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Inactivation of Kupffer Cells by Selenizing Astragalus Polysaccharides Prevents CCl_4-Induced Hepatocellular Necrosis in the Male Wistar Rat

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Abstract Selenizing astragalus polysaccharides-3 ($sAPS_3$) was prepared by nitric acid–sodium selenite method. The effects of $sAPS_3$ on carbon tetrachloride (CCl_4) induced hepatocellular necrosis, and its underlying mechanisms were studied in male Wistar rats. Hepatic damage was induced by intraperitoneal injection of CCl_4 twice a week, for 3 weeks. Meanwhile, the rats in addition to CCl_4 were also exposed to sodium selenite (SS), astragalus polysaccharides (APS), SS + APS or $sAPS_3$, in parallel by oral gavage once a day for 3 weeks. At the end of 3 weeks, blood and liver tissue were taken. Serum was collected to test the levels of alanine aminotransferase, aspartate aminotransferase and antioxidant status parameters. Liver tissue was collected for histopathological examination and determination of messenger RNA (mRNA) expression levels of CD68, TNF-α, IL-1β and ATG7 followed by the measurements of CD68, IL-1β and LC3II by immunohistochemistry assay (IHC), or TNF-α by immunofluorescence assay (IFA). The results showed that $sAPS_3$ effectively ameliorated CCl_4 induced hepatocellular necrosis and inflammation and significantly decreased the levels of aspartate aminotransferase, alanine aminotransferase, malondialdehyde and the expression levels of Kupffer cells (KCs)-specific biomarker CD68 and proinflammatory cytokines produced by activated KCs such as IL-1β and TNF-α ($P < 0.01$). While increasing the levels of total antioxidant capacity, glutathione, glutathione peroxidase and superoxide dismutase ($P < 0.05$) and reduced the expression levels of a key regulator of autophagy in KCs ATG7 or LC3II ($P < 0.05$). These findings indicate that $sAPS_3$ could ameliorate CCl_4-induced hepatocellular necrosis by inactivation of Kupffer cells and its activity may be superior to the application of selenium, APS or combination of selenium with APS.

Keywords CCl_4 · Hepatocellular necrosis · Inflammation · Selenizing astragalus polysaccharides · Rats

Introduction

The liver is considered to be the main organ responsible for the metabolism of all toxic chemicals and drugs; therefore, it represents the primary target organ for nearly all toxic chemicals [1]. Kupffer cells (KCs) are considered as a population of liver resident macrophages, including nearly 90% of the tissue macrophages. KCs remain in the lumen of the liver sinusoids, for phagocytosis of larger particulates and foreign materials [2]. Multiple studies indicate that KCs contribute to the pathogenesis of different liver injuries and diseases including its pathogenetic role in carbon tetrachloride-induced hepatocellular necrosis and fibrosis [3, 4].They also play critical roles in establishing, maintenance and outcome of inflammation by releasing a large amount of inflammatory cytokines and chemokines after its activation. Carbon tetrachloride (CCl_4) intoxication is a common animal model for detecting the mechanism of liver damage as well as the hepatoprotective activity of synthetic and natural compounds [5]. The administration of CCl_4 causes a serious degree of necrosis and inflammation which was owing to more KCs infiltration prominently [6].

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Selenium (Se) is a vital trace element that plays a major role in various physiologic processes including antioxidant system regulation in the body and amelioration of liver injuries [7, 8]. Herbal Chinese medicine was reported to be able to enhance the body immune function, protect the liver, improve blood circulation in the liver, regulate liver function and repair hepatocyte damages effectively [9]. The astragalus polysaccharides, derived from the roots of the *Astragalus membranaceous*, which is demonstrated to have many pharmacological applications, including anti-inflammatory, immunomodulatory, anticancer and antioxidant effects in addition to exhibiting hepatoprotective and antioxidant properties acting as a synergistic effect in CCl_4-induced liver injury in mice [9–11].

Selenium–polysaccharide combination possesses higher and duple biological activities as compared with selenium or polysaccharide, and it is easily absorbed by the body [12, 13]. Selenium polysaccharides were used widely in immunomodulation, antitumor, antioxidation, antiageing and so on [14].

In our laboratory, we prepared selenizing astragalus polysaccharide (sAPS) that has combined properties of astragalus polysaccharides and selenium to ameliorate CCl_4-induced liver injury in rats. The present study aimed to verify if administration of $sAPS_3$ reduced the hepatocellular damage in the rats to a lesser extent, and also to underline the possible mechanisms.

Materials and Methods

Chemicals

Astragalus polysaccharides powder extract with purity 95% was obtained from Pharmagenesis Inc. Sodium selenite and nitric acid (HNO_3) were provided from Shanghai Lingfeng Chemical Reagent Ltd. (China). Kits for glutathione (GSH), total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), SOD, MDA, total protein and CCl_4 were provided from the Institute of Jiancheng Biotechnology (Nanjing, China). TNF-α, CD68, IL1-β and microtubule-associated protein I1 light chain 3 (LC3II) monoclonal antibody were purchased from Abcam (Cambridge, UK).

Preparation of sAPS

Selenizing astragalus polysaccharides (sAPS) were prepared by nitric acid–sodium selenite method [14, 15].We prepared three selenizing molecules ($sAPS_1$, $sAPS_2$ and $sAPS_3$) according to amount of sodium selenite (200, 300, 400 mg sodium selenite) for the whole reaction condition 500 mg of APS in 70 °C for 10 h [16]. The selenium contents of these selenizing molecules were 1.76, 1.89 and 2.21 mg/g, respectively, detected by atomic fluorescence spectrometry method [17, 18] and carbohydrate contents were 47.92, 50.9 and 62.21%, respectively, determined by the phenol–sulphuric acid method. APS and $sAPS_3$ were diluted into 40 mg/mL, and the endotoxins were detected by pyrogen tests (less than 0.5 EU mL^{-1}) according to (Veterinary Pharmacopoeia Commission of the People's Republic of China) [14].

