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Editorial board member of *World Journal of Gastroenterology*, Shinji Tanaka, MD, PhD, Professor, Department of Endoscopy, Hiroshima University Hospital, Hiroshima 734-8551, Japan

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Basic Study

Salvianolic acid B protects hepatocytes from H₂O₂ injury by stabilizing the lysosomal membrane

Xiao-Feng Yan, Pei Zhao, Dong-Yan Ma, Yi-Lu Jiang, Jiao-Jiao Luo, Liu Liu, Xiao-Ling Wang

Xiao-Feng Yan, Pei Zhao, Xiao-Ling Wang, Department of Biology, School of Basic Medical Science, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

Dong-Yan Ma, Women and Infant Hospital of Zhengzhou, Zhengzhou 450000, Henan Province, China

Yi-Lu Jiang, Jiao-Jiao Luo, Liu Liu, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

Author contributions: Wang XL and Yan XF designed the research; Yan XF, Zhao P, Ma DY, Jiang YL, Luo JJ and Liu L performed the research; Yan XF, Zhao P and Ma DY analyzed the data; Wang XL wrote the paper.

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Correspondence to: Dr. Xiao-Ling Wang, Department of Biology, School of Basic Medical Science, Shanghai University of Traditional Chinese Medicine, 1200 Cailun Road, Pudong, Shanghai 201203, China. wangxiaoling1033@shutcm.edu.cn
Telephone: +86-21-51322585

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Abstract

AIM

To investigate the capability of salvianolic acid B (Sal B) to protect hepatocytes from hydrogen peroxide (H₂O₂)/carbon tetrachloride (CCl₄)-induced lysosomal membrane permeabilization.

METHODS

Cell Counting Kit-8 assay was used to measure cell viability. Apoptosis and death were assayed through flow cytometry. BrdU incorporation was used to detect cell proliferation. Serum alanine aminotransferase activity and liver malondialdehyde (MDA) content were measured. Liver histopathological changes were evaluated using hematoxylin-eosin staining. Lysosomal membrane permeability was detected with LysoTracker Green-labeled probes and acridine orange staining. The levels of protein carbonyl content (PCC), cathepsins (Cat)B/D, and lysosome-associated membrane protein 1 (LAMP1) were evaluated through western blotting. Cytosol CatB activity analysis was performed with chemiluminescence detection. The mRNA level of

LAMP1 was evaluated through quantitative real-time polymerase chain reaction.

RESULTS

Results indicated that H₂O₂ induced cell injury/death. Sal B attenuated H₂O₂-induced cell apoptosis and death, restored the inhibition of proliferation, decreased the amount of PCC, and stabilized the lysosome membrane by increasing the LAMP1 protein level and antagonizing CatB/D leakage into the cytosol. CCl₄ also triggered hepatocyte death. Furthermore, Sal B effectively rescued hepatocytes by increasing LAMP1 expression and by reducing lysosomal enzyme translocation to the cytosol.

CONCLUSION

Sal B protected mouse embryonic hepatocytes from H₂O₂/CCl₄-induced injury/death by stabilizing the lysosomal membrane.

Key words: Lysosomal membrane permeabilization; Injury; Salvianolic acid B; Hepatocyte

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Core tip: Lysosomal membrane permeabilization leads to the release of luminal contents, such as proteases and protons, into the cytosol, resulting in cell death. Salvianolic acid B (Sal B), a water-soluble compound extracted from *Salvia miltiorrhiza*, exerts a cell protective effect by anti-oxidation. In the present study, we elucidate that Sal B protects hepatocytes from H₂O₂/CCl₄-induced injury/death by stabilizing the lysosomal membrane.

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INTRODUCTION

Lysosomes are cytoplasmic membrane-enclosed organelles that contain various hydrolytic enzymes; they complete the degradation process of cellular components and macromolecules^[1]. The function of lysosomes is critically dependent on soluble lysosomal hydrolases, including cathepsin (Cat) B and CatD^[2], as well as membrane integrity due to highly glycosylated lysosomal membrane proteins, including lysosome-associated membrane proteins (LAMPs)^[3].

Lysosomes are potentially harmful to cells when damage occurs on the lysosomal membrane under pathological conditions. This occurrence is called lysosomal membrane permeabilization (LMP), which

allows the release of luminal contents, such as proteases, into the cytosol and results in lysosomal alkalization and cell death. The oxidative stress caused by the imbalance between reactive oxygen species (ROS) production and clearance is associated with numerous pathological consequences, including necrosis, autophagy, and apoptosis^[4]. Hydrogen peroxide (H₂O₂) is a key mediator underlying cellular oxidative stress and is involved in various pathological processes^[5,6]. In carbon tetrachloride (CCl₄)-induced liver injury, oxidative stress is one of the main mechanisms^[7].

Hepatocytes make up a systemic hub, integrating the entire body's metabolic demand and detoxification processes, all of which are redox-regulated^[8]. An increase in oxidative stress sensitizes hepatocytes to subsequent necrosis and/or apoptosis and ultimately results in a massive loss of mature hepatocytes with subsequent inflammation, fibrosis, cirrhosis, and even hepatocellular carcinoma^[9]. However, how hepatocyte death is caused by oxidative stress-induced LMP remains unclear.

Salvianolic acid B (Sal B), a water-soluble compound extracted from the herb of *Salvia miltiorrhiza*, has been widely used in many Asian countries for the treatment of cardiovascular disorders and cerebrovascular diseases for thousands of years^[10]. Sal B exerts significant free radical scavenging effects^[11]. Several recent reports have indicated that Sal B produces hepatoprotective effects in chronic alcoholic liver disease^[12], non-alcoholic fatty liver disease^[13], and non-alcoholic steatohepatitis^[14]. Furthermore, Sal B can effectively inhibit hepatocyte apoptosis by regulating death receptor and mitochondrial pathways^[15]. However, whether the anti-oxidation effects of Sal B are related to LMP in hepatocytes still requires elucidation.

In the present study, H₂O₂/CCl₄-induced injury/death in the BNL CL.2 hepatocyte cell line was used to determine if LMP leads to the leakage of lysosomal contents into the cytosol and triggers cell death in hepatocytes and if Sal B protects cells from death.

MATERIALS AND METHODS

Cell culture

The BNL CL.2 mouse embryonic hepatocyte cell line was purchased from the Cell Bank of Shanghai Institutes for Biological Sciences and routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C. The culture media were changed once every 2 d.

Cell viability assay

Cell viability was assessed with the Cell Counting Kit-8 (CCK8) assay (Dojindo, Kumamoto, Japan). Four thousand cells were seeded onto 96-well plates and

incubated for 24 h at 37 °C in 5% CO₂. After incubation with different concentrations of Sal B, 10 µL of the CCK8 reagent was added to each well and incubated for 2 h at 37 °C. The absorbance at 450 nm was measured with a microplate reader (Bio-Tek, Hercules, CA, United States). The mean absorbance values from four wells after each treatment were used to represent the cell viability index. The experiments were repeated at least thrice. Cell viability (%) was obtained as follows: (treated group absorbance - blank group absorbance)/(control group absorbance - blank group absorbance) × 100%.

