Research article

Astragaloside IV attenuates lipopolysaccharide induced liver injury by modulating Nrf2-mediated oxidative stress and NLRP3-mediated inflammation

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A R T I C L E   I N F O

A B S T R A C T

Aims and objectives: Sepsis-associated liver injury is a common public health problem in intensive care units. Astragaloside IV (AS-IV) is an active component extracted from the Chinese herb *Astragalus membranaceus*, and has been shown to have anti-oxidation, anti-inflammation, and anti-apoptosis properties. The research aimed to investigate the protective effect of AS-IV in lipopolysaccharide (LPS)-induced liver injury.

Methods: Male C57BL/6 wild-type mice (6-8 week-old) were intraperitoneally injected with 10 mg/kg LPS for 24 h and AS-IV (80 mg/kg) 2 h before the LPS injection. Biochemical and histo-pathological analyses were carried out to assess liver injury. The RT-qPCR analyzed the mRNA expression of IL-1β, TNF-α, and IL-6. The mRNA and protein expression of SIRT1, nuclear Nrf2, Nrf2, and HO-1 were measured by Western blotting.

Results: Serum alanine/aspartate aminotransferases (ALT/AST) analysis, malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) were showed that AS-IV protected against LPS-induced hepatotoxicity. The protection afforded by AS-IV was confirmed by pathological examination of the liver. Pro-inflammatory cytokines, including interleukin- 1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin 6 (IL-6), were observed to be reversed by AS-IV after exposure to LPS. Western blot analysis demonstrated that AS-IV enhanced the expression levels of Sirtuin 1 (SIRT1), nuclear factor erythroid 2-related factor 2 (Nrf2), and heme oxygenase 1 (HO-1).

Conclusions: AS-IV protects against LPS-induced Liver Injury and Inflammation by modulating Nrf2-mediated oxidative stress and NLRP3-mediated inflammation.

1. Introduction

Sepsis/septic shock has been regarded as a systematic inflammatory dysfunction syndrome and also the main reason for organ failure, including liver injury [1,2]. The most frequent cause of death was recently reported in intensive care units (ICU) due to sepsis [3]. The liver plays an essential role in different physiological and pathophysiological systems, including metabolism, detoxification, homeostasis, and immunity, that make the organ vulnerable at the extreme level during sepsis by endotoxin-induced injury [4]. Acute liver injury (ALI) induced by sepsis is the major cause of fatalities among patients affected with sepsis. Patients afflicted with ALI exhibit clinical symptoms ranging from slightly increased liver enzymes to serious liver injury. Sepsis-induced liver injury directly involves disease progression and fatalities [5]. There are several mechanisms have been reported that may involve the etiology of sepsis-associated ALI, such as cellular hypoxia, inflammatory and immune responses, oxidative stress, and apoptosis [6]. However, the precise underlying mechanism and progression of sepsis-induced liver injury remain unclear.

Several studies demonstrated that gram-negative bacteria generate lipopolysaccharide (LPS) associated with sepsis. It may induce the progression of systematic organ failure, including liver failure [7]. Different experimental animal models have confirmed the association between LPS-induced endotoxemia and liver damage [8]. The liver is the most important immune and detoxification organ in the human body, and LPS performs as an active liver endotoxin, a consequence of liver dysfunction. Moreover, LPS stimulates the hepatic Kupffer cells (macrophages) to produce inflammatory cytokines and activates hepatocyte necrosis or apoptosis, causing ultimate liver damage [9]. However, no study has been carried out on the impact of inflammation and apoptosis on sepsis-associated liver injury.

Astragaloside IV (AS-IV) is a naturally active compound isolated from the Chinese herb *Astragalus membranaceus*. AS-IV has been shown in recent studies to have anti-oxidation, anti-inflammation, and anti-apoptosis properties and the ability to boost immunity [10–12]. Previous studies reported that ASIV protects renal tubular epithelial cells from endoplasmic reticulum stress-induced apoptosis and diabetic cardiomyopathy in T2DM rats [12,13]. One study reported that AS-IV protects against cisplatin-induced damage by suppressing NF-kB activation and lowering levels of some pro-inflammatory cytokines such as TNF-α and IL-1β [14]. However, whether AS-IV has protective effects on LPS-induced liver injury and inflammation is unclear. In the current research, we designed a study to investigate the protective effects of AS-IV against inflammatory injury and related mechanisms in LPS-induced liver injury.

2. Methods

2.1. Animals and treatment

Male C57BL/6 wild-type mice (6-8 week-old) were maintained in the SPF laboratory animal room. All mice were maintained under environmentally controlled conditions (ambient temperature, 22 ± 2 °C; humidity 40%) with a 12 h light/dark cycle and access to water and food ad libitum. Mice were intraperitoneally injected with 10 mg/kg LPS or an equal volume of saline for 24 h. Mice in the AS-IV group were injected with AS-IV (80 mg/kg, HY-N0431, MedChemExpress) 2 h before the LPS challenge. The Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine Ethics Committee approved (SPH NO.18. 5-50) this study. The authors envisaged all standard protocols in accordance with the 1964 Declaration of Helsinki. All methods carried out in this study were in accordance with ARRIVE guidelines.

2.2. Biochemical analysis

Serum was collected to measure ALT (C009-2-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and AST (C010-2-1, Nanjing Jiancheng) activities using commercial kits. The livers were homogenized (10%, w/v) in normal cold saline for measuring MDA (S0131S, Beyotime, Shanghai, China) content, SOD (S0109, Beyotime), and CAT (S0051, Beyotime) activities using commercially available kits.