Dose-Response Experiment for Selenizing Astragalus Polysaccharides

The dose of 0.4 mg/kg for sodium selenite and 200 mg/kg for astragalus polysaccharides were used previously [19, 20] as an effective dose of both sodium selenite and astragalus polysaccharides. In the current study, a preliminary experiment with serum markers enzymes, AST and ALT as liver damage markers and GSH was carried out with three selenizing molecules ($sAPS_1$, $sAPS_2$ and $sAPS_3$) for the selection of the maximum effective dose of sAPS in CCl_4-treated rats. A total of 42 male Wistar rats weighing 190–210 g were obtained from the Center for Laboratory Animals, Yangzhou University (Yangzhou, China). Rats were divided into seven groups (six animals each) as follows: group 1 (control), group 2 (CCl_4), group 3 (CCl_4 + APS), group 4 (CCl_4 + sodium selenite (SS)), group 5 (CCl_4 + APS_1), group 6 (CCl_4 + $sAPS_{2)}$ and group 7 (CCl_4 + $sAPS_3$). Group 1 received an only intraperitoneal injection of olive oil 2 mL/kg BW twice a week for 3 weeks. Groups 2–5 received an intraperitoneal injection with CCl_4 2 mL/kg BW, 1:1 in olive oil twice a week for 3 weeks [7]. Each rat in treated groups was administered orally with 1 mL containing 40 mg/day of APS [20], 80 μg/day of SS [19], 40 mg/day of APS_1, 40 mg/day of $sAPS_2$ and 40 mg/day of $sAPS_3$ along with CCl_4. Blood samples and liver tissues were collected by the end of the experiment and immediately stored at −70 °C.

Induction of Hepatocellular Necrosis and Study Design with the Selected Dose of sAPS

This experiment was approved by the Animal Care and Use Committee of Nanjing Agricultural University, (Certification No.: SYXK (Su) 2011-0036). A total of 48 male Wistar rats weighing 190–210 g were obtained from the Center for Laboratory Animals, Yangzhou University (Yangzhou, China). Rats were maintained under a controlled environmental condition at 25 ± 2 °C, with a normal day/night cycle and fed with normal basal diet and water ad libitum. Animals were divided into six groups (eight animals each) as follows: group 1 (control), group 2 (CCl_4), group 3 (CCl_4 + APS), group 4 (CCl_4 + SS), group 5 (CCl_4 + SS + APS) and group 6 (CCl_4 + $sAPS_3$). Group 1 received an only intraperitoneal injection of olive oil 2 mL/kg BW twice a week for 3 weeks. Groups 2–5 received an intraperitoneal injection with CCl_4 2 mL/kg BW,

1:1 in Olive oil twice a week for 3 weeks. Each rat in treated groups was administered orally with 1 mL containing 40 mg/day of APS, 80 μg/day of SS, 40 mg/day of APS + 80 μg/day of SS or 40 mg/day of $sAPS_3$ along with CCl_4 (The most effective dose from the preliminary study). Blood and liver samples were collected by the end of the experiment and immediately stored at −70 °C.

Determination of Serum Enzymes

ALT and AST were assayed in serum using an automated chemistry analyser. (BS-300, Mindray Medical International Limited).

Determination of Serum T-AOC, GSH-Px, SOD and MDA

T-AOC, GSH-Px, SOD and MDA were assayed in serum using an AF-610A atomic fluorescence spectrometer, according to the instructions of the commercial assay kits.

GSH Activity Assay of Liver Homogenate

A hundred milligrammes of frozen liver tissue in 1 mL of homogenised ice-cold buffer (1 mmol/L EDTA, 0.32 mol/L sucrose and 10 nmol/L Tris-HCl, pH = 7.4) was homogenised on ice with a Polytron-aggregate homogeniser at 12000 rpm. The homogenate was centrifuged at 2500 rpm for 10 min at 4 °C. The activity of GSH was measured from the supernatant using a commercial assay kit, according to the instructions.

Histopathology Examination

Liver tissues were immediately fixed in 10% neutral buffered formalin, processed for paraffin embedding. Haematoxylin–eosin (H&E) was performed using standard procedures [21].

Immunohistochemistry Assay

Liver sections were fixed in 10% neutral-buffered formalin, processed for paraffin embedding and were incubated with mouse monoclonal antibody against CD68, IL-1β and LC3II then incubated with the second antibody peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) then incubated with the streptavidin–peroxidase complex, then subsequently visualised using diaminobenzidine (DAB) solution. Finally, hepatic sections were counterstained with haematoxylin and then mounted on a coverslip.

Immunofluorescence Assay

Liver tissues were immediately fixed in 10% neutral-buffered formalin and processed for paraffin embedding. Liver tissues were then incubated with a specific mouse monoclonal antibody against rat TNF-α diluted at 1:50 with 1% BSA/PBS, followed by incubation with FITC-conjugated rabbit anti-mouse IgG (1:50 dilution) in 1% BSA/PBS. A fluorescent microscope was used to take images.

RNA Extraction and Quantitative Real-Time PCR Assay

The relative messenger RNA (mRNA) expression of TNF-α, IL-β1, CD68, and ATG7 were determined by quantitative real-time PCR assay (qRT-PCR). The primers were designed by online Primer-Blast of NCBI as shown in Table 1. The extraction of RNA and synthesis of complementary DNA (cDNA) were performed as previously reported [22]. Briefly, RNA was extracted from the liver tissue with TRIzol (Invitrogen), according to the instructions. Reverse transcription (RT) was performed to synthesise cDNA with PrimeScript RT Master Mix Perfect Real Time (Takara Co., Otsu, Japan), according to the instructions. The qRT-PCR was carried out on an ABI Prism 7300 Detection System (Applied Biosystems, USA). All cDNA samples were amplified using SYBR Green (Takara Co., Otsu, Japan). The relative levels of gene expression were determined by the $\Delta\Delta^{CT}$ method; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. All reactions were performed in triplicates.

Statistical Analysis

The experimental data were analysed statistically using SPSS 18.0 for Windows. Experimental results were presented as mean ± S.E.M. One-way ANOVA was used to examine the statistical significance. The differences between groups were determined by Duncan's contrasts; $P < 0.01$ was considered to be highly significant difference between the groups, while $P < 0.05$ was considered to be significant between the groups.

Results

Preliminary Study for Selection of the Maximum Effective Dose of sAPS

As indicated by serum ALT, AST and activity of GSH from the liver homogenate (Table 2). All treated groups had significant ($P < 0.05$) decrease in the serum activities of ALT and AST or GSH activity compared with the CCl_4-treated group. $sAPS_3$ showed the best reduction of ALT, AST or GSH compared with other treatments. Accordingly, we selected $sAPS_3$ as the most appropriate dose to complete the main study.

