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ORIGINAL ARTICLE

Salvianolic Acid B Delays Hepatolithiasis through Inhibiting the Fibrosis of Intrahepatic Biliary Epithelial Cells

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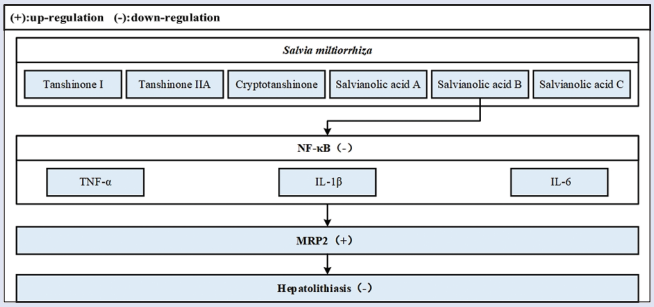
ABSTRACT

Background: Hepatolithiasis (HL) is one of the common diseases in hepatobiliary surgery, and bile duct fibrosis is the key step in the pathogenesis of HL. *Salvia miltiorrhiza* (*S. miltiorrhiza*) has an inhibitory effect on bile duct fibrosis, but its molecular mechanism remains unclear. **Objectives:** Our research aimed to clarify the molecular mechanism of *S. miltiorrhiza* on HL. **Materials and Methods:** The rats were fed with salvianolic acid B and salvianolic acid B-free lithogenic diet (LD) for 4 weeks to observe the degree of bile duct fibrosis. The expression levels of *TNF*, *IL1B*, *IL6*, and *MRP2* in HIBEC stimulated by taurodeoxycholic acid (TDCA) were measured by RT-qPCR and ELISA. E-cadherin and Vimentin expression in HIBEC was detected using immunofluorescence and Western blot. Cell proliferation was detected by CCK8. **Results:** The fibrosis of the bile duct was the key step of HL. The NF-κB pathway was activated and MRP2 was expressed low in intrahepatic biliary epithelial cells surrounding bile duct stones. Through experiments, salvianolic acid B (Sal B) delays HL via the NF-κB/MRP2 axis. **Conclusion:** In this research, we confirmed that salvianolic acid B inhibited the HL via the NF-κB/MRP2 axis.

Key words: Epithelial-mesenchymal transition, hepatolithiasis, MRP2, NF-κB, salvianolic acid B

SUMMARY

- Hepatolithiasis is a common disease of intrahepatic calculi in Asia. *Salvia miltiorrhiza* is a traditional Chinese herb. In this experiment, we found that as a major ingredient in *Salvia miltiorrhiza*, Salvianolic acid B can delay the development of hepatolithiasis by regulating the NF-κB/MRP2 axis.



Abbreviations used: ABC: ATP-binding cassette; CTS: Cryptotanshinone; EMT: Epithelial-mesenchymal transition; HIBEC: Human intrahepatic biliary epithelial cells; HL: Hepatolithiasis; IHC: Immunohistochemical; LD: Lithogenic diet; Sal A: Salvianolic acid A; Sal B: Salvianolic acid B; Sal C: Salvianolic acid C; *S. miltiorrhiza*: *Salvia miltiorrhiza*; Tan I: Tanshinone I; Tan IIA: Tanshinone IIA; TCM: Traditional Chinese medicine; TDCA: Taurodeoxycholic acid.

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INTRODUCTION

Hepatolithiasis (HL) is the presence of gallstones in the biliary ducts of the liver, which is rare in western countries but is prevalent and common in East Asia.[1,2] As a type of gallstone disease, HL is characterized by the presence of gallstones in the left and right hepatic ducts proximal to the intrahepatic bile ducts.[3] The deterioration of HL complications includes biliary tract infection, biliary cirrhosis, and even cholangiocarcinoma.[4,5] Genetic, diet, and environmental factors are suspected for the pathogenesis of HL, but the specific mechanism of HL remains unclear. Although the principles and techniques of surgical treatment of HL continue to develop, residual and recurrence of stones are still the main factors affecting long-term prognosis.[6] The current studies indicate that bile duct stenosis, cholestasis, and bile duct infection have been recognized as the main factors for HL. Among them, bile duct fibrosis has been reported as a key step in the pathogenesis of HL.[7,8]

NF-κB is a crucial transcription factor controlling inflammation, immunity, and apoptosis.[9] The current studies showed that the activation of NF-κB is generally accompanied by the massive production of inflammatory factors.[10-13] Furthermore, the NF-κB signaling pathway accelerates the epithelial-mesenchymal transition (EMT) process through a variety of pathways including up-regulation of the expression of pro-inflammatory factors.[14] Transmembrane transporters from the ATP-binding cassette (ABC) family mediate the entry and elimination of endogenous compounds and exogenous substances from liver cells.[15] MRP2 is one of the superfamilies of ABC transporters located on the side of the hepatocellular biliary canaliculi and functions in biliary transport.[16] The abnormal expression of MRP2 in epithelial cells leads to cholestasis and fibrosis.[17,18] The activation of NF-κB has been reported to significantly inhibits the FXR/RXR transactivation of the MRP2 promoter.[19]

Salvia miltiorrhiza (*S. miltiorrhiza*) as a common traditional Chinese medicine (TCM) has now attracted interest in the therapy of various

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diseases.[20] *S. miltiorrhiza* has been reported to promote blood circulation, eliminate blood stasis, relieve pain, dilate coronary arteries, lower blood sugar, and is an anti-inflammatory.[21,22] Besides, *S. miltiorrhiza* is a compound preparation for treating gallstones, choledocholithiasis, and cholecystolithiasis, but its specific mechanism remains unclear.[23]

Hence, our study revealed the effect of *S. miltiorrhiza* on the development of HL. Salvianolic acid B (Sal B) showed an inhibition effect in taurodeoxycholic acid (TDCA)-induced HIBEC and Sal B-rich diet showed a protective effect against HL. These findings lead us to further understand the regulation mechanism of Sal B on the progress of HL and provide new sights for the therapy of HL.

MATERIALS AND METHODS

Chemicals

S. miltiorrhiza and its ingredients (Salvianolic acid A (Sal A), Salvianolic acid B (Sal B), Salvianolic acid C (Sal C), Tanshinone I (Tan I), Tanshinone IIA (Tan IIA), Cryptotanshinone (CTS)) were purchased from Chengdu PUSH Biotechnology Co. Ltd. (China). The concentrations used in the corresponding experiments were 0, 100, 200, 500, 1,000, 10,000 μM and were freshly diluted using the basal medium.

