ORIGINAL RESEARCH

Emodin Attenuates Sepsis-induced Intestinal Injury by Regulating TRPM7 Expression

Lijuan Wu, MS; Fenqiao Chen, MS; He Zhang, MS; Yanjun Liu, MS; Tane Liu, MS; Pengying Yin, MS; Jianqiang Mei, MS

ABSTRACT

Background • Sepsis is a potentially lethal organ immune dysfunction induced by infection, with the stomach being the first organ to be attacked. Emodin has anti-inflammatory and gastrointestinal functions, but its therapeutic effect on intestinal injury in sepsis remains unclear. This study sought to investigate the role of emodin in treating intestine damage brought on by sepsis.

Methods • Between June 2021 and July 2023, Lipopolysaccharide (LPS) was used to stimulate human intestinal epithelial cells NCM460 to create a septic cell model, and treatment was regulated by rhodopsin. Transient receptor potential melastatin 7 (TRPM7) expression was used to check that the LPS induction conditions were acceptable. About the proliferation of the NCM460 cells, the effects of overexpressing TRPM7 and silencing TRPM7 were assessed. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide test. Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 expression in the cells was detected using enzyme-linked immunosorbent assays. TRPM7 messenger RNA expression was detected using real-time quantitative polymerase chain reaction (RT-qPCR). Western blot determined the levels of TRPM7, Bcl2-associated X (Bax), and B-cell lymphoma-2 (Bcl2) protein expression levels. The terminal deoxynucleotidyl transferase (TdT)-mediated 2'-deoxyuridine 5'-triphosphate (dUTP) nick-end labeling (TUNEL) technique was used to measure the apoptosis rate.

Results • The levels of the inflammatory factors and Bax expression in the cells and the cell apoptosis rate steadily increased as the LPS-induced concentration increased. In contrast, cell viability and the Bcl2 expression levels gradually decreased. In this study, we treated the cells with LPS at a concentration of 25 μg/mL for 12 hours. It was detected that the knockdown of TRPM7 expression decreased the effect of LPS induction, while boosting the expression of TRPM7 boosted the effectiveness. Treatment with emodin lowered TRPM7 expression, increasing cell survival, and Bcl2 expression levels while decreasing the apoptosis rate, inflammatory factors, and Bax expression levels.

Conclusion • Emodin may alleviate sepsis-induced intestinal injury by down-regulating the TRPM7 gene. These findings suggest that emodin may hold promise as a therapeutic agent for treating intestinal injury in sepsis. If further validated through additional research and clinical trials, emodin or similar compounds could potentially be developed into safe and effective medications for sepsis patients. (*Altern Ther Health Med.* [E-pub ahead of print.])

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INTRODUCTION

Sepsis is a clinical illness with a high fatality rate in which an organism becomes infected with pathogenic microorganisms, leading to the dysfunction or collapse of its organs.[1,2] A survey reported that intensive care units in mainland China had a sepsis prevalence rate of 20.6% and hospitalization morbidity and death rates of 29.6% and 32.1%, respectively.[3] Sepsis affects the epithelial cells of organs in the body, particularly the intestinal epithelial cells, resulting in reduced intestinal function in the human body.[4] A damaged intestine further contributes to the translocation of bacteria and toxins, aggravating the systemic inflammatory response and leading to numerous organ failures.[5] The most pressing priority in sepsis treatment is the development of effective and safe medicines.

The inflammatory response plays a vital role in sepsis.[6-8] Transient receptor potential melastatin 7 (TRPM7) channels are bifunctional transmembrane proteins with an ion-channel structure domain and a kinase-structural domain that regulate several inflammatory illnesses in the body.[9] Huang *et al.*[10] showed that the release of inflammatory mediators (interleukin [IL]-13, IL-6, and tumor necrosis factor [TNF]–α) in rat bone marrow-derived dendritic cells was inhibited by TRPM7 suppression. Sun *et al.*[11] discovered that knocking down Kruppel-like factor 2 in rat renal tubular epithelial cells enhanced TRPM7 expression, which in turn increased IL-1β, IL-6, and TNF-α expression, contributing to lipopolysaccharide (LPS)–induced inflammatory dysfunction. Liu *et al.*[12] reported that TNF-α, IL-6, and IL-10 expression levels were higher in patients with high TRPM7 expression, and TRPM7 expression in serum has some diagnostic value in determining the occurrence and progression of sepsis.