BrdU labelling and immunofluorescence

After growing 4000 cells on a 96-well plate treated with 10 µmol/L of Sal B for 24 h and with 500 µmol/L of H₂O₂ for another 2 h, the medium was changed to a final concentration of 10 µmol/L of 5-bromo-2-deoxyuridine (BrdU; Sigma, St. Louis, MO, United States) for 24 h at 37 °C. After fixing with cold 4% paraformaldehyde for 15 min at room temperature and washing with phosphate-buffered saline (PBS), the cells were incubated with 0.2% TritonX-100 for 20 min to permeabilize the membranes and blocked with 10% horse serum in PBS. A primary antibody (CST, Boston, MA, United States) was added at 1:100 dilution to the blocking solution and incubated at 4 °C overnight. After incubating, a secondary antibody (AlexaTM-594 donkey anti-mouse; Jackson ImmunoResearch, West Grove, PA, United States) was added at 1:100 dilution to the blocking solution for 1 h at room temperature. The cells were labelled with 5 µg/mL of Hoechst 33342 (CST) for 1 h at 37 °C. BrdU-positive proliferating cells were expressed as a percentage of the control level.

Annexin V-FITC/propidium iodide (PI) staining with fluorescence-activated cell sorter (FACS)

Cells treated with 10 µmol/L of Sal B for 12 h or 24 h followed by 2 h with 500 µmol/L of H₂O₂ were harvested through trypsinization. After washing with cold PBS three times, the cells were resuspended in 100 µL of binding buffer at a density of 1.2×10^6 cells per mL. Then, 5 µL of Annexin V-FITC (BD Pharmingen, Santiago, CA, United States) and 5 µL of PI (BD Pharmingen) were added to each sample and incubated in the dark for 15 min. Fluorescence was detected immediately after staining by using a flow cytometer (Ex = 488 nm; Em = 530 nm). The results were presented as the percentage of cells that were viable (Ann-V/PI⁻), early apoptotic (Ann-V⁺/PI⁻), late apoptotic (Ann-V⁺/PI⁺), or nonviable (Ann-V/PI⁺).

Western blotting

At the end of the designated treatments, 20 µg of cellular protein was separated *via* sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, United States) blocked with

5% non-fat milk for 2 h. The membranes were probed with the designated primary and secondary antibodies, developed using enhanced chemiluminescence (Thermo Scientific, Waltham, MA, United States), and visualized using an FCM gel imaging device (Protein Simple, San Francisco, CA, United States).

Protein carbonyl content

The protein carbonyl content (PCC) was assayed with an Oxyblot kit (Millipore). Briefly, 5 µL of cell lysates, including 20 µg of protein mixed with 5 µL of 12% SDS, were supplemented with 10 µL of 2,4-dinitrophenylhydrazine in 2 N HCl for 15 min at room temperature to derive carbonyl groups from the protein side chains. Then, the lysates were separated *via* 12% SDS-PAGE. Western blotting was performed using the 2,4-dinitrophenylhydrazine antibody (1:50). Given the presence of multiple bands, the average value of all the bands within each lane was used to provide an overall measure of PCC.

LysoTracker green or acridine orange staining

A total of 10000 cells were seeded on a 60 mm confocal plate. After the designated treatments, the cells were incubated with 75 nmol/L of LysoTracker Green (Invitrogen, Waltham, MA, United States) or 5 mg/mL of acridine orange (AO) (Sigma) for 30 min at 37 °C. Fluorescence was examined with a confocal microscope, with the excitation wavelength set at 488 nm. Two separate emission bands (505-570 nm and 615-754 nm) for AO staining and one for LysoTracker staining (504-511 nm) were obtained, used simultaneously, and photographed.

Cytosolic/particulate separation

Cytosolic/particulate separation was based on the protocol described in the Cytosol/Particulate Rapid Separation Kit (BioVision, Milpitas, CA, United States). Briefly, cells were harvested in 15 mL tubes and centrifuged at 600 × *g* for 5 min. The pellets were resuspended in 40 µL of cell suspension buffer and mixed with 40 µL of cytosol releasing buffer, including 1 mmol/L of PMSF. The cell suspension was carefully layered on 500 µL of an oil layer with 40 µL of a particulate layer at the bottom on ice for 30 s. After centrifugation at 1400 × *g* for 1 min, the cytosol was separated from the membrane particles by the oil layer and transferred to new tubes. The protein concentration was used with a BCA total protein assay kit (Biomiga, San Diego, CA, United States).

Real-time polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from cells (1×10^6) by using the TRIzol reagent (Invitrogen). Equal amounts of RNA were reverse transcribed into cDNA. The cDNA was amplified through RT-PCR, and the housekeeping gene 18S rRNA was used as an internal standard to quantify the levels of the target mRNA. The cycling

conditions were as follows: hold: 95 °C for 10 s; cycling: 95 °C for 5 s, 60 °C for 30 s, 40 cycles; and melt for 65–95 °C. The nucleotide sequences for the primers used were as follows: 18S rRNA: Forward: 5'-GTAACCCGTTGAACCCATT-3' and Reverse: 5'-CCATCCAATCGGTAGTAGCG-3'; LAMP1: Forward: 5'-GATACAGTGGGGTTTGTGGG-3' and Reverse: 5'-CTGTCGAGTGGCAACTTCAG-3'.

CatB activity

A total of 20 µL of cytoplasmic protein was added to 20 µL of CatB reaction buffer (100 mmol/L of sodium acetate, 200 mmol/L of NaCl, 4 mmol/L of EDTA, 10 mmol/L of DTT, and 10 mmol/L of Z-RR-AMC) at 37 °C for 30 min. The fluorescence released was measured at 37 °C for 10 min using a cytofluorimeter plate reader with excitation and emission wavelengths of 355 and 450 nm, respectively. The fluorescence increment value within 10 min was regarded as the relative value of enzyme activity in 20 µL of cytosol protein. Protein concentration was assayed by BCA, and CatB activity in each sample was corrected according to the protein concentration.

Animal experiments

All procedures involving animals were reviewed and approved by the Local Ethics Committee for Animal Research Studies at Shanghai University of Traditional Chinese Medicine (IACUC protocol number: 20140911). The mice were maintained in a specific pathogen-free facility under controlled laboratory conditions (23 °C, 12 h light/12 h dark cycle, 50% humidity, and *ad libitum* access to food and water) for 2 wk prior to experimentation, and were treated humanely. Intra-gastric gavage was performed on conscious animals by using straight gavage needles that were appropriate for the animal size (20 g of body weight: 22 gauge, 1 inch length, 1.25 mm ball diameter). In total, 24 male BALB/c mice were initially and randomly divided into four groups: control group ($n = 6$), CCl₄-treated group ($n = 6$), Sal B/CCl₄-treated group ($n = 6$), and N-acetylcysteine (NAC)/CCl₄-treated group ($n = 6$).

