

# World Journal of *Clinical Cases*

*World J Clin Cases* 2021 December 6; 9(34): 10392-10745



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Thrice Monthly Volume 9 Number 34 December 6, 2021

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**RESPONSIBLE EDITORS FOR THIS ISSUE**

Production Editor: Yan-Xia Xing; Production Department Director: Yun-Jie Ma; Editorial Office Director: Jin-Lei Wang.

**NAME OF JOURNAL**

*World Journal of Clinical Cases*

**ISSN**

ISSN 2307-8960 (online)

**LAUNCH DATE**

April 16, 2013

**FREQUENCY**

Thrice Monthly

**EDITORS-IN-CHIEF**

Dennis A Bloomfield, Sandro Vento, Bao-Gan Peng

**EDITORIAL BOARD MEMBERS**

<https://www.wjnet.com/2307-8960/editorialboard.htm>

**PUBLICATION DATE**

December 6, 2021

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<https://www.wjnet.com/bpg/gerinfo/242>

**STEPS FOR SUBMITTING MANUSCRIPTS**

<https://www.wjnet.com/bpg/GerInfo/239>

**ONLINE SUBMISSION**

<https://www.f6publishing.com>



## Basic Study

## Magnolol protects against acute gastrointestinal injury in sepsis by down-regulating regulated on activation, normal T-cell expressed and secreted

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**Institutional animal care and use committee statement:** All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals (licence No. SYXK(Zhe)2013-0184).

**Conflict-of-interest statement:** We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any

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## Abstract

## BACKGROUND

Sepsis is a major medical challenge. Magnolol is an active constituent of *Houpu* that improves tissue function and exerts strong anti-endotoxin and anti-inflammatory effects, but the mechanism by which it reduces intestinal inflammation in sepsis is yet unclear.

## AIM

To assess the protective effect of magnolol on intestinal mucosal epithelial cells in sepsis and elucidate the underlying mechanisms.

## METHODS

Enzyme-linked immunosorbent assay was used to measure tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, and regulated on activation, normal T-cell expressed and secreted (RANTES) levels in serum and ileal tissue in animal studies. The histopathological changes of the ileal mucosa in different groups were observed under a microscope. Cell Counting Kit-8 and cell permeability assays were used to determine the concentration of drug-containing serum that did not affect the activity of Caco2 cells but inhibited lipopolysaccharide (LPS)-induced decrease in permeability. Immunofluorescence and Western blot assays were used to detect the levels of RANTES, inhibitor of nuclear factor kappa-B kinase  $\beta$  (IKK $\beta$ ), phosphorylated IKK $\beta$  (p-IKK $\beta$ ), inhibitor of nuclear factor kappa-B kinase  $\alpha$  (IkB $\alpha$ ), p65, and p-p65 proteins in different groups *in vitro*.

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**Data sharing statement:** No additional data are available.

**ARRIVE guidelines statement:** The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

**Supported by** Basic Public Welfare Research Foundation of Zhejiang Province, China, No. GD21H290001; and Traditional Chinese Medicine Science and Technology Project Foundation of Zhejiang Province, China, No. 2020ZB072.

**Country/Territory of origin:** China

**Specialty type:** Medicine, research and experimental

**Provenance and peer review:** Unsolicited article; Externally peer reviewed.

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0  
Grade B (Very good): 0  
Grade C (Good): C  
Grade D (Fair): 0  
Grade E (Poor): 0

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**Received:** July 20, 2021

**Peer-review started:** July 20, 2021

**First decision:** August 9, 2021

## RESULTS

In rats treated with LPS by intravenous tail injection in the presence or absence of magnolol, magnolol inhibited the expression of proinflammatory cytokines, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in a dose-dependent manner. In addition, magnolol suppressed the production of RANTES in LPS-stimulated sepsis rats. Moreover, *in vitro* studies suggested that magnolol inhibited the increase of p65 nucleation, thereby markedly downregulating the production of the phosphorylated form of IKK $\beta$  in LPS-treated Caco2 cells. Specifically, magnolol inhibited the translocation of the transcription factor nuclear factor-kappa B (NF- $\kappa$ B) from the cytosol into the nucleus and down-regulated the expression level of the chemokine RANTES in LPS-stimulated Caco2 cells.

## CONCLUSION

Magnolol down-regulates RANTES levels by inhibiting the LPS/NF- $\kappa$ B signaling pathways, thereby suppressing IL-1 $\beta$ , IL-6, and TNF- $\alpha$  expression to alleviate the mucosal barrier dysfunction in sepsis.

**Key Words:** Sepsis; Magnolol; Regulated on activation, normal T-cell expressed and secreted; Anti-inflammation; Lipopolysaccharide; Nuclear factor-kappa B

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**Core Tip:** In this study, it was found that magnolol inhibited the lipopolysaccharide-induced nuclear factor-kappa B signaling pathway in the intestinal mucosal epithelium to regulate the secretion of regulated on activation, normal T-cell expressed and secreted (RANTES) and thus reduce intestinal inflammation in sepsis. Various biological constituents, isolated from traditional Chinese medicine, show multifunctional activities. Magnolol, isolated from Magnolia, has been documented to possess a range of biological activities. The current results for the first time proved that magnolol plays a role in the treatment of sepsis by down-regulating RANTES. Thus, additional studies on its anti-inflammatory mechanism might provide novel ideas and methods for the clinical prevention and treatment of sepsis.

**Citation:** Mao SH, Feng DD, Wang X, Zhi YH, Lei S, Xing X, Jiang RL, Wu JN. Magnolol protects against acute gastrointestinal injury in sepsis by down-regulating regulated on activation, normal T-cell expressed and secreted. *World J Clin Cases* 2021; 9(34): 10451-10463  
**URL:** <https://www.wjgnet.com/2307-8960/full/v9/i34/10451.htm>  
**DOI:** <https://dx.doi.org/10.12998/wjcc.v9.i34.10451>

## INTRODUCTION

Severe sepsis is a life-threatening syndromic response with a 27%-54.1% mortality rate [1,2]. However, the underlying mechanism is not yet understood. It is speculated that mucosal epithelial dysfunction and acute gastrointestinal injury (AGI) are the initial factors of multiple-organ failure [3], which permit translocation of intestinal bacteria and endotoxins to the bloodstream, inducing a strong systemic inflammatory response and leading to AGI [4,5]. Currently, the diagnosis of AGI is poorly understood.