In recent years, active compounds in natural herbs have gained a lot of interest in sepsis pharmacotherapy research. Emodin is a natural compound extracted from rhubarb, thuja, and other Chinese herbs, which has anti-inflammatory effects, improves vascular endothelial function, kidney injury, and myocardial injury, regulates gastrointestinal function, and has a therapeutic impact on sepsis.[13-16] However, the role of Emodin in sepsis-induced intestinal injury has not been observed, and its potential therapeutic mechanism is unknown; whether Emodin regulates the TRPM7 gene requires additional investigation.

In this study, a sepsis cell model was built using LPS to analyze the effects of Emodin on cell viability, apoptosis rate, inflammatory factor levels, and TRPM7 levels in human intestinal epithelial cells, as well as elucidating the mechanism of Emodin's action in regulating sepsis-induced intestinal injury.

MATERIALS AND METHODS

Cell Culture and Transfection

The experimental strain of human intestinal epithelial cells (NCM460) was acquired from the American Therapeutic Collection Center. The cells were placed in a 5%-carbon dioxide, temperature, and humidity-constant (37°C, 70%~80%, RADOBIO, Shanghai, China) incubator. The cells were grown in 10% fetal bovine serum-infused Dulbecco's Modified Eagle Medium (10566024, Gibco, Big Cabin, OK, USA) with 1% penicillin-streptomycin solution (double antibodies, 15140122, Gibco). The solution was changed every other day. The NCM460 cells were transfected with silencing (si)-TRPM7 plasmid generated by Biomics (Nantong, China) and its si-negative control (NC) or with overexpressing TRPM7 plasmid and its NC. The transfection method was executed according to the instructions of the Lipofectamine™ RNAiMAX kit (13778100, Invitrogen, Austin, TX, USA).

To create an in vitro cell model, cells (1×105/mL) were injected into 96-well culture plates (100 μL/well) before or after transfection. The cells were grown for 24 hours before being treated with LPS (0, 5, 10, 25, and 50 μg/mL) for 12 hours. Following the collection of each group's cells, various indicators were measured.

MTT Assays

Well received 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (20 μL, TOX1, Sigma-Aldrich, St. Louis, MO, USA) and was subsequently incubated for four hours after receiving 12 hours of LPS treatment. The supernatant was aspirated and discarded, 150 μL of dimethyl sulfoxide was added, and an enzyme labeler (Multiskan FC, ABI, Foster City, CA, USA) was applied to gauge absorbance at 490 nm. The following formula was used: % viability = $(A_a / A_b) \times 100\%$, where A_a is the experimental absorbance, and A_b is the absorbance of untreated controls.

TUNEL Assays

The TUNEL kit (C1091, Beyotime Biotechnology, Shanghai, China) performed apoptosis quantification. After fixing the cell samples with 4% paraformaldehyde, the cells were successively stained with TUNEL and 4',6-diamidino-2-phenylindole (DAPI) (C1005, Beyotime biotechnology). The cells were rinsed between each staining. After sealing the slices with an anti-fluorescence burst-blocking solution, the TUNEL-positive and DAPI-positive cells were counted by randomly observing five high-magnification fields under the microscope. The apoptosis rate was determined using the formula: % apoptotic = (Number of TUNEL-positive cells / Number of DAPI-positive cells) × 100%.

Inflammatory Indicator Detection

The serum levels of the IL-1β, IL-6, and TNF-α (ab214025, ab178013, and ab181421, Abcam, Waltham, MA, USA) cytokines were assessed using enzyme-linked immunosorbent assay (ELISA) kits. The standards and samples were diluted as directed, and 100 μL of the diluted standards and samples were then added to the assay plates sequentially. Three parallel controls were set up for each group and incubated at 37°C for 1.5 h. Next, 100 μL of biotin-labeled antibody was incubated in each well for 1.5 hours at 37°C, after which 100 μL of affinity hormone-horseradish peroxidase marker was incubated in each well for 0.5 hours at 37°C. Finally, 100 μL of color development solution was applied to each well, which was incubated at 37°C under light protection for 15 minutes before being terminated with 100 μL of termination solution. The absorbance values of each group of samples at 450 nm were recorded using an enzyme meter (Multiskan FC, ABI). The observed data were utilized to generate the standard curve, and the amounts of IL-1β, IL-6, and TNF-α were then computed.

