with the following formula

"Liver (Spleen) index = Liver (Spleen) weight/body weight"

2.7. ALT and AST Measurement

To identify the function of the mouse liver after various treatments, ALT and AST kits were used to detect the levels of ALT and AST in serum following the guidelines. The absorbance was measured using a microplate reader at 510 nm.

2.8. Oxidative Stress Measurement

To detect oxidative stress in the mouse liver, T-AOC, T-SOD, CAT, GSH-PX, and MDA were measured by using a commercial reagent kit. Briefly, 50 mg of liver tissues was added to 1.5 ml tubes containing 500 μl of PBS. After homogenization and centrifugation, the protein concentration was measured. Then, the levels of T-AOC, T-SOD, CAT, GSH-PX, and MDA were measured according to the manufacturer's instructions.

2.9. HE Staining

After fixation, embedding, and sectioning, 5 μm sections were subjected to hematoxylin and eosin (HE) staining. Then, images were captured by using light microscopy.

2.10. Cell Culture

Mouse-derived macrophage RAW264.7 cells were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. Cells were kept at 37°C in a humidified incubator with 5% CO_2 . Cells were pretreated with different concentrations (50, 100, 200 $\mu\text{g}/\text{mL}$) of PSP for 4 h; afterwards, 100 ng/mL LPS was added and treated for 24 h.

2.11. RNA Extraction, cDNA Synthesis, and Quantification

After extraction from cells with TriZOL reagent, total RNA was quantified by using Nano Drop One, and 500 ng of RNA was subjected to cDNA synthesis using a TransScript® II One-Step gDNA Removal and cDNA Synthesis SuperMix kit according to the manufacturer's instructions. Then, a qRT-PCR assay was carried out to measure the level of genes by using the TransStart Top Green qPCR SuperMix Kit according to the guidelines. The relative expression of genes was calculated according to $2^{-\Delta\Delta\text{Ct}}$. 18S rRNA was used as a control. The primers are detailed in Table 1.

Table 1. Primer sequences

Gene	Primers (5'-3')
TNF- α	Fed TGTCCCTTTCACCTACTGGC Rev CATCTTTTGGGGAGTGCCT
IL-1 β	Fed CCATGGCACATTCTGTTCAAA Rev GCCCATCAGAGGCAAGG
iNOS	Fed CCCTTCAATGGTTGGTACATGG Rev ACATTGATCTCCGTGACAGCC
18 SRNA	Fed GAGACCTCAACACCCAGC Rev ATGTCACGCACGATTTC

2.12. Western Blot Assay

Total protein in cells and tissues was isolated by using RIPA lysis buffer containing 1% protease inhibitor, phosphatase inhibitor, and PMSF. After quantification via the BCA method, the protein was boiled and denatured. Then, 15-30 μg of total protein was separated by SDS-PAGE and transferred to PVDF membranes. Subsequently, the membrane was blocked in 5% skim milk diluted in TBST at room temperature for 1 hour. After washing 3 times with TBST, the membrane was incubated with primary antibodies at 4°C for 12-14 h. Afterward, the membrane was washed and incubated with secondary antibody for 1 h at room temperature. Finally, the membrane was detected by ECL, and the level of protein was calculated by Tanon GIS software. ACTB was used as a control.

2.13. NO Detection

The concentration of NO in mouse serum and cells after different treatments was measured by using a NO detection kit according to the manufacturer's instructions at 540 nm by using a microplate reader.

2.14. Immunofluorescence Staining

To detect iNOS in cells, an immunofluorescence assay was performed. After treatment and washing with PBS, cells in 96-well plates were fixed with 4% paraformaldehyde for 30 min at room temperature. Then, the cell membrane was disrupted by incubating with 1% Triton X-100 for 15 min. Subsequently, the cells were blocked with 1% BSA for 1 h at room temperature and then incubated with iNOS antibodies for another 12 h at 4°C. Next, the cells were incubated with Cy3-labeled secondary antibody for 2 h in a dark environment for 2 h. Finally, the cells were washed and stained with DAPI for 5

min. Images were captured using a fluorescence microscope (Carl Zeiss, Axio Vert. A1, Germany). A dual fluorescence staining method was used to measure iNOS levels in mouse liver macrophages. Fresh liver tissues were fixed with 4% paraformaldehyde, embedded in paraffin and sectioned. After deparaffinization, rehydration, and antigen retrieval, tissues were incubated with 3% H_2O_2 to quench endogenous peroxidase. The remaining procedures were the same as those for cell staining.

2.15. Statistical Analysis

All data in our study are presented as the mean $\pm$ SD from replicated assays. ANOVA and LSD Student *t*-test were used to analyze the difference between groups and the two groups by using SPSS 23.0 software, and all statistical graphs were obtained by using GraphPad Prism 8.0 software.

3. Results

3.1. Extraction of PSP and Identification of Polysaccharides

The vacuum dried PSP was a brown powder with a yield of 31.36% and a polysaccharide concentration of 49.6% (Figure 1A). A high performance liquid chromatograph diagram shows the symmetrical peak of PSP (Figure 1B). RT (40.456 min) corresponding to the chromatographic peak was substituted into the correction curve equation, and the number average and weight average molecular weight of PSP were 23341 Da and 31597 Da, respectively. The dispersion coefficient is 1.35 (Figure 1C). Monosaccharide analysis showed that polysaccharides in PSP were mainly composed of glucose (Glu), galactose (Gal) and xylose (Xyl), and the molar ratio of Glu, Gal and Xyl was 8.99:0.54:0.47 (Figure 1D).

