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Preliminary report

Ethyl pyruvate protects against *Salmonella* intestinal infection in mice through down-regulation of pro-inflammatory factors and inhibition of TLR4/MAPK pathway

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ABSTRACT

Salmonella typhimurium is one of the main causes of intestinal diseases, affecting the health of humans and livestock. Ethyl pyruvate (EP), which is ordinarily an edible spice, has been indicated to exert anti-inflammatory effects and preserve intestinal barrier function. In this study, intestinal immune function and signaling pathways activated by EP were investigated *in vivo* in *S. typhimurium*-challenged BALB/c mice and *in vitro* in RAW264.7 cells. EP improved body weight loss and the organ index of the liver and spleen ($p < 0.05$). Serum IgA and IgM levels were significantly increased in EP-treated mice ($p < 0.05$). According to histopathological and immunohistochemical staining, EP significantly increased the villus height, reduced edema in the jejunum and increased the levels of claudin-1, occludin-1 and ZO-1 proteins compared to the *Salmonella*-treated group ($p < 0.05$). In addition, EP decreased the levels of the IL-6, IL-1β and TNF-α mRNA levels in jejunum, liver, spleen and RAW264.7 cells ($p < 0.05$). EP decreased the levels of TLR4, phosphorylated p38MAPK and ERK1/2 in mice infected with *S. typhimurium* ($p < 0.05$). In conclusion, EP effectively protected BALB/c mice from an intestinal *S. typhimurium* infection by improving the activity of the humoral immune system, reducing intestinal barrier damage, and inhibiting proinflammatory cytokine production in the jejunum by modulating the TLR4/MAPK signaling pathway. Based on these findings, EP has the potential to inhibit inflammation or to serve as an immune-enhancing adjuvant.

1. Introduction

Salmonella, a Gram-negative bacterium, is a facultative intracellular parasite of the enterobacteriaceae family. It has received extensive attention due to causing serious harm to humans and the livestock breeding industry [1]. In the United States, 50% of food poisoning cases are caused by bacteria, and approximately 30–50% of cases are caused by *Salmonella typhimurium* [2]. The long-term survival of *S. typhimurium* in the intestine increases the risk of developing enteritis caused by the virulence proteins in the bacteria [3]. *S. typhimurium* mainly infects the liver, spleen, lungs and intestines and causes congestion [4]. In mice, *S. typhimurium* causes typhoid fever as a systemic disease by spreading and replicating in macrophages in the reticuloendothelial system after an intestinal invasion [5]. Therefore, an effective medicament that will reduce the intestinal damage caused by *S. typhimurium* is urgently needed.

Ethyl pyruvate (EP), which is an edible spice, was recently reported to exert anti-inflammatory effects [6,7]. EP is derived from pyruvic acid derivatives. Pyruvate is the main intermediate in energy metabolism and is involved in the tricarboxylic acid cycle through oxidization into acetyl coenzyme A in the presence of adequate oxygen. Pyruvate provides energy through its conversion into lactic acid by dehydrogenases in the absence of adequate oxygen. Thus, pyruvate also exerts an antioxidant effect and reduces the levels of free radicals [8]. However, pyruvate is not used extensively because it is unstable in aqueous solutions and readily induces toxicity. EP, on the other hand, displays higher stability than pyruvate and has the same antioxidant effect [9].

EP exerts a variety of pharmacological functions by improving the tissue and cell injury mediated by redox reactions, reducing the secretion of proinflammatory factors, and enhancing antitumor immunity

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[10–13]. In serum and tissue inflammatory oxidation processes, EP reduces cell infiltration, serum urea and creatinine levels, and malondialdehyde levels in kidney tissues [14]. In addition, EP has been shown to repair intestinal tissue injury by upregulating the expression of the tight junction proteins ZO-1 and occludin [15,16]. EP also maintains intestinal epithelial cells to avoid the dispersion of bacterial endotoxins [17]. LPS is the bacterial endotoxin that is mainly secreted by Gram-negative bacteria. Intestinal barrier dysfunction is associated with LPS, and the host receptors to which LPS mainly binds are TLR2 and TLR4 [18–21]. EP downregulates the TLR4 level in rats with multiple organ dysfunction syndrome caused by LPS [22]. Moreover, LPS from *Salmonella enterica* serovar Typhimurium induces inflammation by activating MAPK pathway [23]. In previous studies, the anti-inflammatory and sepsis effects of EP were achieved by inhibiting the MAPK pathway [24,25]. Thus, TLR2/4 and MAPK may be components of an important pathway regulating the expression of inflammatory cytokines.

Although many studies have reported a function for EP as an anti-inflammatory agent, the EP-induced improvements in specific host immune functions in the context of inflammation have not been specifically researched. In the present study, we infected BALB/c mice with *S. typhimurium* and then administered them a 100 mg/kg EP injection. Serum IgA, IgM and IgG levels were determined using ELISA, and the expression of proinflammatory cytokine mRNAs was assessed using qRT-PCR. Levels of the TLR4, phosphorylated p38MAPK and ERK1/2 proteins were determined using Western blotting. According to the results of the *in vitro* study, EP reduced the expression of proinflammatory cytokines in bacterially infected RAW264.7 cells. The aim of this study was to investigate the modulatory effect of EP on the intestinal immunity in *S. typhimurium*-infected BALB/c mice, particularly its effect on repairing the intestinal barrier and anti-inflammatory mechanisms.

2. Materials and methods

2.1. Chemicals and antibodies

S. typhimurium C7731 and RAW 264.7 cells were obtained from the American Type Culture Collection. EP was purchased from Sigma-Aldrich, USA (E47808). The rabbit p44/42 MAPK (ERK1/2) (137F5) mAb, rabbit phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E) XP® mAb, rabbit anti-p38MAPK (D13E1) XP® mAb and rabbit anti-phospho-p38MAPK (Thr180/Tyr182) (D3F9) XP® mAb were purchased from Cell Signaling Technology (#8690, #4511, USA). The rabbit anti-TLR4 (YT0744) mAb and rabbit anti-actin (YM3028) mAb were obtained from Immunoway. The HRP-labeled goat anti-rabbit IgG (H + L) secondary antibody was purchased from Beyotime (A0208, China). The anti-ZO-1 antibody (21773-1-AP, China) was obtained from Proteintech, and the anti-claudin-1 (WL0662, China) and anti-occludin (WL01996, China) antibodies were obtained from Wanleibio.