qRT-PCR

Cell samples were collected after LPS induction. Total RNA was extracted using TRNzol reagent (DP424, TIANGEN, Beijing, China), which was subsequently reverse-

transcribed to complementary DNA (cDNA) using a reverse transcription kit (RR037Q, TAKARA, Beijing, China). Next, using cDNA as a template, the particular primers for the target gene and its internal reference gene were made by GenePharma, Inc. (Shanghai, China), and a real-time quantitative polymerase chain reaction (RT-qPCR) reaction system was created. Amplification was then carried out using the ABI PRISM 7300 RT-PCR system (7300, ABI, Carlsbad, CA, USA). Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference gene, the $2^{-\Delta\Delta Ct}$ technique was employed to calculate TRPM7 messenger RNA (mRNA) expression. Primer sequences were designed as follows (5'-3'): GAPDH: F: TGTAGGCTCATTTGCAGGGG, R: TCCCATTCCCCAGCTCTCAT; TRPM7: F: CAACTAGGC CTCTGTGCCAA, R: CTGTGAGGTGCAGGCAAAAC.

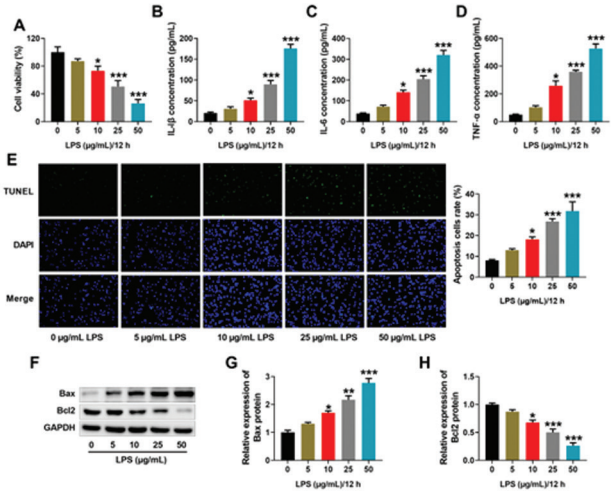
Western Blot

TRPM7, Bcl2-associated X (Bax), and B-cell lymphoma-2 (Bcl2) protein expression in the cells was detected by Western blot [17]. Total proteins were extracted from each cell group, and a protein concentration assay (bicinchoninic acid, BCA) kit was used to calculate the protein concentration (P0012, Beyotime biotechnology). Next, the protein samples were obtained and added to the sampling buffer before being denatured for 10 min in a boiling water bath. The agarose gel was prepared using standard techniques, and the gel was subsequently placed in an electrophoresis tank in which the electrophoresis buffer was poured. Protein samples were added to a polyacrylamide gel (20 μg/well) and subjected to vertical electrophoresis using a gel electrophoresis apparatus (VE-180, Tanon, Shanghai, China) and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. PVDF membranes were washed with Tris-Buffered Saline with Tween (TBST) before being closed at room temperature for one hour with 5% (w/v) skimmed milk powder and then incubated for 24 hours at 4°C with TRPM7 (ab245408, Abcam), Bax (ab32503, Abcam), Bcl2 (ab182858, Abcam) and GAPDH (ab8245, Abcam)–specific primary antibodies, respectively. The membranes were rinsed five times with TBST before being incubated at 25°C for one hour with the secondary antibody. The membranes were then rewashed with TBST (five times). After the membrane washing, protein expression was observed under a developer (Tanon 5200, Tanon) using an enhanced chemiluminescence reagent (P0018S, Beyotime biotechnology). Image J 1.8.0 software from the National Institutes of Health (Bethesda, MD, USA) was used to calculate the absorbance of protein bands. GAPDH was employed as an internal reference to determine the expression of TRPM7, Bax, and Bcl2.

Statistical Analysis

SPSS v 26.0 (SPSS Inc., Chicago, IL, USA) was used for statistical data analysis. The graph plot was created using GraphPad Prism v 9.0 (GraphPad Inc., La Jolla, CA, USA). All experiments were done three times, and findings are reported as mean ± standard deviation (mean ± SD). To compare the mean scores between any two groups, the least significant difference *t* test was used, and a one-way analysis of variance was employed to evaluate data from multiple groups. Statistically significant results ($P < .05$, $P < .01$, and $P < .001$).