Border *et al* [6] first reported an association between bacterial translocation and gut-origin sepsis in 1987. Since then, the gut has been shown to have a major role in the progression of systemic inflammatory response syndrome, sepsis, and multiple organ dysfunction syndrome (MODS). Several studies have reported that increased intestinal permeability in acute bowel injury results in the translocation of large amounts of intestinal bacteria, thus initiating or aggravating systematic inflammatory response syndrome [7]. The current treatment for sepsis to prevent MODS requires rapid diagnosis to allow timely treatment with antibiotics and interventions [8]. Therefore, early and effective inhibition of the production of proinflammatory factors may protect against the intestinal damage caused by excessive inflammation in addition to preventing and treating sepsis and MODS.



Revised: August 24, 2021

Accepted: October 18, 2021

Article in press: October 18, 2021

Published online: December 6, 2021

P-Reviewer: Rodrigues AT

S-Editor: Gao CC

L-Editor: Wang TQ

P-Editor: Gao CC



*Houpu* is a traditional Chinese medicine for phlegm and gas removal. In clinical practice, *Houpu* is widely utilized for treating vomiting and diarrhea, abdominal distension, and constipation. Some studies suggested that *Houpu* improves gastrointestinal ischemia, inhibits bacterial migration, and reduces endotoxin absorption, and could be the favored approach in adjusting immunity[9,10]. Magnolol, an active constituent of *Houpu*, improves tissue function, exerts strong anti-endotoxin and anti-inflammatory effects, and acts as an oxygen-free-radical scavenger[11]. However, the exact mechanism underlying the magnolol effect on intestinal inflammation is yet unclear.

A subset of CC motif chemokines and cytokines is increased in sepsis patients compared to normal controls[12]. Various interactive and dynamic chemokines involved in sepsis are used in diagnosis, prognosis, etiology, and evaluation of response to therapy[13]. Regulated on activation, normal T-cell expressed and secreted (RANTES) is a critical member of the chemokine superfamily; its specific receptor is the transmembrane G protein-coupled receptor (CCR1, CCR2, CCR3, CCR4, and CCR5). CCR1 and CCR5 are high-affinity receptors, while CCR4 is a low-affinity receptor[14]. RANTES and its receptors affect chemotaxis or stimulation of T lymphocytes, monocytes, eosinophils, and basophils, especially in lymphocyte CD4<sup>+</sup>/CD45RO<sup>+</sup> memory type, to induce T cell activation and proliferation, regulate Th cell and cytotoxic cell immune response, and stimulate eosinophils, basophils, and degranulation[15]. Ajuebor *et al*[16] found that RANTES was significantly expressed in Sprague-Dawley (SD) rats with chronic colitis. The same study suggested that the administration of the receptor antagonist reduced the colon tissue damage along with the number of mononuclear cells, mast cells, and neutrophils in the lesion. Furthermore, Kucuk *et al*[17] treated colitis with Met-RANTES and found less damage and bacterial translocation. Previous studies have shown that the promoters of RANTES genes contain the binding sites for the transcription factor nuclear factor-kappa B (NF-κB)[18].

NF-κB is a nuclear transcription factor in cells involved in the inflammatory and immune responses of the body and regulates cell apoptosis, stress response, and NF-κB overactivation. NF-κB has been associated with several human diseases, such as rheumatoid arthritis and inflammatory changes in the heart and brain diseases. NF-κB is a major transcription factor in the inflammatory response, and its accumulation in the nucleus influences transcription by binding to the promoter of the RANTES gene, thereby inducing its production in large quantities[19]. Magnolol significantly suppresses the expression of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), phosphorylated extracellular regulated protein kinases (p-ERK), phosphorylated C-jun N-terminal kinase (p-JNK), and p-p38 in lipopolysaccharide (LPS)-induced mouse uterine epithelial cells (MUECs), which is associated with the inhibition of Toll-like receptor 4 (TLR4)-regulated NF-κB signaling[20]. Therefore, whether magnolol can inhibit the LPS/NF-κB signaling pathway in the intestinal mucosal epithelium to regulate the secretion of RANTES and thus reduce intestinal inflammation in sepsis needs to be further investigated.

## MATERIALS AND METHODS

### Animals

All animal studies (including mouse euthanasia) were carried out in compliance with the regulations and guidelines of Zhejiang Chinese Medical University institutional animal care and according to the Institutional Animal Care and Use Committee (IACUC) guidelines. Healthy male SD rats ( $n = 55$ ), weighing  $200 \pm 20$  g, were obtained from the Experimental Animal Center of Zhejiang Chinese Medical University, China (certification number SCXK [Hu] 2017-0005) and housed for 2 wk under normal conditions (certification number SYXK [Zhe] 2013-0184) at  $20 \pm 1$  °C and 50%-60% humidity, under 12:12-h light/dark cycle, with a ventilation rate of 8-15 times/h.

Using a random number table, the rats were divided into five groups ( $n = 11$ /group): Control group (Control), severe sepsis group (Model), low-dose magnolol (Tongtong Biologicals, Shanghai, China) group (LM, 5 μg/kg), middle-dose magnolol group (MM, 10 μg/kg), and high-dose magnolol group (HM, 20 μg/kg).

### Animal model establishment and treatment

A rat model of severe sepsis was established by intravenous injection of LPS via the tail vein. Briefly, all rats were deprived of food but had free access to water for 12 h before surgery. The Control rats received 15 μg/kg normal saline plus 4 mg/kg normal

saline. The Model animals received 15 µg/kg normal saline plus 4 mg/kg LPS. The rats in the LM, MM, and HM groups received 5 µg/kg magnolol plus 4 mg/kg LPS, 10 µg/kg magnolol plus 4 mg/kg LPS, and 20 µg/kg magnolol plus 4 mg/kg of LPS, respectively. Blood was collected from each group after 6, 12, and 24 h. After 24 h, the animals were euthanized using an intraperitoneal injection of chloral hydrate, and their mesenteric lymph nodes, liver, spleen, and terminal ileum tissues were collected under aseptic conditions.