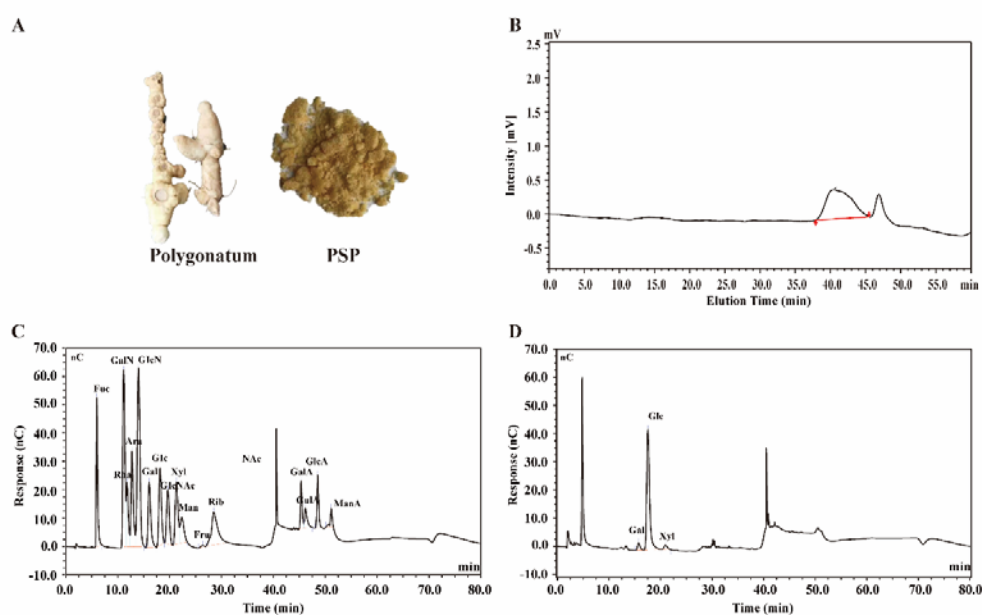


Figure 1. Extraction of PSP and constitution identification. Pictures of dried polygonatum sibiricum and PSP powder (A). HPGPC graph of PSP (B). HPIEC graph of monosaccharide standards (C). HPIEC graph of PSP (D)

3.2. PSP Protects Against Acute Liver Injury Induced by CCl₄

To investigate the protective effect of PSP against acute liver injury, we injected different concentrations of PSP into mice for 30 days and then established an acute liver injury model with CCl₄ (Figure 2A). Liver and spleen indexes were significantly elevated in the CCl₄ injured group, indicating congestion and edema in the liver and spleen, but these increases were attenuated by PSP (Figure 2B-C). The elevation of serum ALT and AST is a common index to evaluate liver damage. The results showed that the levels of ALT and AST in serum of mice with CCl₄

injury were significantly increased, indicating that the liver injury model was successfully established. PSP combined treatment significantly inhibited CCl₄-induced upregulation of serum ALT and AST levels, and the inhibitory effect was proportional to dose concentration (Figure 2D-E). In addition, when the concentration of PSP reaches 10 g/kg, it can show the same effect as bifendate tablets. HE staining showed that PSP combined treatment significantly reduced hepatocyte necrosis, degeneration, and inflammatory cell infiltration compared with CCl₄ injury group (Figure 2F). In summary, PSP has a good protective effect against CCl₄-induced injury in vivo.

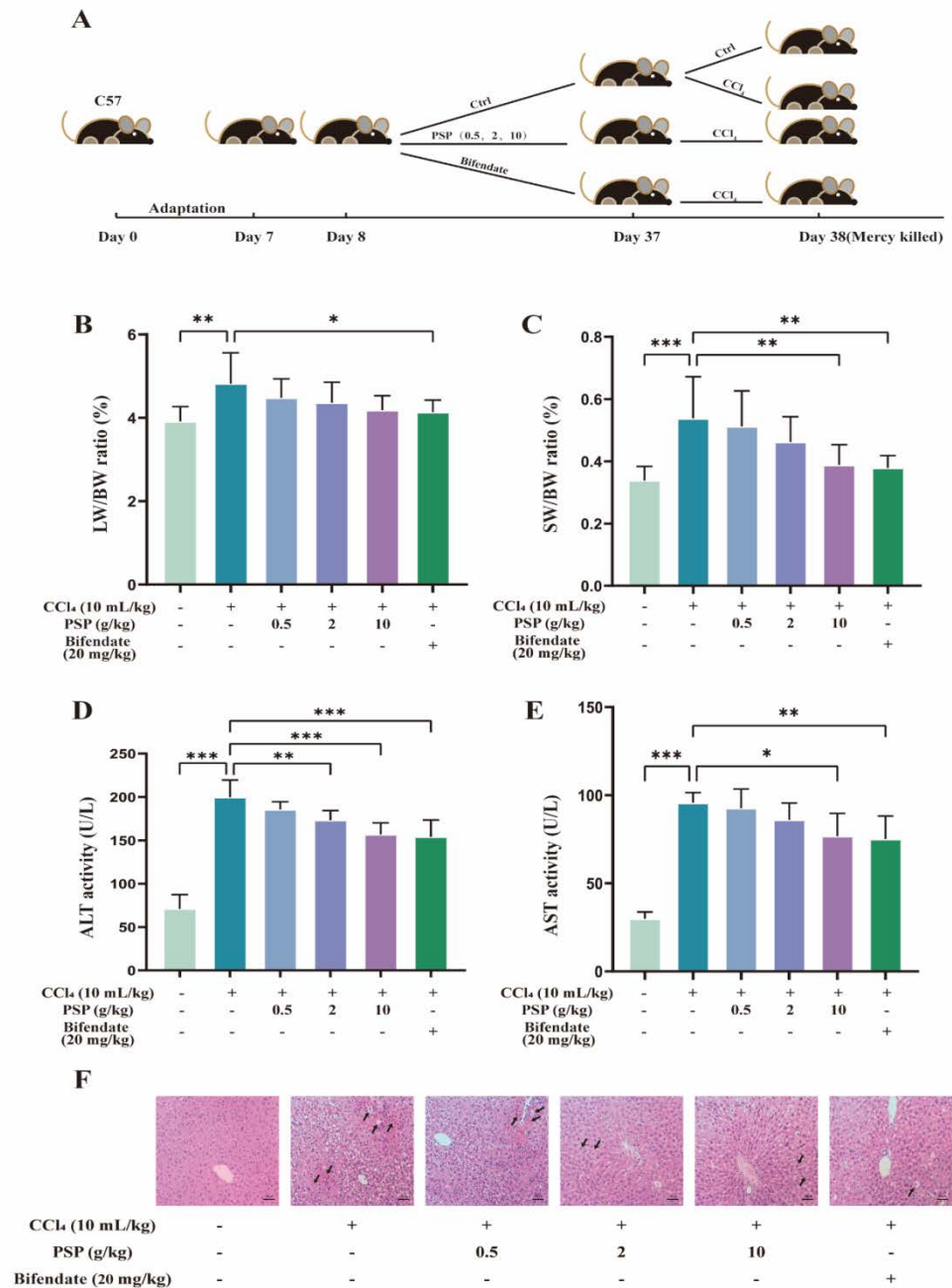


Figure 2. Pretreatment with PSP protects against acute liver injury induced by CCl₄. Mouse model construction (A). Effects of PSP on the liver index and spleen index (B-C) and liver function in mice (D-E). Liver sections stained with HE (F). Observation and photographs were captured by optical microscope. Scale bar=100 μm. *p < 0.05, **p < 0.01, ***p < 0.001, compared to CCl₄-treated mice