Figure 1. LPS-induced sepsis intestinal epithelial cell model. Sepsis cell models were created using various doses (0, 5, 10, 25, and 50 μg/mL) of LPS-induced NCM460 cells for 12 hours. (A) MTT assays were used to assess cell viability. (B–D) ELISA kits were used to assess the expression of the inflammatory factors. (E) TUNEL staining was used to assess the apoptosis rate of the cells. (F–H) Western blot was used to determine the expression of apoptosis-related proteins Bax and Bcl2.



*$P < .05$,
**$P < .01$
***$P < .001$.

RESULTS

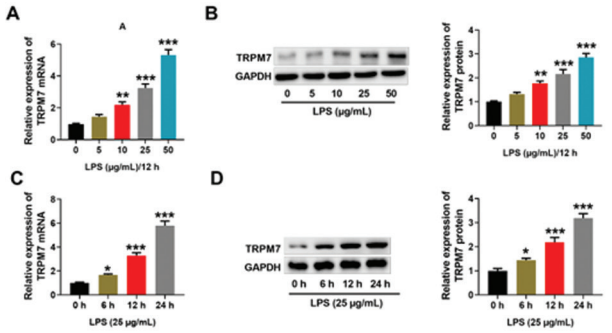
Effect of LPS Concentration on NCM460 Cells

The cell viability of the sepsis cell model produced by LPS induction decreased gradually as concentrations of LPS treatment increased (see Figure 1A). The three inflammatory factors were significantly improved after LPS induction (0, 5, 10, 25, and 50 μg/mL) (see Figures 1B–1D), indicating that a large number of inflammatory factors were released from the NCM460 human intestinal epithelial cells after LPS induction. Additionally, the apoptosis rate increased as the quantity of LPS treatment increased (see Figure 1E), and 25 μg/mL LPS appeared to alter cell viability and apoptosis significantly. The anti-apoptotic protein Bcl2 expression level steadily decreased with LPS-induced concentration, and the pro-apoptotic protein Bax expression level gradually increased (see Figures 1F–1H). Following LPS induction, NCM460 cell apoptosis increased, proliferation ability decreased, and an inflammatory response occurred.

Effect of LPS on TRPM7 Expression

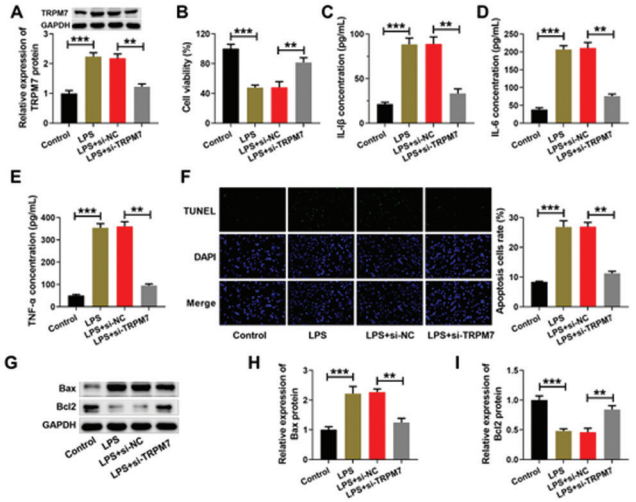
As LPS concentration increased, the relative expression of TRPM7 mRNA and protein in cells increased (see Figures 2A–2B). TRPM7 mRNA and its protein content were

Figure 2. TRPM7 was upregulated in the LPS-induced cell model. (A, B) TRPM7 mRNA and protein content after LPS treatment at 0, 5, 10, 25, and 50 μg/mL. (C, D) TRPM7 mRNA and protein content after LPS treatment at 0, 6, 12, and 24 h.



*$P < .05$
**$P < .01$
***$P < .001$.

Figure 3. Silencing of TRPM7 ameliorated LPS-induced cell damage. (A) Western blot was used to determine the expression level of TRPM7. (B) MTT assays were used to assess cell viability. (C–E) ELISA kits were used to assess the expression of the inflammatory factors. (F) TUNEL staining was used to detect the apoptosis rate of the cells. (G–I) Western blot was used to determine the expression of apoptosis-related proteins Bax and Bcl2.