### **Detection of serum and ileal inflammatory reaction**

TNF-α, IL-1β, and IL-6 levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Medical Equipment Co., Ltd (80-2), Shanghai, China).

### **Pathological changes in the ileal mucosa**

The paraffin sections of the rat terminal ileum were stained with hematoxylin and eosin and observed under an XZT-302 microscope (Shanghai Yuguang Detection Equipment Co., Ltd, Shanghai, China) at × 100 magnification to assess the histopathological changes.

### **Expression of RANTES in serum and ileal tissue**

Peripheral blood samples and serum were isolated. Serum RANTES levels were measured using ELISA kits following the manufacturer's instructions (Shanghai Medical Equipment Co., Ltd (80-2), Shanghai, China).

### **Caco2 cell culture and treatment**

Caco2 cells were cultured in RPMI-1640 medium containing 20% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco, Waltham, United States). The cells were incubated (Thermo Scientific, Waltham, United States) at 5% CO<sub>2</sub>, 37 °C, and saturated humidity. The medium was changed every 2-3 d until the cells reached 80% confluency. Under a microscope (Olympus, Tokyo, Japan), Caco2 cells were observed to be adherent like paving stones.

Subsequently, cells were divided into four groups: Group A (Solvent), treated with solvent only; group B (Magnolol), treated with different concentrations of magnolol (2 µmol/L, 5 µmol/L, and 10 µmol/L); group C (LPS), treated with solvent and LPS (100 µg/mL); group D (Magnolol plus LPS), treated with magnolol (2, 5, and 10 µmol/L) and LPS (100 µg/mL). Groups B/C/D were pretreated with magnolol or solvent for 8 h, followed by LPS for 24 h.

### **Cell counting kit-8 assay**

After 24 h of culture, cells were digested with trypsin-EDTA (Solarbio, Beijing, China) and collected by centrifugation at 1000 rpm for 5 min (Eppendorf, Hamburg, Germany) and resuspended in complete fresh medium (GIBCO, Waltham, United States). The cell suspension was inoculated in a 96-well plate and incubated at 37 °C and 5% CO<sub>2</sub>. Then, 10 µL of cell counting kit-8 (CCK-8) (Biyuntian, C0037, Shanghai, China) was added to each well and incubated at 37 °C for another 2 h. The absorbance was measured at 450 nm using a microplate reader (Thermo Scientific, Waltham, United States).

### **Cell permeability detection**

Caco2 cells in the logarithmic phase were inoculated in 24-well Transwell (Corning, 3415, Shanghai, China) plate. Epithelial cells were inoculated in the upper chamber. The filtration membrane embedded in the chamber was a polycarbonate membrane, on which the cells grew to form a monolayer. After the epithelial cells were overgrown, phenol-free red minimum essential medium (MEM) (GIBCO, Waltham, United States) containing fluorescein isothiocyanate isomer (FITC)-labeled glucan (10 kDa, 100 µg/mL) (Santa Cruz, Sc-263323), magnolol, and LPS was added to the upper chamber, and phenol-free red MEM containing glucan, magnolol, and LPS was added to the lower chamber. After treatment for 12 h, 100 µL of the lower chamber medium was placed in 96-well plates, and the fluorescence intensity of FITC was detected by an enzyme marker (excitation wavelength of 490 nm and emission wavelength of 520 nm) (Thermo Scientific, Waltham, United States).

### **ELISA**

According to the CCK-8 and cell permeability detection, we found that 10 µmol/L of magnolol had a maximal inhibitory effect on increased permeability of Caco2 cells after LPS induction. Caco2 cells were divided into four groups again and treated with

the appropriate concentrations of magnolol. RANTES levels were measured using ELISA kits, according to the manufacturer's instructions (Shanghai Medical Equipment Co., Ltd (80-2), Shanghai, China).

### **Western blot analysis**

Caco2 cells were lysed in 200 mL of radio-immunoprecipitation assay (RIPA) (Beyotime Biotechnology, Shanghai, China) buffer and homogenized. The protein concentration was determined using the bicinchoninic acid method. The cells were then washed with ice cold phosphate buffered saline (PBS) (Sinopharm Chemical Reagent, Shanghai, China) and re-centrifuged at 5500 rpm for 5 min at 4 °C. The cell pellets were re-suspended in a lysis buffer [100 mmol/L phenylmethylsulfonylfluoride (PMSF) (Aladdin, Shanghai, China), 10 µL phosphatase inhibitor (Beyotime Biotechnology, Shanghai, China)] for 30 min. The lysate was centrifuged at 12000 rpm for 5 min at 4 °C before the supernatant was collected. We performed the Bradford assay (Biorad, Hercules, CA, United States) and UV spectrophotometry (Bio-wave II; Biochrom WPA, Cambridge, United Kingdom) to equalize loading protein. Equal amounts of protein (40 µg) were transferred to vinylidene fluoride membranes (Milipore, Billerica, MA, United States). The membranes were incubated with blocking buffer [5% non-fat dry-milk in TBS containing 0.1% Tween-20 (TBST)] for 2 h at room temperature. The membranes were probed with anti-RANTES (1:2000; AF5151, Affinity Biosciences, United States), anti-IKKβ (1:2000; AF6009, Affinity Biosciences, United States), anti-p-IKKβ (1:2000; AF3010, Affinity Biosciences, United States), anti-IKBA (1:2000; AF5002, Abcam, United Kingdom), anti-p65 (1:2000; ab16502, Affinity Biosciences, United States), and anti-p-p65 (1:2000; AF2006, Affinity Biosciences, United States) polyclonal antibodies overnight at 4 °C. Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (1:50000, BA1054, Dr. DE Biological Engineering Co. Ltd, Wuhan, China) at 37 °C for 2 h. GAPDH (AB-P-R 001, Xianzhi Biology Co. Ltd, Hangzhou, China) was used as the internal control and detected on the membrane. The signals were detected on an X-ray imaging system (6535876, Ruike Medical Equipment Co., Ltd, Xiamen, China), and the gray value of the immunoreactive bands was analyzed using BandScan.