2.2. Experimental animals

Sixty BALB/c male mice (LiaoNing ChangSheng Co., Ltd., China) weighing 20–25 g and aged 4–5 weeks were randomly divided into the following 4 groups ($n = 15$) [26]: 1) control group, 2) *S. typhimurium* group (ST), 3) *S. typhimurium* + EP group (ST + EP), and 4) EP group. All mice were provided water containing 5 mg/mL streptomycin for 2 days followed by regular water for 1 day [27]. Mice were fasted overnight before being administered the bacterial solution by oral gavage. The control group was administered 0.2 mL/d of PBS (gastric administration) in all trial periods and injected with 0.2 mL/d of PBS during the last three days. We created an inflammatory model by administering 0.2 mL of 1×10^8 CFU/mL/d *S. typhimurium* in PBS (gastric administration) for 6 days. The ST + EP group was administered the same bacterial treatment and injected with 0.2 mL of a 100 mg/mL/d EP solution in PBS starting on the fourth day [6]. The EP group was administered PBS (gastric administration) using the same procedure as that described for the control group and EP using the same procedure as that described for the ST group. After the administration of the last dose, the mice were sacrificed by isoflurane anesthesia after a 12 h-fast. Animal care and treatments complied with the standards described in the guidelines for the care and use of laboratory animals of Northeast Agricultural University (NEAU-[2011]-9).

2.3. Determination of immunoglobulin by ELISA

IgA, IgG and IgM levels in serum samples were assayed with a commercial ELISA kit (m1037606, ML-Biology, China) according to the manufacturer's instructions. Different concentrations of standards (50 μL/well) and 10 μL of samples in 40 μL of sample diluent were added to the appropriate wells. Blank wells contained 10 μL of diluent instead of samples. Then, we added 100 μL of enzyme labeling reagent to each well except for the blank wells and incubated the plate at 37 °C for 1 h. The liquid was removed from the wells, and the wells were washed with buffer 5 times for 30 s each then dried. Fifty microliters of chromogenic agent A were added to each well, followed by the same volume of chromogenic agent B. The solutions were mixed gently, and the plate was incubated at 37 °C for 15 min; then, we stopped the reaction with stop solution. After zeroing against the blank wells, the absorbance values were determined with an ELISA microplate reader at 450 nm. We constructed a standard curve and calculated the concentrations of the samples.

2.4. Histology

Samples of the jejunal tissue were fixed with 10% buffered formaldehyde and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H and E), dehydrated with ethyl alcohol and then cleared with xylene. The pathological changes in the jejunum were observed under an optical microscope. Histopathological examinations were performed by pathologists who were blinded to the study design. The United States Moticam 3000 microphotograph imaging system was used to capture images at 100× magnification, and the intestinal villus height and crypt depth were measured using the Motic Images Advanced 3.2 Pathological Image Analysis System. The pathological scoring criteria for jejunal tissue injury described by Caplan MS were used to divide H and E-stained pathological sections of jejunal tissues into four grades according to the histological damage to the intestinal tissue, such as submucosal and/or lamina propria separation, neutrophil infiltration, vasodilation, interstitial edema, villus exfoliation and necrosis. Noninvasive damage received a score of 0 points; micromucosal and/or lamina propria separation received a score of 1 point; moderate submucosal and/or lamina propria separation and/or submucosal and muscular edema received a score of 2 points; severe submucosal and/or lamina propria separation and/or submucosal and muscular edema with local villus shedding received a score of 3 points; and a lack of intestinal villi with intestinal necrosis received a score of 4 points [28].

2.5. Immunohistochemistry

Unfolded tissue sections were placed on slides and dried. Slices were dehydrated with xylene I and II, followed by 95%, 85% and 75% ethanol. Then, the sections were heated in the antigen retrieval solution for 10 min and cooled in PBS for 15 min. Slices were incubated with hydrogen peroxide for 15 min and with normal goat serum for 15 min. Next, slices were incubated with primary and HRP-labeled secondary antibodies. One hundred microliters of the DAB color reagent were dropped onto the sections until the color deepened and was deemed exactly right. The sections were stained with hematoxylin and then dehydrated. Neutral gum was applied, and the slide was coverslipped

Table 1
Primer sequences for the quantitative reverse transcriptase polymerase chain reaction.

Gene	Primer (5’-3’)	Accession
IL-6	F: TGGAGTACCATAGCTACCTGGA R: TCTCTCTGAAGGACTCTGGCT	NM_001314054.1
IL-1β	F: TCGCAGCAGCACATCAACAAGAG R:TGCTCATGTCCTCATCCTGGAAGG	NM_008361.4
TNF-α	F:GCCTGGTGGTGGAACTTGCTC R:CTATGGCCCAGACCCTCACA	NM_001278601.1
β-actin	F:GGAGATTACTGCCCTGGCTCCTA R:GACTCATCGTACTCCTGCTTGCTG	AY618569.1
TLR-2	F:GCGACATCCATCACCTGACTCTTC R:GCCTCGGAATGCCAGCTTCTTC	AF165189.1
TLR-4	F:ACAAGGCATGGCATGGCTTACAC R:TGTCTCCACAGCCACCAGATTCTC	NM_021297.3

and dried at room temperature. Finally, the staining was observed under a microscope.

2.6. *Quantitative real-time PCR*

Total RNA was extracted from ground tissues with TRIzol reagent and reverse-transcribed into cDNAs with a PrimeScript™ RT reagent kit (RR037A, Takara Bio Inc., Shiga, Japan) to assess the mRNA levels of various inflammatory factors. Then, mRNA expression was measured as a reference using TB green Premix Ex Taq as the fluorescent dye (RR420A, Takara Bio Inc., Shiga, Japan). The PCR cycling parameters were 95 °C (30 s) for 1 cycle, followed by 95 °C (5 s) and 58 °C (34 s) for 40 cycles. Relative quantification of the mRNA expression of the inflammatory factor genes is shown in the form of a comparative threshold cycle number for each sample ($2^{-\Delta\Delta CT}$). Gene expression was compared with the corresponding β-actin level. All primer pairs used for qRT-PCR were designed and synthetized by Sangon (China) and are described in Table 1.