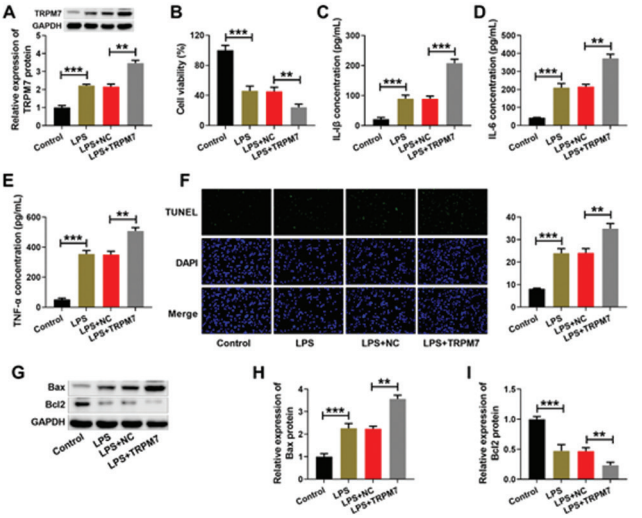
**$P < .01$
***$P < .001$.

significantly altered by the LPS induction at a concentration of 25 μg/mL. TRPM7 mRNA and protein expression levels gradually increased (see Figures 2C–2D). LPS (25 μg/mL) was used to stimulate the NCM460 cells for 12 hours, resulting in a notable change in TRPM7 mRNA and protein expression. An LPS induction condition of 25 μg/mL for 12 hours was used for the subsequent experiments.

Effect of Silencing TRPM7 Expression on Cells

TRPM7 expression was successfully knocked down in the NCM460 cells by silencing TRPM7 (si-TRPM7) (see Figure 3A), and si-TRPM7 transfection increased cell viability (see Figure 3B), decreased IL-1β, IL-6 and TNF-α levels (see Figures 3C–3E), decreased the rate of apoptosis and Bax expression (see Figures 3F–3G) and increased Bcl2 expression (see Figure 3I). This suggests that si-TRPM7 expression increased cell proliferation, decreased apoptosis and lowered the inflammatory response.

Figure 4. TRPM7 OverexpressIon aggravated LPS-induced cell damage**.** (A) Western blot was used to determine the expression level of TRPM7. (B) MTT assays were used to assess cell viability. (C–E) ELISA kits were used to assess the expression of the inflammatory factors. (F) TUNEL staining was used to detect the apoptosis rate of the cells. (G–I) Western blot was used to determine the expression of apoptosis-related proteins Bax and Bcl2.



**$P < .01$
***$P < .001$.

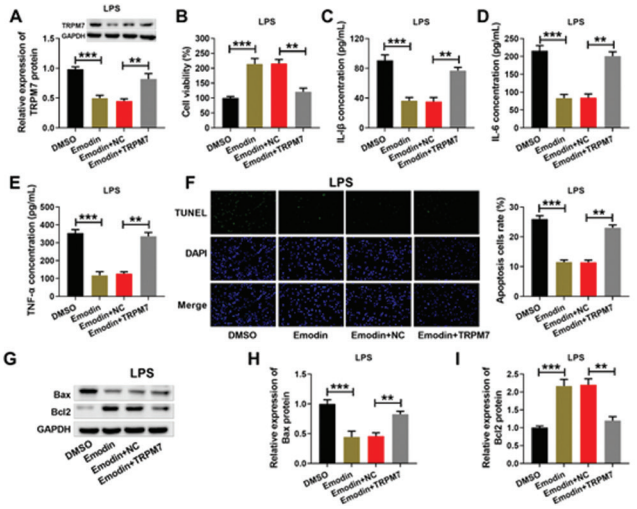
Effect of Overexpression of TRPM7 Expression on Cell Proliferation

After transfection of overexpressed plasmid TRPM7, the level of TRPM7 in NCM460 cells was elevated (see Figure 4A), cell viability was decreased (see Figure 4B), the expression levels of the inflammatory factors were all elevated (see Figure 4C–4E), and the apoptosis rate was promoted (see Figure 4F), while the Bax and Bcl2 expression levels were down-regulated and increased, respectively (see Figure 4G–4I). The above findings revealed increased TRPM7 expression in NCM460 cells boosted the LPS induction effect and reduced NCM460 cell growth.