Table 1 Primer pairs used for real-time PCR

Target genes	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
CD68	ATGGTTCCCAGCCATGTGTT	TTTCCACCCTGGGTCAGGTA
TNF-α	CGTCAGCCGATTTGCCATTT	TCCCTCAGGGGTGTCCTTAG
IL-1β	GCACAGTTCCCCAACTGGTA	AAGACACGGGTTCCATGGTG
ATG7	GAGAGCCGATGGCTTCCTAC	CCTGGAGCCACCACATCATT
GAPDH	AAGGTCGGTGTGAACGGATT	CTTTGTCACAAGAGAAGGCAGC

Determination of Serum Enzymes

Assays of ALT and AST in animal serum were given in (Fig. 1a, b). The CCl_4-treated group significantly ($P < 0.01$) had elevated serum activities of ALT compared with the control group. The serum activities of ALT in rats treated with APS, SS, SS + APS or $sAPS_3$ significantly decreased ($P < 0.05$) compared with the CCl_4-treated group. The SS + APS and $sAPS_3$ groups showed a significant decrease ($P < 0.05$) in the serum activities of ALT compared with the APS or SS group. There is no significant difference between SS and APS groups.

The CCl_4-treated group significantly ($P < 0.01$) had elevated the serum activities of AST compared with control group. The serum activities of AST in rats treated with APS, SS, SS + APS or $sAPS_3$ significantly decreased ($P < 0.05$) compared with the CCl_4-treated group. $sAPS_3$ was more effective than APS, SS or SS + APS group ($P < 0.05$). While SS and SS + APS showed a better result compared to APS.

T-AOC, GSH-Px, SOD and MDA Levels

The T-AOC, SOD and GSH-Px activities, decreased significantly in the CCl_4-treated group compared with that of the control group ($P < 0.05$). The groups treated with APS, SS, SS + APS or $sAPS_3$ improved the T-AOC, SOD and GSH-Px activities (Fig. 2a–c) compared to the CCl_4-treated group ($P < 0.05$). There was no significant difference in the T-AOC activities between the SS + APS and $sAPS_3$ groups. However, the $sAPS_3$ group showed further increase in the SOD and GSH-Px activities, compared with the APS, SS and SS + APS supplementation ($P < 0.05$). MDA levels significantly increased in serum of the CCl_4-treated group compared to the control group ($P < 0.05$; Fig. 2d). However, APS, SS, SS + APS or $sAPS_3$ supplementation significantly lowered the MDA levels compared with the CCl_4-treated group. The SS + APS and $sAPS_3$ groups showed a better result compared to APS or SS ($P < 0.05$).

Table 2 Preliminary study for selection of the most appropriate dose of sAPS by measuring serum activity of ALT, AST and levels of GSH

Groups	ALT (U/mL)	AST (U/mL)	GSH mmol/g prot
Control	40.7a ± 3	82.9d ± 1	47a ± 3
CCl4	170.2 a ± 3	264.3a ± 1	18.3d ± 2
CCl4 + APS	120.8b ± 2	175.7b ± 9	24c ± 3
CCl4 + SS	104.7c ± 2	130.6c ± 5	29.3c ± 3
CCl4 + $sAPS_1$	91.2d ± 3	117.1c ± 6	30.7c ± 2
CCl4 + $sAPS_2$	69.1e ± 2	96.6d ± 4	31.3c ± 2
CCl4 + $sAPS_3$	57.3f ± 1	85.7d ± 2	39b ± 1

Data are represented as mean ± SEM ($n = 6$). Different letters beside the values within each test parameter indicate significant differences between groups ($P < 0.05$)

Effects of APS, SS, SS + APS or $sAPS_3$ on GSH Levels in Liver Tissues

The level of hepatic GSH was an important indicator of liver oxidative damage. The activities of GSH were showed in (Fig. 1c). CCl_4-treated group significantly had reduced ($P < 0.05$) antioxidant activities of GSH compared with the control group. While the levels of GSH were highly increased in the APS, SS, SS + APS or $sAPS_3$ group compared with CCl_4-treated group ($P < 0.05$).

Histopathological Examination

The histopathological sections of the liver of control and treated rats were showed in (Fig. 3).The liver histopathological appearance of the CCl_4-treated group showed massive necrosis, hepatocyte ballooning, and inflammation associated with hepatotoxicity (Fig. 3b). In the control group, no significant inflammation and necrosis are observed (Fig. 3a). APS and SS groups revealed moderate necrosis, inflammation and hepatocyte ballooning (Fig. 3c, d). SS + APS group revealed mild necrosis and inflammation (Fig. 3e). While $sAPS_3$ administration ameliorated the hepatic necrosis and inflammation induced by CCl_4 (Fig. 3f).

Effects of $sAPS_3$ on the Expression of KCs Specific Biomarker CD68

To clarify the effect of $sAPS_3$ on the activation degree of KCs, we measured specific biomarker of KCs CD68 (Fig. 4a).The mRNA expression levels of CD68 in CCl_4-

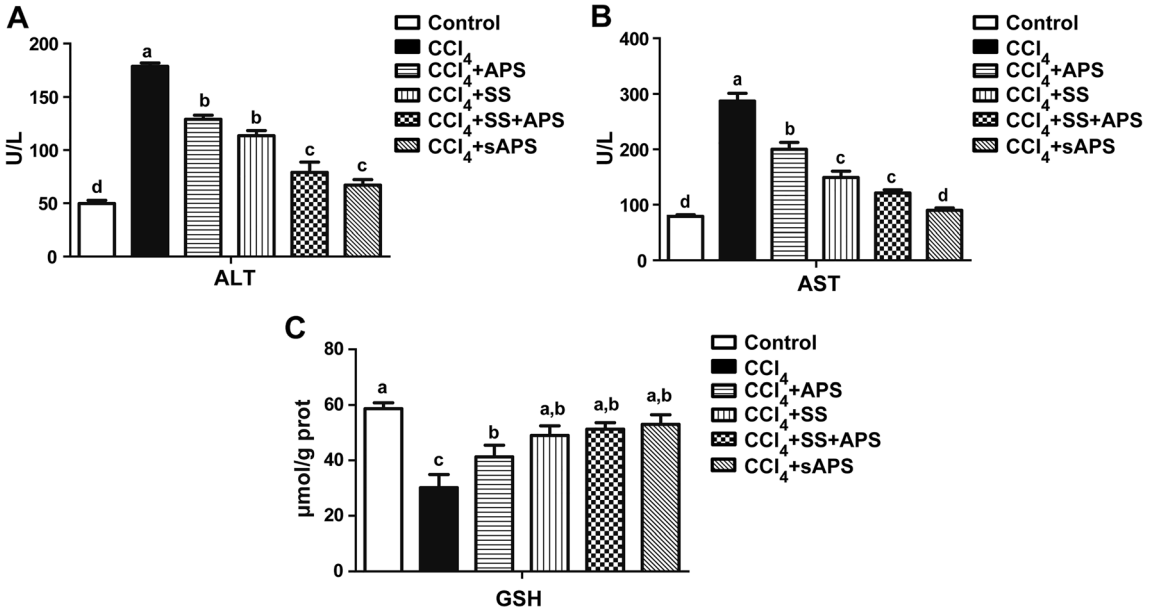

Fig. 1 Effects of APS, SS, SS + APS, and $sAPS_3$ on liver marker enzymes in the serum and glutathione (GSH) levels in liver tissues **a** serum alanine aminotransferase; **b** serum aspartate aminotransferase and **c** the levels of GSH. Data are represented as mean ± SEM (n = 8). *Columns with different letters* differ significantly ($P < 0.05$)