2.7. *Cell-based assay*

RAW264.7 cells (American Type Culture Collection) were cultivated in modified RPMI medium (SH30027.01, HyClone, USA). Murine monocyte/macrophage RAW264.7 cells (1×10^5 cells/well) were seeded in 24-wells plates and incubated for 12 h. Cells were incubated for 6 h with medium containing viable bacteria (MOI = 0.1), and then we added EP to the medium to a final concentration of 5 mM and continued to incubate the cells for 6 h [29]. Cells were incubated for 12 h in a 37 °C incubator. Total RNA was collected in TRIzol for qRT-PCR.

2.8. *Western blotting analysis*

For this assay, 0.1 g of tissue samples were ground, homogenized, ultrasonicated and then treated with 1 mL of RIPA buffer containing 10 μL of PMSF (Beyotime catalog no. P0013B) to assess the phosphorylation of target protein. The lysate was centrifuged at 10,000*g* for 30 min at 4 °C, and then the supernatant was transferred to a new tube. All samples underwent denaturation after being mixed with the commensurable buffer and were boiled for 10 min. The expression levels of target protein were detected by separating the proteins on 12% SDS-polyacrylamide gels for 15 min at 80 V and 90 min at 120 V. We transferred the total protein from the gel to the polyvinylidene fluoride (PVDF) membrane at 75 V for 90 min, and then the PVDF membranes were blocked with 5% fat-free milk powder at 37 °C for 2 h. The membranes were incubated with the primary antibody (dilution, 1:1000) at 4 °C overnight and then washed with TBST 3 times for 10 min each. Next, the membranes were incubated with the corresponding HRP-labeled secondary antibodies (dilution, 1:1000) for 1 h at 37 °C and washed with TBST as described above. The protein bands were observed using a superenhanced chemiluminescence (ECL) Plus detection system (P0018, Beyotime) and documented with X-ray film (Clinx Science Instruments Co., Ltd., Shanghai, China).

2.9. *Statistical analysis*

Data are reported as the means ± SD. One-way ANOVA was used to analyze the significance of differences between the groups. A comparison of data between each pair of groups was performed using unpaired Student's *t*-test or General Linear Model of Statistical Analysis System (GLM). All analyses were performed using SPSS 20.0. Statistical, significance was defined as $p < 0.05$.

3. Results

3.1. *Effect of EP on body, liver and spleen weights*

We determined the body weight of mice after the infection. The body weight of the infected mice was significantly decreased compared with that of the control group, and EP significantly inhibited the decrease in body weight (Fig. 1A). Additionally, EP prevented increases in the liver and spleen weights caused by edema in the infected mice.

3.2. *EP promoted host immunity stimulated by S. typhimurium by increasing the secretion of IgA and IgM*

Serum immunoglobulin levels are shown in Fig. 2. The *S. typhimurium* infection significantly increased IgA and IgM levels. The EP treatment increased the IgA and IgM levels compared with those of the infected group. In particular, treatment with EP alone increased the IgA level compared with that of the control group. However, the IgG level did not show a significant change after either the *S. typhimurium* infection or EP treatment.

3.3. *EP prevented S. typhimurium-induced decreases in claudin-1, occludin-1 and ZO-1 expression*

S. typhimurium increased villus edema and cellular infiltration, as evidenced by an increase in the number of blue-stained cells in histological sections, compared with the control group (Fig. 3A). Inflammatory cells were mostly stained blue in a punctate pattern, while the other cells were larger in size with a pinker cytoplasm and smaller nucleus stained blue or dark blue. EP reduced villus edema. According to the pathological score, EP reduced the edema of villi in the jejunum (Fig. 3A and B). A significantly shorter villus height was observed in the jejunum of the ST group than in the other groups, and the crypt depth was increased by the *S. typhimurium* infection. Based on Western blotting and immunohistochemical staining (Fig. 4), EP protected the mouse jejunum by preventing the degradation of the tight junction proteins claudin-1, ZO-1 and occludin-1 induced by the *S. typhimurium* infection.

3.4. *Inhibitory effect of EP on the production of proinflammatory factors in the jejunum, liver and spleen of BALB/c mice and RAW264.7 cells infected with S. typhimurium*

The expression of inflammatory factors in the jejunum, spleen and liver was detected using qRT-PCR, as shown in Fig. 5. EP decreased the expression of the IL-6, IL-1β and TNF-α mRNAs in the jejunum, liver and spleen of mice infected with *S. typhimurium* compared with the control mice. RAW264.7 cells infected with *S. typhimurium* to investigate whether EP acts on macrophages. The EP treatment alone did not produce significant changes, and the EP treatment of *S. typhimurium*-infected macrophages significantly decreased the levels of the IL-6, IL-1β and TNF-α mRNAs compared with the control (Fig. 6).