Animals

All experimental procedures were authorized by the Harrison International Peace Hospital and were conducted based on the instructions of care and use for laboratory animals. Healthy male SD rats aged 8 weeks were obtained from the Beijing HFK Biotechnology Company Limited (Beijing, China) and placed in environmentally controlled plastic cages (22 ± 2°C, a 12 h light cycle). We arbitrarily divided the rats into two groups after partial ligation of the common bile duct: lithogenic diet (LD) in rats (n = 5) and LD with Sal B in rats (n = 5). The concentration of each component was calibrated, and the rats were weighed every day before administration. After 4 weeks of treatment with a different component diet, rats were euthanatized according to the AVMA Guidelines for the Euthanasia of Animals 1 day after the last dose. The liver tissues of rats were harvested, and the fibrotic degree of the intrahepatic bile duct was evaluated using histological analysis.

Cell culture

All cell lines were acquired from ATCC (USA). We cultured HEK293 and HIBEC in DMEM (USA) and added 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, USA) and nurtured them in a 5% CO_2 incubator at 37°C.

CCK-8 assays

Cell Counting Kit-8 (CCK-8) was acquired from Dojindo. HEK293 cells and HIBEC were cultured (2×10^3 per well) in 96-well plates overnight, respectively. We treated cells with *S. miltiorrhiza* for 24 or 72 h. According to the manufacturers' instructions, CCK-8 was used to perform cell viability at 24 and 72 h. Then 96-well plate reader at 450 nm was used to measure the absorbency of cells to evaluate the cell viability.

RT-qPCR assays

Taking out the culture plates, the cells were washed with PBS. After treatment, we extracted total RNA from glioma cells by Trizol reagent (Life Technologies, NY, USA). The reverse transcription of cDNA was performed by the PrimeScript™RT kit (Takara, Japan). PCR primers were synthesized by GenePharma (ShangHai Gene Pharma, Shanghai, China) and the sequences were listed. The expression detected by SYBR Premix Ex Taq II (TaKaRa, Japan).

ELISA

After ventilation, the right lung tissue was collected and ground, followed by centrifugation to collect the supernatant. Concentrations of TNF-α (RTA00), IL-1β (RLB00), IL-6 (R6000B), and MRP2 were detected by ELISA kits (R&D Co., Ltd., Minneapolis, MN, USA). All operations were strictly as per kit instructions.

Western blot assays

The protein samples were extracted and separated by 10% SDS-PAGE gel, and then transferred to a PVDF membrane (Millipore, USA). The membrane was then blocked with 5% skim milk and cultured overnight at 4° with the following primary detection antibodies (Abcam, UK). We washed three times with TBS-T and the membranes were cultured with the secondary antibody at 24°C for 1 h. Western blots were pictured using an ECL reagent (Pierce, USA) and the density was verified using ImageJ software (NIH, USA).

Immunofluorescence

We detect the expression of E-cadherin and Vimentin in HIBEC by using immunofluorescence. The slides were washed with PBS three times in the culture plate for 3 min each time. Then, it was fixed with 4% paraformaldehyde for 35 min and permeabilized with 0.5% Triton X-100 for 10 min. Overnight staining was done with E-cadherin rabbit antibody (Novus) (1:100) or Vimentin rabbit antibody (Abcam) at 4°C, followed by 1 h staining at normal temperature with a fluorescence secondary antibody (Bitianyun Company, China). We washed the cells with PBS among antibody staining. Finally, DAPI (Bitianyun Company, China) staining solution was added at room temperature for more than 15 min and was installed on the slides with an anti-fading patch (Bi Tianyun Company, China). The slides were stored at −20°C. The images were taken from a fluorescence microscope (Leica).

Histopathological and Immunohistochemical (IHC) studies

The experiment rats were euthanized and then the needed tissues were removed, put into washing, and fixed (4% paraformaldehyde). We fixed the tissue in paraffin and sectioned it after fixation. Before IHC, we dewaxed and heated all sections in sodium citrate buffer (pH 6.0) diluted 100 times. We treated all sections with 3% H_2O_2 at room temperature (15 min). After inactivation, we cultured the serum at room temperature and blocked it for 10 min. IHC staining was performed with primary antibody E-cadherin and primary antibody Vimentin. After incubation with the secondary antibody, it was inoculated with the SABC kit (ZSGB-Bio, Beijing, China) and diaminobenzidine (DAB; Sigma, St. Louis, Mo., USA) to examine the expression of these markers in cells. The sections were stained with hematoxylin. Pictures were taken by Baumer digital camera with Olympus BX51 microscope (Olympus, Tokyo, Japan). The German semiquantitative method was used to evaluate the IHC scores.

Statistical analysis

The data were analyzed and visualized using GraphPad Prism 8.0 (USA). Comparisons between the two groups were performed using the Student's t-test. Comparisons between multiple groups were performed using one-way ANOVA. Each experiment was repeated at least three times independently. The data were expressed as average ± SD, (** $P < 0.01$).

RESULTS

EMT is closely involved in TDCA-induced HIBEC fibrosis

The previous studies have shown that the fibrosis of the bile duct is a critical event during the occurrence and progression of HL.[8] Here, we used HIBEC as a research cell model, and logarithmic growth phase cells were chosen for the experiment. TDCA was used to induce the fibrosis of HIBEC to mimic the early stage of HL. The expressions of E-cadherin and Vimentin in HIBEC after TDCA stimulation were evaluated by immunofluorescence staining. The fluorescence intensity of E-cadherin was significantly reduced in TDCA-induced HIBEC, while that of Vimentin was significantly enhanced, suggesting the EMT process was accelerated in TDCA-induced cells [Figure 1a and b]. Western blot also revealed a substantial reduction expression in E-cadherin, whereas Vimentin was significantly increased in TDCA-induced HIBEC [Figure 1c]. The results showed that EMT is integrally involved in the TDCA-induced HIBEC fibrosis. Fibrotic HIBEC lost epithelial features and acquired a mesenchymal phenotype.