Before CCl₄ injection, the mice in the control and CCl₄ groups were injected intragastrically with 0.9% NaCl. The mice in the Sal B/CCl₄-treated and NAC/CCl₄-treated groups were intragastrically administered with Sal B and NAC at a dosage of 10 mg/kg body weight and 100 mg/kg body weight, respectively, for 3 d. The mice in the CCl₄-treated, Sal B/CCl₄-treated, and NAC/CCl₄-treated groups were given intraperitoneal injections of 5% CCl₄ (10 µL/g weight), and the mice in the control group were intraperitoneally injected with olive oil (10 µL/weight). All mice were sacrificed on day 7 of the experiment. The livers were excised, and samples were obtained for the following investigation.

Hematoxylin and eosin staining

The fresh liver tissue samples were fixed in 10% formalin and embedded in paraffin. Then, the samples

were cut into 4 µm thick sections and stained with hematoxylin and eosin (H and E) staining solution. The stained sections were subject to visualization of histopathological changes under a light microscope.

Liver function and malondialdehyde

Serum alanine aminotransferase (ALT) activity was assayed according to the recommendations of the Institute of Biological Products Nanjing Jiancheng (Nanjing, China). The malondialdehyde (MDA) in the tissues was determined according to the manufacturer's instructions (Cayman, Ann Arbor, MI, United States).

Statistical analysis

All data are presented as mean ± SD. Statistical analysis was performed using Student's *t*-test and one-way analysis of variance. Statistical significance was set to $P < 0.05$. At least three independent experiments were performed for each condition.

RESULTS

Sal B attenuates H₂O₂-induced apoptosis/death and restores the inhibition of proliferation

Cells were exposed to Sal B at concentrations of 0, 1, 10, 100, 200 and 400 µmol/L for 24 h to assess the safety of Sal B. The results revealed that Sal B concentrations from 1 µmol/L to 400 µmol/L were not cytotoxic to cells (Figure 1A). The inhibition rate of 500 µmol/L of H₂O₂ in cell viability was approximately 50%. However, Sal B effectively revised H₂O₂-inhibited cell viability at different concentrations, ranging from 5 µmol/L to 40 µmol/L (Figure 1B).

As shown in Figure 1C, in the untreated control, only 3.98% of the cells were late apoptotic cells and 0.45% were dead cells. By contrast, in the H₂O₂-treated group, 19.38% of the cells were late apoptotic cells and 3.72% were dead cells. This result indicates an approximately 5-fold increase in the number of late apoptotic or dead cells in the H₂O₂-treated group compared with the untreated control group. After 12 h of treatment with 10 µmol/L of Sal B and 500 µmol/L of H₂O₂, 6.82% of the cells were late apoptotic cells and 0.92% were dead cells. At the 24 h time point, 5.94% of the cells were late apoptotic cells and 0.75% were dead cells in the Sal B treatment group. These results demonstrate that Sal B protected hepatocytes from H₂O₂-induced apoptosis and death.

Instead of thymidine, BrdU was incorporated into the nuclear DNA during the S-phase of the cell cycle to evaluate cell proliferation^[16]. As shown in Figure 2, after exposure to H₂O₂, the number of BrdU-positive cells significantly decreased to 50.1% of the control. However, pretreatment with 10 µmol/L of Sal B significantly increased the number of BrdU-positive cells to 68.6% of the control.

Sal B exerts an anti-oxidative effect

Proteins are the major target of ROS because amino

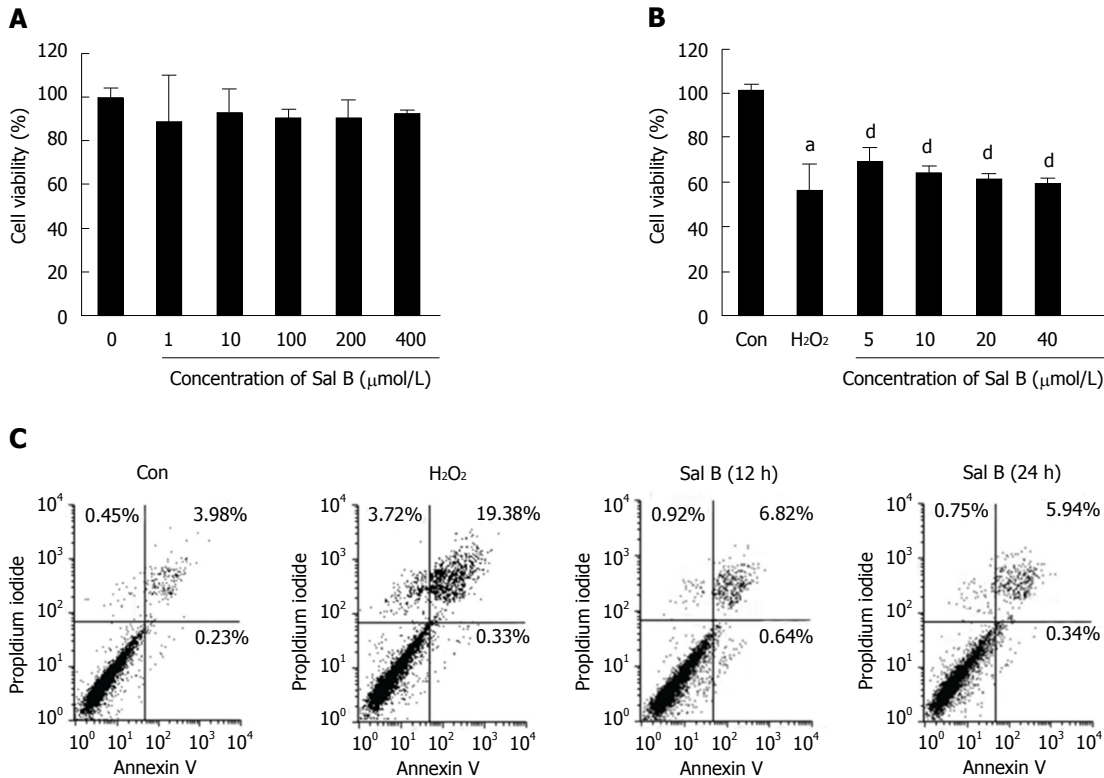


Figure 1 Effect of Sal B on apoptosis and death. A: The viability of hepatocytes treated with different concentrations of Sal B for 24 h; B: The viability of hepatocytes treated with different concentrations of Sal B for 22 h following induction by H₂O₂ for 2 h; C: The Annexin V/PI assay with FACS. All data are presented as mean ± SD. Statistical analysis was performed using Student's *t*-test (^a*P* < 0.05 vs Con; ^d*P* < 0.01 vs H₂O₂).

acids are susceptible to oxidation. Carbonylation is the most common oxidative alteration of proteins. The results in Figure 3 demonstrate that the amount of PCC in cells increased by nearly 2-fold after H₂O₂ treatment. However, with or without H₂O₂, Sal B reduced the content of PCC in the cells. Evidently, Sal B diminished the oxidative stress of cells.