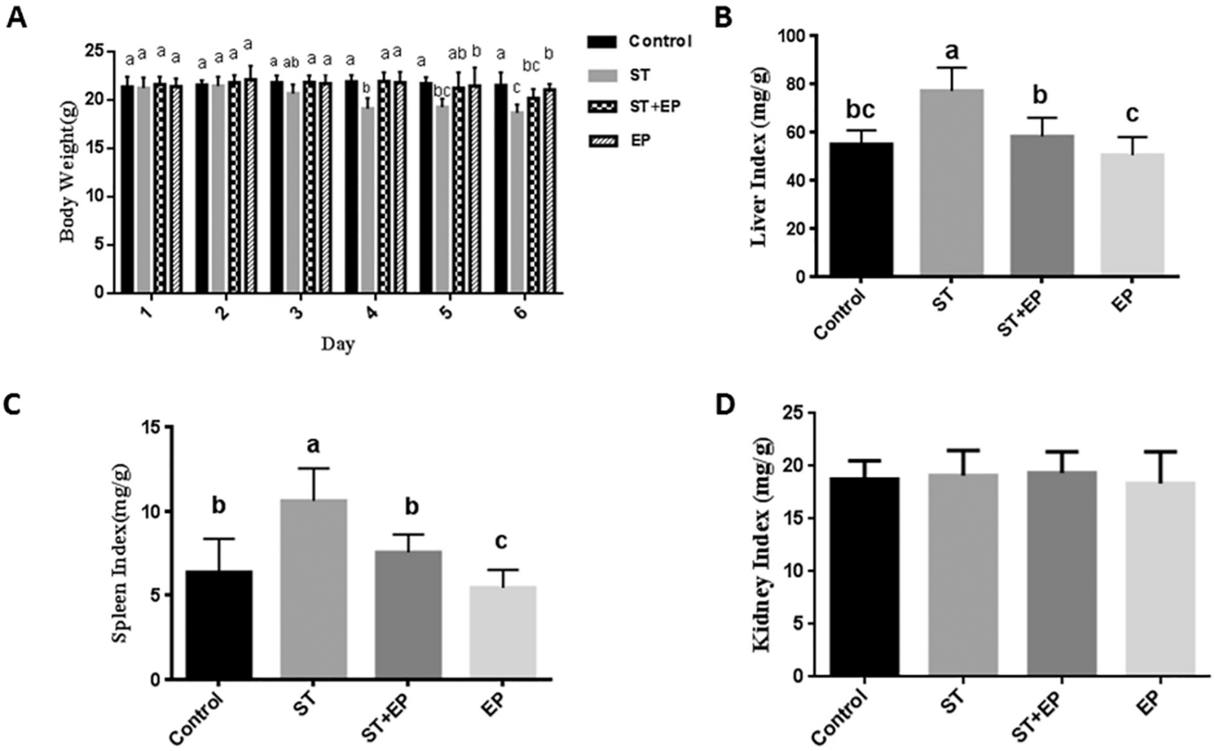

Fig. 1. Effects of EP on body weight and immunity organs in infected BALB/c mice. (A) Body weight change BALB/c mice (n = 10). (B–D) Organ index of spleen, liver and kidney in BALB/c mice (n = 10). Control: normal mice treated with PBS. ST: Mice infected with 1×10^8 CFU/mL *Salmonella typhimurium* in PBS. ST + EP: *Salmonella typhimurium*-infected mice with 100 mg/kg PBS solution of EP treatment. EP: Mice with EP treatment alone. (a, b, c; $p < 0.05$) Values are mean ± SD.

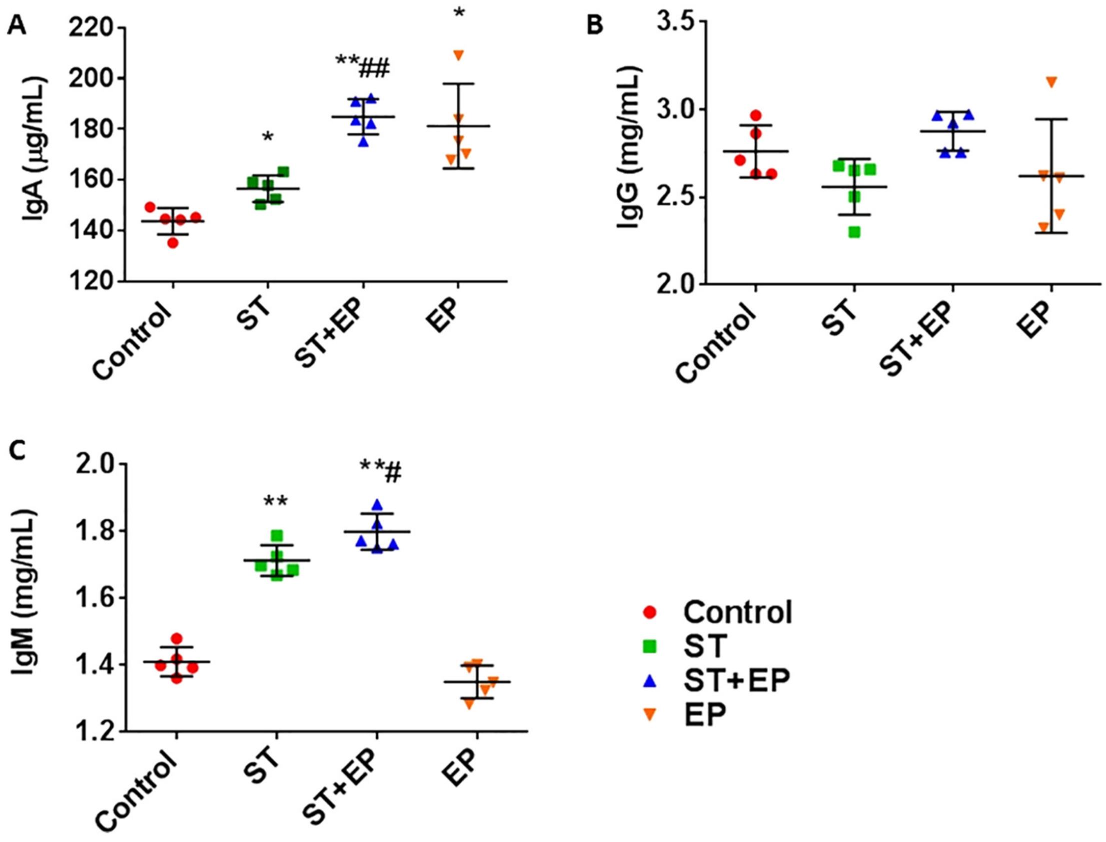

Fig. 2. Effect of EP on serum IgA, IgM and IgG levels in *Salmonella Typhimurium*-infected BALB/c mice (n = 5). Serum from each mouse was isolated. IgA (A), IgG (B) and IgM (C) levels were determined by ELISA and emission at 450 nm. *p means significant compared with control and $^{\#}p$ means significant between the ST and ST + EP groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Values are mean ± SD.