3.3. PSP Inhibits Liver Inflammation and Macrophage M1 Polarization

PSP has been shown to have a protective effect against acute liver injury, but whether it ameliorates acute liver injury by inhibiting macrophage M1 polarization and reducing inflammation needs further analysis. The results showed that PSP treatment significantly inhibited the increase of NO level in the liver tissue with acute injury, and when PSP concentration reached 10 g/kg, compared with the CCl₄ injury group, the PSP combined treatment reduced NO level by 47.7% (Figure 3A). In addition, IL-1 β and TNF- α were significantly reduced in the PSP combined treatment group, and the reduction was

proportional to the PSP concentration, suggesting the inhibitory effect of PSP on inflammation after injury. Next, we examined the effect of PSP on M1 polarization of liver macrophages. The results showed that M1-type polarized macrophages were significantly elevated in the acutely injured liver tissue, which could be demonstrated by up-regulated iNOS expression and iNOS positive macrophages. However, PSP treatment significantly inhibited M1 polarization of macrophages in the liver (Figure 3B-I). Therefore, these results suggest that PSP plays a role in protecting the liver by inhibiting the polarization of macrophage M1 and reducing the occurrence and development of inflammation in acute liver injury.

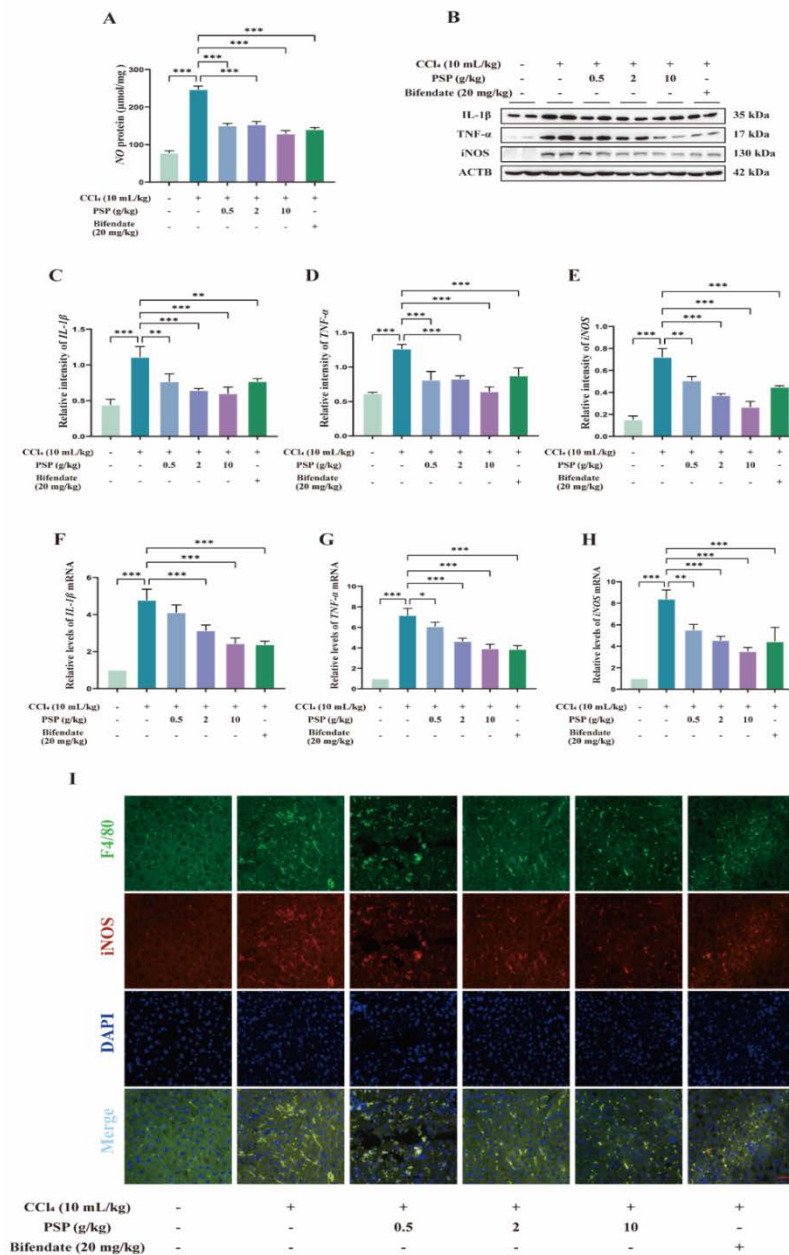


Figure 3. Pretreatment with PSP inhibits liver inflammation and macrophage M1 polarization induced by CCl₄. NO levels in mouse serum (A). The protein levels of inflammation- and M1 polarization-associated proteins in liver tissue (B-E). The mRNA levels of inflammation- and M1 polarization-associated genes (IL-1 β , TNF- α , iNOS) in liver tissue (F-H). The expression of CCl₄-induced M1 macrophages observed by fluorescence microscope (I). Scale bar=50 μ m. *p < 0.05, **p < 0.01, ***p < 0.001, compared to CCl₄-treated mice

3.4. PSP Prevents Liver Oxidative Stress Induced by CCl₄

Many studies have shown that oxidative stress is closely related to inflammation, and oxidative stress is a pathogenic factor of chronic inflammatory diseases [24]. In order to prove whether the protective effect of PSP is related to oxidative stress, the levels of oxidative stress markers T-AOC, T-SOD, CAT, GSH-PX and MDA in liver tissues of each group were detected. The results showed that CCl₄ treatment impaired the antioxidant capacity of mouse liver, and was associated with the

decrease of T-AOC, T-SOD, CAT, GSH-PX and the increase of MDA. However, PSP combined treatment significantly upregulated T-AOC, T-SOD, CAT and GSH-PX levels, while inhibiting MDA levels. When the concentration of PSP reached 10 g/kg, the combined treatment of PSP reduced the MDA level by 29.4% compared with the CCl₄ injured group, showing similar antioxidant effects as biphenylate tablets (Figure 4A-E). Taken together, these phenomena suggest that PSP can inhibit oxidative stress in acute liver injury and has antioxidant effects.