S. miltiorrhiza inhibits the fibrosis of HIBEC in a dose-dependent manner

To explore the effect of *S. miltiorrhiza* on HIBEC fibrosis, we first performed CCK-8 assay to evaluate the effect of *S. miltiorrhiza* on HIBEC cell viability at different concentrations. The results showed that *S. miltiorrhiza* were non-toxic to HIBEC within the concentration of 10,000 μM for 1 day [Figure 2a]. Similarly, this non-toxicity did not alter when the treatment was extended for 3 days [Figure 2b]. Next, TDCA-stimulated HIBEC was treated with different concentrations of *S. miltiorrhiza* for 24 h. Six different groups were set according to the screening concentration of *S. miltiorrhiza*. Western blot analysis showed that the expression of E-cadherin in TDCA-stimulated HIBEC was significantly increased

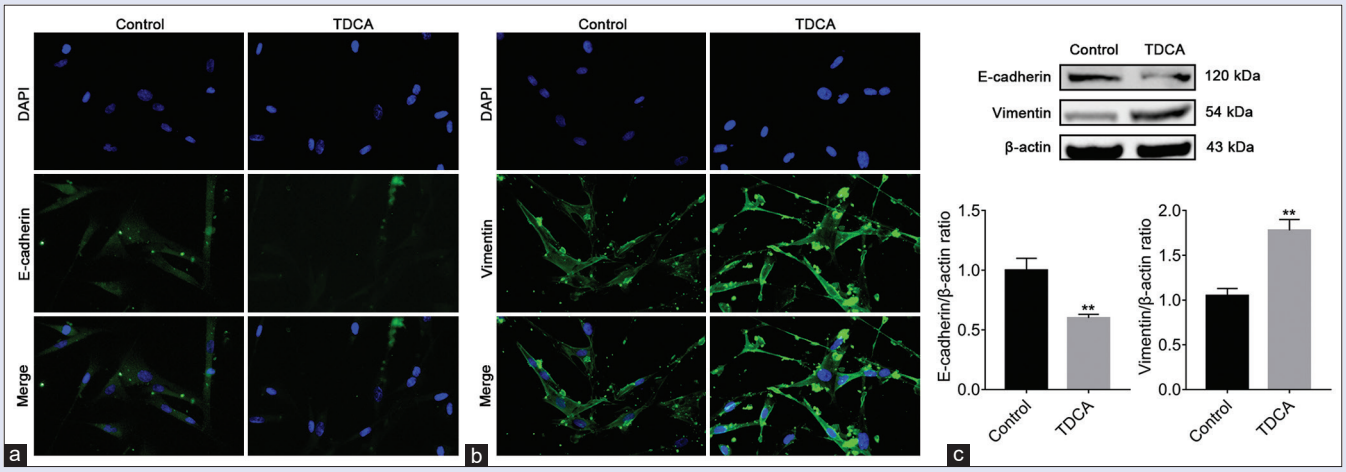

Figure 1: EMT is closely involved in TDCA-induced HIBEC fibrosis**.** The expression of (a) E-cadherin and (b) Vimentin in HIBEC specimens was detected by immunofluorescence assay. (c) Western blot analysis of E-cadherin and Vimentin in TDCA-induced HIBEC. The data are expressed as average ± SD, (** $P < 0.01$)

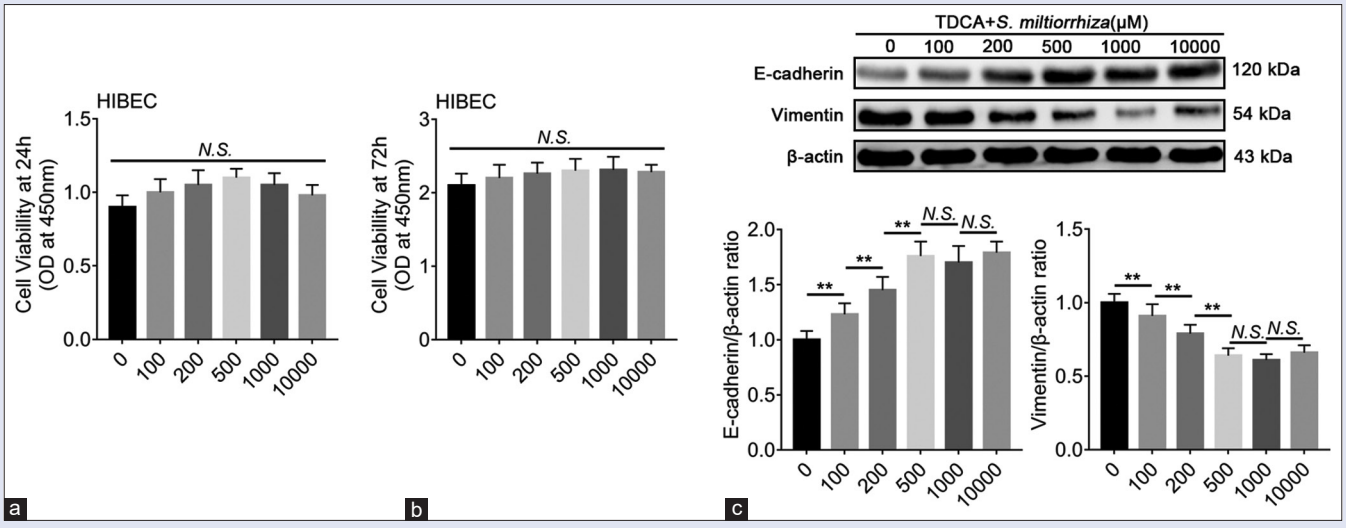

Figure 2: *S. miltiorrhiza* inhibits the fibrosis of HIBEC in a dose-dependent manner. CCK-8 analysis of cell viability at (a) 24 h or (b) 72 h of HIBEC treated with six different dosages of *S. miltiorrhiza* (0, 100, 200, 500, 1,000, and 10,000 μM). (c) Western blot analysis of E-cadherin and Vimentin in HIBEC induced by TDCA treated with different concentrations of *S. miltiorrhiza*. The data are expressed as average ± SD, (** $P < 0.01$)

in the range of 0 to 500 μM *S. miltiorrhiza*, while it stopped in the range of increasing from 500 to 10,000 μM *S. miltiorrhiza*. Notably, the expression of Vimentin showed the opposite trend to that of E-cadherin [Figure 2c]. Together, our data suggested that within a concentration of 500 μM, *S. miltiorrhiza* inhibited the fibrosis of HIBEC in a dose-dependent manner. This response reached saturation after the concentration of *S. miltiorrhiza* was greater than 500 μM.