2.3. Hematoxylin and eosin (H&E) staining

The Hematoxylin and Eosin (H&E) assay were implemented to detect liver tissue injury. Fresh liver tissues were first stained for 6 h at 60–70 °C with hematoxylin solution (HHS80, Sigma-Aldrich) and then washed in tap water until colorless. The liver tissues were then cleaned with tap water after being separated 2 times for 2 h and once for 10 h using 10% acetic acid and 85% ethanol in water. The liver tissue was immersed in a saturated lithium carbonate solution for 12 h before being washed with tap water as part of the bluing process. Eosin Y solution (HT110116, Sigma-Aldrich) was then used to stain the sample for 48 h [15,16]. The stained fixed sections (4 μm-thick) were observed under the light microscopy (Olympus). The degrees of liver injury were scored on a scale of 0–3 in a double-blind fashion, and two slides were studied from each mice.

2.4. Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is a method to recognize the location and distribute target antigens in cells or tissues by using specific antibodies to stain the samples. First, fresh liver tissues were fixed in 4% paraformaldehyde fixative, gradually dehydrated, and then embedded in paraffin. The fixed section was sectioned into 4 μm-thick slices. The next step was accomplished with the biotin-
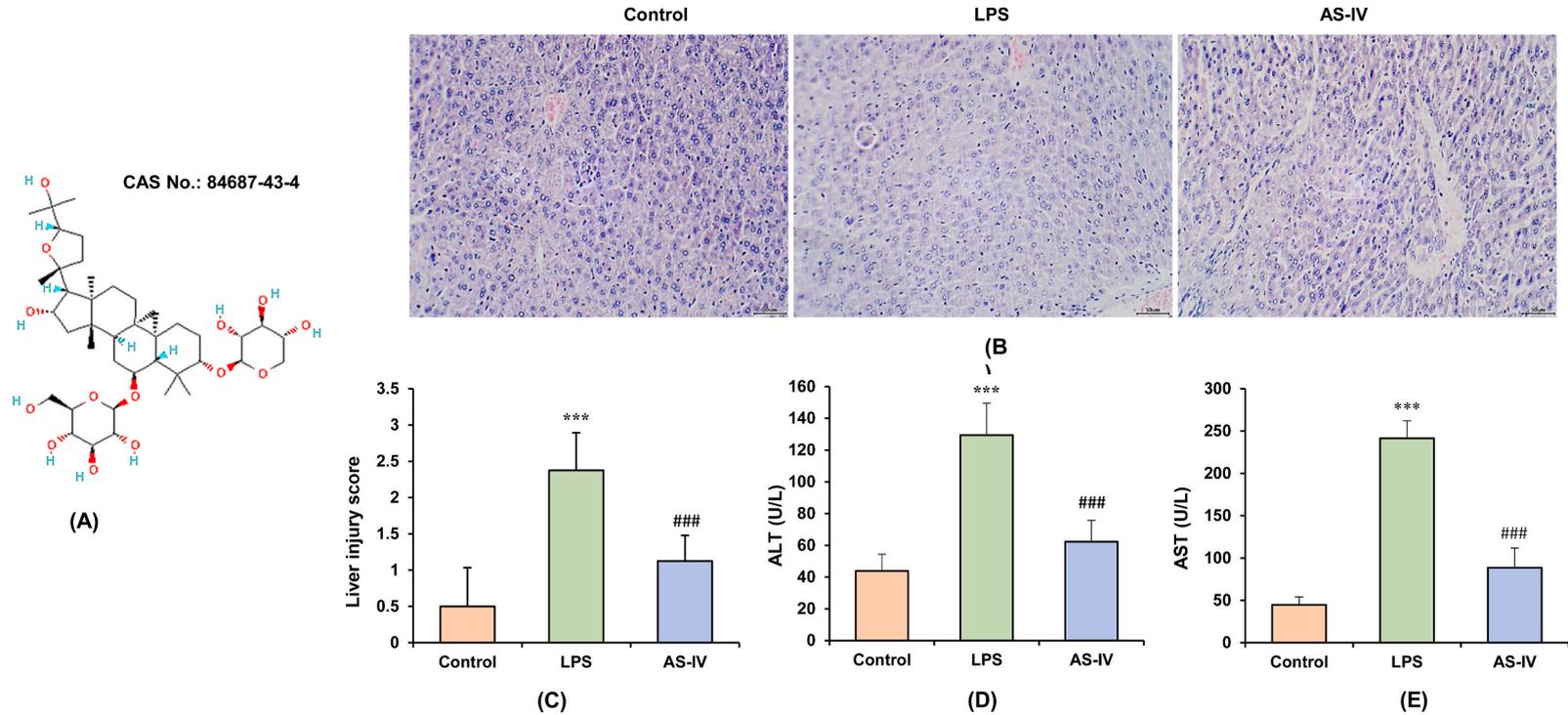
Fig. 1. AS-IV alleviates LPS-induced liver injury. Male C57BL/6 mice (6–8 weeks old) were pre-treated with AS-IV (80 mg/kg) for 2 h and then injected intraperitoneally with LPS (10 mg/kg). Mice were sacrificed 24 h after LPS administration, and liver tissues and serum were collected for further analysis. **A** The chemical structural formula of Astragaloside IV. **B** Representative images of liver HE staining (Magnification, 200 × ; Scale bar: 50 μm). **C** The liver injury scores were calculated based on the HE staining images. **D, E** ALT and AST level in serum of mice. Data are presented as mean ± SD. N = 8 mice per group. ***P < 0.001 vs. control group; ###P < 0.001 vs. LPS group.