Sal B protects the lysosome membrane

LAMP1 is highly expressed in lysosomes, and its primary localization is at the endosome-lysosomal membrane^[17]. The results of this study revealed that LAMP1 mRNA and protein levels significantly decreased with H₂O₂ treatment. By contrast, for cells pretreated with Sal B or NAC, either the LAMP1 mRNA level or the protein level increased (Figure 4A-C). LysoTracker green, a lysosomal marker that is selectively retained in the partially acidic lysosome and reflects lysosomal membrane permeability^[18], was used to evaluate the integrity of the lysosome membrane. The intensity of green fluorescence decreased with H₂O₂ treatment. However, the fluorescence intensity increased with Sal B pretreatment. NAC was used as a positive antioxidant and showed a similar result as Sal B (Figure 4D and E).

Sal B antagonizes H₂O₂-induced LMP and CatB/D leakage

To further investigate whether catalytic enzymes in the lysosome leaked into the cytosol and caused the inhibition of cell viability, the content and activity of

CatB and CatD in the cytosol and entire cells were assessed. The contents of CatB and CatD in the cytosol increased significantly after exposure to H₂O₂ compared with that in the control (Figure 5A and C). By contrast, the content detected in the entire cells remained unchanged (Figure 5B and D). Sal B and NAC suppressed cytoplasmic CatB and CatD contents (Figure 5A-D). The CatB activity in the cytosol obviously increased with H₂O₂ treatment. However, either Sal B or NAC suppressed cytoplasmic CatB activity (Figure 5E).

AO is a cell-permeable fluorescent dye with a concentration-dependent fluorescence emission that ranges from red (when the concentration is high in the lysosome) to green (when the concentration is low in the cytosol). AO is positively charged at a low pH, which consequently hinders the ability of AO molecules to cross the vesicular membrane and escape into the surrounding cytoplasm. In the case of LMP, AO is released from the lysosomes into the cytosol, where it emits an enhanced green fluorescence that can be monitored by fluorescence microscopy^[18]. After the delivery of H₂O₂ to the cells, a significant reduction in red fluorescence was observed, suggesting that H₂O₂ induced LMP at the cellular level (Figure 6A and C). By contrast, Sal B and NAC were associated with an increase in red fluorescence and concomitant suppression of green fluorescence compared with the situation in the H₂O₂-treated cells (Figure 6A-C).

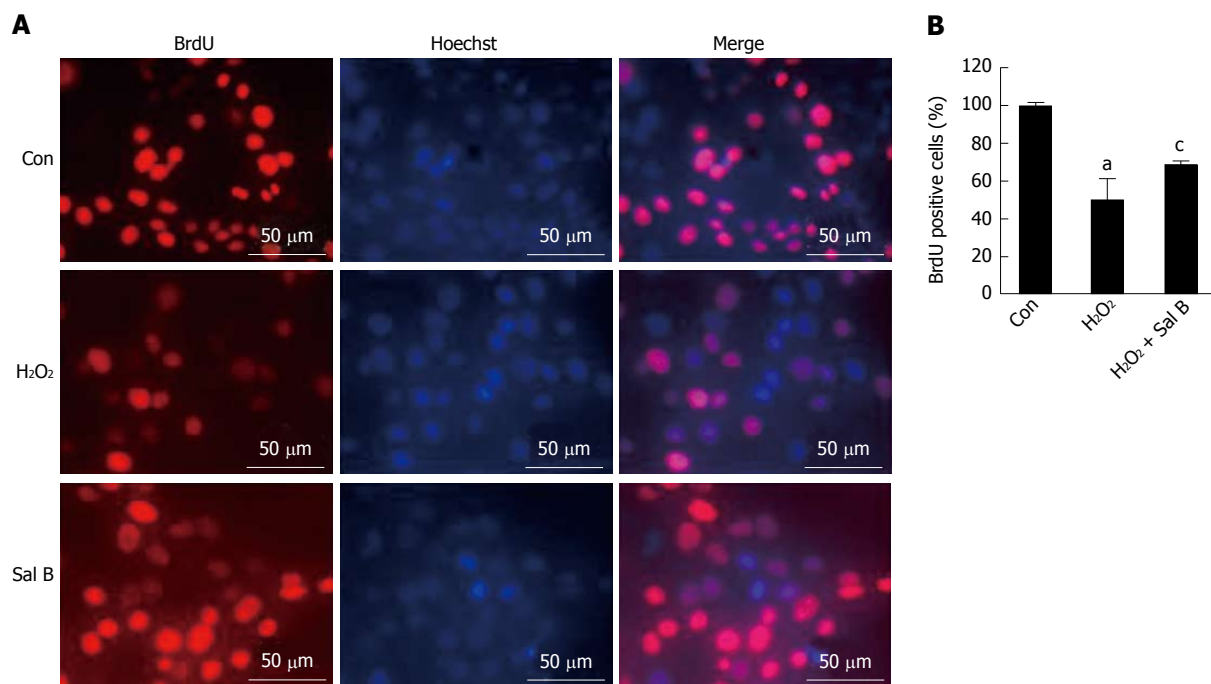


Figure 2 Effect of Sal B on cell proliferation. A: BrdU incorporation assay, with 4000 cells seeded in a 96-well plate being treated with 10 $\mu\text{mol/L}$ of Sal B for 24 h and with 500 $\mu\text{mol/L}$ H₂O₂ for an additional 2 h. A final concentration of 10 $\mu\text{mol/L}$ BrdU was supplemented in the medium for 24 h (scale bar = 50 $\mu\text{mol/L}$); B: Quantification of BrdU incorporation assay. All data are presented as mean \pm SD. Statistical analysis was performed using Student's *t*-test (^a $P < 0.05$ vs Con; ^c $P < 0.05$ vs H₂O₂).

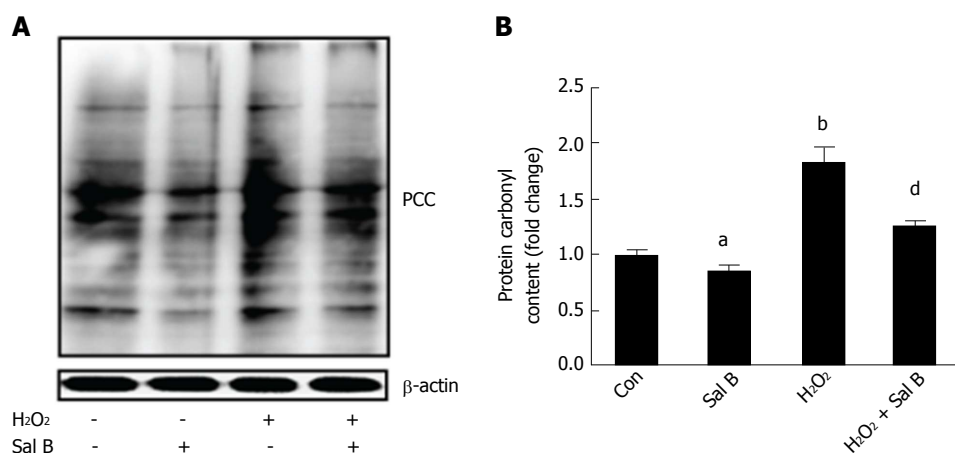


Figure 3 Effect of Sal B on protein carbonyl content. A: The expressions of protein carbonyl content in hepatocytes detected by western blot; B: Quantitative analysis of optical densities of protein carbonyl content and normalized to the expression of β -actin (^a $P < 0.05$, ^b $P < 0.01$ vs Con; ^d $P < 0.01$ vs H₂O₂).