S. miltiorrhiza alleviates the fibrosis of HIBEC by inhibiting NF-κB/MRP2 axis

The previous studies demonstrated that MRP2 is an important factor regulated by NF-κB pathway.[24] NF-κB pathway-activating is generally accompanied by overexpression of *TNF, IL1B, and IL6*.[11] To determine whether TDCA-induced HIBEC affects the NF-κB/MRP2 axis, we first performed RT-qPCR to detect the mRNA expression

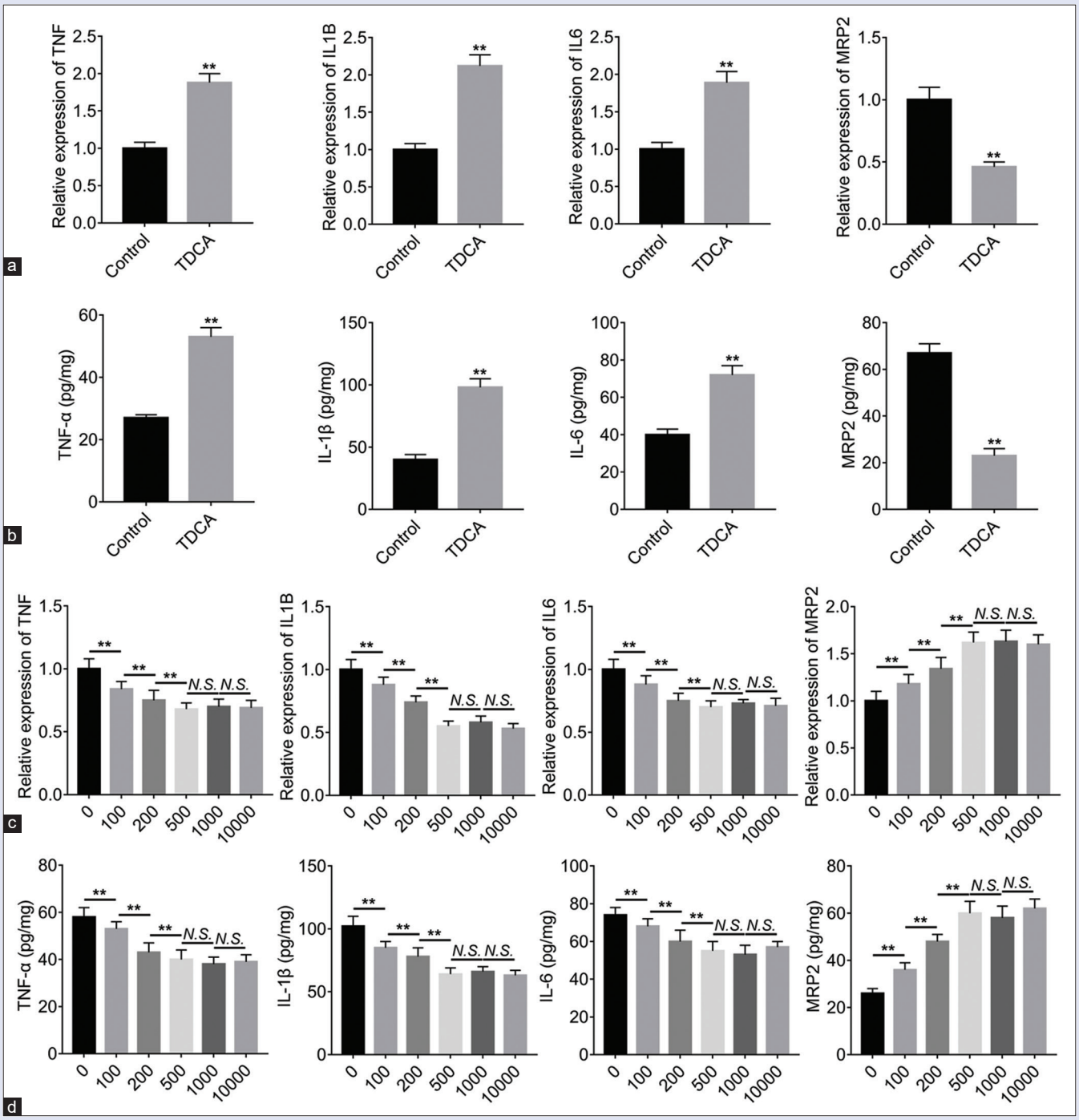

Figure 3: *S. miltiorrhiza* alleviates the fibrosis of HIBEC by inhibiting the NF-κB/MRP2 axis. (a) RT-qPCR analysis of TNF, IL1B, IL6, and Mrp2 in HIBEC and HIBEC induced by TDCA. (b) TNF-α, IL-1β, IL-6, and MRP2 levels in HIBEC and HIBEC induced by TDCA were evaluated by ELISA. (c) RT-qPCR analysis of TNF, IL1B, IL6, and MRP2 in HIBEC induced by TDCA treated with different dosages of *S. miltiorrhiza*. (d) TNF-α, IL-1β, IL-6, and MRP2 levels in HIBEC induced by TDCA treated with different dosages of *S. miltiorrhiza* were evaluated by ELISA. The data are expressed as average ± SD, (** $P < 0.01$)

of NF-κB-related inflammatory factors, and *Mrp2* of TDCA-treated HIBEC. The results indicated that the expression of *TNF, IL1B,* and *IL6* was upregulated, while *Mrp2* was downregulated in the TDCA group [Figure 3a]. Similarly, ELISA indicated that TDCA treatment significantly aggravated the levels of TNF-α, IL-1β, and IL-6, whereas the levels of MRP2 were alleviated, suggesting the NF-κB/MRP2 axis was activated in HIBEC after TDCA treatment [Figure 3b]. To explore the effect of *S. miltiorrhiza* on the activation of NF-κB/MRP2 axis, six different concentrations of *S. miltiorrhiza* were performed to treat TDCA-induced HIBEC. The RT-qPCR analysis indicated *S. miltiorrhiza* significantly downregulated the mRNA expression of *TNF, IL1B, and IL6*, while *Mrp2* was upregulated [Figure 3c]. We also found downregulated NF-κB-related inflammatory factors levels and upregulated MRP2 levels in the supernatant after *S. miltiorrhiza* treatment using ELISA [Figure 3d]. Notably, *S. miltiorrhiza* inhibited the NF-κB/MRP2 axis under a certain dose within a concentration of 500 μM. Nevertheless, the inhibitory effect reached saturation when the concentration of *S. miltiorrhiza* was over 500 μM. These findings indicated that *S. miltiorrhiza* alleviates HIBEC fibrosis progression through the NF-κB/MRP2 axis.

