Title:

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The effect of *Salvia miltiorrhiza* in a mouse model of hepatic sinusoidal obstruction syndrome induced by *Gynura segetum*

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ABSTRACT

Background and aims: to investigate the potential effect and mechanism of *Salvia miltiorrhiza* in *Gynura segetum*-induced hepatic sinusoidal obstruction syndrome (HSOS).

Methods: the mice were gavaged with PBS, *Gynura segetum* or *Gynura segetum,* along with 100 or 200 mg/kg *Salvia miltiorrhiza.* Histological scoring and liver function were performed. The expression of tumor necrosis factor-alpha (TNF-α), vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and nuclear transcription factor P65 (NF-κBp65) were determined by reverse transcriptase polymerase chain reaction (RT-PCR) and western blot.

Results: liver function were effectively improved in the *Salvia miltiorrhiza* groups. The levels of TNF-α, VCAM-1, ICAM-1 and NF-κBp65 were significantly lower in the *Salvia miltiorrhiza* groups than in the *Gynura segetum* group.

Conclusions: *Salvia miltiorrhiza* has a therapeutic effect on *Gynura segetum*-induced HSOS.

INTRODUCTION

Gynura segetum is a common Chinese medical material containing pyrrolizidine alkaloids (PA) (1,2). In our previous study, we found that *Gynura segetum* could trigger HSOS and contribute to its development (3,4). Clinical research confirmed that *Salvia miltiorrhiza* could prevent hepatic sinusoidal obstruction syndrome (HSOS) after transplantation (5,6). However, the therapeutic effect and mechanism of *Salvia miltiorrhiza* in the prevention of HSOS induced by *Gynura segetum* remains unclear. Therefore, this study aimed to demonstrate the therapeutic effect of *Salvia miltiorrhiza* in *Gynura segetum*-induced HSOS in an animal model and determined the underlying mechanism *in vivo*.

MATERIALS AND METHODS

Preparation of *Gynura segetum*

The root of *Gynura segetum* (Identification of Traditional Chinese Medicine Herbs Company, Haozhou, Jiangsu, China) was placed in a ceramic pot and soaked for one hour. Subsequently, 200 ml water was added and the preparation was boiled for 0.5 hours. After boiling, the residual matter was filtered out and the decoction remained. The concentration of the decoction was equal to 1 g/ml of raw medicine.

Animals and treatment

Experimental procedures and animal care were performed in accordance with the Animal Care and Use Committee of the Hunan Normal University. Female KM mice from The Second Hospital of Xiangya Animal Center, Changsha, China, were used for the exepriments that weighed 20.7 ± 0.4 g at eight weeks of age. The animals were randomly divided into four groups and received a daily gavage of vehicle PBS (30 ml/kg/d, n = 10), *Gynura segetum* (30 g/kg/d, n = 30), *Gynura segetum* (30 g/kg/d) with 100 mg/kg/d *Salvia miltiorrhiza* (n = 30) or 200 mg/kg/d *Salvia miltiorrhiza* (n = 30). All animals were sacrificed 30 days later by cervical dislocation under isoflurane

anesthesia.

Liver function measurement

The mice were monitored daily for ascites and body and liver weight were measured. Blood samples were collected and liver function was tested via alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), total bilirubin (TBIL) levels that were determined by spectrophotometric analysis using commercial kits (Hitachi Company, Japan).

Histological analysis

Livers were stained with hematoxylin and eosin (H&E) and Masson trichrome to assess the endothelial damage of the central vein (CV), coagulative necrosis of hepatocytes, sinusoidal hemorrhage and subendothelial hemorrhage, subendothelial fibrosis, adventitial fibrosis and inflammation of CV. Overall scores were calculated as follows: 1-5 points were classified as mild, 6-8 points were classified as moderate and 9-14 points were classified as severe HSOS (7). The pathologic diagnosis was made by an experienced pathologist blinded to the trial.