treated group was highly increased ($P < 0.01$), compared with the control group. While the mRNA expression levels of CD68 in the APS, SS, SS + APS or $sAPS_3$ group were significantly decreased compared with the CCl_4-treated group ($P < 0.01$).$sAPS_3$ was more effective than APS, SS or SS + APS group in the mRNA expression levels of this gene ($P < 0.05$). SS + APS showed a better result than APS or SS. However, there was no significant difference in the mRNA expression levels of CD68 between APS and SS groups. This result was confirmed by staining CD68 in liver tissue sections by immunohistochemistry (IHC) assay. As shown in (Fig. 5). The CD68 expression did not show in the liver tissue of the control rats (Fig. 5a), while CCl_4-treated group showed extensive expression of CD68 mainly around portal tracts and liver sinusoid (Fig. 5b). SS and APS groups showed moderate expressions of hepatic CD68 (Fig. 5c, d). SS + APS showed mild expressions of hepatic CD68 (Fig. 5e). Hepatic expression of CD68 has been completely reduced by $sAPS_3$ administration (Fig. 5f).

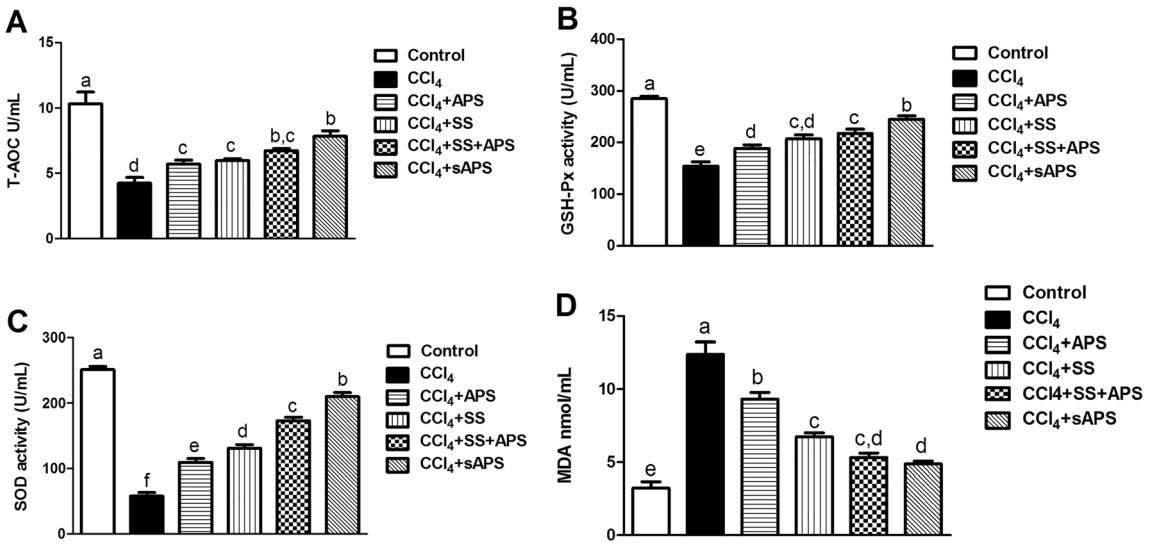

Fig. 2 Effect of APS, SS, SS + APS, and $sAPS_3$ on serum oxidative stress parameters. **a** The levels of T-AOC. **b** The activities of GSH-Px. **c** The activities of SOD. **d** the levels of MDA. Data are represented as mean ± SEM (n = 8). *Columns with different letters* differ significantly ($P < 0.05$)