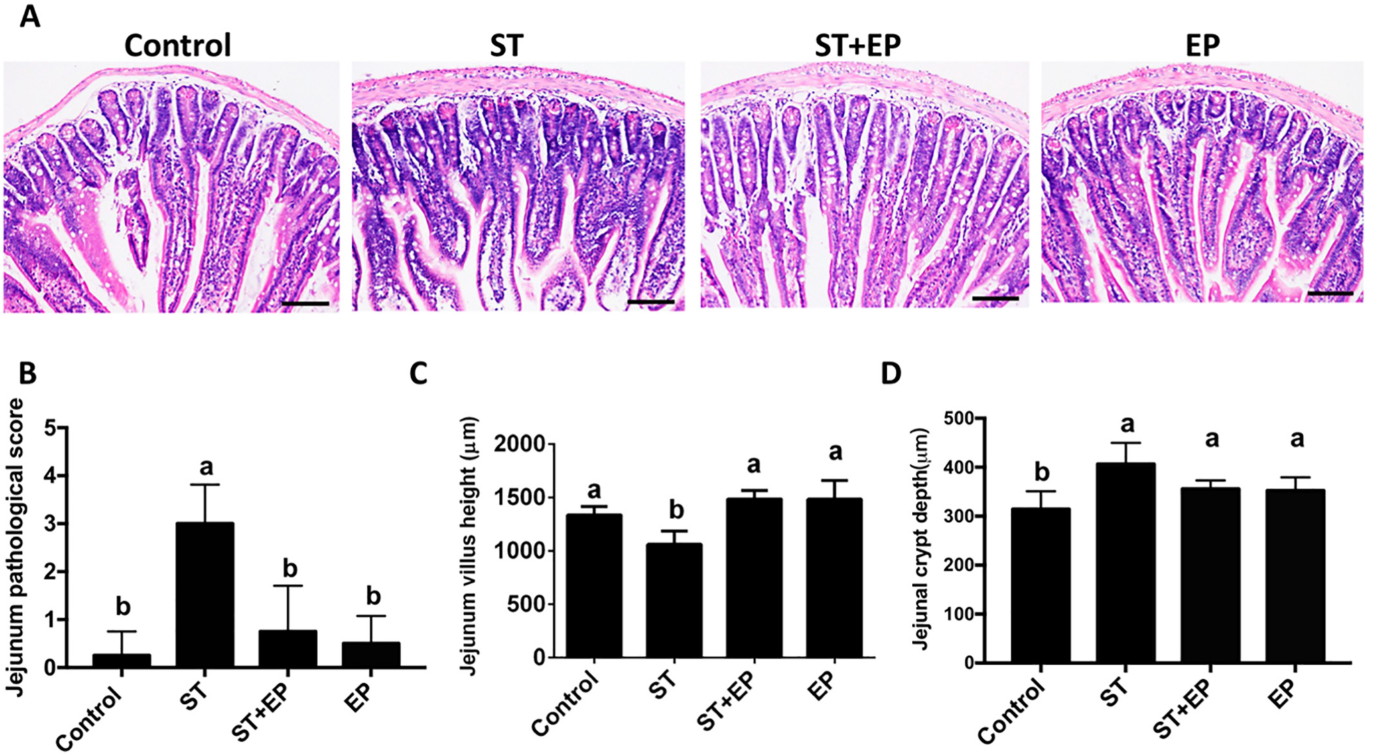

Fig. 3. BALB/c Mice Jejunum Barrier Morphology. (A) Histopathologic examination of BALB/c mice jejunum fluff; (B) Jejunal histopathological score (C) Effect of EP on jejunum villus height (D) Effect of EP on jejunum crypt depth. Mice jejunums were fixed with formaldehyde solution, embedded in paraffin and dyed by HE. The United States moticam 3000 microphotograph imaging system was used with a 100× photograph, and the intestinal villus height and crypt depth were measured using the motic images advanced 3.2 pathological image analysis system. Different values between groups are shown with capital letters (a, b; $p < 0.05$). Values are mean ± SD.

3.5. TLR4, p38 and ERK1/2 are the key signaling nodes in the jejunal immune response

The levels of the TLR4 mRNA and protein were significantly increased after the *S. typhimurium* infection. In addition, infected mice that were treated with EP displayed a decreased expression of TLR4 compared with that of the control group (Fig. 7A). However, TLR2 levels were not significantly altered among the groups. As shown in Fig. 7, EP also significantly reduced the *S. typhimurium*-induced increases in the levels of the TLR4 and phospho-p38 and phospho-ERK1/2 proteins.

4. Discussion

The prevalence of gastrointestinal pathogenic bacteria in patients with diarrhea seriously affects human health and the development of animal husbandry. In particular, *S. typhimurium* causes great harm by transferring infections between humans and animals [30]. *S. typhimurium* not only invades the body through the respiratory tract and then attacks the liver and spleen but also easily pollutes feed and rendering industries [31,32]. The virulence factors of *S. typhimurium* invade host tissues and induce intestinal inflammation after surviving in monocytes [33]. New drugs designed to ensure animal intestinal health are urgently needed to solve this problem. In the current study, we employed EP to treat BALB/c mice infected with *S. typhimurium* as an inflammation model *in vivo* and mouse RAW264.7 macrophages *in vitro*. Using the *S. typhimurium* inflammation model, the role of the TLR4/MAPK pathway was further investigated in the *in vivo* experiments employing the pharmacological EP treatment.

S. typhimurium enters the mouse intestinal tract following a gastric administration; then, *S. typhimurium* adheres to intestinal epithelial cells and promotes the colonization of bacteria in the intestinal tract through its type I pilus [34]. As the phagocytic cells migrate from the mesenteric lymph nodes, they ultimately spread throughout the body and invade different organs or tissues, such as the liver and spleen, causing various clinical symptoms [35]. EP relieves the damage to immune organs by enhancing host immunity through a reduction in the apoptosis of immune cells and inhibition of T cell function and TNF secretion [36,37]. In our study, mice showed mental fatigue and diarrhea during the *S. typhimurium* infection period. Oral infection with *S. typhimurium* (0.2 mL 1×10^8 CFU/mL/d) reduced the survival rate of mice in 6 days. Each sample included intestinal tissues of all the surviving mice in each group, the appropriate number of samples was selected for the execution of the experiment. In the present study, the organ indexes of the liver and spleen (organ weight/body weight) were significantly increased in the *Salmonella*-infected group. The significant increase in the organ index was due to swelling of the mitochondria, resulting in organ edema [38]. EP significantly reduced the liver and spleen indexes of the infected mice, suggesting that EP relieves the edema in the liver and spleen induced by *S. typhimurium* (Fig. 1B and C). *S. typhimurium* mainly targets the lymph nodes, liver and spleen [39]. Thus, the EP-induced reduction in the spleen and liver indexes in the ST group observed in the present study indicates that the liver and spleen lesions were alleviated.