Reverse transcription polymerase chain reaction

RNA was isolated from liver samples using the Trizol® reagent (Invitrogen™, Carlsbad, CA, USA) and then reverse transcribed with a first-stand cDNA synthesis kit (Fermentas, Burlington, Canada). The gene-specific primers used for the polymerase chain reaction (PCR) were: β-actin (forward: 5'-ATGGATGACGATATCGCT-3', reverse: ATGAGGTAGTCTGTCAGGT); tumor necrosis factor-alpha (TNF-a) (forward: 5'-ACTCAACAAACTGCCCTTCTGAG-3', reverse: 5'-TTACAGCTGGTTTCGATCCATTT-3'); intercellular adhesion molecule-1 (ICAM-1) (forward: 5'-CAACTGGAAGCTGTTTGAGCTG-3', reverse: 5'-TAGCTGGAAGATCGAAAGTCC-3'); vascular cellular adhesion molecule-1 (VCAM-1) (forward: 5'-CCTCACTTGC AGCACTACGGGCT-3', reverse: 5'-TTTTCCAATATCCTCAATGACGGG-3'); and nuclear transcription factor P65 (NF-κBp65) (forward: 5'-TGGCGAGAGAAGCACAGATA-3', reverse: 5'-TGTTGGTCTGGATTCGCTG-3'). The polymerase chain reaction (PCR) was

performed using a PCR mix (Fermentas) and the amplification was performed for 35 cycles. The PCR reaction products were separated on a 2% agarose gel and detected under ultraviolet transillumination. The sequences of primer pairs are shown in table 1. The target gene expression levels were quantified after normalization with β-actin.

Western blot analysis

Liver lysates were resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked and incubated with antibodies overnight (TNF-α 1:200, ICAM 1:1:100, VCAM-1 1:500 [Santa Cruz, CA, USA], NF-κBp65 1:500 [Abcam, Cambridge, UK], β-actin 1:2000 [Sigma, St. Louis, MO, USA]). The protein bands were subsequently incubated with secondary antibodies (Santa Cruz) and detected with chemiluminescence detection reagents (Beyotime, Jiangsu, China). The target protein expression levels were quantified after normalization with the β-actin.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Multiple comparisons were performed by one-way ANOVA and pairwise comparisons were evaluated by the least significant difference (LSD) test or Dunnett's T3, where appropriate. p-values < 0.05 were considered as statistically significant.

RESULTS

Clinical findings

Twenty-four mice developed HSOS after *Gynura segetum* treatment and five of these suffered massive ascites. While *Salvia miltiorrhiza* intervention inhibited the onset of HSOS (Table 1), only eight mice in group C and three in group D developed HSOS, without ascites. Compared with group A, group B displayed significant differences in the liver index (liver weight/body weight) (Table 1). Mice treated with *Salvia miltiorrhiza* showed significantly improved liver function compared to group B mice (p < 0.05) (Fig. 1).

Light-microscopic observations

According to the scoring following histological analysis, 80% (24/30) of mice were diagnosed with HSOS induced by *Gynura segetum.* While the ratio of HSOS in the *Salvia miltiorrhiza* groups was significantly lower than in the *Gynura segetum* group (Table 2). As shown in figure 1, *Gynura segetum* administration induced collapsed liver lobules and complete endothelial coagulative necrosis, sinusoidal hemorrhage and obstruction of sinusoids *in vivo.* For its part, *Salvia miltiorrhiza* obviously reduced endothelial coagulative necrosis, sinusoidal hemorrhage and obstruction of sinusoids in a dosage-dependent manner.

Expression of the TNF-α, ICAM-1, VCAM-1 and NF-κBp65

Reverse transcriptase polymerase chain reaction (RT-PCR) demonstrated higher levels of TNF-α, ICAM-1, VCAM-1 and nuclear NF-κBp65 mRNA in group B compared to controls. The expression of TNF-α, ICAM-1, VCAM-1 and NF-κBp65 mRNA reduced significantly after intervention with *Salvia miltiorrhiza*, especially in group D ($p < 0.05$) (Fig. 2). These findings were confirmed by western blot analysis (Fig. 2).

DISCUSSION

Common causes of HSOS include the complication of hematopoietic stem cell transplantation, ingestion of alkaloid toxins or chemotherapeutic agents (8,9). To date, there is no uniformly effective treatment for HSOS and supportive care is the cornerstone of patient management. *Salvia miltiorrhiza* has been used to prevent HSOS after hematopoietic stem cell transplantation at the China transplantation center (10). Our study showed that *Salvia miltiorrhiza* could effectively improve liver function and had therapeutic effects in *Gynura segetum*-induced HSOS in a dose-dependent manner *in vivo*.

