Review Article
Salidroside alleviates acute liver injury through mitochondrial protection and anti-oxidative effects

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Abstract: Accumulating evidence has indicated the anti-oxidative and anti-inflammatory effect of salidroside (SAL) on acute liver injury; however, the cytoprotection and underlying mechanisms of SAL remained elusive. The aim of our present research was to evaluate the hepatoprotective effect of SAL through carbon tetrachloride (CCl₄)-induced hepatic injury model and H₂O₂-induced oxidative injury model in a normal human liver cell line L-02. In vivo, the results revealed that SAL reduced serum alanine aminotransferase (ALT) and alanine aminotransferase (AST) levels, accompanied with ameliorated histopathological changes. In vitro, SAL increased cell viability, inhibited cell apoptosis, reduced reactive oxygen species (ROS) production and recovered mitochondrial membrane potential when compared to the H₂O₂ group. In addition, SAL treatment up-regulated the expressions of antioxidant-related genes including hemeoxygenase-1 (HO-1), nuclear factor erythroid 2 related factor (Nrf2), manganese superoxide dismutase (Mn-SOD) and catalase (CAT), and down-regulated NADPH oxidase isoform 2 (NOX-2). Taken together, these results demonstrated that SAL could relieve CCl₄-induced liver damage and H₂O₂-induced oxidative injury by mitochondrial protection and oxidative stress suppression, suggesting that SAL could be a potential agent for treating or preventing acute liver injury.

Keywords: Salidroside, acute liver injury, oxidative injury, hydrogen peroxide, carbon tetrachloride, hepatoprotective, L-02 cells

Introduction
Due to its increasing incidence, liver disease has become a serious health problem. It can gradually develop into cirrhosis and carcinoma which threaten human life [1]. Causative factors such as exposure to specific chemical drugs, or radiation damage can induce inflammation, cell necrosis and apoptosis, leading to acute liver damage [2]. As the major contributor to the pathogenesis and progression of liver diseases, oxidative injury is accumulative by an imbalance of antioxidant defenses and oxidative stress, consequently resulting in a more oxidized environment [3]. ROS, including hydrogen peroxide (H₂O₂), anion radicals (O₂⁻) and reactive free radical species hydroxyl (OH); so this is a double-edged sword. Low concentration ROS can promote the activation of transcription factors, cell proliferation and differentiation. While excessive ROS is harmful to normal liver cells. By initiating an ROS-mediated cascade, ROS causes cell apoptosis and necrosis through oxidative stress, and ultimately gives rise to hepatocyte death and acute hepatic damage [4].

Salidroside (SAL), a phenylpropanoid glycoside compound, is the main active ingredient of Rhodiola rosea. It grows at high altitude zones and has been used as a precious traditional medicine for hundreds of years to treat high altitude sickness [5]. In addition to its anti-autophagy, anti-hypoxia, anti-inflammatory, anti-depressive, neuroprotective and cardio protective properties [6-9], SAL also is a natural antioxidant product [10]. It was reported that the hepatoprotective mechanism of SAL in treatment of fulminant hepatic failure induced by D-galactosamine and lipopolysaccharide appeared to be related to antioxidant activity and inhibition of hypoxia-inducible factor-1a [11]. A recent study proved that SAL could protect the liver from High-Fat Diet-Induced
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Nonalcoholic Fatty Liver injury by resisting oxidative stress and protecting mitochondrial function [12]. Although the anti-oxidative ability of SAL has been demonstrated to be closely correlated with liver diseases, quite a few questions are still elusive and need more investigation, such as how it functions in the progress of acute liver injury, its underlying mechanism of protection on normal liver cells, and whether it is suitable for clinical application.

In the current study, we used a liver oxidative stress model induced by CCl₄ in mice and H₂O₂ in L-02 cells to study the hepatoprotective effect of SAL on hepatic injury and its underlying mechanisms. The findings of our study may have potential clinical prevention or usage for acute liver injury.

Materials and methods

Dilution

Salidroside powder (purity >98%, Meilunbio, Dalian, China) dissolved in 1 mL PBS (phosphate buffer solution) or saline solution as the stock solution and kept in dark place at -20°C. One mL 3% H₂O₂ (Sigma-Aldrich, St. Louis, MO, USA) was diluted in 8 mL PBS at a concentration of 200 μM as working solution.