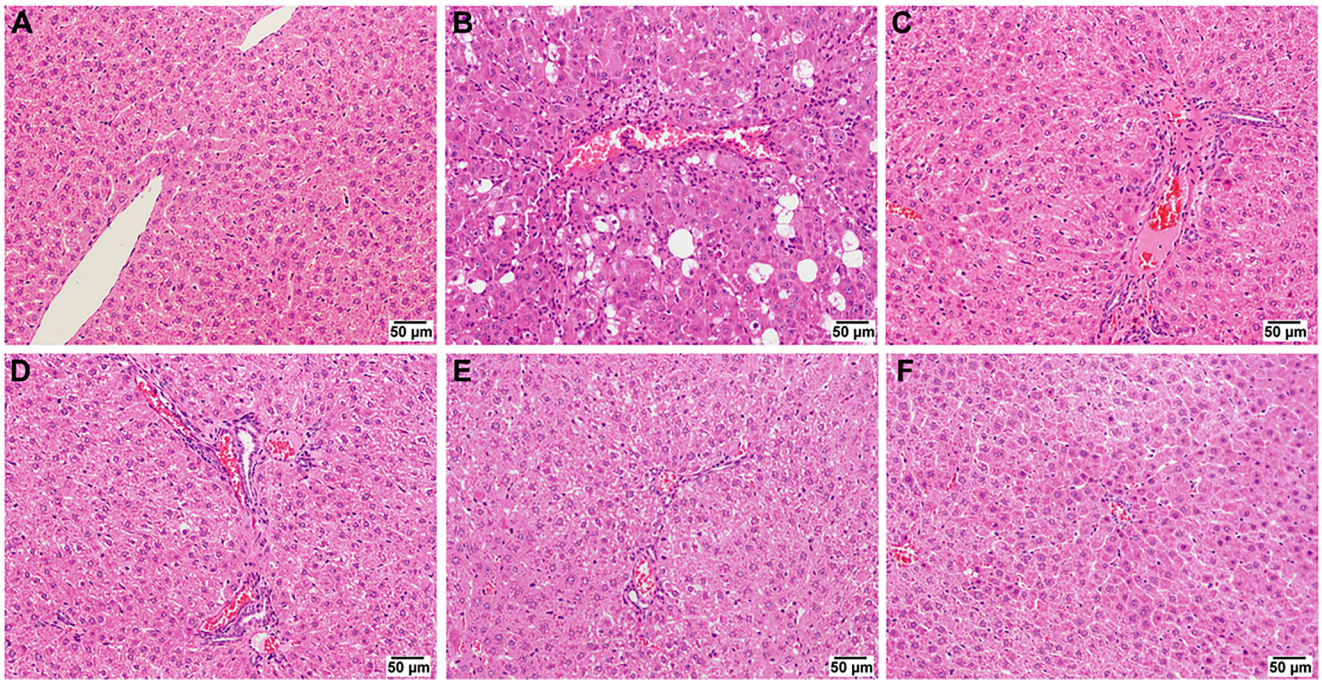

Fig. 3 Liver sections stained with H&E (*scale bar* = 50 μm). **a** Control group, **b** CCl_4 group, **c** CCl_4 + APS group, **d** CCl_4 + SS group, **e** CCl_4 + SS + APS group and **f** CCl_4 + $sAPS_3$ group

Effects of $sAPS_3$ on the Expression of Proinflammatory Cytokines Produced by Activated KCs

To assess the effect on the activation degree of KCs, we measured the mRNA expression levels of TNF-α and IL-β1 in the liver tissues (Fig. 4b, c). CCl_4-treated group had highly increased mRNA expression levels of TNF-α and IL-β1 ($P < 0.01$) compared with the control group. The groups treated with APS, SS, SS + APS or $sAPS_3$ showed reduced mRNA expression levels of TNF-α and IL-β1 compared with the CCl_4-treated group ($P < 0.05$). The $sAPS_3$ group showed a further reduction in the mRNA expression levels of IL-β1 ($P < 0.05$) compared with APS, SS or SS + APS group. While SS + APS and $sAPS_3$ more effective than APS and

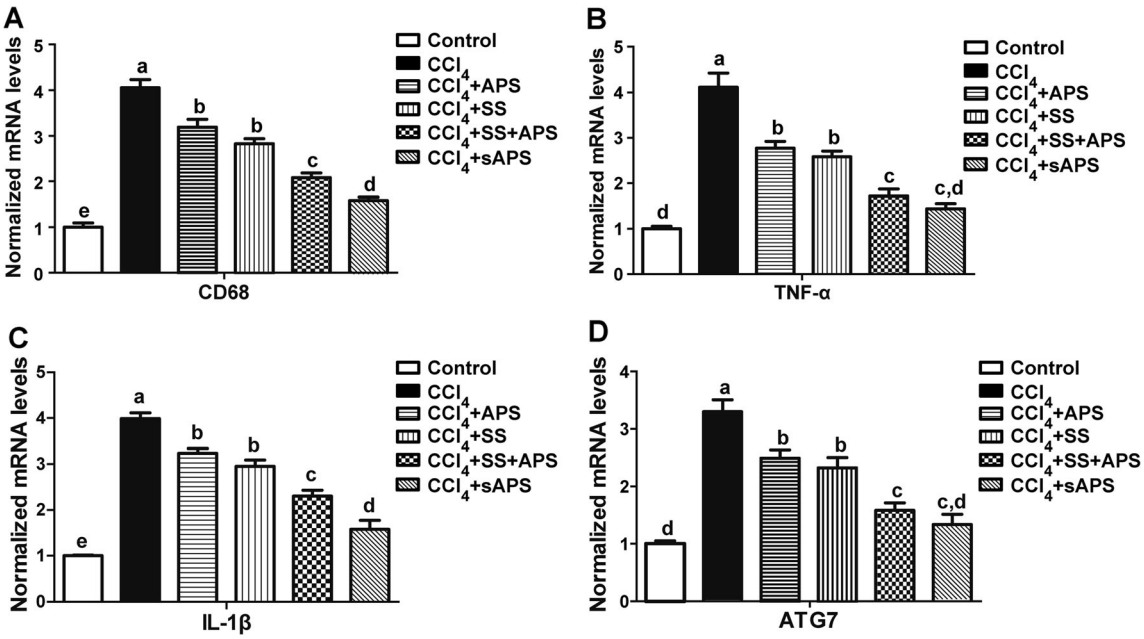

Fig. 4 Effects of APS, SS, SS + APS and $sAPS_3$ on the genes expressions. Quantitative real-time PCR (qRT-PCR) analysis detected the mRNA expression levels of CD68, TNF-α, IL-1β and ATG7 in the liver. GAPDH served as an internal control. Data are represented as mean ± SEM ($n = 8$). *Columns with different letters* differ significantly ($P < 0.05$)