TNF-α induces the shape and motility changes of endothelial cells, which contributes to vascular leakage of inflammation and increases the expression of cell adhesion molecules (11). Studies have reported that TNF-α stimulation could promote the cell surface expression of adhesion molecules, such as ICAM-1 and VCAM-1 in endothelial cells (12,13). Our study found that the expression of TNF-α, ICAM-1 and

VCAM-1 was lower in the *Salvia miltiorrhiza* groups than the *Gynura segetum* group. Thus, suggesting that *Salvia miltiorrhiza* down-regulated the expression of TNF-α, ICAM-1 and VCAM-1 and therefore prevented HSOS in a dose-dependent manner.

The NF-κBp65 signaling pathway has been reported to be involved in endothelial cell damage (14). NF-κB p50/p65 heterodimers plays an important role in mediating the cytokine-dependent transcription of ICAM-1 and VCAM-1 in human and bovine vascular endothelial cells (15). The expression of NF-κBp65 in the liver was examined and the expression of NF- Bp65 decreased along with ICAM-1 and VCAM-1. This suggests that *Salvia miltiorrhiza* may prevent HSOS by down-regulating ICAM-1 and VCAM-1 expression *via* inhibition of NF-κBp65 signaling.

In summary, our study provides evidence that *Salvia miltiorrhiza* plays a role in preventing *Gynura segetum*-induced HSOS in a dose-dependent manner. *Salvia miltiorrhiza* intervention can improve liver function and relieve sinus endothelial cell damage and hepatic fibrosis *in vivo*. The mechanism of *Salvia miltiorrhiza* may rely on the suppression of TNF-α, ICAM-1 and VCAM-1 via inhibition of NF-κBp65 signaling. Further research is needed to define the mechanisms of action and clarify whether there are other mechanisms of action.

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16. **Table 1. Number of HSOS mice with ascites and changes in weight and liver index**

Group	Development HSOS	Development ascites	Base weight (g)	Final weight (g)	Liver index
A (n = 10)	0	0	20.68 ± 0.39	23.51 ± 0.34*	0.0297 ± 0.0013*
B (n = 30)	24	5	20.16 ± 0.37	26.51 ± 0.66[†]	0.0471 ± 0.0015[†]
C (n = 30)	8	0	20.32 ± 0.43	21.27 ± 0.42*	0.0354 ± 0.0011*
D (n = 30)	3	0	20.39 ± 0.46	21.21 ± 0.43*	0.0314 ± 0.0014*

Liver index = liver weight/body weight. *Compared to group B $p < 0.05$. [†]Compared to control group $p < 0.05$.

Table 2. Scoring of light microscopy

Group	Mild HSOS	Moderate HSOS	Severe HSOS	Endothelial damage of the CV	Coagulative necrosis of hepatocytes	Subendothelial hemorrhagic of the CV	Sinusoidal hemorrhage	Subendothelial fibrosis of the CV	Adventitial fibrosis of the CV	Inflammation of the CV	Mean score ± SD
A	/	/	/	0	0	0	0	0	0	0	0*
B	6	11	7	1-3	2-3	2-3	1-2	1-2	1-2	1-2	7.75 ± 0.14
C	3	4	1	1-2	1-2	1-2	1-3	0-1	0-1	0-1	6.75 ± 0.21*†
D	2	1	0	1-2	0-1	0-1	0-1	0	0	0-1	5.00 ± 0.08*