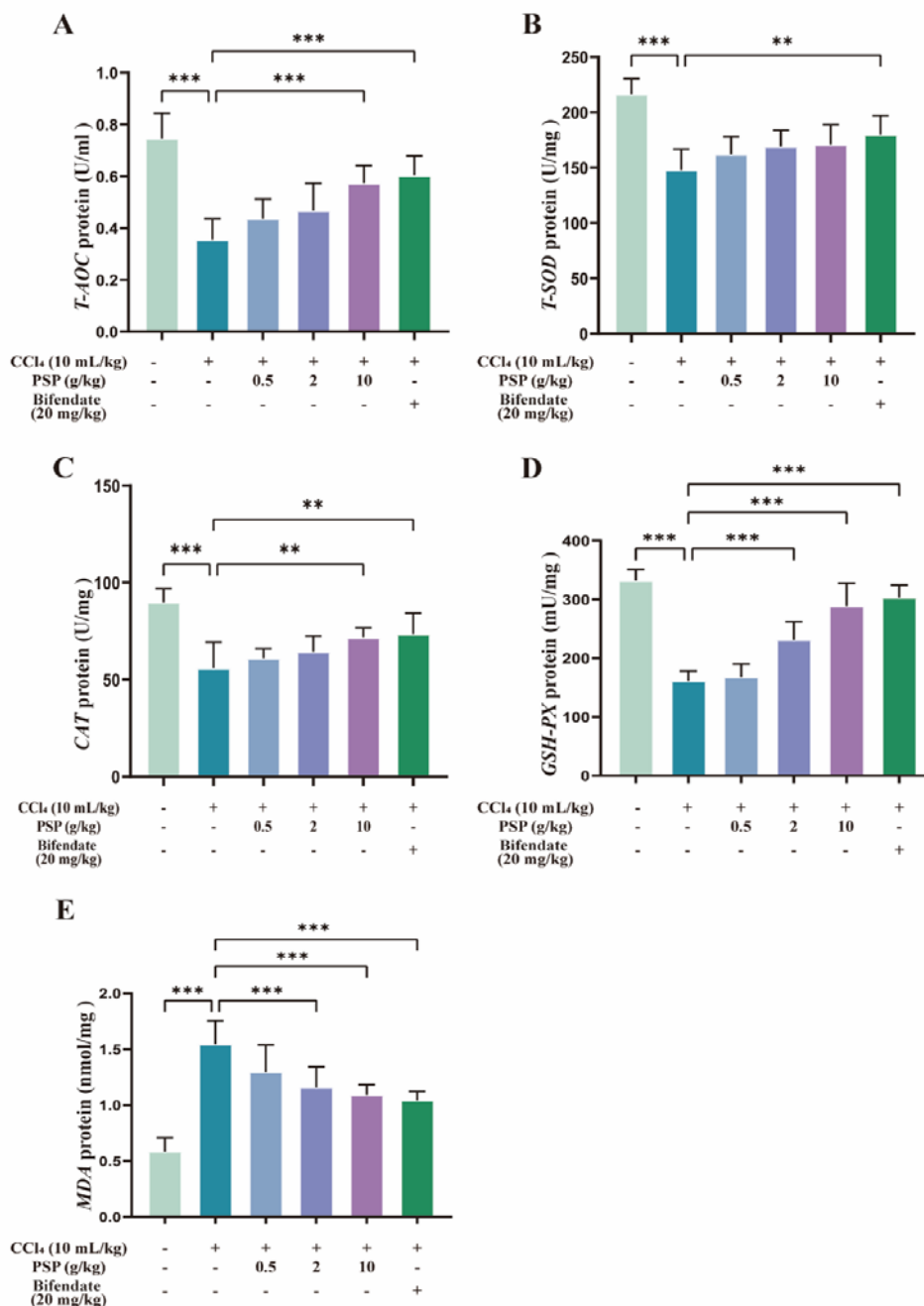


Figure 4. Pretreatment with PSP prevents liver oxidative stress induced by CCl₄. T-AOC level (A) and T-SOD level (B) and CAT level (C) and GSH-PX level (D) and MDA level (E) in liver tissue. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, compared to CCl₄-treated mice

3.5. PSP Suppresses LPS-induced Inflammation in RAW 264.7 Cells

The above results have proved that PSP has anti-inflammatory and antioxidant effects *in vivo*, but the specific mechanism of action is not very clear. Therefore, we will further explore the possible molecular mechanisms at the cellular level. First, LPS was applied to RAW264.7 cells, and the M1 polarization model of macrophages was successfully established. The results showed that the NO level was up-regulated by 76.9%, the

iNOS expression was up-regulated at both gene and protein levels, and the cells were iNOS positive (Figure 5A-E). At the same time, the results of gene and protein levels showed that inflammation-related markers IL-1 β and TNF- α were significantly up-regulated in LPS-treated cells (Figure 6A-E), while PSP combined treatment significantly inhibited M1 polarization and IL-1 β and TNF- α levels in RAW264.7 cells (Figure 5A-E and 6A-E). In conclusion, PSP treatment can inhibit M1 polarization of macrophages, which is consistent with the results of *in vivo* studies.