Sal B attenuates lysosomal damage after CCl₄-induced acute liver injury

Hematoxylin-eosin staining revealed a normal hepatic architecture within the clear hepatic lobule and the absence of dead hepatocytes in the control group of mice. By contrast, a large area of dead hepatocytes was observed in the central vein area of the CCl₄-treated mice. Compared with the CCl₄-treated group, the CCl₄ group treated with Sal B or NAC exhibited improvements in CCl₄-associated pathological changes (Figure 7A). ALT has been used as an index of liver injury in clinics and *in vivo* studies^[19]. The mice in the CCl₄-treated group exhibited signs of liver injury. The ALT activity in these mice was significantly higher than

that in the control group ($P < 0.01$), although Sal B or NAC interventions reduced the activity of ALT ($P < 0.01$) (Figure 7B).

MDA level has been widely used as a marker of lipid peroxidation in cells and body fluids in clinical and experimental studies^[20]. The MDA concentration in the CCl₄-treated group was significantly higher than that in the control group ($P < 0.01$). Pretreatment with Sal B or NAC reduced the MDA levels ($P < 0.01$) (Figure 7C).

LAMP1 is used as a lysosomal marker and contributes to the protection of the lysosomal membrane and its proteins from hostile constituents, such as hydrogen ions and proteases^[21]. LAMP1 protein levels decreased significantly after CCl₄ treatment ($P < 0.01$),

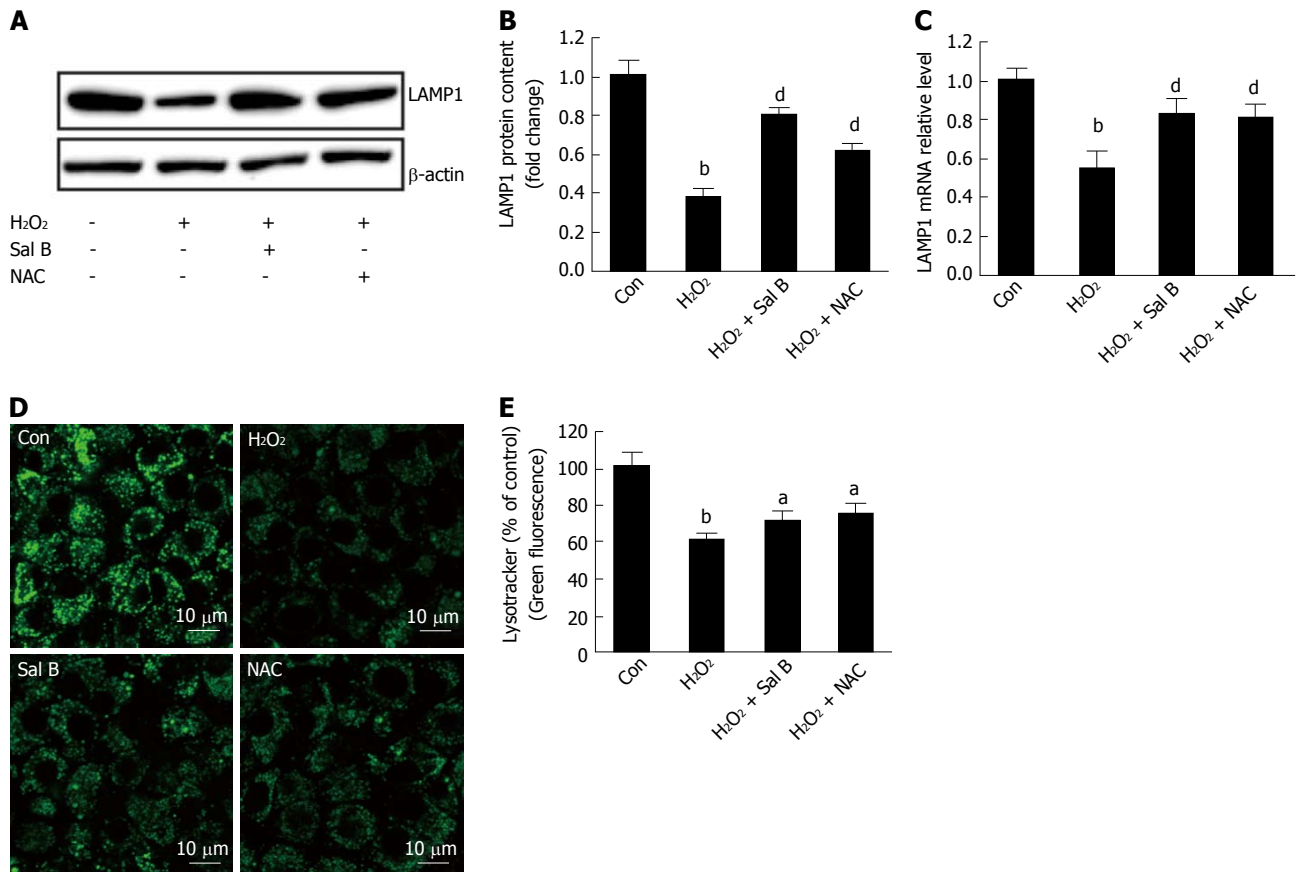


Figure 4 Effect of Sal B on lysosomal membrane permeability. A: The expressions of LAMP1 in hepatocytes detected by western blot; B: Quantitative analysis of optical densities of LAMP1 and normalized to the expression of β -actin; C: Levels of LAMP1 mRNA were measured in cells by qRT-PCR; D: LysoTracker green staining; E: Quantification of the LysoTracker green staining (^b $P < 0.01$ vs Con; ^a $P < 0.05$; ^d $P < 0.01$ vs H₂O₂).

and Sal B or NAC pretreatment increased the protein levels ($P < 0.01$) (Figure 8A and D).

To further confirm if CCl₄ induces liver injury triggered by lysosomal disintegration, the leakage of lysosomal enzymes into the cytosolic fractions was investigated. Surprisingly, the contents of CatB and CatD in the cytosol increased significantly after CCl₄ treatment ($P < 0.01$), and Sal B or NAC pretreatment decreased the protein levels ($P < 0.01$) (Figure 8B and E). By contrast, the content detected in the entire cells remained unchanged (Figure 8C and F).