Regulation mechanism of Sal B on HIBEC fibrosis

To explore the main components of *S. miltiorrhiza* that attenuated the fibrosis of HIBEC, we investigated several major tanshinones (Tan I, Tan IIA, CTS) and salvianolic acids (Sal A, Sal B, Sal C) ingredients of *S. miltiorrhiza*. Each of the main components was used to treat HIBEC in the presence of TDCA for 24 h, then the expression levels of inflammatory factors and MRP2 in HIBEC were detected using the RT-qPCR analysis [Figures 4 and 5]. The results showed that treatment of Tan I and Sal C had little effect on the *TNF*, *IL1B*, *IL6,* and *Mrp2* expression [Figures 4a and 5c]. Treatment of Tan IIA and CTS merely suppressed the expression of *TNF* [Figures 4b and c]. Treatment of Sal

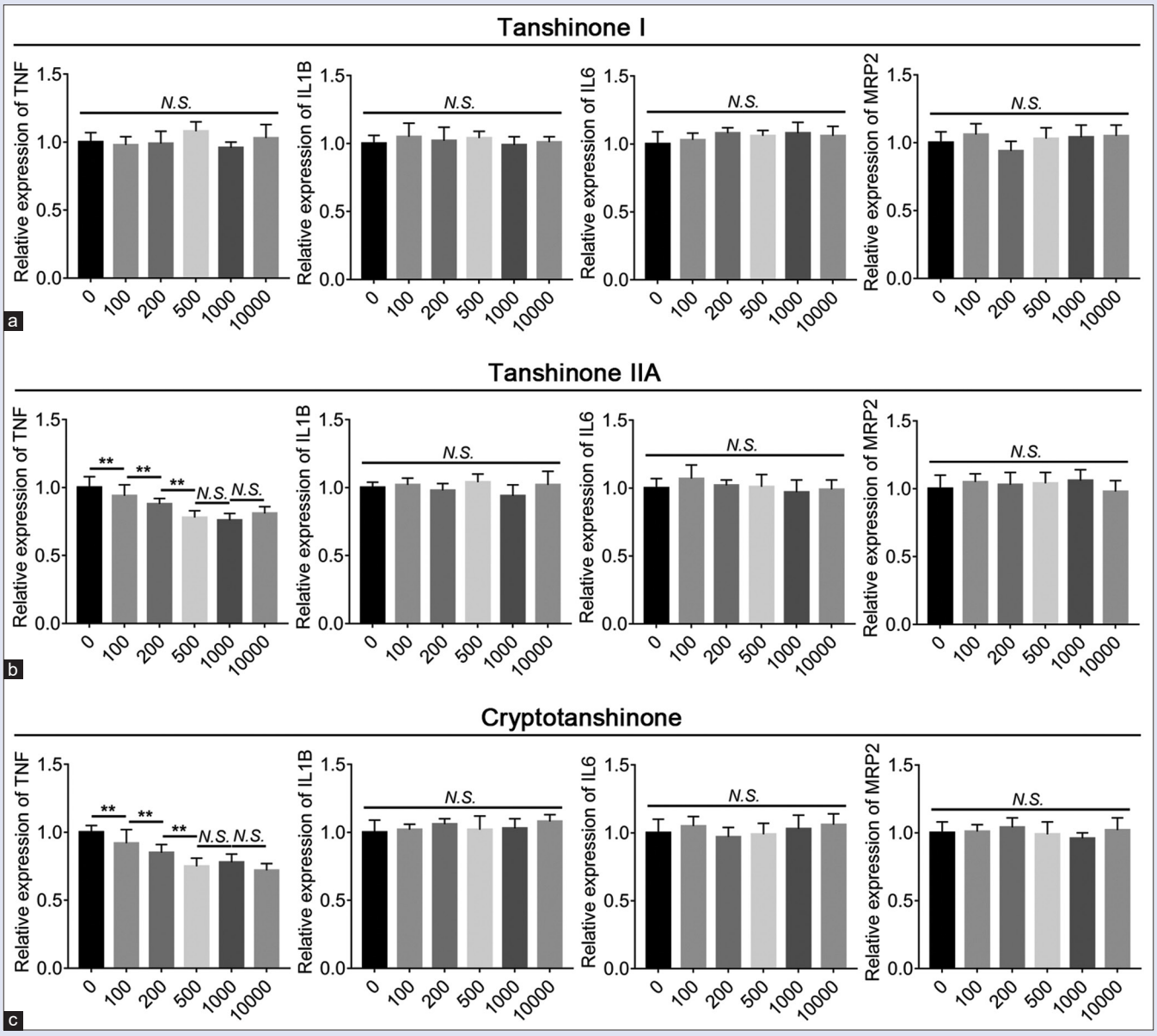

Figure 4: Effects of main tanshinones of *S. miltiorrhiza* on the NF-κB/MRP2 axis. RT-qPCR analysis of *TNF, IL1B, IL6,* and *Mrp2* in HIBEC and HIBEC induced by TDCA treated with different dosages of (a) Tan I, (b) Tan IIA, (c) CTS. The data are expressed as average ± SD, (** $P < 0.01$)