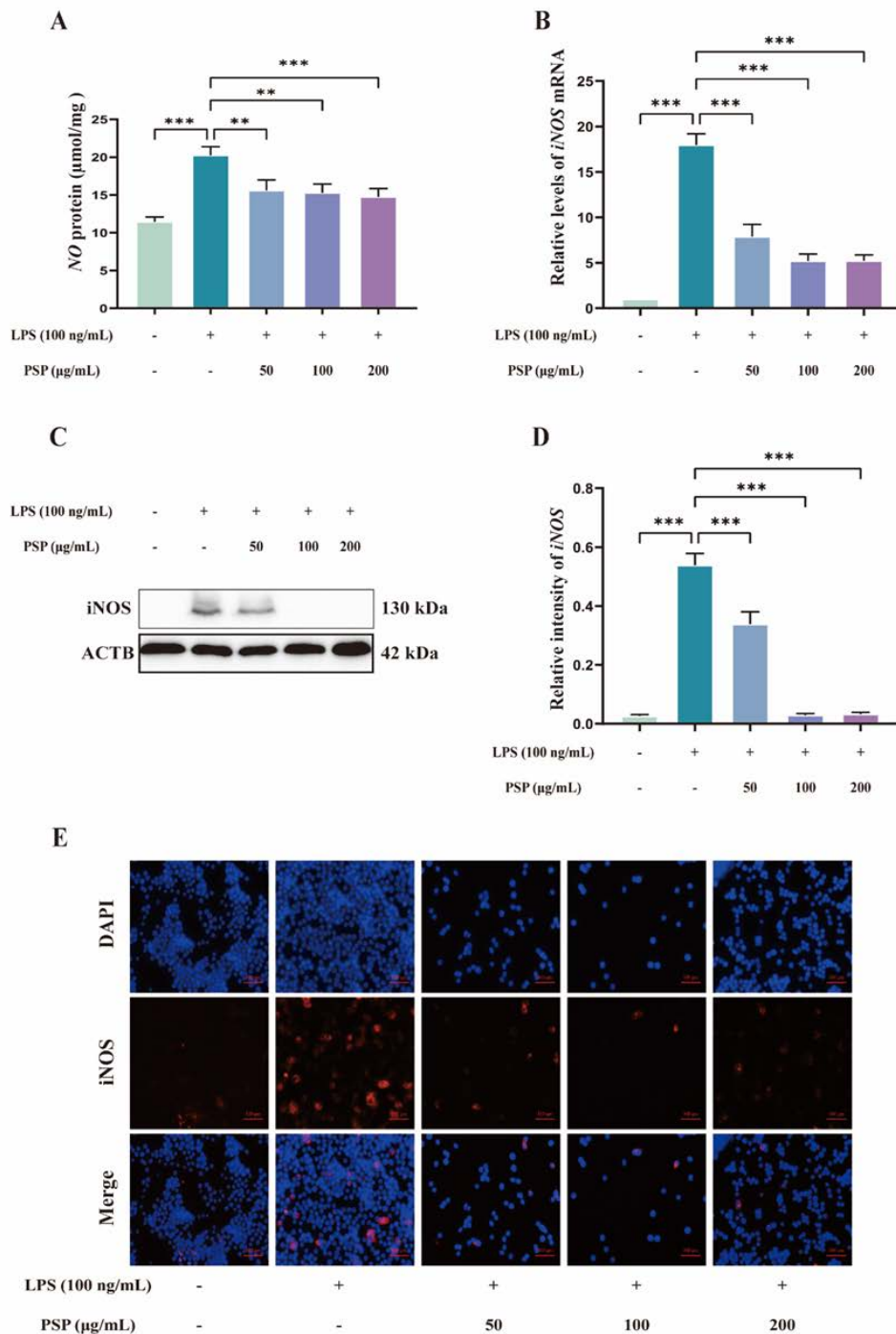


Figure 5. Pretreatment with PSP suppresses the inflammatory factor level in RAW264.7 cells induced by LPS. NO levels in RAW264.7 cells (A). The mRNA and protein levels of the M1 polarization marker iNOS in RAW264.7 cells (B-D). The expression of LPS-induced M1 macrophages observed by fluorescence microscope (E). Scale bar= 100 μ m. * p < 0.05, ** p < 0.01, *** p < 0.001, compared to LPS-treated cells

3.6. PSP Reversed the Activation of the JAK2/STAT3/NF- κ B Signaling

Studies have shown that the activation of JAK₂/STAT₃ pathway promotes M1-type polarization of macrophages and then activates NF- κ B, leading to massive secretion of inflammatory factors [25]. To clarify whether the JAK₂/STAT₃/NF- κ B signaling pathway mediates the anti-inflammatory and hepatoprotective effects of PSP,

western blot analysis was performed on LPS-treated RAW264.7 cells and CCl₄-treated mouse liver tissues. The results showed that levels of p-JAK₂, p-STAT₃, and p-NF- κ B were elevated in both in vivo and in vitro model groups, and were significantly suppressed after combined treatment with PSP (Figure 7A-D). In conclusion, PSP can inhibit the M1 polarization of macrophages by attenuating the JAK₂/STAT₃/NF- κ B pathway, and play an anti-inflammatory and hepatoprotective role.