### **Immunofluorescence**

Cells were dehydrated, cleared, waxed, embedded, sliced, and incubated with primary antibody (Abcam, United States) and secondary antibody (San Eagle, Wuhan, China). Subsequently, the cells were stained with 4,6-guanidine-2-phenyl indole (DAPI) and observed under a confocal microscope (Perkin Elmer & Olympus, Tokyo, Japan).

### **Statistical analysis**

Data were analyzed using IBM SPSS Statistics version 22. Measurement data following a Gaussian distribution are expressed as the mean ± SD. The comparison between groups was conducted using the LSD method (minimum significance method) in one-way ANOVA or *t*-tests as appropriate. *P* values < 0.05 indicated statistical significance.

## **RESULTS**

### **Establishment of a rat model of sepsis**

During the 24-h postoperative observation period, no rats died in the Control group. Two rats each died in the Model group and the low-dose LM group, and one rat each died in the MM and the HM groups, corresponding to the death rates of 0%, 18.18%, 18.18%, 9.09%, and 9.09%, respectively, which did not differ significantly between the groups.

The rats in the Control group behaved normally, were sensitive, and had glossy hair, normal stool, and no hyperemia or edema in the intestine. Within 6 h post-intervention, the other four groups of mice exhibited a series of abnormalities, such as piloerection, shortness of breath, listlessness, loss of appetite, diarrhea, loss of interest in the surrounding environment, the disappearance of self-cleaning behavior, and increased secretion from the eyes. The symptoms of the HM, MM, and LM groups were milder compared to those of the Model group. After 12 h, the HM, MM, and LM groups showed signs of mental improvement and self-cleaning behaviors, while the Model group did not.

**Protective effects of magnolol on intestinal mucosal barrier of rats with sepsis**

Compared to the Control group, the levels of serum and ileal TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were significantly higher those of the Model group ( $P < 0.01$ ; Tables 1 and 2), while they were decreased slightly in the LM, MM, and HM group ( $P < 0.05$ ; Tables 1 and 2).

**Magnolol improves intestinal mucosal morphology**

In the Control group, the ileal mucosal villi were arranged in an orderly manner, and the structure of the surrounding blood vessels was normal. In the Model group, the ileal mucosal villi were damaged. The intestinal mucosal epithelial cells were degenerated and wiped off, the subepithelial capillaries exhibited dilation and congestion, and inflammatory cells were diffused in the lamina propria. Compared to the Model group, the morphology of the ileal mucosal villi in the three magnolol groups was improved, although slight damage to the ileal mucosal villi, local necrosis, and some bleeding were observed. A pronounced improvement in ileal mucosal morphology was observed in the HM group (Figure 1A-E).

**Magnolol decreases RANTES release in serum and expression in ileal tissue of sepsis rats**

Compared to the Control group, the RANTES release in serum was significantly increased in the Model group ( $P < 0.05$ , Table 1). Magnolol decreased the RANTES release in the LM, MM, and HM groups compared to the Model group ( $P < 0.05$ , Table 1). Moreover, the RANTES expression levels in ileal tissue were significantly decreased in the LM, MM, and HM groups compared to the Model group ( $P < 0.05$ , Table 2).

**Effects of different concentrations of magnolol on Caco2 permeability induced by LPS**

CCK-8 cells were used to detect cell permeability. The relative absorbance value of Caco2 cells treated with solvent was 1.0. No significant change was detected in cell permeability after adding different concentrations of magnolol (Figure 2). Conversely, the permeability of Caco2 cells increased significantly in the LPS-treated Caco2 cells ( $P < 0.01$ , Figure 2). However, compared to the LPS-treated Caco2 cells, the permeability decreased significantly when Caco2 cells were treated with magnolol before LPS treatment ( $P < 0.01$ , Figure 2).

**Magnolol decreases RANTES expression in Caco2 cells induced by LPS**

The RANTES expression in Caco2 cells in each group was determined by ELISA and Western blot analysis (Figure 3A and B). The expression of RANTES in Caco2 cells without LPS treatment was low, and magnolol had little effect on the expression of RANTES. On the other hand, LPS increased the level of RANTES in Caco2 cells, which was significantly inhibited by magnolol ( $P < 0.01$ , Figure 3A).

**Magnolol inhibits LPS-induced activation of NF- $\kappa$ B signaling pathway**

No significant difference was observed in the expression of inhibitor of nuclear factor kappa-B kinase (IKK) and p65 in different groups (Figure 4A). LPS increased the phosphorylated level of inhibitor of nuclear factor kappa-B kinase  $\beta$  (IKK $\beta$ ) and p65 in Caco2 cells and decreased the expression of inhibitor of nuclear factor kappa-B kinase  $\alpha$  (IKK $\alpha$ ). Magnolol inhibited the increased phosphorylation of IKK and p65 in LPS-treated Caco2 cells and reversed the down-regulation of IKK $\alpha$ . Compared to the Solvent group of Caco2 cells, the p65 distribution in the nucleus of Caco2 cells treated with 10  $\mu$ mol/L of magnolol did not change significantly ( $P > 0.05$ ), while LPS enhanced the translocation of p65 to the nucleus. Compared to the LPS-treated Caco2 cells, 10  $\mu$ mol/L of magnolol reduced the nuclear p65 abundance in Caco2 cells (Figure 4B).