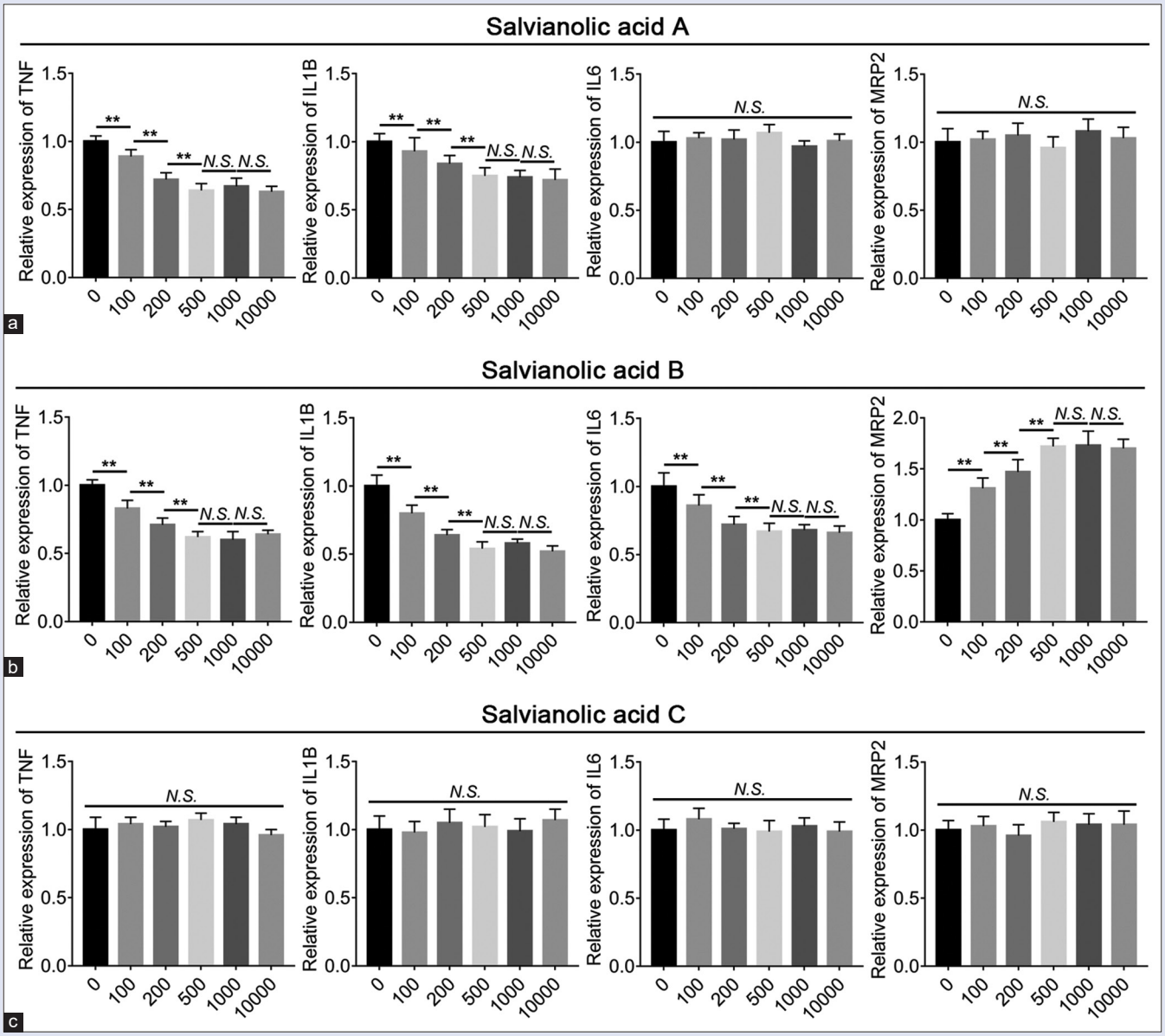

Figure 5: Effects of main salvianolic acids of *S. miltiorrhiza* on the NF-κB/MRP2 axis. RT-qPCR analysis of *TNF, IL1B, IL6,* and *Mrp2* in HIBEC and HIBEC induced by TDCA treated with different dosages of (a) Sal A, (b) Sal B, (c) Sal C. The data are expressed as average ± SD, (** $P < 0.01$)

A downregulated the expression of *TNF* and *IL1B* but had little effect on *IL6* and *Mrp2* [Figure 5a]. Interestingly, Sal B significantly impaired *TNF*, *IL1B,* and *IL6* expression levels and elevated *Mrp2* expression levels. Sal B significantly increased the *Mrp2* expression level and decreased the expression levels of *TNF*, *IL1B,* and *IL6* [Figures 5b]. Besides, the influence of Sal B on *Mrp2* and its downstream molecules showed a dose-dependent manner within 500 μM and reached saturation when the concentration exceeded 500 μM. We also performed ELISA to detect the expression concentrations of inflammatory factors and MRP2 in cell culture supernatant after treatment with each of the main components. The results were consistent with the RT-qPCR analysis, suggesting that Sal B significantly upregulated the levels of the Mrp2 via inhibited the activation NF-κB pathway [Figures 6 and 7]. Together, these results confirmed that Sal B acted as a main functional component of *S. miltiorrhiza* in delaying fibrosis through NF-KB/MRP2 axis under a certain dose.

Sal B-rich diet alleviates the fibrosis of hepatic bile ducts in SD rats

To further confirm the role of Sal B on bile duct cells *in vivo*, 10 male rats were randomly arranged with LD or LD with Sal B (LD + Sal B) after partial ligation of the common bile ducts. After 4 weeks of treatment with a different component diet, we performed histological analysis to assess the fibrotic degree of intrahepatic bile ducts. The findings revealed that Sal B markedly reduces the infiltration of inflammatory cells in the portal vein and the proliferation of fibrous tissue. In addition, the biliary dilation and necrosis were alleviated after treatment with Sal B [Figure 8a]. Furthermore, IHC staining and semiquantitative analysis revealed that treatment of Sal B significantly increased E-cadherin levels and decreased Vimentin levels, suggesting the decreased fibrotic degree of the intrahepatic bile duct [Figure 8b]. Collectively, these findings suggested that Sal B prevented HL by inhibiting intrahepatic bile duct fibrosis.