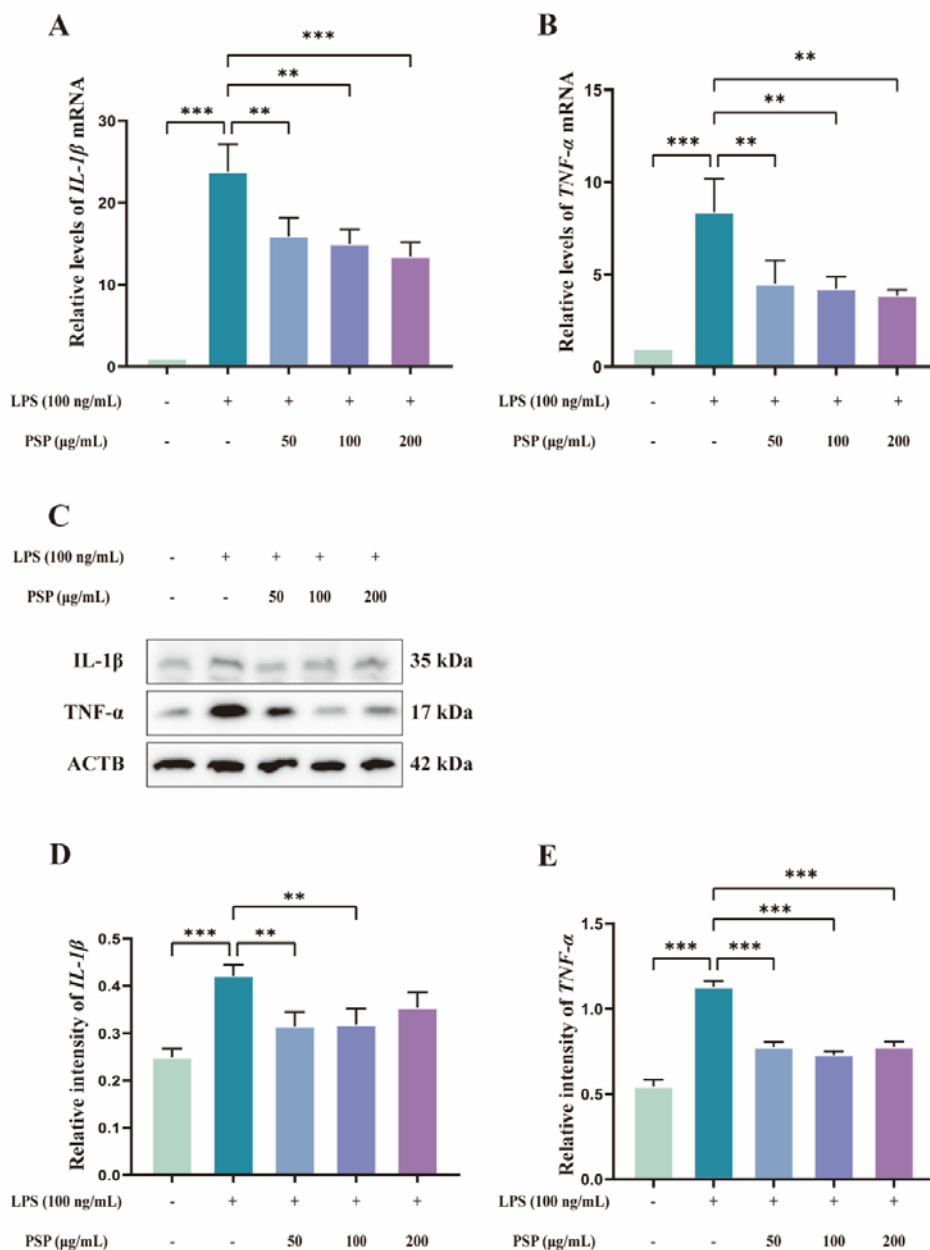


Figure 6. Pretreatment with PSP inhibits the polarization of M1 macrophages induced by LPS. The mRNA levels of IL-1 β and TNF- α in RAW264.7 cells (A-B). The protein levels of IL-1 β and TNF- α in RAW264.7 cells (C-E). * p < 0.05, ** p < 0.01, *** p < 0.001, compared to LPS-treated cells