DISCUSSION

Hepatocytes, which are in charge of the detoxification of exogenous xenobiotics, drugs, viral infections and chronic alcoholism, have the largest amount of cells in the liver. Regardless of etiology (*e.g.*, toxic chemicals, drugs and virus), hepatocyte injury/death is involved in all pathological stages of hepatitis, fibrosis, cirrhosis, and even liver cancer^[22]. Therefore, hepatocytes act as a common therapeutic target in various liver diseases. Sal B is a major water-soluble polyphenolic antioxidant in *Radix Salviae Miltiorrhizae* and has been extensively used in clinics in China^[23-25]. Sal B possesses efficient radical scavengers and antioxidants^[14,26,27]. Previous

reports have shown that Sal B exerts therapeutic effects in treating liver fibrosis^[28,29]. In this study, the effect of Sal B on H₂O₂-induced hepatocytes was investigated *in vitro*.

CCl₄ is a potent hepatotoxicant that is commonly used to generate liver injury in animal models^[30]. In hepatocytes, CCl₄ is metabolized by cytochrome P450 to produce highly reactive free radicals^[31]. Therefore, oxidative stress is one of the main mechanisms of CCl₄-induced liver injury^[7]. The present results suggest that the levels of lipid peroxidation in livers and serum ALT activity in hepatocytes after cell injury/death increased after CCl₄ treatment. The results of hepatic histological staining in CCl₄-treated mice also showed large areas of dead hepatocytes.

LAMP1 is a highly glycosylated protein located in the lysosomal membrane; it controls the integrity of the lysosomal membrane^[21]. In this study, LAMP1 detected in the livers was low, and CatB and CatD levels in the cytoplasm increased but were unchanged in whole tissues after CCl₄ treatment. However, after Sal B treatment, the structure of the liver tissues returned to normal. Meanwhile, serum ALT decreased, the levels of lipid peroxidation diminished, and the levels of LAMP1 were enhanced in the livers. Thus, the hepatotoxicant effect of CCl₄ is attributed to its induction

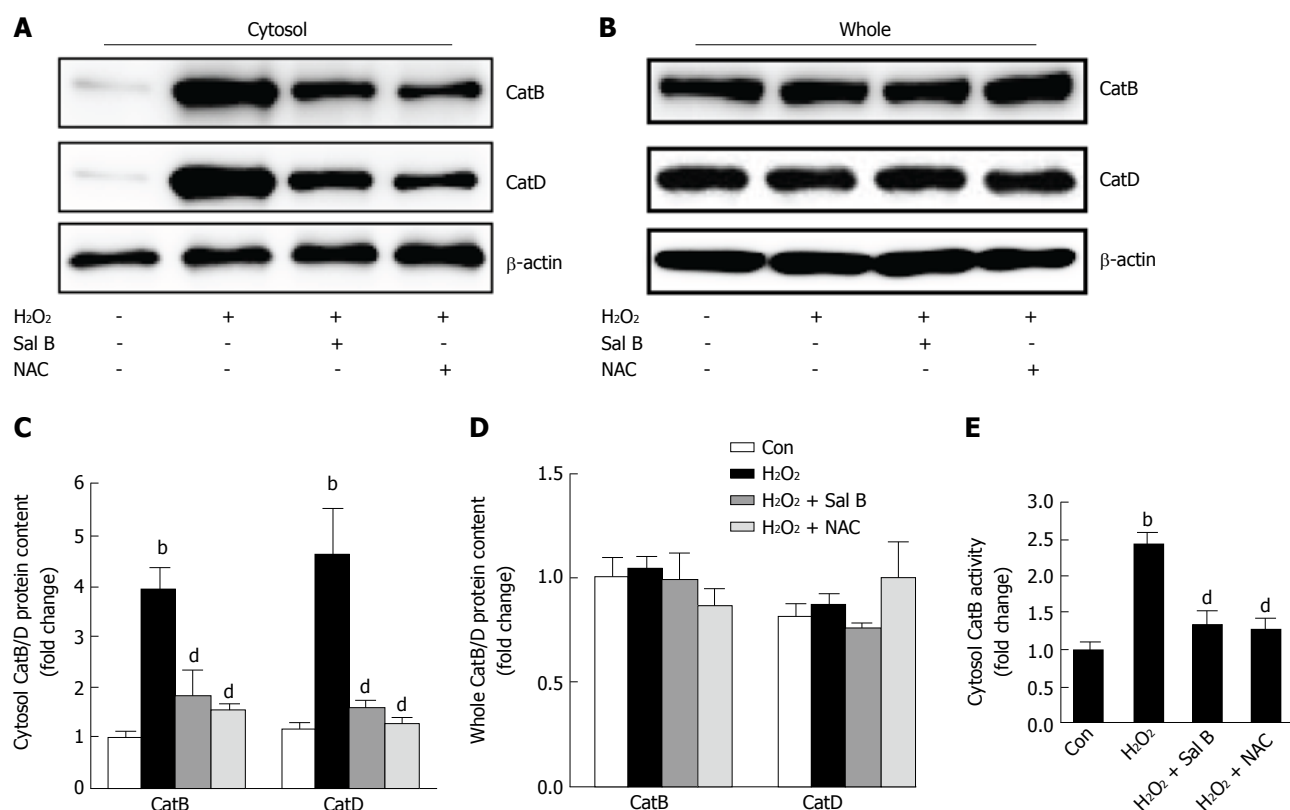


Figure 5 Effect of Sal B on the leakage of CatB/D into the cytosol. A and B: CatB/D levels in the cytosol/whole lysate were measured by western blot; C and D: Quantitative analysis of optical densities of CatB/D in the cytosol/whole lysate and normalized to the expression of β -actin; E: CatB activity analysis was performed with chemiluminescence detection. (^b $P < 0.01$ vs Con; ^d $P < 0.01$ vs H_2O_2).