For animal treatment, mice were injected with different doses of SAL stock solution in the light of body weight. For cell treatment, 2 or 4 μL stock solution of SAL was diluted in 2 mL serum-free 6-well plates to the final concentration of 50 μM or 100 μM, 2 μL working solution of H₂O₂ was diluted to 200 μM.

Animals and treatment

Eight-week-old male C57BL/6 mice (average weight 20.0±1.0 g) were purchased from Jinan Pengyue Experimental Animal-Breeding (Jinan, Shandong, China). The animals were housed in a controlled environment conditions at 22±2°C, 50±5% relative humidity and 12 h light-dark cycle with free access to water and standard rodent diet. The mice were kept for one week to acclimatize to the conditions before experiment. All animal experiments were performed in accordance with the Local Animal Use Committee. Thirty male mice were randomly divided into five groups (n=6 per group) to induce acute liver injury. 1) Control group: mice were given sterile saline only; 2) 0.5% CCl₄ group: mice were given CCl₄ (1 mL/kg, diluted in olive oil); 3) 0.5% CCl₄+SAL (50 mg/kg) group; 4) 0.5% CCl₄+SAL (100 mg/kg) group. Due to the pharmacokinetics and preliminary experiment of SAL, it was injected intraperitoneally 2 h before CCl₄ administration. Retro-orbital blood was collected 24 h after CCl₄ injection to detect serum ALT, AST. Then mice were killed under anesthesia using chloral hydrate to take the liver tissue samples for histopathological examination. The administration dosage of SAL and CCl₄ and sampling time were based on previous work [13, 14].

Biochemical assay

Serum levels of ALT and AST were measured using an ELISA Kits (Nanjing Jiancheng Biotechnology Institute, Jiangsu, China) according to the manufacturer’s instructions.

Histological examination

Liver specimens embedded in paraffin were sliced into 5-8 μm for hematoxylin and eosin (H&E) stain. The degree of CCl₄-induced inflammation and lesions were assessed by pathological changes under light microscopy in a double-blind manner.

Cell culture and treatment

Normal human hepatic cell strain-L-02 (Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultivated in RPMI 1640 medium (HyClone, Shanghai, China) supplemented with 10% FBS (fetal bovine serum, ThermoFisher Scientific, Massachusetts, USA), with 1% antibiotic at 37°C in a humidified 5% CO₂ environment. The culture medium was changed every other day until the cells were in good condition then plated onto 6-well plates with the same serum medium. When L-02 cells reached over 70% confluence, they were washed twice by ice-cold PBS and incubated in 2 mL fresh serum-free 1640 medium for experiments to be conducted. For our study, control group were incubated in serum-free medium without any treatment while the experimental group (H₂O₂, H₂O₂+SAL50, H₂O₂+SAL100) were exposed to 200 μM H₂O₂ either with or without 50/100 μM SAL (as previously mentioned).
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for 6 h, with reverse transcription-polymerase chain reaction (RT-PCR) analysis. The time and dose of \( \text{H}_2\text{O}_2 \) that could induce an oxidative injury were guided by relevant research [15, 16].

**LDH release assay**

Lactate dehydrogenase (LDH) released from \( \text{H}_2\text{O}_2 \)-induced dead L-02 cells was assessed by Cytotoxicity Assay Kit (Beyotime, Shanghai, China) according to the manufacturer’s protocol.

**Mitochondrial membrane potential assay**

Measurement of mitochondria membrane potential was done using JC-1 staining (Beyotime). According to manufacturer’s instruction, L-02 cells were treated with \( \text{H}_2\text{O}_2 \) in 6-well plates as stated above. The original medium was replaced by 1 mL fresh medium containing JC-1 (5 mg/mL) and incubated at 37°C for 20 min. After washing twice with JC-1 staining solution (1 mg/mL), images were acquired by fluorescent microscopy (Nikon, Tokyo, Japan) and analyzed for green and red fluorescence using Image-Pro Plus 6.0 software. The reduced red/green fluorescence intensity ratio represented mitochondrial depolarization.

**Mitochondrial ROS in liver cells**

MitoSox Red (Molecular Probes, Shanghai, China) assay was used to monitor ROS generation of intracellular mitochondrial. In the Mitochondrial, MitoSox Red reagent can be oxidized by superoxide and showed red fluorescence. L-02 cells were applied with 1-2 mL of 5 μM MitoSox reagent and incubated for 10 min at 37°C, protected from light. After washing gently three times with warm buffer, images of red fluorescence were collected under fluorescent microscopy (Nikon).