(A)

(B)

(C)

(D)
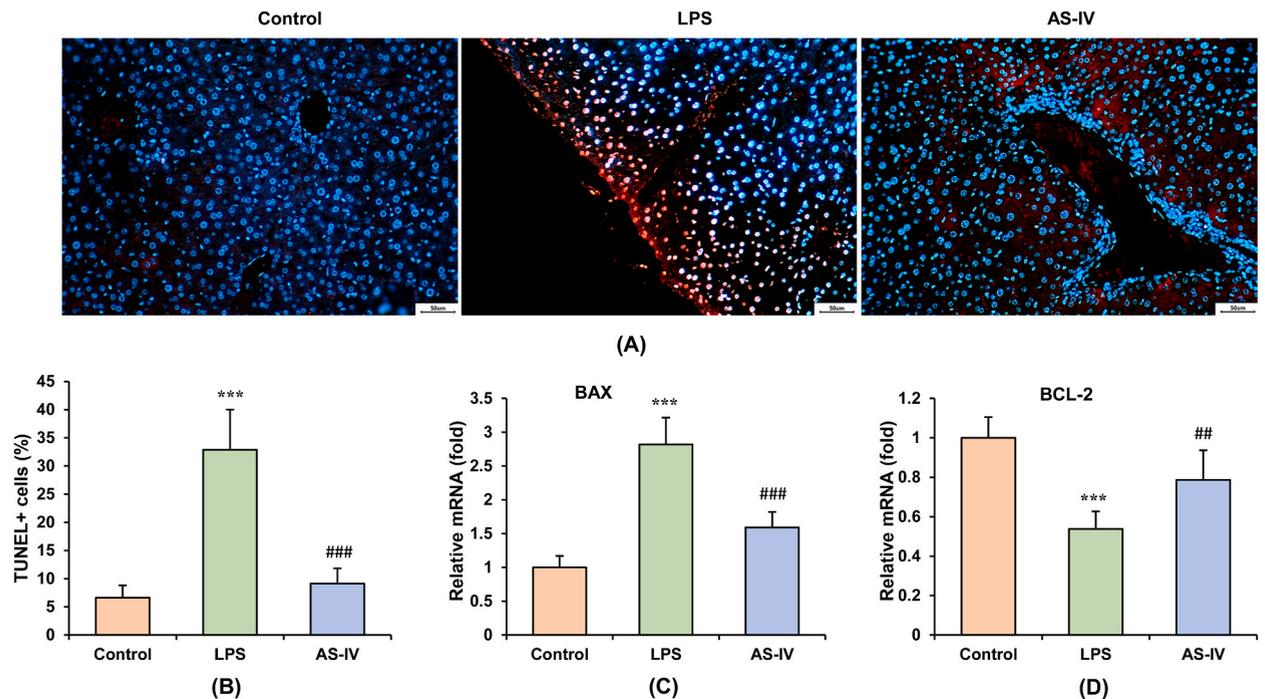
Fig. 2. AS-IV inhibits LPS-induced apoptosis in liver tissue. **A** Representative image of TUNEL staining in liver tissue of mice (Magnification, $100 \times$; Scale bar: 50 μm). **B** The quantification of TUNEL positive cells. RT-qPCR was performed to determine the mRNA expression of two apoptosis-related genes **C** BAX and **D** BCL-2 in liver tissue. Relative mRNA levels were normalized to GAPDH. Data are presented as mean $\pm$ SD. N = 8 mice in per group. ***$P < 0.001$ vs. control group; ##$P < 0.01$, ###$P < 0.001$ vs. LPS group.

streptavidin peroxidase method (SPlink Detection Kit, ZSGB-Bio). According to the developer's instructions, the method was performed. Finally, the slides were observed under the optical microscope (IX51, Olympus, Japan).

2.5. Immunofluorescence (IF)

Frozen sections of the liver tissue from mice were obtained. After being washed with phosphate-buffered saline (PBS) three times, the sections were stained with TUNEL and DAPI and observed under an inverted microscope (IX51, Olympus, Japan). The Immunofluorescence was performed as previously described [17].

2.6. RT-qPCR

TRizol (Invitrogen, USA) used to extract total RNA from mice atrial tissues. Reverse transcription was carried out to convert total RNA into complementary DNA (cDNA). The mRNA was amplified by Real-Time Quantitative PCR (RT-qPCR) using SYBR Green reagent (TaKaRa, Japan) in an ABI Prism 7700 Real-Time PCR system (Applied Biosystems, USA). The list of primer sequences was used as follows: BAX (forward: 5-GCC TCC TCT CCT ACT TCG G-3′; reverse: 5′-AAA AAT GCC TTT CCC CTT C-3′); BCL-2 (forward: 5′-CTC GTC GCT ACC GTC GTG ACT TCG-3′; reverse: 5′-CAG ATG CCG GTT CAG GTA CTC AGT C-3′); IL-1β (forward: 5′-CTC AAC TGT GAA ATG CCA CC-3′; reverse: 5′-GAG TGA TAC TGC CTG CCT GA-3′); TNF-α (forward: 5′-CGT CGT AGC AAA CCA CCA A-3′; reverse: 5′-GGG CAG CCT TGT CCC TTG A-3′); IL-6 (forward: 5′-TGT ATG AAC AAC GAT GAT GCA C-3′; reverse: 5′-CTG GCT TTG TCT TTC TTG TT-3′); Nrf2 (forward: 5′-AGC CCC ATT CAC AAA AGA CA-3′; reverse: 5′-GAA GTC ATC AAC AGG GAG GTT A-3′); HO-1 (forward: 5′-AAG CCG AGA ATG CTG AGT TCA-3′; reverse: 5′-GCC GTG TAG ATA TGG TAC AAG GA-3′); GAPDH (forward: 5′-TCA ACA GCA ACT CCC ACT CTT CCA-3′; reverse: 5′-ACC CTG TTG CTG TAG CCG TAT TCA-3′). Each Ct value was normalized with GAPDH to determine relative expression, and experiments were carried out in triplicate. The data were analyzed with the 2-ΔΔCT method.