**DISCUSSION**

The intestinal tract is the most commonly involved organ in sepsis. According to statistics, up to 60% of sepsis cases are accompanied by gastrointestinal tract dysfunction[21]. The intestinal tract is also the second-largest immune organ of the body. The gut has long been considered the “motor” of the systemic inflammatory response[22]. The common factors, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-1 $\beta$ , are mainly secreted by activated mononuclear macrophages and partially by other nucleated cells[23]. IL-6

**Table 1 Serum regulated on activation, normal T-cell expressed and secreted, tumor necrosis factor  $\alpha$ , interleukin 1 $\beta$ , and interleukin 6 levels in rats of different groups**

Group	RANTES (ng/mL)	TNF- $\alpha$ (pg/mL)	IL-1 $\beta$ (pg/mL)	IL-6 (pg/mL)
Control ( <i>n</i> = 11)	0.65 $\pm$ 0.25	124.91 $\pm$ 11.29	185.87 $\pm$ 24.97	183.69 $\pm$ 43.02
Model ( <i>n</i> = 9)	5.17 $\pm$ 0.70 <sup>a</sup>	256.89 $\pm$ 19.55 <sup>a</sup>	383.01 $\pm$ 41.85 <sup>a</sup>	380.99 $\pm$ 44.11 <sup>a</sup>
LM ( <i>n</i> = 9)	4.07 $\pm$ 0.67 <sup>a,b</sup>	167.39 $\pm$ 20.92 <sup>a,b</sup>	318.83 $\pm$ 74.06 <sup>a,b</sup>	308.21 $\pm$ 73.22 <sup>a,b</sup>
MM ( <i>n</i> = 10)	3.13 $\pm$ 0.45 <sup>a,b,c</sup>	143.87 $\pm$ 15.22 <sup>a,b,c</sup>	256.79 $\pm$ 25.37 <sup>a,b,c</sup>	246.14 $\pm$ 41.73 <sup>a,b,c</sup>
HM ( <i>n</i> = 10)	2.52 $\pm$ 0.28 <sup>a,b,c,d</sup>	144.49 $\pm$ 18.02 <sup>a,b,c</sup>	225.07 $\pm$ 17.46 <sup>a,b,c,d</sup>	215.18 $\pm$ 12.49 <sup>a,b,c,d</sup>

The contents of regulated on activation, normal T-cell expressed and secreted and cytokines in serum of septic rats after magnolol intervention were determined using enzyme-linked immunosorbent assay. All data are represented as the mean  $\pm$  SD.

<sup>a</sup>*P* < 0.01 vs Control group.

<sup>b</sup>*P* < 0.05 vs Model group.

<sup>c</sup>*P* < 0.05 vs LM group.

<sup>d</sup>*P* < 0.05 vs MM group.

TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; IL-1 $\beta$ : Interleukin-1 $\beta$ ; IL-6: Interleukin-6; RANTES: Regulated on activation, normal T-cell expressed and secreted; Control: The Control group; Model: Severe sepsis group; LM: Low-dose magnolol group; MM: Middle-dose magnolol group; HM: High-dose magnolol group.

**Table 2 Ileal regulated on activation, normal T-cell expressed and secreted, tumor necrosis factor  $\alpha$ , interleukin 1 $\beta$ , and interleukin 6 levels in rats of different groups**

Group	RANTES (g/mL)	TNF- $\alpha$ (pg/mL)	IL-1 $\beta$ (pg/mL)	IL-6 (pg/mL)
Control ( <i>n</i> = 11)	3.40 $\pm$ 1.45	347.04 $\pm$ 60.66	240.4 $\pm$ 37.08	195.11 $\pm$ 28.60
Model ( <i>n</i> = 9)	29.29 $\pm$ 4.07 <sup>a</sup>	1015.73 $\pm$ 90.96 <sup>a</sup>	453.52 $\pm$ 54.82 <sup>a</sup>	436.01 $\pm$ 46.29
LM ( <i>n</i> = 9)	22.47 $\pm$ 3.72 <sup>a,b</sup>	720.82 $\pm$ 88.16 <sup>b</sup>	330.89 $\pm$ 49.06 <sup>a,b</sup>	337.66 $\pm$ 73.79 <sup>b</sup>
MM ( <i>n</i> = 10)	17.39 $\pm$ 2.59 <sup>a,b,c</sup>	487.48 $\pm$ 115.29 <sup>a,b</sup>	301.43 $\pm$ 44.51 <sup>a,b</sup>	271.25 $\pm$ 36.82 <sup>b,c</sup>
HM ( <i>n</i> = 10)	14.10 $\pm$ 1.59 <sup>a,b,c,d</sup>	428.79 $\pm$ 48.71 <sup>a,b,c</sup>	294.27 $\pm$ 68.2 <sup>a,b</sup>	237.75 $\pm$ 16.05 <sup>b,c,d</sup>

The expression levels of regulated on activation, normal T-cell expressed and secreted and cytokines in ileal tissue of septic rats after magnolol intervention were determined using Western blot assay. All data are represented as the mean  $\pm$  SD.

<sup>a</sup>*P* < 0.01 vs Control group.

<sup>b</sup>*P* < 0.05 vs Model group.

<sup>c</sup>*P* < 0.05 vs LM group.