Effect of Emodin on TRPM7 Expression

In the sepsis cell culture, emodin administration reduced TRPM7 expression while reducing the LPS induction effect (see Figure 5A), enhanced NCM460 cell viability (see Figure 5B), decreased inflammatory factor levels (see Figure 5C–5E), reduced the apoptosis rate (see Figure 5F) and inhibited and promoted the expression of Bax and Bcl2, respectively (see Figure 5G–5I). Similarly, Chen *et al.* [18] found

Figure 5. Emodin alleviated LPs-induced cell injury through the down-regulation of TRPM7. (A) TRPM7 expression levels in the cells in the emodin-treated and untreated groups. (B) Viability of the cells in the emodin-treated and untreated groups. (C–E) Expression of the three inflammatory factors in the cells of the emodin-treated and untreated groups. (F) The apoptosis rates of the cells in the emodin-treated and untreated groups. (G–I) The expression of apoptosis-related proteins Bax and Bcl2 were determined by Western blot.



$^{**}P < .01$
$^{***}P < .001$.

that emodin inhibited the inflammatory reaction and preserved the intestinal tissues of septic rats in the cecum ligation and puncture (CLP) model, which is congruent with the present study's findings.

DISCUSSION

The systemic inflammatory response and its fast onset are the key pathogenetic features of sepsis, which leads to multiple organ dysfunction or shock and is a leading cause of death in severely ill individuals.[19-22] TNF-α, IL-1β, and IL-6 produced by monocytes and macrophages are critical pro-inflammatory factors in the development of sepsis, and a large number of inflammatory factors will destroy the integrity of the intestinal mucosal barrier and increase permeability, allowing pathogenic bacteria to invade the intestinal lumen and toxins to be absorbed, exacerbating the development of sepsis.[23,24] In our study, a sepsis cell model was established using LPS induction, and the results revealed that the model was consistent with changes in septic cell model indicators observed in previous studies.[25-27] The results also showed that the sepsis cell model was successfully established in our research.

TRPM7 regulates cell migration and proliferation as a bifunctional membrane protein with ion-channel and kinase activity.[28] Previous investigations have demonstrated that TRPM7 is crucial for the regulation of inflammation. An *et al.*[29] discovered that lowering TRPM7 expression TRPM7 inhibited the toll-like receptor 4/ nuclear factor kappa-light-chain-enhancer of activated B cells (TLR4/NF-κB) pathway and activated the mitogen-activated extracellular signal-regulated kinase/extracellular signal-regulated kinase (MEK/ERK) pathway in enterocytes, preventing apoptosis and attenuating inflammatory responses. Other studies have similarly found that blocking TRPM7 inhibited the expression of inflammatory factors, consistent with the present study's findings.[30,31]

Currently, several studies have focused on emodin effects on sepsis. Gao *et al.* [32] found that emodin inhibits the CLP-induced inflammatory response in mice by up-regulating BDNF/TrkB signaling, which helps to alleviate cognitive impairment and pathological damage in mice. Emodin has also been found to reverse cardiac insufficiency and improve myocardial condition in septic rats by inhibiting the activation of inflammatory vesicles.[33] Guo *et al.* [34] found that emodin attenuates sepsis-induced lung tissue edema by regulating aquaporin (AQP) and tight junction (TJ), inflammatory factors, and pulmonary apoptosis. Studies have also used emodin to treat septic bowel injury,[35-37] but these studies did not report on the modulation of TRPM7 by emodin. In this study, we discovered that emodin treatment significantly reduced TRPM7 expression in LPS-induced cell models, down-regulated inflammatory factors in cells, increased cell viability, and decreased the apoptosis rate, which suggests that the modulation of TRPM7 may be one of the mechanisms of action by which emodin inhibits intestinal inflammatory response in sepsis.

In summary, emodin may exert a protective effect against intestinal injury in sepsis by decreasing the expression of TRPM7 in NCM460 cells, reducing cellular inflammatory response, enhancing cell survival, and slowing apoptosis. However, Emodin has limited solubility, high permeability, and extensive metabolism, making it unsuitable for clinical application. The development of Emodin medications must be paired with novel methods such as nanoemulsions, hydrogels, and electrostatic spinning to improve Emodin bioavailability and extend its action time in patients. Furthermore, the effect of Emodin administration concentration on in vivo toxicities should be investigated further to identify the best concentration range applicable to all persons and facilitate clinical translation.

DISCLOSURE OF INTEREST

The authors have no financial conflicts of interest to declare.

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