2.7. Western blotting

Using RIPA lysis buffer and Nuclear Extraction Reagents (Thermo Scientific, CA, USA), we extracted total and nuclear proteins from mice atrial tissues by following the kit's instructions. A BCA protein assay kit (Beyotime Biotech) was used to measure the protein concentration. Then protein samples (50 μg) were loaded for electrophoresis on 10% SDS-PAGE, and the protein was transferred to a PVDF membrane (Millipore, Bedford, MA). Then, the membrane was blocked with 5% skimmed milk in a TBS solution. The membrane was incubated overnight at 4 °C against SIRT1 (1:400, sc-74465, mouse monoclonal, Santa Cruz), Nrf2 (1:500, ab92946, rabbit
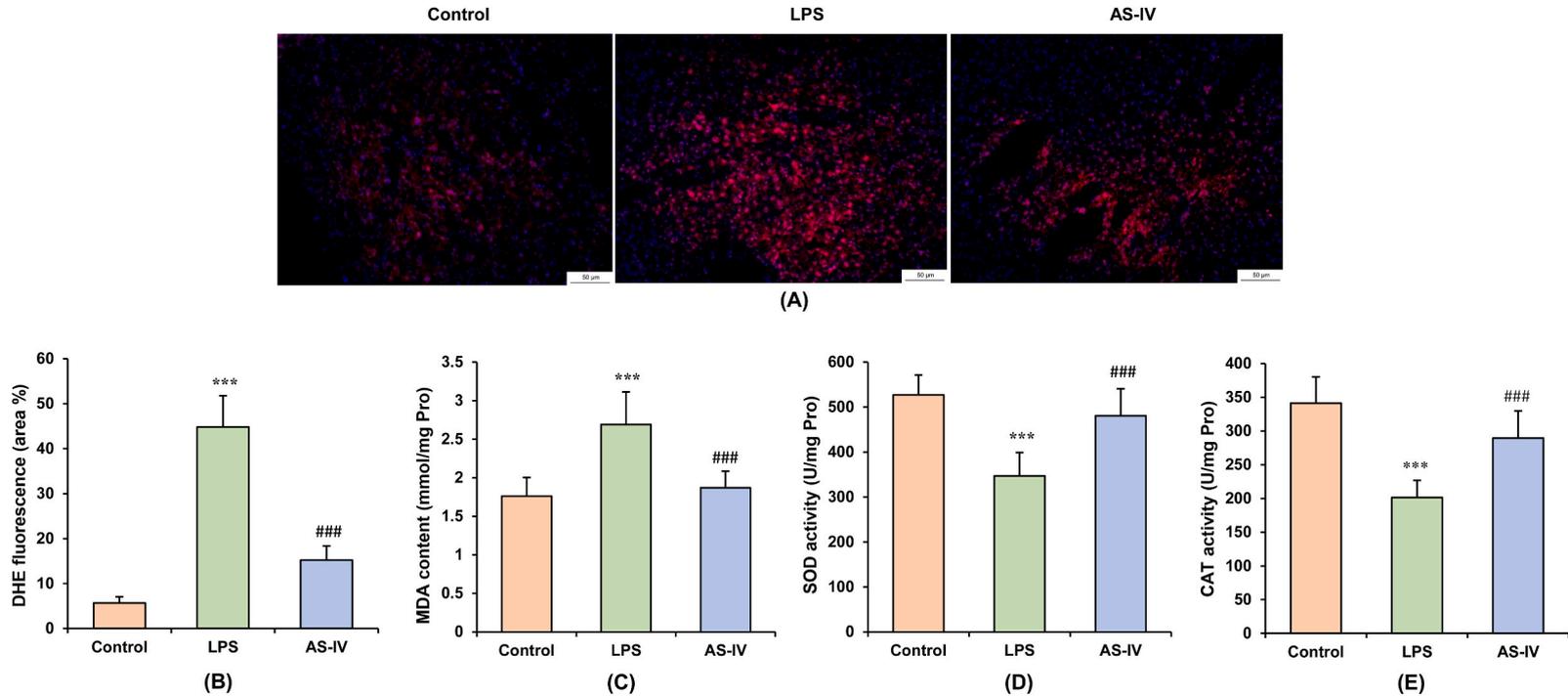
Fig. 3. AS-IV reduces ROS injury in sepsis-induced liver injury. **A** DHE fluorescence staining of the liver (scale bars: 50 μm). **B** The quantification of fluorescence intensity. The levels of **C** malon-dialdehyde (MDA), **D** superoxide dismutase (SOD), and © catalase (CAT) were measured in liver tissue (normalized to protein). Data are presented as mean ± SD. N = 8 mice per group. ***P < 0.001 vs. control group; ###P < 0.001 vs. LPS group.