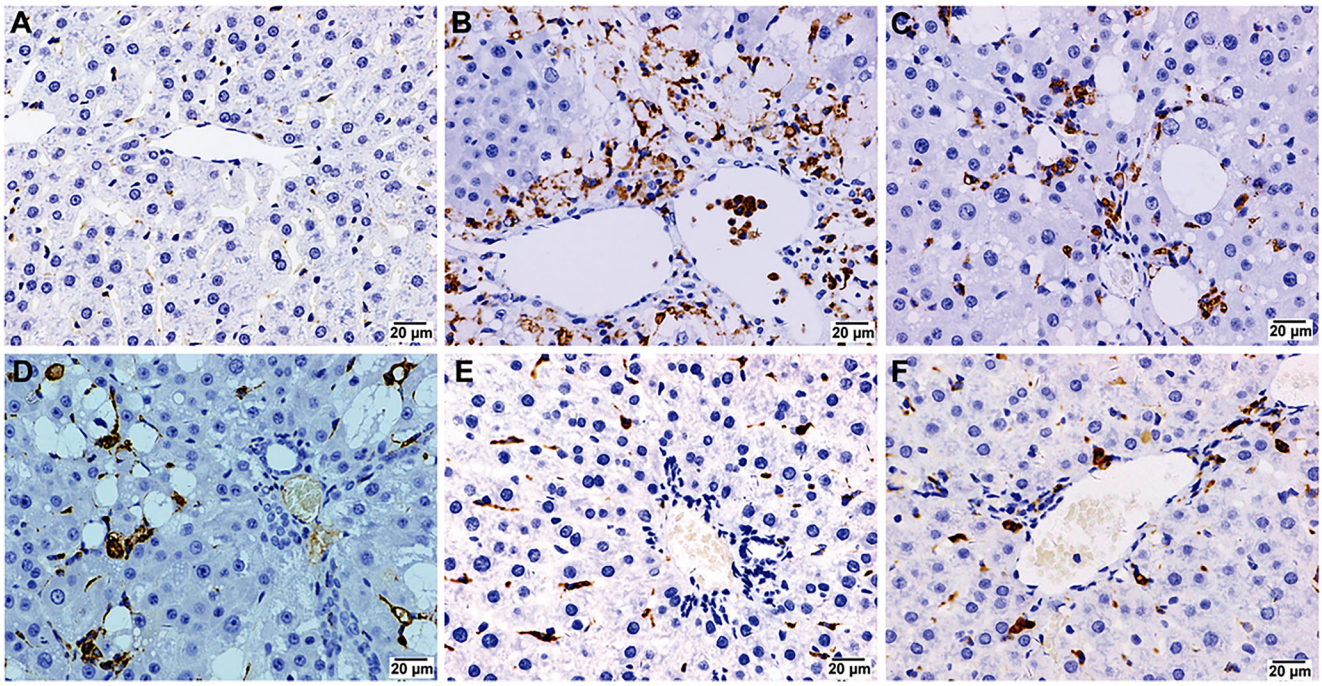

Fig. 5 Immunohistochemical staining for CD68 in liver sections (*scale bar* = 20 μm). **a** Control group, **b** CCl_4 group, **c** CCl_4 + APS group, **d** CCl_4 + SS group, **e** CCl_4 + SS + APS group and **f** CCl_4 + $sAPS_3$ group

SS in reducing mRNA expression levels of TNF-α. However, there was no significant difference in the mRNA expression levels of these genes between the SS and APS groups. This result was confirmed by staining IL-β1 in liver tissue sections by immunohistochemistry (IHC) assay. As shown in (Fig. 6), the ccl_4-treated group showed massive expressions of IL-β1 especially around degenerated hepatocytes of the liver (Fig. 6b), compared with the control group (Fig. 6a). SS, APS, and SS + APS groups showed sporadically expressions of IL-β1 (Fig. 6c–e) compared with the CCl_4-treated group, while administration of $sAPS_3$ markedly reduced the degree of IL-β1 in the liver tissue (Fig. 6f). We also investigate the

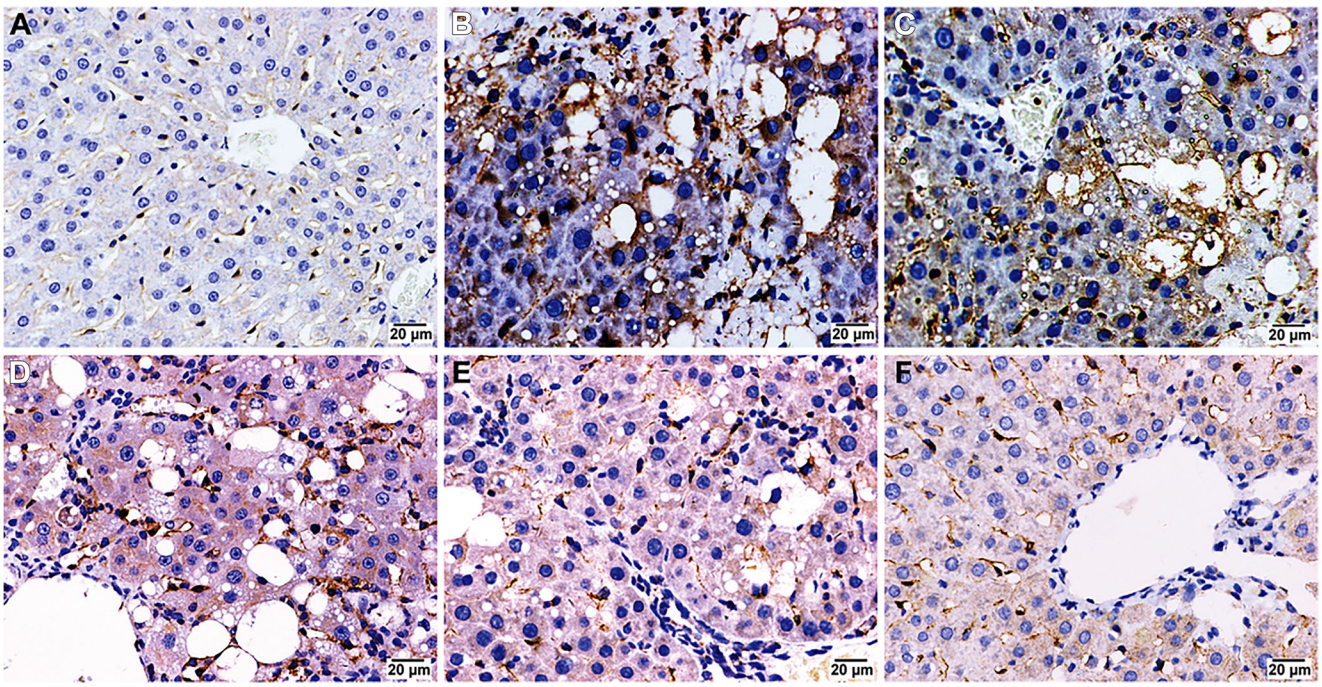

Fig. 6 Immunohistochemical staining for IL-1β in liver sections (*scale bar* = 20 μm). **a** Control group, **b** CCl_4 group, **c** CCl_4 + APS group, **d** CCl_4 + SS, **e** CCl_4 + SS + APS group and **f** CCl_4 + $sAPS_3$ group

expressions of TNF-α in liver tissue sections by immunofluorescence (IFA) assay. As shown in (Fig. 7), immunofluorescence analysis showed that the green signals representing TNF-α were highly localised in the cytoplasm of hepatocytes of the CCl_4-treated group (Fig. 7b) compared with the control group (Fig. 7a). The moderate density of TNF-α was shown in the SS and APS groups (Fig. 7c, d), while the density of TNF-α in the cytoplasm of hepatocytes was reduced in the SS + APS or $sAPS_3$ group (Fig. 7e, f).