**ROS assay**

Reactive Oxygen Species Assay Kit (Beyotime) was used to detect amount of intracellular ROS by DCFH-DA probes according to the manufacturer’s instruction. L-02 cell were treated with \( \text{H}_2\text{O}_2 \) in 6-well plates as described in the text and incubated at 37°C for 20 min in the medium that contained DCFH-DA working solution (10 μM/L). After washing with PBS three times to fully remove DCFH-DA that did not enter the cells, images were scanned directly under fluorescent microscopy (Nikon). In general, ROS positive controls can be significantly increased after 20 to 30 min stimulation of cells.

**TUNEL stain**

DAPI was used to label all cells and displayed the blue fluorescence. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) can detect DNA fragmentation of a cell nucleus in the early apoptosis that showed a green fluorescence. After treating with 200 μM \( \text{H}_2\text{O}_2 \), and 50 or 100 μM SAL 24 h in 6-well plates, L-02 cells were carried out by TUNEL apoptosis detection kit (Beyotime) per manufacturer’s explanations. The representative images were observed under fluorescent microscopy (Nikon) and quantified by green/blue fluorescent ratio with Image-Pro Plus software 6.0.

**RT-PCR**

Total RNA was extracted from L-02 cells with GeneJET RNA Purification Kit (ThermoFisher Scientific) as instructed by manufacturer. RT-PCR test was performed using PrimeScript RT Reagent kit (TaKaRa, Dalian, China) and the procedure was implemented using a SYBR Green PCR Master Mix (Roche, Switzerland). The expressions of related genes were normalized by Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and relative quantity were

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**Table 1. The primer sequences used in RT-PCR**

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Tm</th>
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<tbody>
<tr>
<td>GAPDH-F</td>
<td>CATGTTCGTATGGGTTGAGAA 58</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>GGATCGACTGCTGTTCAAGAG 58</td>
</tr>
<tr>
<td>h-NOX2-F</td>
<td>ACACATATGCCTTCTGAGTTT 58</td>
</tr>
<tr>
<td>h-NOX2-R</td>
<td>CATATGTCGACAGACGAAAT 58</td>
</tr>
<tr>
<td>h-HMOX1-F</td>
<td>ACACACGGCGAGAGAATGGCT 58</td>
</tr>
<tr>
<td>h-HMOX1-R</td>
<td>CGAAGACTGGGCTCTTCTGTG 59</td>
</tr>
<tr>
<td>h-Nrf2-F</td>
<td>AGATCGCCCCACCTGCTGA 58</td>
</tr>
<tr>
<td>h-Nrf2-R</td>
<td>GATAAGATCGCGGAGTCGAAATC 59</td>
</tr>
<tr>
<td>h-Mn-SOD-F</td>
<td>CATATGTCGACAGACGAAATGGCT 58</td>
</tr>
<tr>
<td>h-Mn-SOD-R</td>
<td>TCCAGCCTTTCCTGTTGTTG 59</td>
</tr>
<tr>
<td>h-CAT-F</td>
<td>CATATGTCGACAGACGAAATGGCT 58</td>
</tr>
<tr>
<td>h-CAT-R</td>
<td>GCACCGTAGGGACAGTCACCA 60</td>
</tr>
</tbody>
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Statistical analysis

Data were expressed as mean ± standard deviation (SD). The analysis of statistical differences was determined by Student’s t-test using Graph Prism 5.0. “*P<0.05” was considered to be statistically significant.

Results

SAL had protective effects on CCl₄-induced acute liver injury in mice

In the normal liver tissues, there are central veins and thin sinusoids, which are radially arranged around the central vein. Hepatic cells are large and round with a clear and prominent nucleus as shown in the control group, Figure 1A. In contrast, liver sections in the CCl₄ group calculated using 2^ΔΔCt method. The primer sequences (Invitrogen, Shanghai, China) are shown in Table 1.