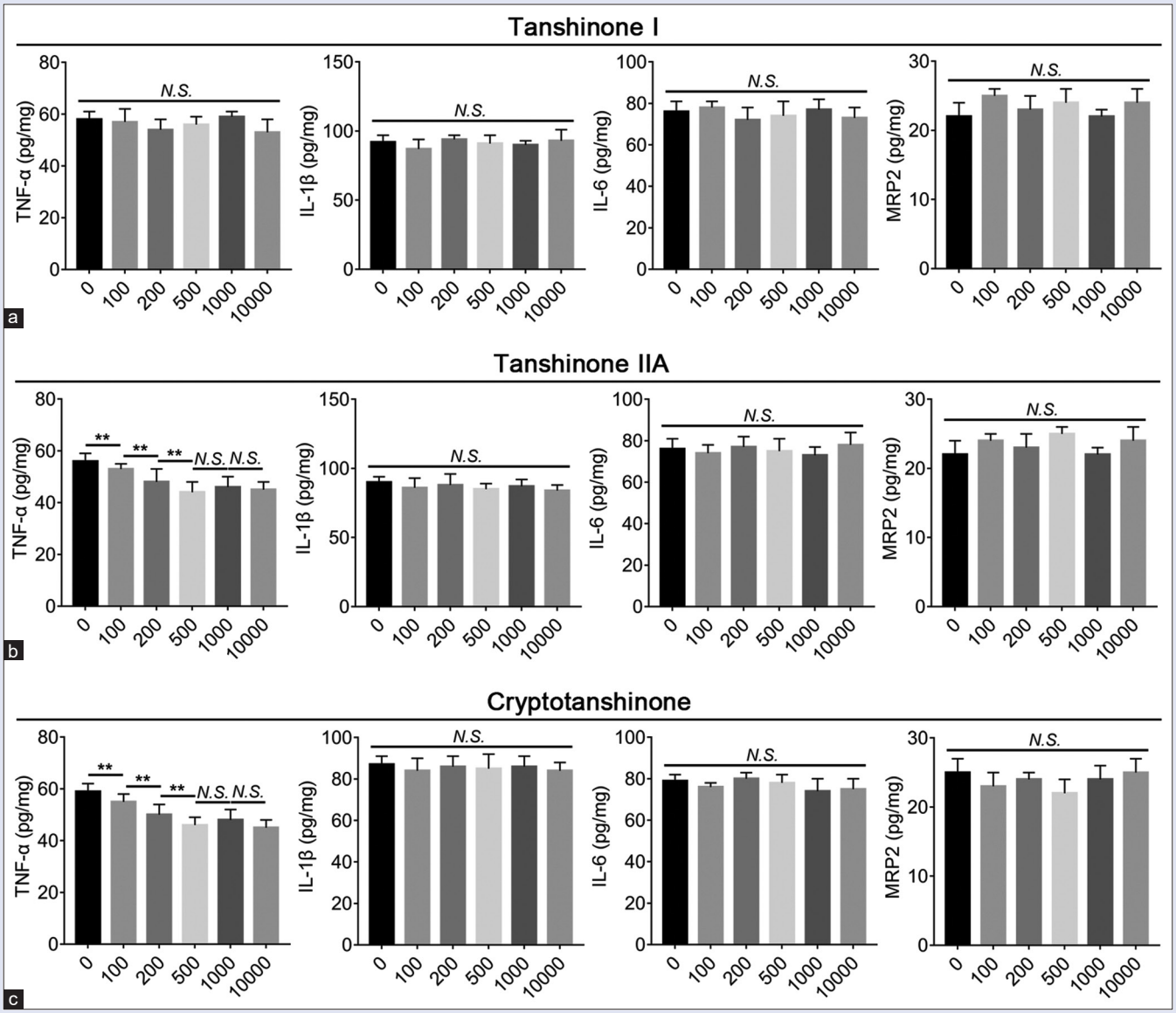

Figure 6: Effects of main tanshinones of *S. miltiorrhiza* on NF-κB/MRP2 axis. TNF-α, IL-1β, IL-6, and MRP2 levels in HIBEC induced by TDCA treated with different dosages of (a) Tan I, (b) Tan IIA, (c) CTS were evaluated by ELISA. The data are expressed as average ± SD, (** $P < 0.01$)

DISCUSSION

Biliary tract obstruction, biliary tract infection, and hepatic parenchyma destruction are the basic pathological changes of HL, and bile duct fibrosis is the key step in causing these basic pathological changes.[25,26] EMT is the transformation of epithelial cells to mesenchymal cells, manifested by the loss of epithelial marker E-cadherin and the acquisition of mesenchymal marker Vimentin.[27] As a transient and dynamic procedure regulated by NF-κB, Wnt, ErbB, and other cellular signaling pathways, EMT is closely attached to chronic inflammation, cancer metastasis, and fibrotic diseases.[28,29] Besides, cholestasis has also been revealed to stimulate EMT processes in biliary epithelial cells.[30] As one of the ABC transporters, MRP2 contributes to the clearance of both endogenous and exogenous toxic compounds in bile. Furthermore, current studies suggest that low MRP2 expression, which is associated with NF-κB activation, contributes to cholestasis.[17-31] However, our research provided novel evidence to support the low expression of MRP2 in fibrotic intrahepatic biliary epithelial cells, which is also associated with NF-κB activation.

S. miltiorrhiza is considered a kind of TCM with high medicinal value for the reason of its ability involved in the treatment of multiple diseases. Its main components are composed of tanshinone and salvianolic acid. Recently, *S. miltiorrhiza* has been reported to treat a variety of cardiovascular diseases, cirrhosis, and modulate the EMT process.[32-35] Meanwhile, *S. miltiorrhiza* is also an ingredient of compound preparations used in the therapy of intrahepatic bile duct stones and participates in the treatment of gallstones and HL. *S. miltiorrhiza* has the function of anti-bile duct cell fibrosis and inhibiting primary bile duct gallstones.[36] Further studies have shown that different components of *S. miltiorrhiza* are involved in treating multiple diseases. Tan IIA has been revealed to improve myocardial function by activating AMPK-mTOR signaling pathway and Sal A strengthens the transfer of anti-tumor drugs to brain tumor tissues via PKB/Src/Cav-1 axis.[37,38] The previous studies have demonstrated that Sal B can attenuate EMT