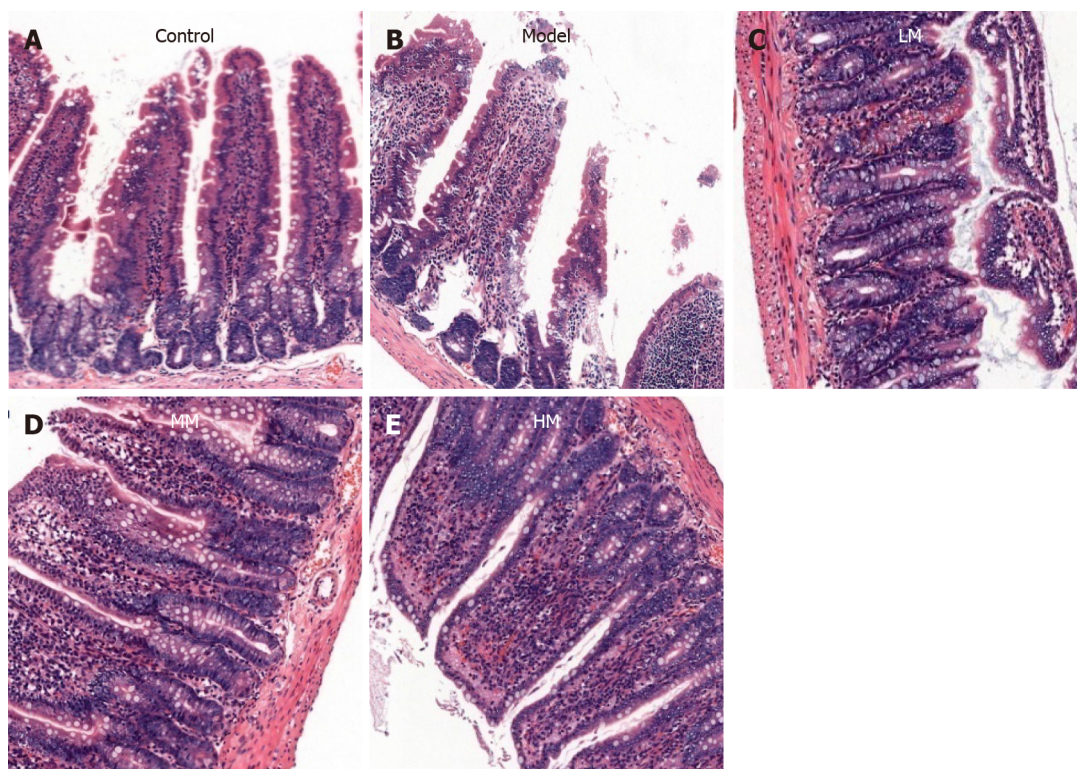
<sup>d</sup>*P* < 0.05 vs MM group.

TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; IL-1 $\beta$ : Interleukin-1 $\beta$ ; IL-6: Interleukin-6; RANTES: Regulated on activation, normal T-cell expressed and secreted; Control: The Control group; Model: Severe sepsis group; LM: Low-dose magnolol group; MM: Middle-dose magnolol group; HM: High-dose magnolol group.

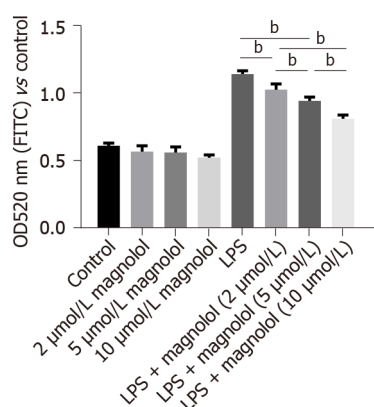
is secreted by mononuclear macrophages, fibroblasts, and endothelial cells. The primary function of the intestinal tract is to promote the acute phase protein synthesis and participate in inflammation. In the hemorrhagic shock model, IL-6 gene knockout protected the rats from intestinal barrier dysfunction[24]. Therefore, IL-6 has been suggested as a common inflammatory indicator of intestinal barrier dysfunction[25]. TNF- $\alpha$  is mainly secreted by activated mononuclear macrophages, and its main function is to heat and dilate blood vessels, and promote the chemotactic adhesion of neutrophils[26]. In the sepsis model, the increase in serum and tissue TNF- $\alpha$  has been closely related to mortality[27]. TNF- $\alpha$  is released at the initial stage of inflammation. It promotes the secretion of IL-1 $\beta$  and IL-6 and also stimulates the expression of adhesion molecules on the surface of intestinal microvascular endothelial cells and neutrophils, accelerating neutrophil aggregation and releasing the reactive oxygen and proteolytic enzymes. These phenomena disrupt the intestinal mucosal epithelium and accelerate apoptosis of intestinal mucosal epithelial cells[28].

In this study, we quantitatively detected the differences in the expression levels of inflammatory factors in each group, such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . In the severe sepsis group, the expression levels of all the three inflammatory factors were significantly increased, which were consistent with previous studies[29]. After magnolol administration, the release of inflammatory cytokines was reduced in a dose-dependent manner, and the apoptosis of LPS-induced ileal epithelial cells was





**Figure 1 Hematoxylin and eosin staining of ileal tissues.** A: Control group shows orderly arranged ileal mucosal villi; B: Model group shows damaged ileal mucosal villi. The epithelial cells at the villus tips were wiped off, the subepithelial capillaries exhibited congestion, the central lacteals were expanded, the lamina propria was exposed and disintegrated, and blood capillaries were bleeding, with ulcer formation and widened intracellular tight junctions; C-E: Ileal mucosal structure in rats of the LM (C), MM (D), and HM (E) groups, respectively. The morphology of ileal mucosal villi and intestinal glands was improved to varying degrees compared to panel B. The ileal mucosal morphology was improved in panel E compared to D and in D compared to C. Magnification,  $\times 100$ . Control: The control group; Model: Severe sepsis group; LM: Low-dose magnolol group; MM: Middle-dose magnolol group; HM: High-dose magnolol group.