(A)

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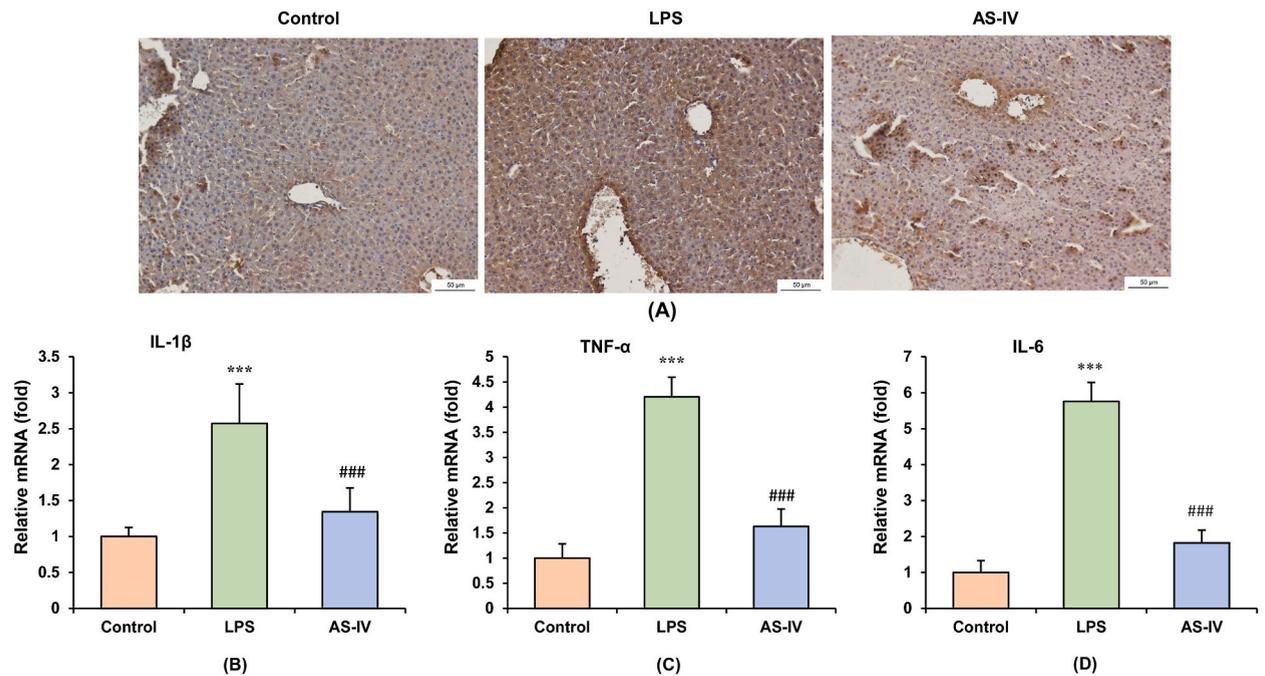
Fig. 4. AS-IV suppresses inflammation in sepsis-induced liver injury. **A** Representative image of IL-1β by immunohistochemistry in liver tissue. RT-qPCR was performed to determine the mRNA expression of three proinflammatory cytokines in liver tissue, **B** IL-1β, **C** TNF-α, and **D** IL-6. ***P < 0.001 vs. control group; ###P < 0.001 vs. LPS group.

polyclonal, Abcam), TXNIP (1:500, ab188865, rabbit monoclonal, Abcam), NLRP3 (1:400, ab214185, rabbit polyclonal, Abcam), ASC (1:500, sc-514414, mouse monoclonal, Santa Cruz), Cleaved caspase-1 (1:400, #89332, rabbit monoclonal, Cell Signaling Technology), GAPDH (1:1000, ab9485, rabbit polyclonal, Abcam), and PNCA (1000, ab18197, rabbit polyclonal, Abcam). HRP-conjugated secondary antibodies (1:2000) were used to detect the immune reactivity of these target proteins. The protein band was visualized by ECL (Thermo, Waltham, MA, USA), and band density was determined using Image Software (Bio-Rad, Hercules, CA, USA).

2.8. Statistical analysis

Data are expressed as mean ± SD and analyzed by SPSS20.0 statistical software. One-way ANOVA was used to compare the difference between three or more groups, followed by Student-Newman-Keuls tests. P < 0.05 was considered for a significant criterion for the difference.

3. Results

3.1. AS-IV protected mice from sepsis-induced liver injury

Sepsis is a systemic inflammatory response to injury displayed by the immune system. To explore the effects of AS-IV on liver injury caused by sepsis, we performed a liver histological analysis. We measured two serum biochemical markers of liver function, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). AS-IV ameliorated pathological changes in liver tissue, including inflammatory cell infiltration, necrosis, and degeneration (Fig. 1A). Similarly, the liver injury score of AS-IV-treated mice was markedly decreased compared with that of the LPS-only mice (Fig. 1B). As a result of LPS-induced liver injury, ALT and AST levels in the serum were significantly elevated compared to control mice. We found that AS-IV reduced the levels of ALT and AST in LPS-induced liver injury (Fig. 1C and D).