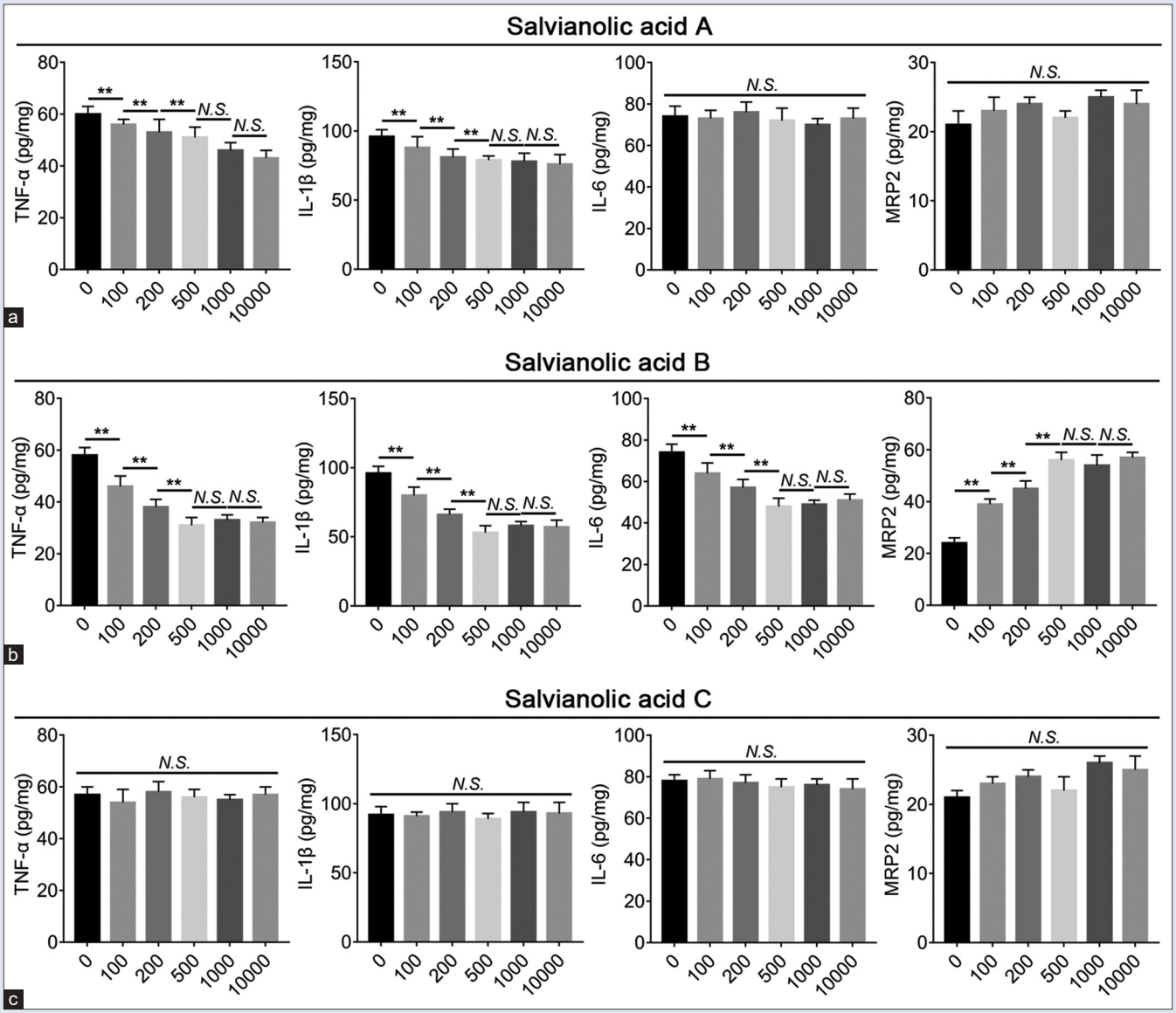

Figure 7: Effects of main salvianolic acids of *S. miltiorrhiza* on the NF-κB/MRP2 axis. TNF-α, IL-1β, IL-6, and MRP2 levels in HIBEC induced by TDCA treated with different dosages of (a) Sal A, (b) Sal B, (c) Sal C were evaluated by ELISA. The data are expressed as average ± SD, (** $P < 0.01$)

progression by activating Sirt1-mediated autophagy or modulating TGF-β1/Smads pathway.[39,40] In our study, we found that Sal B reduces HL by inhibiting bile duct fibrosis caused by cholestasis.

CONCLUSION

In our research, we first found that the NF-κB signaling pathway was activated in fibrotic HIBEC, while MRP2 was inhibited. We demonstrated that compared with other ingredients of *S. miltiorrhiza*, Sal B has the effect of inhibiting TDCA-induced fibrotic HIBEC. Sal B was found to have a significant effect on the inhibition of HL via the NF-κB/MRP2 axis. Interestingly, Sal B upregulated MRP2 via inhibiting the NF-κB signaling pathway. Moreover, a diet rich in Sal B could significantly prevent HL in SD rats. In summary, our results suggested that supplementary Sal B may have potential therapeutic value for HL.

Acknowledgements

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Ethics approval and consent to participate

The present study was approved by the Institutional Ethics Committee of China Harrison International Peace Hospital. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki.

Authors' contribution

Hao Yao designed study, analyzed data, wrote the initial draft of manuscript and revised manuscript. Wenpin Xu performed experiments. Zhaoming Liu performed experiments and contributed to the revision

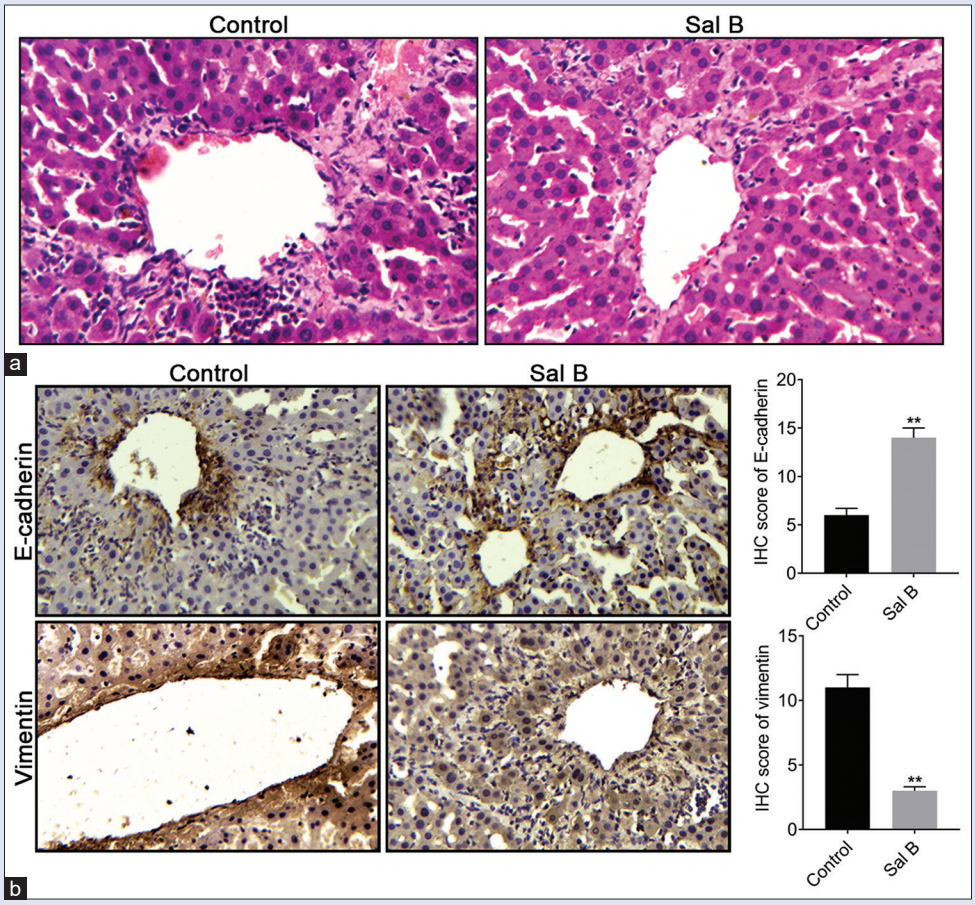

Figure 8: Sal B-rich diet alleviates the fibrosis of hepatic bile ducts in SD rats. Histopathological evaluation of HL in SD rats. (a) The histopathological assessment (hematoxylin-eosin staining) was performed in both the control group (LD) and the Sal B group (LD + Sal B). (b) Immunofluorescence assays evaluated the levels of E-cadherin and Vimentin expression in the control group (LD) and Sal B group (LD + Sal B). The data are expressed as average ± SD, (** $P < 0.01$)

of manuscript. Dawei Ma prepared the experiment resources. Hongbin Bao provided supervised the progress of study, provided experimental platform and financial support.

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Conflicts of interest

There are no conflicts of interest.

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