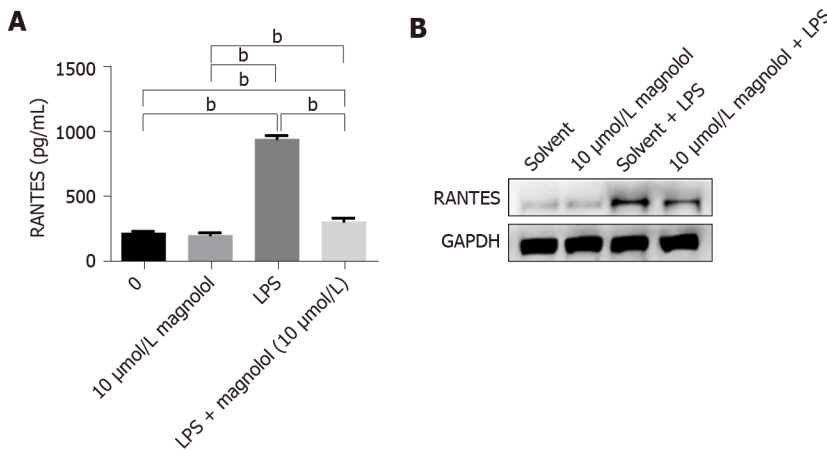


**Figure 2 Effects of different concentrations of magnolol on Caco2 cell permeability induced by lipopolysaccharide.** Cell permeability was evaluated to determine the concentration of magnolol that inhibited the lipopolysaccharide (LPS)-induced increased permeability of Caco2 cells. After adding LPS, the permeability of the cells was significantly increased ( $^*P < 0.01$ ). After adding magnolol to LPS-treated Caco2 cells, the permeability of the cells was decreased in a dose-dependent manner ( $^*P < 0.01$ ). Control: Treated with solvent only; 2 μmol/L, 5 μmol/L, and 10 μmol/L magnolol: Treated with different concentrations of magnolol; LPS: Treated with solvent and LPS (100 μg/mL); LPS + magnolol (2 μmol/L, 5 μmol/L, and 10 μmol/L): Treated with magnolol (2 μmol/L, 5 μmol/L, and 10 μmol/L) and LPS (100 μg/mL); OD520nm: Optical density at 520 nm; FITC: Fluorescein isothiocyanate isomer. LPS: Lipopolysaccharide.

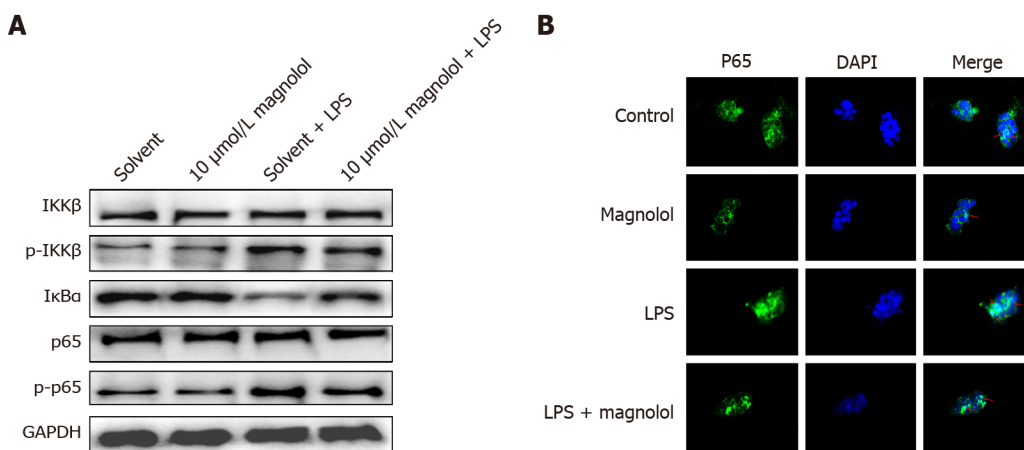
pronounced. Therefore, we speculated that magnolol prevents the aggravation of inflammation and the damage of intestinal mucosal tissues.

RANTES, a member of the chemotactic cytokine CC subgroup, also known as CCL5, is secreted by epithelial cells[25,30]. RANTES plays a critical role in chemotactic and stimulating T cells, accumulating a large number of T cells in the inflammatory lesions, activating T cells to produce inflammatory factors, and aggravating the tissue damage. Several studies have shown that RANTES is highly superficial in the intestinal





**Figure 3 Expression of regulated on activation, normal T-cell expressed and secreted protein in Caco2 cells in each group.** A: The cells were pretreated with magnolol or solvent for 8 h, followed by lipopolysaccharide (LPS) (100 μg/mL) for 24 h. The content of RANTES in the supernatant of the four groups of cells was determined by ELISA. Data are presented as the mean ± SD; the comparison between different groups was performed using the LSD method in one-way ANOVA ( $^*P < 0.01$ ); B: The cells were pretreated with magnolol or solvent for 8 h and then with LPS (100 μg/mL) for 24 h. The expression of RANTES protein in Caco2 cells in each group was detected by Western blot analysis. 0 or Solvent: Treated with solvent only; 10 μmol/L magnolol: Treated with 10 μmol/L magnolol; LPS: Treated with solvent and LPS (100 μg/mL); LPS + magnolol 10 μmol/L: Treated with magnolol 10 μmol/L and with LPS (100 μg/mL); GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RANTES: Regulated on activation, normal T-cell expressed and secreted.



**Figure 4 Nuclear factor-kappa B pathway-related protein expression and p65 nucleation in Caco2 cells in each group.** A: The cells were treated with magnolol or solvent for 8 h and then with lipopolysaccharide (LPS) (100 μg/mL) for 24 h. The protein levels of phosphorylated inhibitor of nuclear factor kappa-B kinase β and p65 were assessed by Western blot; B: Fluorescence distribution of p65 in the nucleus of Caco2 cells treated with magnolol or solvent for 8 h and then with LPS (100 μg/mL) for 24 h. Control or Solvent: Treated with solvent only; 10 μmol/L magnolol: Treated with 10 μmol/L concentration of magnolol; LPS: Treated with solvent and LPS (100 μg/mL); LPS + magnolol 10 μmol/L: Treated with magnolol 10 μmol/L and with LPS (100 μg/mL); GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; DAPI: 4,6-guanidine-2-phenylindole; IKKβ: Inhibitor of nuclear factor kappa-B kinase β; p-IKKβ: Phosphorylated inhibitor of nuclear factor kappa-B kinase β; IκBα: Inhibitor of nuclear factor kappa-B kinase α; LPS: Lipopolysaccharide.

mucosal tissues of patients with ulcerative colitis[31]. Also, its high expression is an indicator of sepsis in premature infants and neonates[32,33]. In the current study, RANTES was significantly increased in both serum and ileal tissue after LPS treatment as well as inflammatory factors. This phenomenon proved that the RANTES has a positive role in inflammation-induced LPS models. However, the serum secretion was consistent with the decreased expression of RANTES in ileal tissue after administration of magnolol, which further indicated its role in resisting the inflammatory response in sepsis.