3.2. AS-IV suppressed LPS-induced apoptosis in liver tissue

To investigate whether AS-IV-inhibited liver injury is associated with apoptosis, we applied TUNEL staining to the liver tissue of mice. Compared to the LPS group, the AS-IV group showed markedly reduced green fluorescence (TUNEL+) in the liver tissue of mice (Fig. 2A). Quantification analysis showed that the AS-IV significantly reduced the percentage of TUNEL-positive cells in the liver tissue of LPS-treated mice (Fig. 2B). Furthermore, RT-qPCR was carried out to determine two apoptosis-related genes. The mRNA level of BAX was downregulated, and that of BCL-2 was upregulated after AS-IV administration compared with mice with LPS alone (Fig. 2C and D).
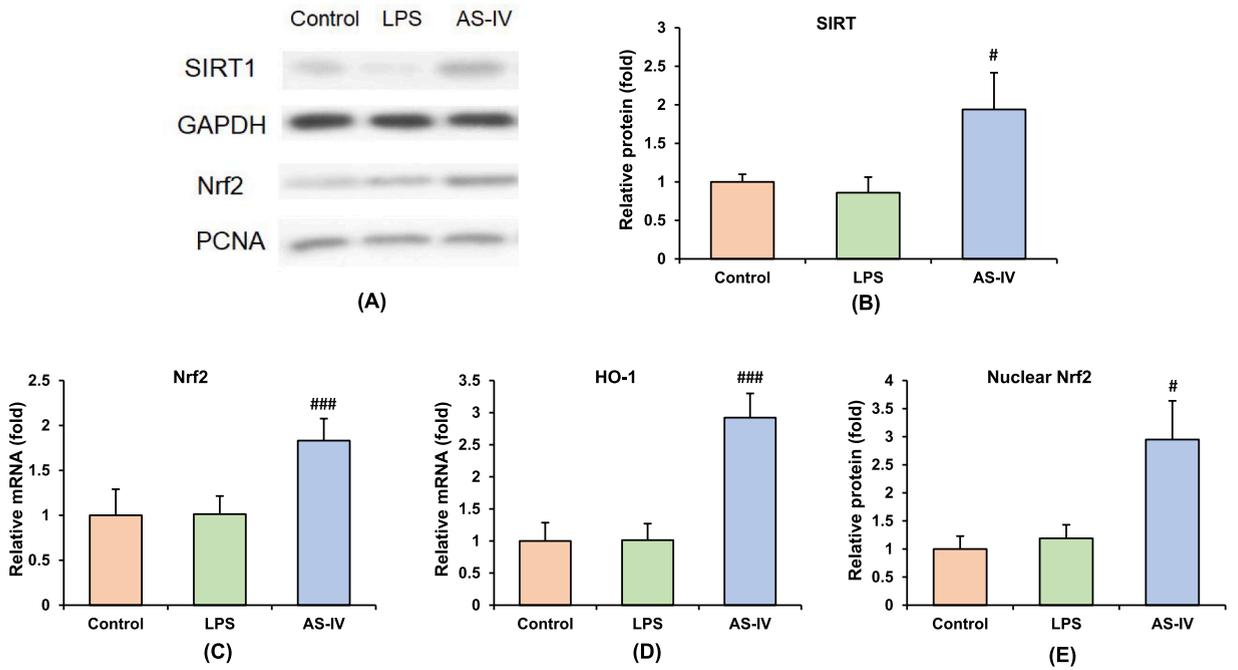
Fig. 5. Effect of AS-IV on the SIRT1/Nrf2 pathway in sepsis-induced liver injury. **A** Representative gel blots by Western blot. Quantification of optical density value for **B** SIRT1 (normalized to GAPDH), **C, D** mRNA expression of Nrf2, its downstream gene HO-1 are normalized to GAPDH, and **E** nuclear Nrf2 normalized to PCNA. **P < 0.01 vs. control group; ###P < 0.001 vs. LPS group.
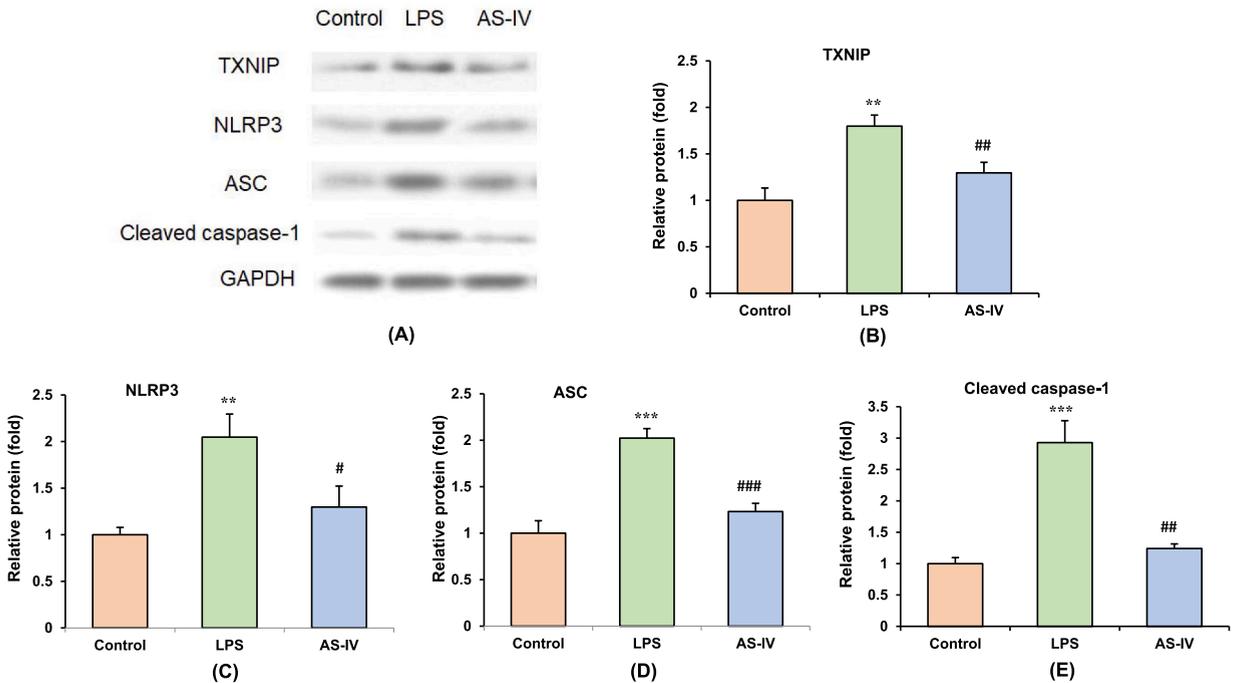
Fig. 6. Effect of AS-IV on pyroptosis activity in LPS-treat mice. **A** Representative gel blots by Western blot for pyroptosis-related proteins. Quantification of optical density value was performed for **B** TXNIP, **C** NLRP3, **D** ASC, and **E** Cleaved caspase-1. GAPDH was used as a loading control. ***P < 0.001 vs. control group; ###P < 0.001 vs. LPS group.