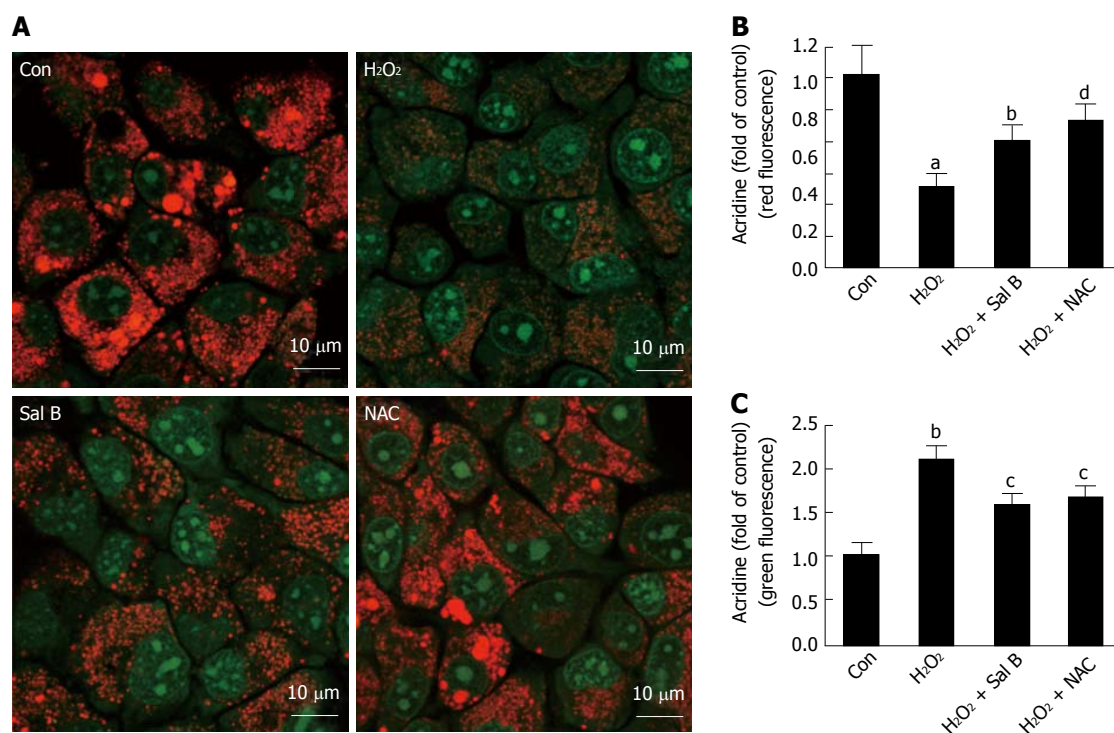


Figure 6 Effect of Sal B on lysosomal PH. A: AO staining; B: Quantification of the AO staining (^a $P < 0.05$, ^b $P < 0.01$ vs Con; ^c $P < 0.05$, ^d $P < 0.01$ vs H_2O_2).

of oxidative stress followed by lysosomal membrane permeabilization, which mediates hepatocyte injury/death. Sal B protected the livers by interfering with

lysosome membrane permeabilization.

Consistent with the *in vivo* results, H_2O_2 -induced cytotoxicity is a common method used for measuring

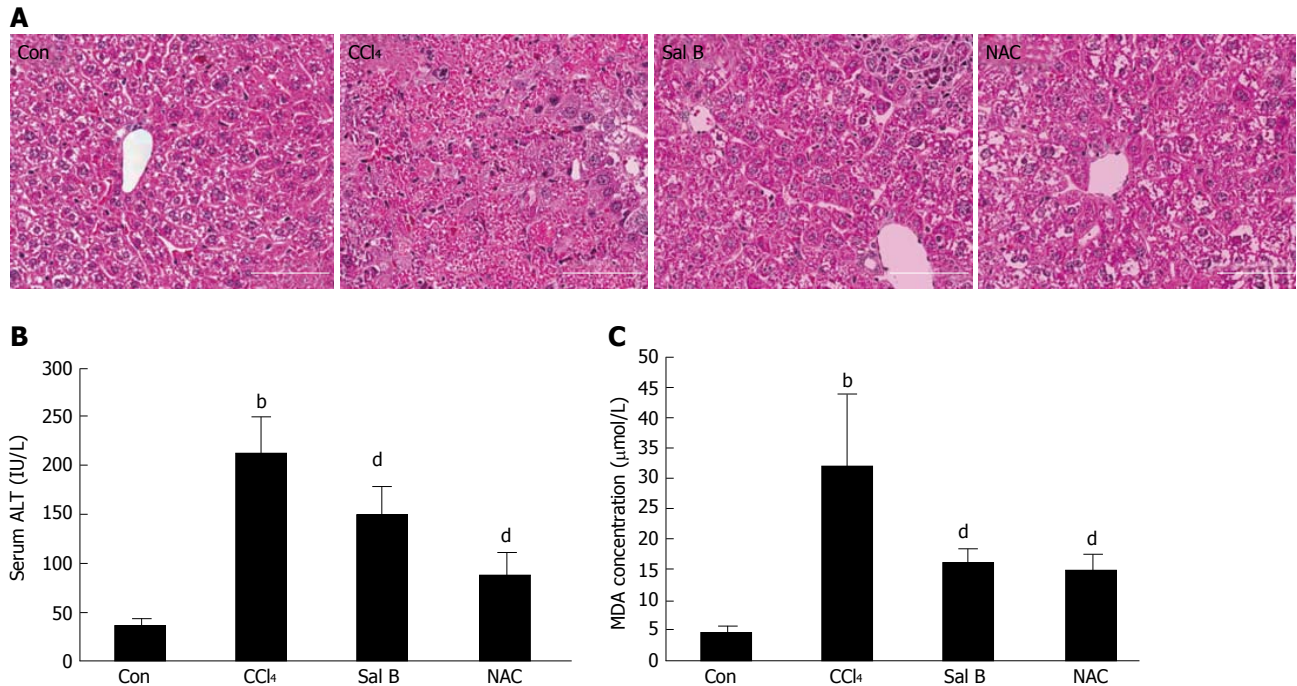


Figure 7 Effect of Sal B on CCl₄-induced liver injury. A: Hematoxylin-eosin staining is shown (scale bar = 100 μm). Con: control group ($n = 6$), CCl₄: CCl₄ injection group ($n = 6$), Sal B: CCl₄ injection and Sal B administration group ($n = 6$), NAC: CCl₄ injection and NAC administration group ($n = 6$); B: Serum ALT activity in mice; C: MDA content of liver tissues in mice. Statistical analysis was performed using Student's *t*-test (^b $P < 0.01$ vs Con; ^d $P < 0.01$ vs CCl₄). ALT: Alanine aminotransferase; MDA: Malondialdehyde.