The mechanisms by which magnolol regulates RANTES to reduce damage in intestinal mucosal epithelial cells are not well-understood. It has been hypothesized that the NF-κB signaling pathway is closely related to the production of various cytokines, chemokines, and adhesion factors in oxidative stress and inflammatory response. In normal cells, NF-κB is retained in the cytoplasm due to binding to its inhibitory protein IκB and kinase IKKα/β. When inflammation occurs, phosphorylated

I $\kappa$ Bs and IKK $\alpha/\beta$  may activate the NF- $\kappa$ B signaling pathway, thus leading to nucleation of p65 and p50 and promoting transcriptional activation of the relevant target genes[34]. When NF- $\kappa$ B is dislodged from the complex, it translocates rapidly from the cytoplasm to the nucleus and aggregates in the nucleus to induce RANTES by binding to its promoter[19]. Several studies have shown that patients with inflammatory bowel disease exhibit high expression of p65 in the nucleus of cells in intestinal mucosal tissue. Abnormal expression of intracellular p65 was also detected in the dextran sodium sulfate (DSS)-induced colitis model. Simultaneously, PDTC, an inhibitor of NF- $\kappa$ B, significantly upregulates the expression of the dense protein in DSS-induced colon cancer rats, restores the normal intestinal mucosal permeability, and reduced the inflammatory response[35]. The current results revealed that after magnolol treatment, the permeability of Caco2 cells induced by LPS, the phosphorylation of IKK $\beta$ , and the nucleation of p65 was decreased, and the expression of RANTES was down-regulated. The abnormal activation of the NF- $\kappa$ B signaling pathway was closely correlated with the expression of RANTES during inflammation, and magnolol inhibited the up-regulation of RANTES expression in the NF- $\kappa$ B signaling pathway.

We found that in our study, inhibition of serum TNF- $\alpha$  levels by magnolol was only partial, *i.e.*, 50%-60% inhibition *vs* Model group and there may be enough circulating TNF- $\alpha$  for host defense. It appears that magnolol has more than a single anti-inflammatory effect in the treatment of sepsis. Some studies have reported that drugs with anti-inflammatory actions, such as antioxidative agents, could regulate immunity dysfunction with apparent safety in sepsis[36,37]. As we know, pathogen-associated molecular patterns (PAMPs) can lead to the initiation of innate immune and inflammatory responses. TLRs are a group of evolutionarily conserved and membrane-bound pattern recognition receptors that recognize various PAMPs including microbial nucleic acids, lipids, proteins, lipoproteins, and glycoproteins[38,39]. We have found that magnolol could down-regulate RANTES levels by inhibiting the TLR4-regulated NF- $\kappa$ B signaling pathways. Taken together, these findings suggest that magnolol may have an immunomodulating role in sepsis. We will test this idea in subsequent experiments.

## CONCLUSION

Overall, magnolol significantly alleviates inflammatory response and pathological changes in the intestinal tissue and the levels of RANTES expression in rats with sepsis. The abnormal activation of the NF- $\kappa$ B signaling pathway is closely correlated with the expression of RANTES during inflammation, and magnolol inhibits the up-regulation of RANTES expression *via* the NF- $\kappa$ B signaling pathway. Thus, this study provided the pharmacological proof for use of magnolol and suggested that it is a potential agent in the prevention and treatment of sepsis. Further studies should be conducted to clarify the underlying mechanism(s) of the anti-inflammatory and immunomodulating role of magnolol in sepsis.

## ARTICLE HIGHLIGHTS

### Research background

Sepsis is a major medical challenge, and finding specific targets and effective drugs is a scientific concern. Currently, various biological constituents are isolated from traditional Chinese medicine and have been confirmed to possess multifunctional activities.

### Research motivation

Magnolol is an active constituent of *Houpu*, which improves tissue function and exerts strong anti-endotoxin and anti-inflammatory effects. Thus, we aimed to identify the role of magnolol in the treatment of sepsis.

### Research objectives

To assess the protective effect of magnolol on intestinal mucosal epithelial cells in sepsis and elucidate the underlying mechanisms.

### Research methods

We carried out animal studies and cell studies *in vitro*, respectively. In animal studies, enzyme-linked immunosorbent assay was used to measure the differentially expressed inflammatory factors and regulated on activation, normal T-cell expressed and secreted (RANTES) levels in serum and ileal tissue. In the *in vitro* experiments, Cell Counting Kit-8 and cell permeability assays were employed to determine the concentration of drug-containing serum that did not affect the activity of Caco2 cells but inhibited lipopolysaccharide (LPS)-induced permeability reduction. Immunofluorescence and Western blot assays were used to detect the protein levels of RANTES, inhibitor of nuclear factor kappa-B kinase  $\beta$  (IKK $\beta$ ), phosphorylated IKK $\beta$  (p-IKK $\beta$ ), inhibitor of nuclear factor kappa-B kinase  $\alpha$  (IkB $\alpha$ ), p65, and p-p65 in different groups.

### Research results

In animal studies, magnolol inhibited the expression of proinflammatory cytokines tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin 1 $\beta$  (IL-1 $\beta$ ), and IL-6 in a dose-dependent manner and suppressed the production of RANTES in sepsis rats. In the *in vitro* studies, magnolol inhibited the increase of p65 nucleation and down-regulated the production of the phosphorylated form of IKK $\beta$  in LPS-treated Caco2 cells. Moreover, magnolol inhibited the translocation of the transcription factor nuclear factor-kappa B (NF- $\kappa$ B) from the cytosol into the nucleus and downregulated the expression level of the chemokine RANTES in LPS-stimulated Caco2 cells.

### Research conclusions

Magnolol downregulates RANTES levels by inhibiting the LPS/NF- $\kappa$ B signaling pathway, resulting in the suppression of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  expression that in turn, alleviates the mucosal barrier dysfunction in sepsis.

### Research perspectives

This study, for the first time, proved that magnolol plays a role in the treatment of sepsis by down-regulating RANTES, and further studies on the anti-inflammatory mechanism might provide an in-depth insight into novel methods for the clinical prevention and treatment of sepsis.

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