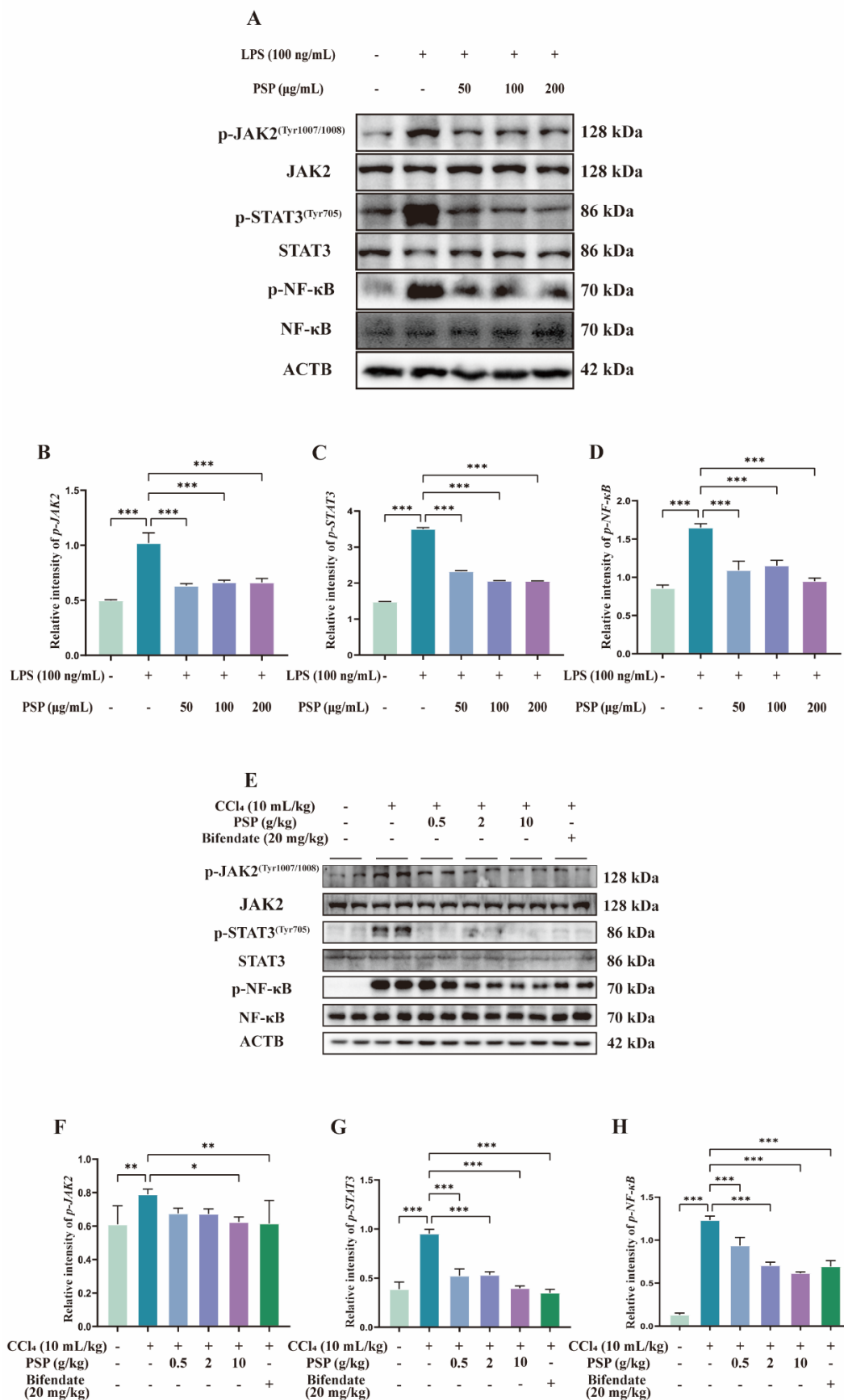


Figure 7. Pretreatment with PSP reversed the activation of the JAK2/STAT3/NF- κ B signaling. JAK2/STAT3/NF- κ B signaling pathway protein levels in RAW264.7 cells (A-D) and liver tissue (E-H). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to CCl₄-treated mice (A-D) or LPS-treated cells (E-H)

4. Discussion

These above results confirmed that PSP inhibited the occurrence and development of inflammation and oxidative stress through the JAK₂/STAT₃/NF- κ B signaling pathway, and alleviated the acute liver injury induced by CCl₄ in mice.

Polygonatum sibiricum is a kind of traditional Chinese medicine for both medicine and food. In Chinese medicine, *Polygonatum sibiricum* has the functions of invigorating qi and nourishing Yin, moistening lung, invigorating spleen and invigorating kidney. Because of its good medical role in preventing and treating diseases, the rhizome was included in the latest Chinese pharmacopoeia in 2020. As a chemical component with the highest content and important medicinal value, PSP has become a hot spot in the field of flavonoid extract, purification and functional exploration. Zhao et al. obtained the crude polysaccharides of *Xanthophylla* by hot water extraction and ethanol precipitation, with a content of up to 75%. Further analysis showed that the crude polysaccharides of *Xanthophylla* mainly consisted of fructose, galactose, galacturonic acid and a small part of rhamnose-xylose and arabinose [26]. By using fractional precipitation combined with chromatographic column separation method, Wang et al. obtained 3 polysaccharides from the extract of *C. yunnanensis*, whose relative molecular weights were 85017.83 Da, 266084.76 Da, 474799.22, respectively. Monosaccharide compositions include D-mannose, D-galacturonic acid, D-glucuronic acid, d-galactose, D-arabinose, L-fucose, D-ribose, and L-rhamnose [27]. The purity of PSP used in this study was 49.6%, the relative molecular weight was 31597Da, 23341Da, and the monosaccharide composition included glucose, galactose and xylose. Previous studies have found that PSP can regulate oxidative stress in the liver of mice and regulate the disorder of glucose and lipid metabolism [28,29]. Subsequent experiments focused on the protective effect and mechanism of PSP on acute liver injury.

As an important substantive organ of the human body, liver plays an important role in the process of participating in the body's metabolism and maintaining the homeostasis of the internal environment. At the same time, the liver is often exposed to different endogenous or exogenous toxic and harmful substances, which can easily cause acute or chronic liver injury and eventually lead to liver failure. It is of great significance to provide effective treatment or prevention measures in the early stage of liver injury and disease to inhibit or alleviate the progression of liver disease [30].

Acute liver injury is a common liver disease that, without adequate protection, can eventually progress to cirrhosis and liver cancer, resulting in a significant health burden. Therefore, providing effective treatment or preventive measures in the early stage of liver injury and disease is of great significance to inhibit and alleviate the progression of liver disease. In recent years, in order to further explore the occurrence and development of liver diseases, researchers have established a variety of animal models of liver injury to simulate acute liver injury. Common models of acute liver injury include: canavalin A [31], galactosamine/lipopolysaccharide induced immune

acute liver injury [32]; Drug-induced acute liver injury induced by acetaminophen [33]; Alcoholic acute liver injury induced by alcohol feeding [34] and chemical acute liver injury induced by CCl₄ [35]. CCl₄, as a cytotoxic substance with high affinity to the liver, has a strong toxic effect on the liver and is often used as an acute liver injury model at home and abroad. In this study, three doses of PSP and bifendate were used for 30 consecutive days by gavage, followed by intraperitoneal injection of CCl₄ for 12 h and short-term exposure to establish an acute liver injury model to study the protective effect of PSP on acute liver injury. Under normal physiological conditions, ALT and AST are located in cytoplasm and mitochondria respectively. When liver injury occurs, the cell morphology in the liver tissue changes irregularly, resulting in cell rupture and nuclear deformation, and cell rupture will cause ALT and AST to enter the systemic circulation, resulting in a sharp increase of ALT and AST in serum [36]. In this study, compared with the control group, ALT and AST levels in the serum of the model group were increased, and H&E results showed that the cell arrangement of the liver tissue in the model group was disordered, the cell size was different, and there were many vacuole-like structures between the cells. These results indicate that the liver of mice in the model group of this study has met the liver injury criteria, and the acute liver injury model has been successfully established. PSP pretreatment can effectively alleviate the acute liver injury caused by CCl₄ in mice, and the high level of ALT and AST caused by CCl₄ are significantly inhibited. After PSP, the cells in the liver were arranged in an orderly manner and the vacuole-like structure was significantly reduced, indicating that PSP can play a protective role in the liver.