Effects of $sAPS_3$ on the Autophagy Impairment in KCs after CCl_4 Treatment

Autophagy is considered to be a key regulator of inflammation and its reduction can trigger various inflammatory conditions [6]. To detect the autophagy activation in KCs, we measured the mRNA expression levels of ATG7, a key regulator of autophagy activation (Fig. 4d). The mRNA expression levels of ATG7 in CCl_4-treated group was significantly increased ($P < 0.05$), compared with the control group. However, the mRNA expression levels of ATG7 in the APS, SS, SS + APS or $sAPS_3$ group were highly decreased compared with the CCl_4-treated group ($P < 0.05$). SS + APS and $sAPS_3$ were more effective in the reduction of ATG7 mRNA expression levels ($P < 0.05$) compared with the APS or SS group. While there was no significant difference in the mRNA expression levels of ATG7 between the APS and SS groups. This result was confirmed by staining microtubule-associated protein I1 light chain 3 (LC3II) in liver tissue sections by immunohistochemistry (IHC) assay. As shown in (Fig. 8), the CCl_4-treated group showed strong expressions of LC3II all around the hepatic lobules (Fig. 8b) compared with the control group (Fig. 8a). SS and APS groups showed relatively low expressions of LC3II (Fig. 8c, d). No expression was observed in the administration of SS + APS or $sAPS_3$-treated group (Fig. 8e, f).

Discussion

Liver damage is one of the major health problems worldwide, frequently occurring as the result of oxidative damage and intoxication [23]. KCs are resident macrophages of the liver and play a major role in its homoeostasis and involved in the acute and chronic responses of the liver to various toxic agents including CCl_4. Activation of KCs directly or indirectly by toxic compounds results in the release of a number of inflammatory cytokines, growth factors, and reactive oxygen species. This activation is commonly associated with acute hepatocellular damage as well as chronic liver conditions including hepatic cancer [24]. Therefore, inactivation of KCs may results in amelioration of hepatocellular necrosis to a lesser extent. The studying of hepatoprotective activity of medicinal plants and foods and their bioactive ingredients s is one of the main goals of medical research today [23]. Selenium (Se) is a fundamental nutrient element that can ameliorate the progress of liver injury and reduce the hepatic fibrosis [25]. However, some investigations showed that APS had hepatoprotective

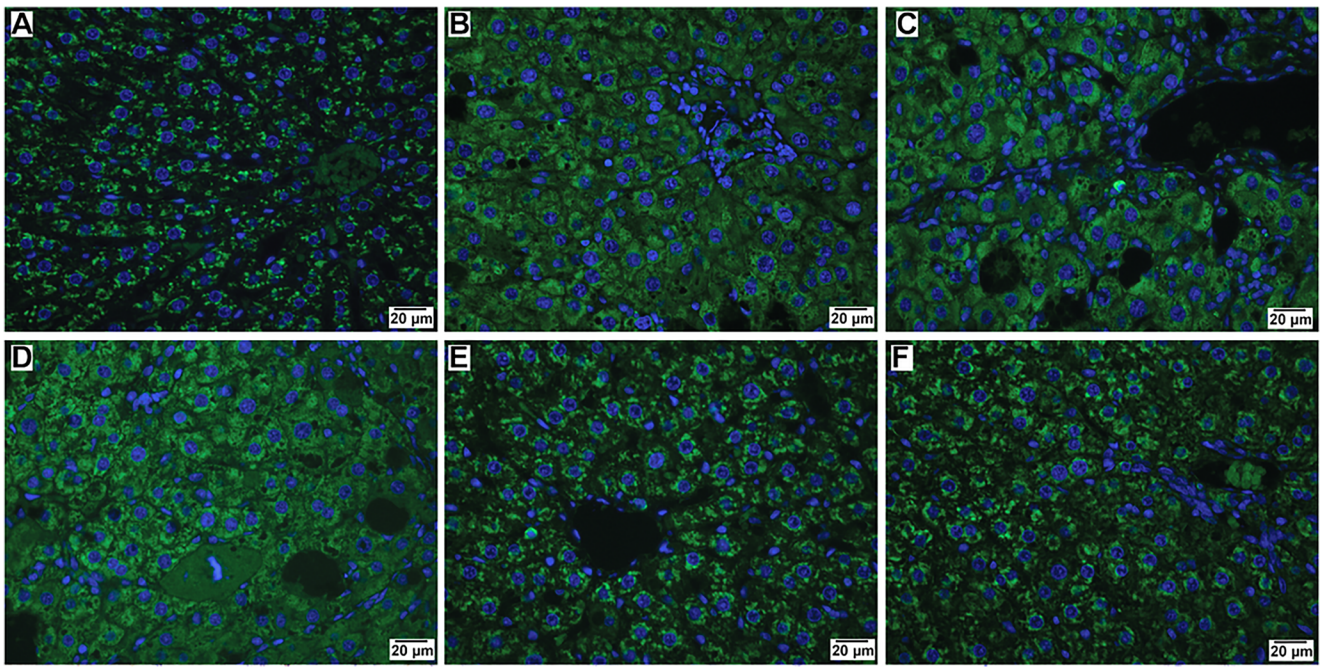

Fig. 7 Immunofluorescence staining for TNF-α in liver sections (*scale bar* = 20 μm). **a** Control group, **b** CCl_4 group, **c** CCl_4 + APS group, **d** CCl_4 + SS group, **e** CCl_4 + SS + APS group and **e** CCl_4 + $sAPS_3$ group