The pathogenic bacteria will destroy the intestinal barrier and increase its permeability, allowing the bacteria to enter the circulation [40]. The increase in serum immunoglobulin levels, including IgA and IgM, is a response to the stimulation of cellular immunity by gut bacteria [41]. Serum IgA and IgM antibodies may remove bacteria by binding to surface proteins on *Salmonella* in infected mice [42]. In the present study, EP increased the serum IgA and IgM levels compared with those of the *S. typhimurium*-treated group (Fig. 2), suggesting that EP improves host humoral immunity by further increasing serum immunoglobulin levels.

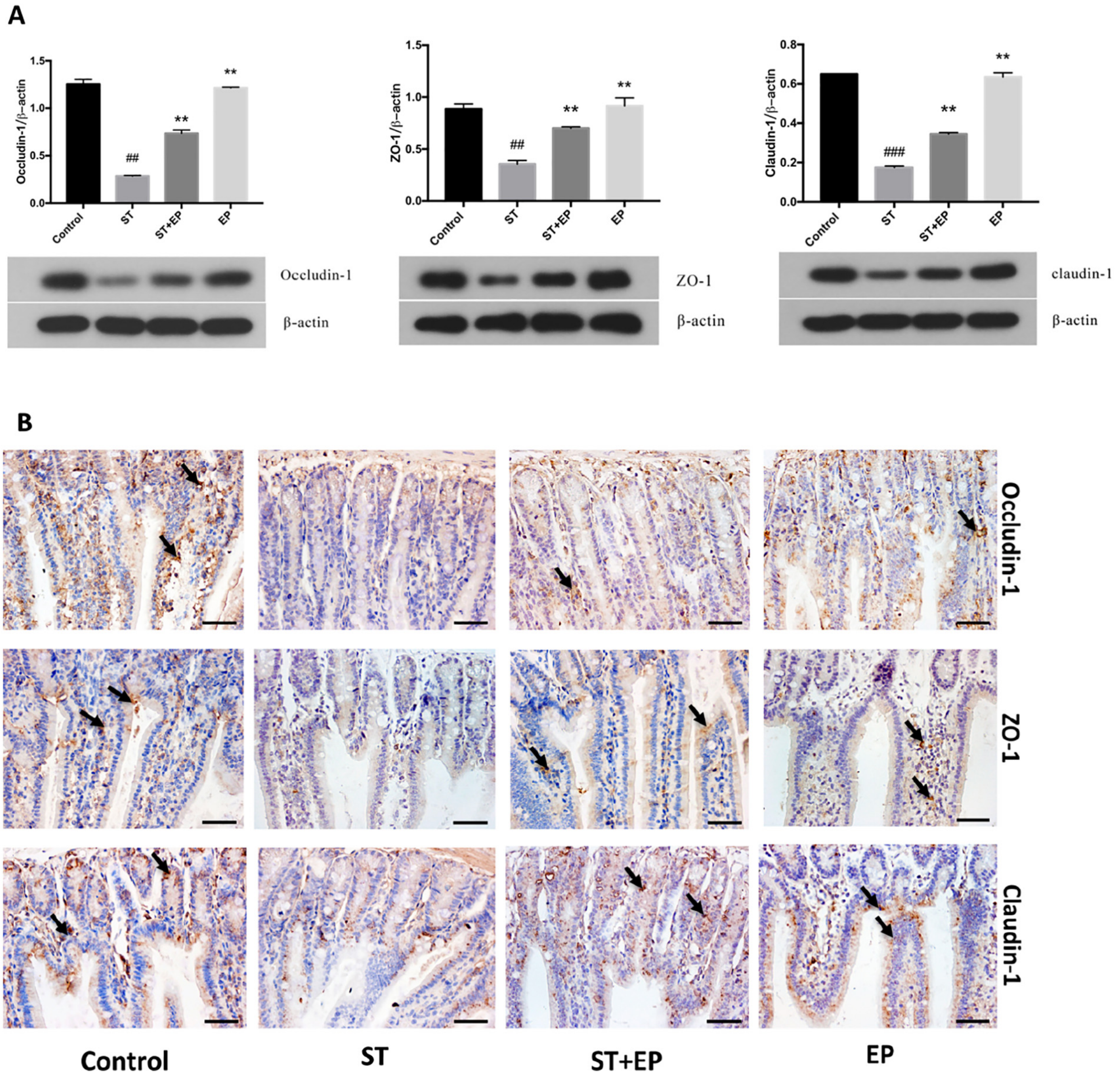

Fig. 4. Effect of EP on ZO-1, occludin-1and claudin-1 expression in *Salmonella typhimurium*-infected mouse jejunum (n = 4). (A) ZO-1, occludin-1and claudin-1 expression protein level. (B) Immunohistochemistry of jejunum ZO-1, occludin-1and claudin-1. **p* means significant compared with control and #*p* means significant between the ST and ST + EP groups. $^{**}p < 0.01$, $^{\#\#\#}p < 0.001$. Values are mean ± SD.

The intestinal mucosal barrier function plays an important role in transporting nutrients and maintaining host intestinal immune homeostasis [43]. Restoration of the intestinal barrier is also key to ensuring intestinal resistance to pathogens. A decrease in villus height will affect the ability of the small intestine to digest and absorb food [44]. In previous studies, *S. enterica* serovar Typhimurium decreased the intestinal villus height but did not significantly change the crypt depth [45]. However, the villus height and ratio of villus/crypt were both decreased in chickens challenged with *S. typhimurium* [46]. The base of the dividing crypts is necessary to maintain the structure of the villi. As the crypt depth increases, the number of villi will increase [47]. *S. typhimurium* increased the number of inflammatory cells, which were mainly neutrophils, lymphocytes and monocytes (Fig. 3A). Compared with the infected mice, the infected mice treated with EP displayed an increased villus height and a reduced edema, suggesting that EP protected the mechanical barriers in the intestine (Fig. 3B and C).

EP has been shown to exert a protective effect on a damaged gut barrier and to increase the expression of the mRNAs encoding the tight junction proteins ZO-1 and occludin [16,48]. Tight junction proteins are important for maintaining the intestinal barrier and maintaining mucosal permeability [49]. The permeability of the intestinal epithelial cells is increased when occludin-1 has been mutated or when its expression is reduced or lacking [50]. *S. typhimurium* reduces the expression of ZO-1 to promote its uptake and distribution in the host by regulating the molecular composition of cell junctions [51]. *S. typhimurium* invades intestinal epithelial cells by targeting and destroying cell junctions and induces a decrease in intestinal occludin mRNA levels [52]. In our research, *S. typhimurium* also reduced claudin-1, occludin-1 and ZO-1 expression in BALB/c mice, while EP prevented these changes (Fig. 4).