3.3. AS-IV reduced ROS injury in sepsis-induced liver injury

To clarify the inhibitory effect of AS-IV on ROS production and oxidative stress, we used DHE staining to measure ROS levels in liver tissues after LPS administration. The DHE fluorescence intensity was significantly increased after LPS. AS-IV significantly attenuated this effect (Fig. 3A and B). Moreover, the AS-IV group showed a more significant decrease in malondialdehyde (MDA) content than the LPS group (Fig. 3C). The superoxide dismutase (SOD) and catalase (CAT) levels were significantly increased in the AS-IV group compared to the LPS group (Fig. 3D and E).

3.4. AS-IV inhibits inflammation in sepsis-induced liver injury

The effects of AS-IV on inflammation were evaluated in liver tissue of mice. Immunohistochemistry was carried out and showed AS-IV significantly attenuated the IL-1β staining intensity cells in mice liver tissue (Fig. 4A). RT-qPCR revealed that the mRNA expression of IL-1β, TNF-α, and IL-6 was significantly increased after LPS injection, but their mRNA levels were reduced considerably by AS-IV administration (Fig. 4B, C, 4D).

3.5. AS-IV activated the SIRT1/Nrf2 pathway in sepsis-induced liver injury

Western blot was performed to analyze the protein expression of SIRT1 and Nrf2 in liver tissue of mice (Fig. 5A and Fig. S1). LPS decreased SIRT1 expression in the liver tissue. In addition, AS-IV recovered the reduced protein levels of SIRT1 and other enhanced SIRT1 expressions (Fig. 5B). The transcription of Nrf2 and its target gene HO-1 were increased in AS-IV-treated liver tissues compared to tissue with LPS alone (Fig. 5C and D). Then the Nrf2 protein was determined in the extracted nuclear protein of liver tissue and revealed that AS-IV markedly increased protein expression of nuclear Nrf2 (normalized to PCNA) (Fig. 5E).

3.6. AS-IV suppressed LPS-promoted NLRP3 inflammasome

We performed Western blot to measure protein levels of NLRP3 and its related proteins (Fig. 6A and Fig. S2). LPS activated NLRP3 inflammasome, as evidenced by increased protein expression of TXNIP, NLRP3, ASC, and cleaved caspase-1, compared with control cells. AS-IV cotreatment reversed LPS-stimulated increased expression of these proteins (Fig. 6B, C, 6D, 6E).

4. Discussion

AS-IV is one of the most important bioactive components of Astragali Radix, which is commonly used in China to boost immunity and cure diabetes. Growing research evidence suggests that AS-IV is useful in treating organ fibrosis, oxidative damage, inflammatory injury, apoptosis, and immunoregulation [18]. In the current study, we observed that AS-IV pre-treatment (80 mg/kg) markedly attenuated LPS-induced liver injury, as evidenced by the decreased levels of ALT and AST (Fig. 1C and D), and the histopathological abnormalities were reduced (Fig. 1A). According to the growing number of research, herbal medications generated from plant extracts are increasingly being used to treat liver damage [19]. Plant extracts help alleviate inflammatory responses in hepatocytes through a number of intracellular signaling pathways. However, the anti-oxidative capabilities of numerous natural products, such as andrographolide, chlorogenic acid, and quercetin, have been found to prevent drug-induced liver injury (DILI) [20–22]. Similarly, AS-IV also shows the reduction of oxidative stress. Our data demonstrated that AS-IV potentially increased the superoxide dismutase (SOD) and catalase (CAT) levels and reduced the MDA level induced by LPS (Fig. 3), indicating that AS-IV might be attenuated LPS-induced liver injury by decreasing the oxidative liver damage.