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displayed a loss of hepatic architecture with severe inflammatory foci and diffuse centrilobular necrosis that composed of inflammatory and apoptotic cells. However, seen in Figure 1A, 1B, the degree of liver lesions and the percent of necrotic area were prominently decreased in SAL-pre-treated group and the maximum improvement was in the SAL100 group that showed the minor histopathology changes with less infiltration of inflammatory cells and well-defined hepatic cells. This can almost be comparable to the control group, implying that SAL could relieve liver damage induced by CCl₄.

This result was next confirmed by serum level of ALT and AST. The activity of which are important biochemical indicators of liver function. This rise reflects damage and inflammation of liver tissue. Compared with the control group, serum AST and ALT activity in CCl₄ treatment group were significantly elevated. However, in Figure 1C, 1D, there was a clear reduction of ALT, AST in mice that were pretreated with SAL in comparison with those CCl₄-treated mice and CCl₄+SAL100 group had lower level of serum ALT, AST than in CCl₄+SAL50.

SAL protected L-02 cells from H₂O₂-induced oxidative injury

Oxidative stress can cause hepatic apoptosis [17]. Here, we determined the effect of SAL on hepatocytes viability by the amount of released LDH. In Figure 2, LDH in H₂O₂-incubated L-02 cells had a remarkable increase compared to the control group, but decrease in SAL-treated group (50 and 100 μM) groups.
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H$_2$O$_2$ exposure induced more positive cells that had a high green/blue fluorescent intensity ratio than the control group. But after addition with SAL, this increased ratio was reduced and H$_2$O$_2$+SAL100 group showed the most significant reduction.

SAL decreased ROS generation and maintained the mitochondrial function in H$_2$O$_2$-treated L-02 cells

Oxidative stress begins with the overproduction of ROS that can cause dysfunction and apoptosis of mitochondrial [18]. Here, ROS was estimated in L-02 cells by DCFH-DA probe which can be hydrolyzed by intracellular esterase to DCFH. Intracellular ROS can oxidize non-fluorescent DCFH into fluorescent DCF. Accordingly, the level of ROS in cells can be found by detecting the fluorescence of DCF. Results shown in Figure 4 revealed a decreased ROS after SAL addition compared with H$_2$O$_2$-exposed group. In addition, we investigated the effect of SAL on mitochondrial function using JC-1 staining and MitoSox Red. As seen in Figure 5, normal L-02 cells were fluorescent red in JC-1 stain assay, while more cells were fluorescent green in the H$_2$O$_2$-treated group due to a loss of mitochondrial membrane potential that was a landmark event of early apoptosis. In addition with SAL, the ratio of green/red fluorescence was clearly decreased, demonstrating a reversal of oxidative stress-induced mitochondrial membrane potential and the alleviation of cells.
Hepatoprotective effect of SAL via mitochondrial protection

CCl₄-induced hepatotoxicity is the most widely used model of studying the liver disease in vivo. Studies show that oxidative stress is vital for liver toxicity caused by CCl₄ that can result in acute liver damage [20, 21]. H₂O₂ is one of the main non-radical species of ROS which can react with DNA, proteins, lipids and potentially form lipid peroxidation, DNA damage, cell apoptosis or death [22]. So, we evaluated the hepatoprotective effect of SAL on hepatic injury induced by CCl₄ in mice and H₂O₂-injured L-02 cells, respectively. We observed that SAL attenuated CCl₄-induced liver damage via decreasing serum ALT, AST and ameliorated the pathological changes of liver tissues in mice. In H₂O₂-injured L-02 cells, SAL promoted cell viability, inhibited early apoptosis, reduced ROS production as well as regulated mRNA level of genes related to oxidative stress such as NOX-2, HO-1, Nrf2, Mn-SOD and CAT.

LDH is regarded as an important indicator of membrane integrity. The destruction of cell membrane structure caused by apoptosis or necrosis will trigger the release of intracytoplasmic enzymes such as LDH into the culture.

**Figure 5.** Protection of SAL on mitochondrial function. After treating with or without different dose of SAL (50 or 100 μM) for 24 h, H₂O₂-injured L-02 cells were stained with MitoSox Red or JC-1, separately. There were greater red fluorescence represented more ROS of mitochondria in MitoSox Red. Green fluorescence implied monomer and red implied aggregate. An increased green/red fluorescence ratio indicated a decrease mitochondrial membrane potential.