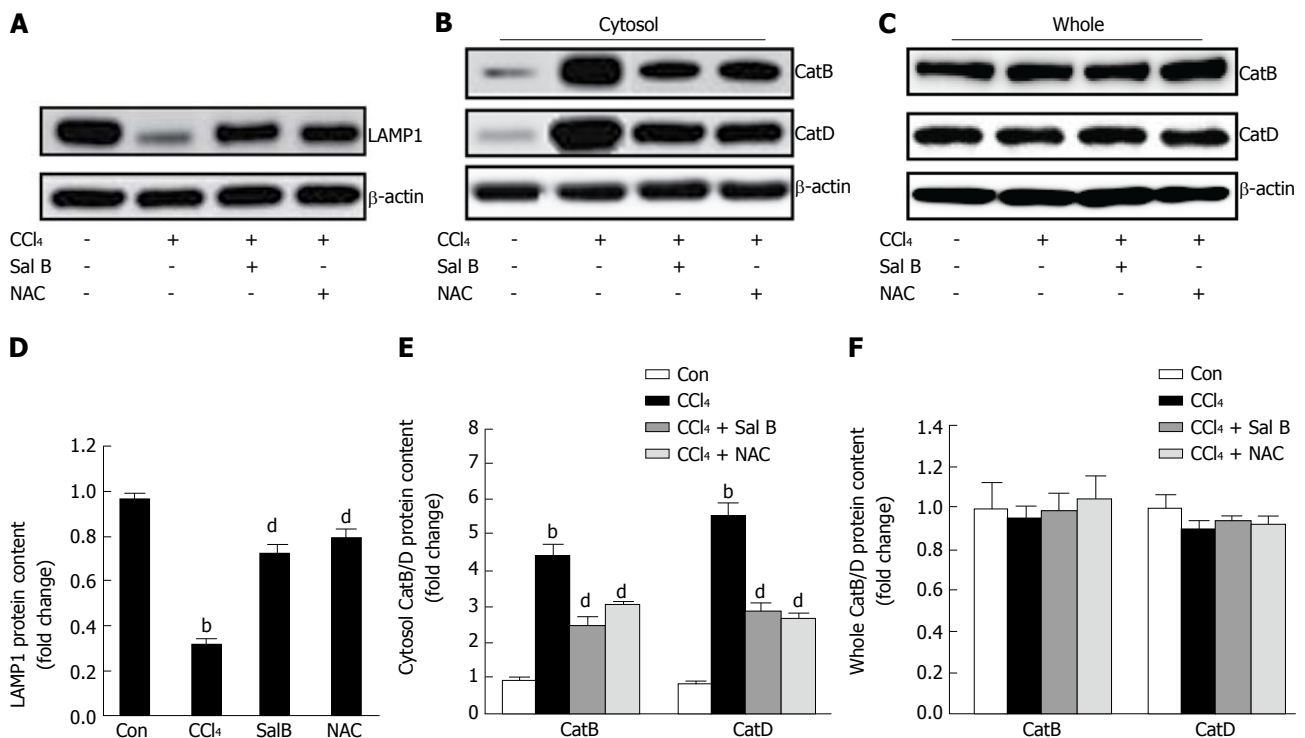


Figure 8 Effect of Sal B on LAMP1 and leakage of CatB to the cytosol. A: Expressions of LAMP1 in liver detected by western blot; B, C: CatB/D levels in the cytosol/whole lysate were measured by western blot; D: Quantitative analysis of optical densities of LAMP1 and normalized to the expression of β-actin. E, F: Quantitative analysis of optical densities of CatB/D in the cytosol/whole lysate and normalized to the expression of β-actin. All data are presented as mean ± SD. Statistical analysis was performed using Student's *t*-test (^b $P < 0.01$ vs Con; ^d $P < 0.01$ vs CCl₄).

potential antioxidants. In the *in vitro* study, treatments with 500 μmol/L of H₂O₂ for 2 h induced a decrease in cell viability, and the rate of inhibition was approximately

50%. This concentration was used for the subsequent experiments. After oxidative damage, cell viability was inhibited, and cell proliferation diminished. Sal B

decreased the number of apoptotic and necrotic cells and restored cell proliferation.

In cultured cells, a large amount of H₂O₂ diffuses into the lysosome and results in LMP^[32-34], which is attributed to the reduced H₂O₂-induced expression of LAMP1^[35]. Upon LMP, hydrolytic enzymes^[36,37] and protons^[38,39] in the lysosomes are released into the cytoplasm, resulting in acidification and triggering lysosome-induced cell death^[40]. Hepatocyte death is accompanied by an increase in LMP in acute liver injury^[41]. According to the cell type and dosages of the inducing factors, lysosomal apoptosis or necrosis occurs^[42]. Cathepsins, including CatB and CatD, exhibit catalytic activity for a few hours even under near-neutral pH conditions^[43]. CatB leakage from the lysosomes is also associated with apoptosis^[44,45].

The current study found that the levels of CatB and CatD in the cytoplasm increased considerably after exposure to H₂O₂ for 2 h. The activity of CatB in cytoplasm increased and reached nearly 2.5-fold. Meanwhile, the results of LysoTracker and AO staining confirmed that LMP occurred during H₂O₂ treatment. After Sal B treatment, the levels of CatB/D and CatB enzyme activity in cytoplasm decreased, and the fluorescence intensity of LysoTracker and AO in the lysosome was enhanced. Thus, leakage of lysosome contents was improved, lysosome membrane integrity was protected, and cell injury/death was inhibited.

NAC is a sulfhydryl donor antioxidant that contributes to the regeneration of glutathione^[46] and commonly used as a positive antioxidant control. (-)-epigallocatechin-3-gallate (EGCG) promotes the production of intracellular ROS upstream of LMP and cell death, and NAC protects against EGCG-mediated LMP by decreasing the level of ROS^[47]. Bxpc3 cell death is also involved in LMP and oxidative stress, which is protected against by NAC^[21]. In the present study, the content of CatB/D and CatB enzyme activity in the cytosol after NAC treatment showed a significant decrease compared with that in the H₂O₂ treatment group. The fluorescence intensity of LysoTracker Green and the LAMP1 level in the cells treated with NAC increased. These results show that NAC protected hepatocytes from H₂O₂-induced apoptosis and death by inhibiting LMP. The effects of NAC were similar to those of the antioxidant Sal B.

In sum, Sal B protected the integrity of the lysosomal membrane by up-regulating the expression of LAMP1, which reduced CatB/D leakage into the cytosol and protected hepatocytes from death.

COMMENTS

Background

Highly glycosylated lysosome-associated membrane proteins (LAMPs), especially LAMP1, on the inner surface of the lysosomal membrane serve as a barrier to soluble hydrolases and keep the integrity of membrane. The loss of membrane integrity leads to lysosomal membrane permeabilization (LMP), allowing release of the luminal contents into the cytosol, resulting in

cellular injury/death. Oxidative stress causes LMP in hepatocytes, leading to cellular injury/death. Salvianolic acid B (Sal B) performs anti-inflammatory and antioxidant functions and elicits protective effects on hepatocytes.

Research frontiers

Lysosomes filled with more than 50 soluble acid hydrolases are the cellular recycling center and play an important role in cells. Upon LMP, the leakage of enzymes from lysosomes into cytosol causes cellular injury/death. Hepatocytes are the most frequent cells in the liver and the target of oxidative stress, and the resulting injury/death is involved in all liver diseases. Herein, hepatocytes served as a target of therapy.

Innovations and breakthroughs

Upon H₂O₂ attacking, the expression of LAMP1 was significantly decreased and destroyed the integrity of the lysosome membrane in hepatocytes and contributed to cellular injury/death. Sal B elicits protective effects on hepatocytes by scavenging reactive oxygen species and enhancing LAMP1 expression.

Applications

Sal B can protect lysosome membrane integrity by diminishing oxidative stress and can elicit a protective effect on hepatocytes. This study provided a theoretical basis for the clinical application of Sal B to treat liver diseases.

Terminology

LMP is the leakage of lysosome contents due to the destruction of lysosome membrane integrity and causes cell injury/death. Sal B is a major water-soluble polyphenolic antioxidant in *Radix Salviae Miltiorrhizae* and also elicits protective effects on hepatocytes.

Peer-review

The paper is very interesting. Moreover, whereas the authors target the effect of Sal B on the protection of the integrity of the lysosomal membrane by upregulating the expression of LAMP1, via reduction of cathepsin B/D leakage into the cytosol, and protects hepatocytes from apoptosis.

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