EP enhances host immunity and inhibits inflammatory responses induced by multiple inflammatory models, such as bacterial endotoxins, sepsis induced by live *E. coli* and inorganic compounds [53–56]. *S. typhimurium* infection initiates an intense inflammatory reaction and produces a large number of cytokines (TNF-α, IFN-γ, IL-12, *etc.*) [57]. In the present study, EP inhibited the expression of the inflammatory factors IL-6, IL-1β and TNF-α in the jejunum, liver and spleen, compared to the high levels of inflammatory factors detected in the *S.*

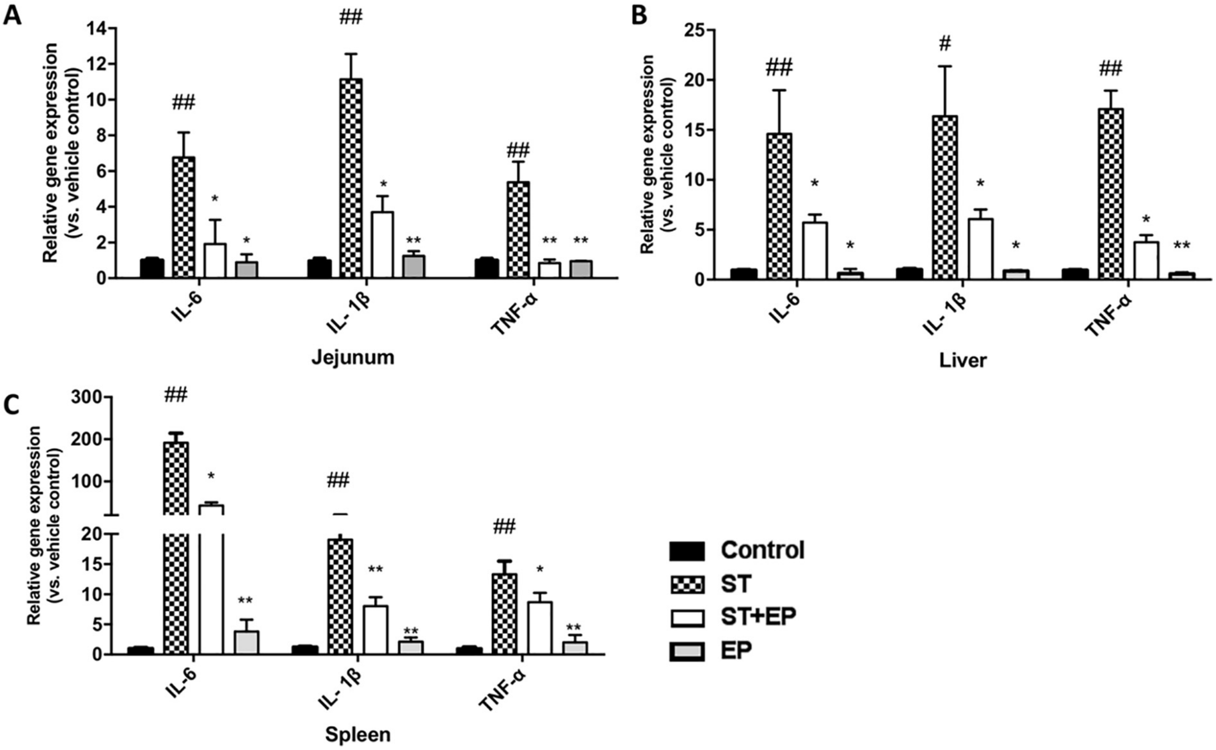

Fig. 5. Effects of EP on expression of inflammatory factors in the jejunum, spleen and liver of *Salmonella typhimurium*-infected BALB/c mice (n = 6). (A) IL-6, IL-1β and TNF-α mRNA expression in mouse jejunum tissue. (B) IL-6, IL-1β and TNF-α mRNA expression in mouse livers. (C) IL-6, IL-1β and TNF-α mRNA expression in mouse spleens. $^{\#}p$ means significant between control and ST group. $^{*}p$ means significant between the ST and ST + EP group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. Values are mean ± SD.

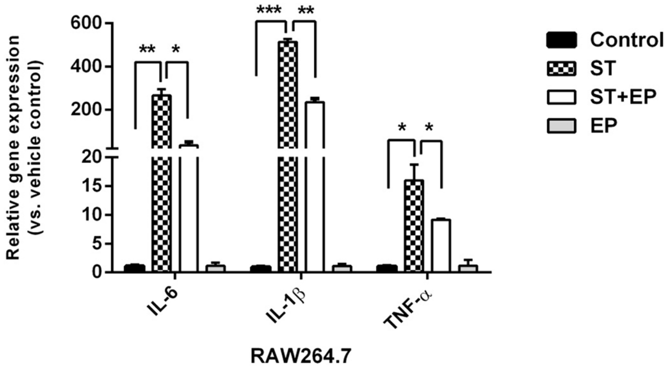

Fig. 6. Effect of EP on IL-6, IL-1β and TNF-α mRNA expression in *Salmonella typhimurium*-infected RAW 264.7 cells. The cells were incubated for 6 h in medium with viable bacteria (MOI = 0.1), then we added EP to a final concentration of 5 mM and continued to incubate them for 6 h. $^{*}p$ means significant in different groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Values are mean ± SD.

typhimurium-treated group. Macrophages also secrete IL-6, IL-1β and TNF-α in a typical enteritis case [58]. Three types of phagocytic cells interact with *S. typhimurium*: neutrophils, macrophages that differentiated from inflammatory monocytes and dendritic cells [59]. We studied the effect of EP on proinflammatory cytokine levels in mouse macrophages. In previous studies, EP inhibited HMGB1-induced enteritis in mice by activating macrophages and dendritic cells. In the present study, the mononuclear macrophage cell line RAW264.7 was infected with *S. typhimurium*, and we also showed that EP relieved inflammation in these cells.