**Discussion**

Liver damage, ranging from high-fat diet-caused nonalcoholic fatty liver disease to ischemia reperfusion injury, and subclinical icteric hepatitis, has been known to be associated with oxidative stress [19]. CCl₄-induced hepatotoxicity is the most widely used model of studying the liver disease in vivo. Studies show that oxidative stress is vital for liver toxicity caused by CCl₄ that can result in acute liver damage [20, 21]. H₂O₂ is one of the main non-radical species of ROS which can react with DNA, proteins, lipids and potentially form lipid peroxidation, DNA damage, cell apoptosis or death [22]. So, we evaluated the hepatoprotective effect of SAL on hepatic injury induced by CCl₄ in mice and H₂O₂-induced oxidative injury in L-02 cells, respectively. We observed that SAL attenuated CCl₄-induced liver damage via decreasing serum ALT, AST and ameliorated the pathological changes of liver tissues in mice. In H₂O₂-injured L-02 cells, SAL promoted cell viability, inhibited early apoptosis of cells, reduced ROS production as well as regulated mRNA level of genes related to oxidative stress such as NOX-2, HO-1, Nrf2, Mn-SOD and CAT.
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Therefore, we examined the protective effect of SAL by LDH release assay in L-02 cells. We found that SAL can increase cell viability and inhibit cell apoptosis. Hepatocyte mitochondria and endoplasmic reticulum are the main sites of ROS generation. ROS-induced oxidative stress has been known to be associated with various liver diseases. An overproduction of ROS then depletes the endogenous antioxidants, impairs mitochondrial function and activates the signaling cascades that subsequently lead to cellular apoptosis or cell death [23]. By detecting the fluorescence in ROS Assay and TUNEL, we found that SAL decreased ROS production with less green fluorescence and inhibited apoptosis with lower percentage of TUNEL positive cells when compared with H₂O₂-exposed L-02 cells. This result was further confirmed by MitoSox Red stain and JC-1 which exhibited less red fluorescence and decreased ratio of green to red. Taken together, SAL can restrain oxidative stress and recover mitochondrial function in H₂O₂-injured L-02 cells.

The cellular mechanism that controls oxidative stress is crucial for intracellular environmental stability. There are non-enzymatic antioxidant system such as vitamin C, vitamin E, Zn, carotenoid and another critical enzymatic antioxidant system including Mn-SOD, CAT, glutathione peroxidase (GSH-Px) in our body. SOD can transform harmful superoxide radicals into hydrogen peroxide in the reaction, which is then decomposed by CAT into completely harmless oxygen and water, thus forming a complete

Figure 6. Modification of SAL on mRNA expression in L-02 cells. The mRNA levels of oxidative stress-related genes (A) NOX-2, (B) HO-1, (C) Nrf2, (D) Mn-SOD, (E) CAT were measured by RT-PCR. L-02 cells were exposed to H₂O₂ (200 μM) with or without SAL (50 or 100 μM) for 6 h in serum-free medium. The results were expressed as means ± SD, n=3, *P<0.05, compared with control or H₂O₂-treated group.
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anti-oxidation chain \[24\]. Mn-SOD as a kind of SOD that mainly exists in the mitochondria of eukaryotic cells, is the main scavenger of ROS.

Some researchers suggest that HO-1 can effectively prevent the oxidative damage to L-02 cells \[25, 26\]. Given that Nrf2 are responsible for the production of HO-1 to protect cells from \( \text{H}_2\text{O}_2 \)-induced oxidative injury \[27, 28\], we next examined the mRNA level of HO-1 and Nrf2 in L-02 cells. We observed SAL not only up-regulated the expression of HO-1 mRNA but also the Nrf2 level compared to \( \text{H}_2\text{O}_2 \)-treated group that was compliance with other previous studies. In addition to those antioxidant factors, we also tested NOX-2 that plays a positive role in oxidative stress. As the major source of cellular ROS, NOX-2 has been known to be associated with liver damage \[29\]. Our data indicated that SAL exerted its antioxidant property characterized by inhibiting mRNA expressions of NOX-2 and elevating HO-1, Nrf2, Mn-SOD, CAT in \( \text{H}_2\text{O}_2 \)-induced L-02 cells.

In conclusion, SAL alleviates hepatic injury induced by \( \text{CCl}_4 \) in mice and protects L-02 cells from oxidative injury. Hence, SAL may have possible efficacy for clinical usage against liver oxidative damage.

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Disclosure of conflict of interest

None.

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References


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