Inflammation and oxidative stress are the common pathological basis of various liver diseases, Mao et al. found that alcohol-induced liver injury in mice would induce severe oxidative stress and inflammatory response and release a large number of inflammatory factors [37]. Yu et al. found that CCl₄ could induce liver damage in mice (elevated ALT and AST levels) and significantly increase the levels of oxidative stress-related indexes (SOD, MPO, MDA, GPX-2) and inflammatory factors (TNF- α , IL-6, IL-10) [38]. Therefore, targeting inflammation and oxidative inhibition may be one way to improve liver damage. Guo et al. found that shikonin inhibits paracetamol-induced acute liver injury by reducing inflammation and oxidative stress [39]. Wu et al.'s study showed that salvianolic acid C inhibited inflammation and oxidative stress through Keap1/Nrf2/HO-1 signaling, thus significantly improving acute liver injury [40]. Similarly, this study found that PSP can significantly inhibit inflammation and oxidative stress, reduce NO concentration, down-regulate IL-1 β and TNF- α mRNA and protein levels, significantly increase T-AOC, T-SOD, CAT, GSH-PX levels, and inhibit MDA levels. These data suggest that PSP's protective effect against CCl₄-induced acute liver injury is achieved by reducing inflammation and oxidative stress.

Recent studies have shown that macrophages play a key role in regulating inflammation and oxidative stress [41,42]. Macrophages residing in the liver have a high plasticity, and when the body is under stress, they will undergo different phenotypic transformations under the

stimulation of endogenous or exogenous substances to play different functions, and can be polarized into two different types. Among them, the pro-inflammatory polarization of macrophages (M1 type) can induce inflammation and oxidative stress in tissue injury, and there is a significant correlation with liver injury [43,44,45]. Liu et al. found that aging would destroy liver homeostasis and damage hepatocyte regeneration, thus aggravating acute liver injury; while inhibition of autophagy in the aging process would promote M1-type polarization of liver macrophages and release inflammatory related factors, thus aggravating acute liver injury, targeting liver macrophages and inhibiting their M1-type polarization has a significant liver protective effect [46]. Gong et al. confirmed that the phenylethylamine analogue FC-99 could promote the LPS-induced transformation of M1 macrophages into M2 type, thus alleviating liver injury induced by LPS [47]. In this study, the M1-type polarization of macrophages in CCl₄-induced acute liver injury was demonstrated through the increase of iNOS level and iNOS positive macrophages. Consistent with this, LPS-treated RAW264.7 cells also showed increased M1-type macrophage activation and inflammation. These results suggest that M1-type polarization of macrophages can promote the development of inflammation. Thus, acute liver injury can be mitigated by preventing M1 polarization of macrophages. In this study, PSP pretreatment significantly inhibited M1 polarization of macrophages in CCl₄-induced liver injury and LPS-treated RAW 264.7 cells, suggesting the inflammatory regulatory potential of PSP and its association with M1 polarization of macrophages.

At present, researches on the mechanism of regulating M1-type polarization of macrophages mainly focus on MAPK, PI3K/Akt and mTOR signaling pathways [48,49,50,51,52]. Recently, it has been found that estrogen receptors can regulate SOCS3's participation in bisphenol A-induced M1-type polarization of macrophages through the activation of JAK₂/STAT₃ pathway [53]. STAT3 is a cell signaling and transcriptional activator that receives cytokine and growth factor signaling in vivo. Upon receiving cytokine stimulation, the receptor dimerization is induced and the receptor-related Janus(JAK) is activated, and the JAK phosphorylates itself and the receptor. The phosphorylation of JAK and receptor can act as a binding site for a variety of cytokines, the most important of which is STAT3. After binding to phosphorylated JAK₂, STAT₃ is phosphorylated and transferred to the nucleus to function as a transcription factor [22]. Some studies have found that JAK₂/STAT₃ is closely related to inflammation in the body, but whether JAK₂/STAT₃ pathway plays a role in the mouse model of acute liver injury induced by CCl₄ and the M1-type polarization model of RAW264.7 cells induced by LPS is not clear [23,54]. The results of this study showed that PSP pretreatment significantly reduced the activation of JAK₂/STAT₃/NF-κB pathway in CCl₄-treated mouse liver tissue and LPS-treated RAW264.7 cells.

In conclusion, PSP alleviates CCl₄-induced acute liver

injury in mice by inhibiting JAK₂/STAT₃/NF-κB mediated M1-type polarization and oxidative stress of macrophages, and plays a hepatoprotective role. This study revealed that PSP can control acute liver injury in the early stage of liver disease progression, and can effectively prevent or delay the progression of liver disease, which has important public health significance for reducing the burden of liver disease, and provides a new direction for the functional development of PSP and the development of related products. Although this study demonstrated the protective effect of PSP against acute liver injury, the protective effect of PSP against other liver injuries has not been confirmed. In addition, the polysaccharide content of PSP in this study was low, and the extraction process needs to be further improved.

ACKNOWLEDGMENTS

Declared none.

Sources of Support

This work was supported by the Anhui Academician Workstation Research Project (grant number JHHJYSGZZ19001) and the Major Projects of Science and Technology of Anhui Province (202103a06020003).

Author Contributions

W. J. Chen and P. Wang conceived and designed the study and helped polish the manuscript. C. S. Shao, G. J. Wang, D. Liang, J. C. Yu, Z. X. Liu, C. C. Xiao and F. D. Zhou helped polish the manuscript, analyzed the data, and were responsible for the animal experiments. J. J. Li and T. Wang carried out the experiments, analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

Author Declarations

The authors declare no conflict of interest.

Institutional Review Board Statement

Animal experiments were approved by the Animal Care and Use Committee, Anhui medical university (Number: LLSC20211182).

Data Availability Statement

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to a partner wanting to continue the research.

Abbreviations

Abbreviation	English full name
ALT	Alanine aminotransferase
AST	Aspartate transaminase
CCl ₄	Carbon tetrachloride
CAT	Catalase
GSH-PX	Glutathione peroxidase
HE	Hematoxylin and eosin staining
HPGPC	High-performance gel permeation chromatography
HPIEC	High-efficiency gel ion exchange method
iNOS	Inducible nitric oxide synthase
IL-1 β	Interleukin-1 β
JAK ₂	Janus kinase 2
KCs	Kupffer cells
LPS	Lipopolysaccharide
MDA	Malondialdehyde
NF- κ B	Nuclear factor- κ B
NO	Nitric oxide
PSP	<i>Polygonatum sibiricum</i> polysaccharides
ROS	Reactive oxygen species
STAT ₃	Signal Transducers and Activators of Transcription 3
T-AOC	Total antioxidant capacity
T-SOD	Total superoxide dismutase
TNF- α	Tumor necrosis factor- α

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