The current study confirmed that TLR2 and TLR4 are the main receptors that regulate inflammation caused by gram-negative bacteria [60]. The mRNA levels of the possible regulatory proteins TLR2 and TLR4 were detected using qRT-PCR to investigate the pathway through which EP regulated the inflammatory response. Only the TLR4 mRNA was significantly increased in the jejunum of mice infected with *S. typhimurium*. EP decreased the expression of the TLR4 mRNA following infection with *S. typhimurium* (Fig. 7A). Furthermore, in this study, lower levels of the TLR4, p-p38 and p-ERK1/2 proteins were detected in the jejunum of the infected mice treated with EP than in the control group (Fig. 7B–E). EP inhibits the phosphorylation of p38 and ERK1/2 induced by the inflammatory response. Moreover, TLR4 is the main receptor by which EP inhibited inflammation by EP. MAPK binds to cell scaffold proteins when *S. typhimurium* invades eukaryotic cells and induces the rearrangement of the host cell cytoskeleton [61]. The host immune mechanism resists invasion of bacteria by inducing the activation of p38 and ERK1/2 [62,63]. Thus, the inhibition of p38 and ERK1/2 phosphorylation is an important goal of anti-inflammatory therapy. This study provides evidence that EP has become a new class of anti-inflammatory drugs. However, the optimal concentration of EP within the scope of safety and the duration of action are worthy of further detailed research. Current researches indicate that EP is only administered by intraperitoneal injection, and there is no detailed study of other methods. Injection of EP may cause mechanical damage to diseased mice to weaken their immunity. At the same time, the injection method will also increase the cost of treatment in the practical application of the livestock industry. Furthermore, the molecular mechanism of EP action needs to be further explored. The degree of effect of *S. typhimurium* on infection in mice is also related to individual differences in mice, so the repeatability of the *S. typhimurium* infection model needs further study.

5. Conclusions

In our study, EP relieved inflammation by inhibiting body weight loss and reducing the liver and spleen indexes in infected mice. EP

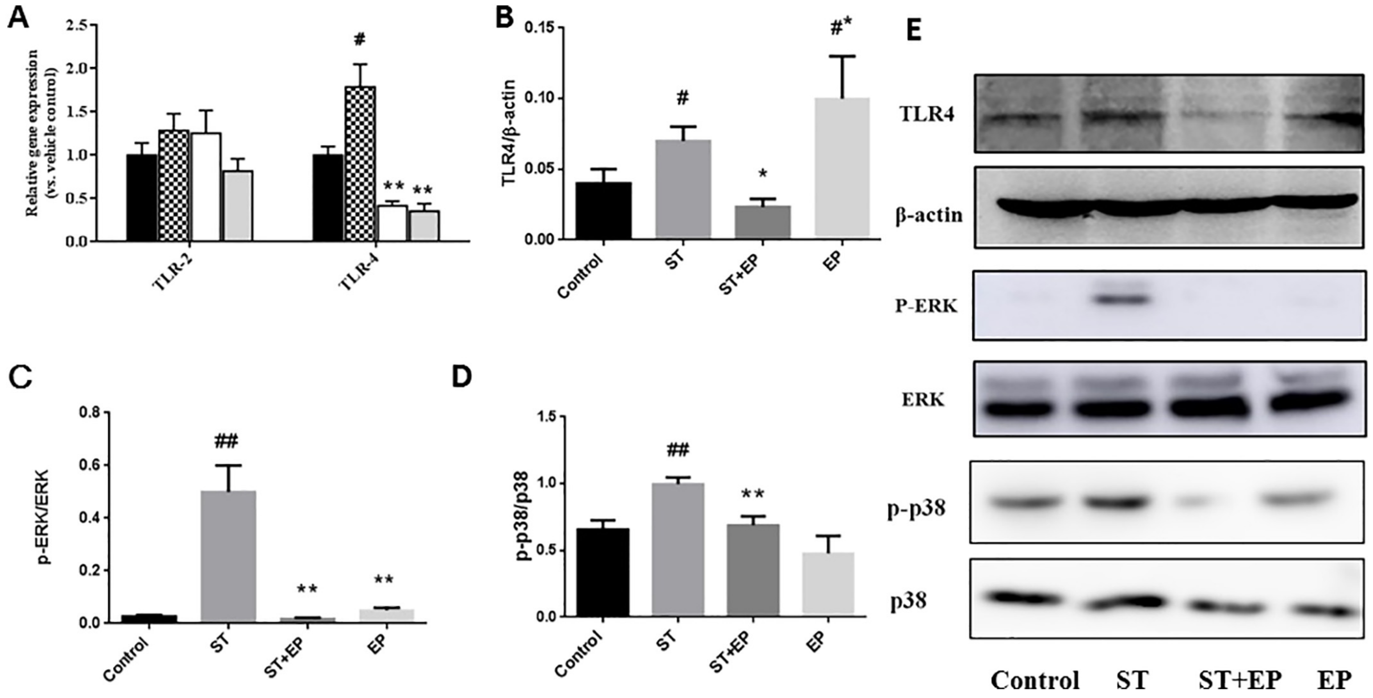

Fig. 7. Role of TLR4, p38MAPK and ERK1/2 of EP against jejunum inflammation in *Salmonella typhimurium*-infected BALB/c mice (n = 3). (A) Effect of EP on TLR2, TLR4 mRNA fold change in *Salmonella typhimurium*-infected mouse jejunum. (B–E) Inhibition of TLR4, phosphorylation of p38 and ERK1/2 expression by EP. $^{\#}p$ means significant between control and ST group. $^{*}p$ means significant between the ST and ST + EP group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. Values are mean ± SD.

reduced inflammatory cell infiltration and decreased claudin-1, occludin-1 and ZO-1 expression. Additionally, the EP treatment increased serum IgA and IgM levels to promote host immunity. Furthermore, the expression of the proinflammatory cytokines IL-6, IL-1β and TNF-α was reduced in the jejunum, liver and spleen of BALB/c mice and in RAW264.7 cells infected with *S. typhimurium*. EP decreased the levels of TLR4 and phosphorylated p38MAPK and ERK1/2 in the mouse jejunum. In summary, EP relieved the *S. typhimurium*-induced inflammation in the jejunum of BALB/c mice by repairing the barrier damage and inhibiting the production of proinflammatory cytokines and the phosphorylation of p38 and ERK1/2. Thus, the study reveals the potential of EP as a treatment for enteritis.

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