Septic shock is caused by the multiorgan dysfunction caused by LPS, which is a primary component of Gram-negative bacteria's outer wall and a crucial modulator of the body's response to infection [23]. Endotoxic shock is a dangerous symptom of sepsis and may be caused by injecting LPS into animals. As a result, the LPS-induced endotoxic model is frequently employed in the research for medications and therapy strategies for sepsis caused by Gram-negative bacterial infections [24]. Through the cell signaling system in the body, mononuclear macrophages and endothelial cells can be activated, stimulating them to synthesize and produce a range of inflammatory cytokines [25], which then induce a number of responses in the body. IL-6 and TNF-α are pro-inflammatory cytokines that have a role in developing and modulating the inflammatory response [26]. It has been reported that macrophages exposed to LPS generate a considerable amount of TNF-α and IL-6 [27]. In this study, IL-1β, TNF-α, and IL-6 levels in serum were considerably higher in mice treated with LPS. However, AS-IV significantly attenuated the production of IL-1β, TNF- a, and IL-6 levels in serum (Fig. 4), speculating that its protecting activity on endotoxic might be associated with its anti-inflammatory effects.

Nrf2 is an essential transcriptional factor that mediates the expression and regulation of anti-oxidative proteins and detoxifying enzymes by regulating multiple genes [28]. The cytoplasmic Nrf2 is separated from the Kelch-like ECH-associated protein-1 (Keap-1). At the same time, the cells are subjected to high levels of oxidative stress and moved to the nucleus, stimulating several gene expressions associated with the antioxidant response element (ARE) [29]. HO-1 is an antioxidant enzyme that is a downstream target of Nrf2. A recent study reported that epicatechin therapy attenuated hypertension and proteinuria in a deoxycorticosterone acetate salt-associated preeclampsia (PE) mice model via activating the Nrf2 pathway [30]. Our study observed that AS-IV significantly affects sepsis-induced liver injury by activating the SIRT1/Nrf2 pathway. AS-IV potentially increased SIRT1 and nuclear Nrf2 protein expression and also considerably enhanced the transcription of Nrf2 and its target gene, HO-1 (Fig. 5).

The NLRP3 protein, caspase-1, and apoptosis-associated speck-like proteins containing a CARD (ASC) make up the nucleotide-
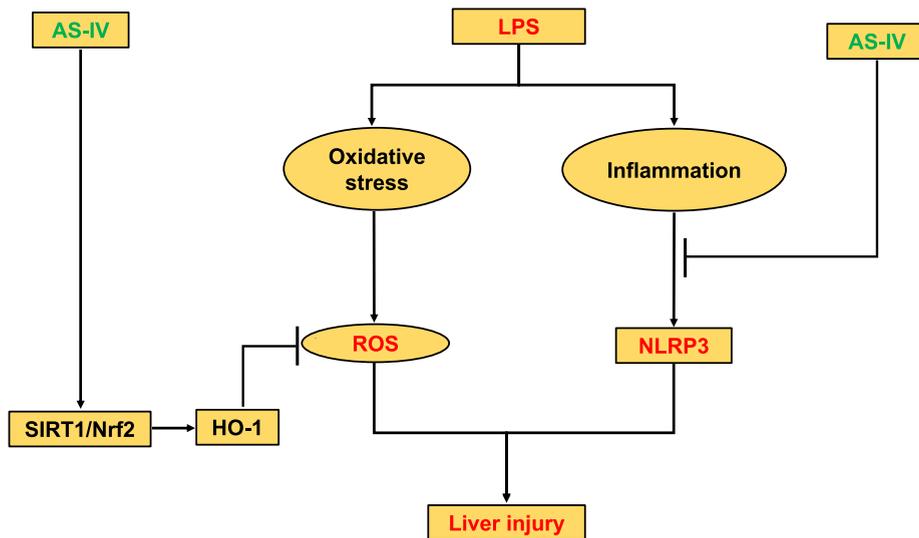
Fig. 7. Schematic illustration of the protective effects of LPS-induced inflammation, oxidative stress, apoptosis, and liver damage are due to AS-IV-mediated control of Nrf2-mediated oxidative stress and NLRP3-mediated inflammation.

binding domain (NOD)-like receptor protein 3 (NLRP3) inflammasome, which is crucial in the emergence of various inflammatory diseases, including liver injury [31]. Pro-inflammatory mediators like LPS can be stimulated the NLRP3 inflammasome, which increases the production of cytokines [32]. The importance of the NLRP3 inflammasome in the emergence of a number of liver diseases, including NAFLD, liver fibrosis, and acute liver damage, has been demonstrated by accumulating evidence [33]. In order to effectively treat various types of liver diseases, including liver injury, targeting the NLRP3 inflammasome has been investigated [34]. However, our study results demonstrated that TXNIP, NLRP3, ASC, and cleaved caspase-1 protein expression were higher in LPS-treated cells than in control cells, indicating that LPS activated the NLRP3 inflammasome. LPS-induced upregulation of these proteins' expression was reversed by AS-IV treatment (Fig. 6).

5. Conclusion

In this study, we explored the protective effects of AS-IV on LPS-induced liver injury. Our data demonstrate that its high anti-oxidative and anti-inflammatory effects through activating the SIRT1/Nrf2 pathway induced the hepato-protective impact of AS-IV (Fig. 7). Accumulating evidence speculates that AS-IV may be a potential therapeutic candidate for treating the liver injury. However, the comprising data of this study is not verified in the preclinical/clinical contexts. Therefore, these results need to be further evaluated in preclinical and clinical settings in the future.

Data availability statement

Due to confidential issues, the datasets generated and/or analyzed during the current work are not publicly available but are available from the corresponding author upon reasonable request.

Declaration of interest statement

The authors declared no conflict of interest with other people or organizations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15436.

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