Each parameter was graded on a 4-point system (0: absent; 1: mild; 2: moderate; 3: severe). 1-5 points were classified as mild, 6-8 points were classified as moderate and 9−14 points were classified as severe HSOS. *$p < 0.05$, compared with group B; †$p < 0.05$, comparing group C with D.
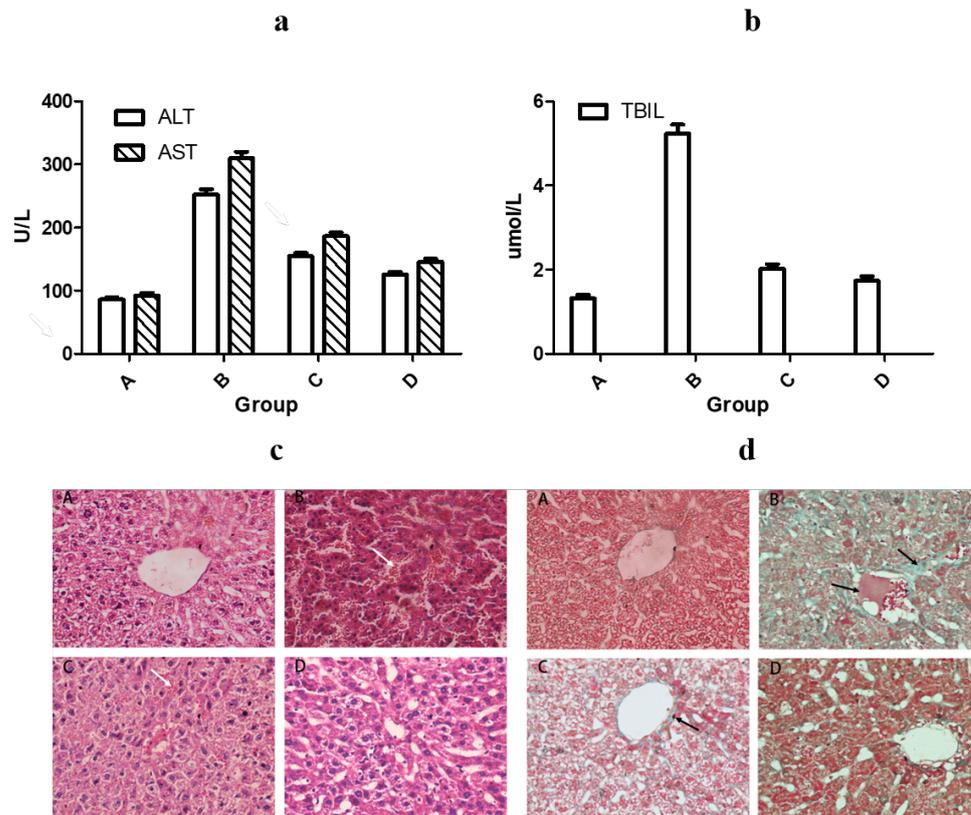
Fig. 1. Effect of *Salvia miltiorrhiza* on ALT, AST (a) and TBIL levels (b) in mice. Features of histopathologic changes in the different groups (c) (H&E, ×200). Fibrosis changes of the different groups (d) (Masson, ×200).
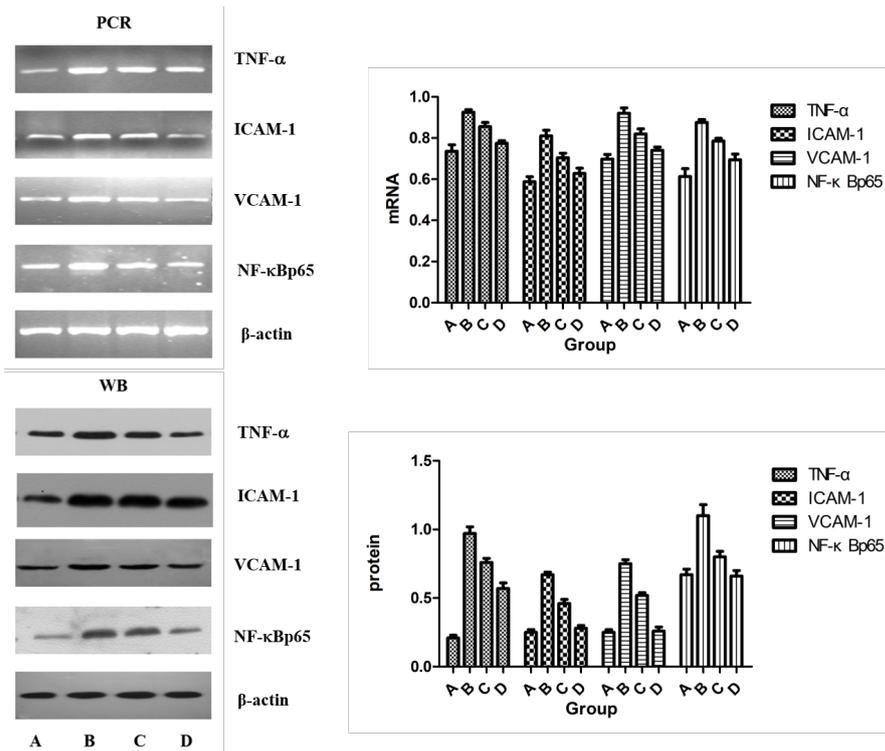
Fig. 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis of the tumor necrosis factor-alpha (TNF-α), intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1) and nuclear transcription factor P65 (NF-κBp65) in the liver ($\overline{x} \pm s$).