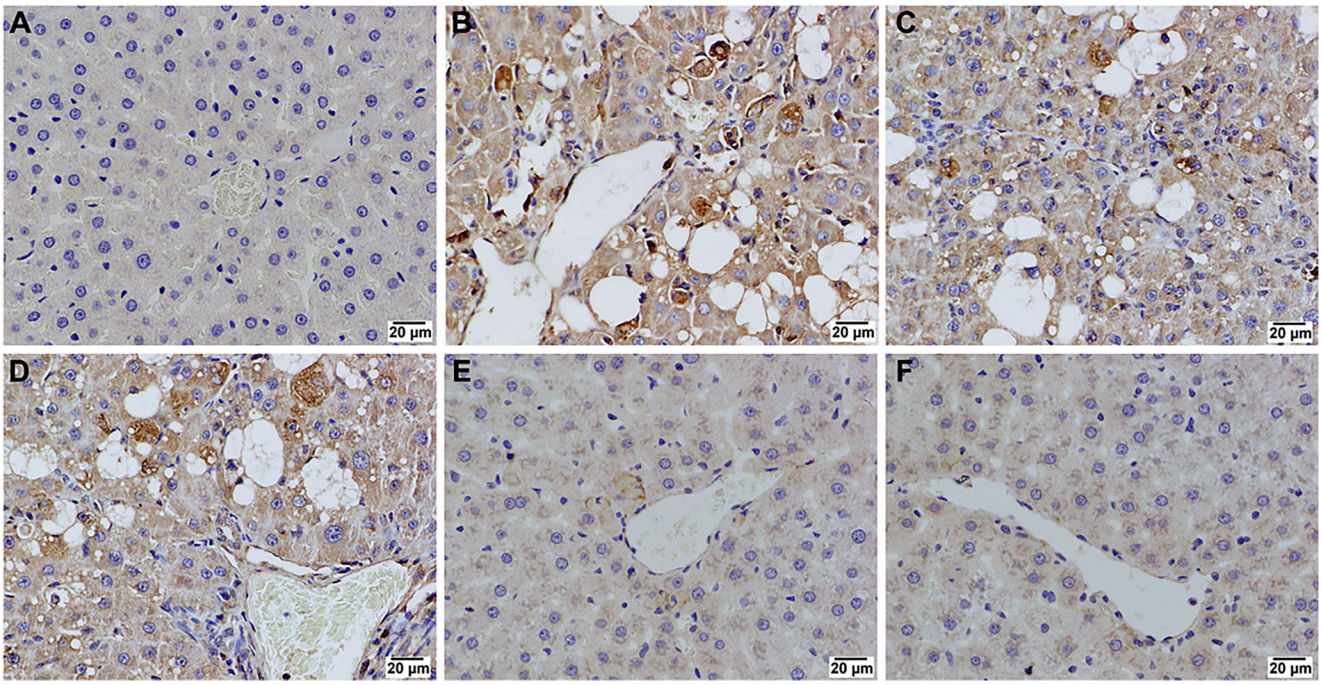

Fig. 8 Immunohistochemical staining for LC3II in liver sections (*scale bar* = 20 μm). **a** Control group, **b** CCl_4 group, **c** CCl_4 + APS group, **d** CCl_4 + SS, **e** CCl_4 + SS + APS group and **f** CCl_4 + $sAPS_3$ group

effects on liver damage induced by CCl_4 in mice [26]. The combination of selenium with polysaccharides into organic selenium polysaccharides as selenizing polysaccharides may improve the activity compared to polysaccharide and selenium. Therefore, this study was conducted to prepare a sAPS product that has the combined effects of selenium and APS. Our results showed that administration of $sAPS_3$ was effective in reducing CCl_4-induced hepatocellular necrosis via inactivation of KCs. This study is the first to determine the effect of $sAPS_3$ on CCl_4-induced hepatocellular necrosis in rats and elucidate the underlying mechanisms.

Specific serum marker enzymes, such as ALT and AST, are commonly recognised as indicators of liver damage and they are increased in hepatocellular necrosis and liver dysfunction [7, 27, 28]. The present findings showed that the levels of ALT and AST were highly elevated in the serum of CCl_4-treated rats, suggesting severe damage of hepatocytes. Administration of $sAPS_3$ significantly reduced the levels of the of the above-mentioned serum enzymes. These results were confirmed by the finding of histopathological examination that revealed the damage of hepatic lobule with severe hepatocytes necrosis, ballooning, and inflammation in the CCl_4-treated rats. There is some evidence indicating that CCl_4 causes massive hepatocellular necrosis and inflammation [7, 25] while $sAPS_3$ could effectively reduce the histopathological alterations induced by CCl_4. These finding indicated that $sAPS_3$ could prevent the liver from hepatocellular necrosis.

Oxidative stress was a major contributor in the pathophysiologic process of liver injury [29]. Free radicals accumulation in oxidative stress process can damage the hepatocytes membrane mainly through lipid peroxidation [30]. Nevertheless, antioxidants have protective effects against induced liver injury by lessening oxidative stress in cells [31]. Glutathione (GSH) is one of the important non-enzymatic antioxidants involved in the protection of cells from oxidative stress. However, activation of KCs results in the release of reactive oxygen species and driving more oxidative stress in the injured liver by CCl_4 [24, 29]. The levels of T-AOC, GSH, GSH-Px, and SOD significantly decreased in CCl_4-treated rats and were elevated toward normal values by $sAPS_3$. The peroxidative status showed inhibited by $sAPS_3$ according to the lower lipid peroxide (MDA). These findings indicate that $sAPS_3$ was able to ameliorate the deleterious effect of oxidative stress.

CD68 is the major specific marker of KCs; it has been used to monitor KCs. Activation when increased may indicate hyperfunction of KCs in the injured liver tissue [6, 32, 33]. The expression levels of CD68 were significantly increased in CCl_4-treated rats, while administration of $sAPS_3$ reduced the expression of this marker. These results indicate that $sAPS_3$ was able to ameliorate the hepatocellular necrosis by inactivation of KCs.

KCs are the primary source of inflammatory cytokines when activated by toxic compounds including CCl_4 secreted variety of these cytokines including IL-β1 and TNF-α. These cytokines can act on hepatocytes to cause cell death [24, 34]. The expression levels of IL-β1 and TNF-α were significantly increased in CCl_4-treated rats, while administration of $sAPS_3$

reduced the expression of these inflammatory cytokines. These findings indicate that $sAPS_3$ was able to ameliorate the liver damage by inhibition of KCs and their cytokines production.

Autophagy is a major lysosomal catabolic pathway of eliminating intracellular components and also recycling damaged macromolecules, including dysfunctional organelles, proteins and lipids. Many studies indicate that autophagy deficiency increases inflammation as a key regulator of inflammation. Under toxic stimuli, excess autophagy can eliminate essential cellular components and may lead to cellular death. [6, 35]. Recent studies have indicated that impaired autophagy induces necrosis and thus stimulates the inflammatory response [36]. Others indicated that CCl_4 could stimulate autophagy activation significantly in young KCs [6]. Our study indicated that CCl_4 stimulates autophagy in KCs by increasing the expression of a key regulator of autophagy activation ATG7 in addition to a biological marker of autophagy microtubule-associated protein I1 light chain 3 (LC3II). Meanwhile, administration of $sAPS_3$ decreased the expression of ATG7 and LC3II in the liver tissue. These findings indicated that autophagy in KCs might increase due to strong stimulation of CCl_4 and hyperactivation of KCs compared with KCs of $sAPS_3$ rats, but these results need a further study.

In conclusion, $sAPS_3$ shows strong protective effects against CCl_4-induced hepatocellular necrosis by inactivation of KCs. In addition, the hepatoprotective activities of $sAPS_3$ may be superior to that of selenium, APS or selenium +APS and the combination of selenium with APS was more effective than APS or selenium alone. Further study is required to investigate the role of CCl_4 in the autophagy in KCs. Further analysis is also needed to determine the quality and standard of the materials and about clinical application of $sAPS_3$ in animals.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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