Am J Transl Res 2022;14(6):4278-4294
www.ajtr.org /ISSN:1943-8141/AJTR0141500

Original Article

Moxibustion attenuates inflammation in intestinal mucosal by regulating RAGE-mediated TLR4-NF-κBp65 signaling pathway in vivo and in vitro

Bingrong Li[1,2*], Long Yuan[1*], Peng Liu[1*], Zixiang Geng[1], Kaiyong Zhang[1], Huiru Jiang[1], Hua Sui[3], Bimeng Zhang[1]

[1]Department of Acupuncture and Moxibustion, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200080, People's Republic of China; [2]Shanghai Key Laboratory of Acupuncture Mechanism and Acupoint Function, Fudan University, Shanghai 200433, People's Republic of China; [3]Medical Experiment Center, Jiading Branch of Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201803, People's Republic of China. [*]Equal contributors.

Received January 3, 2022; Accepted May 20, 2022; Epub June 15, 2022; Published June 30, 2022

Abstract: This study was performed to investigate the effect of moxibustion on the RAGE/TLR4-NF-κBp65 pathways and mucosal damage in rat model of 5-fluorouracil (5-Fu)-induced intestinal mucositis (IM) and the underlying mechanisms. 5-Fu treatment significantly increased the expression of the receptor for advanced glycation end products (RAGE) and its ligand, thehigh-mobility group box 1 protein (HMGB1), in the rat intestinal tissue. The inhibition of RAGE could induce the repair of intestinal mucosal damage and downregulate the expression of Toll-like receptor (TLR)-4 and nuclear factor kappa-B (NF-κB) p65 in intestinal tissues of 5-Fu-treated rats. Moxibustion treatment significantly improved the physical symptoms and repaired the intestinal mucosal damage of IM rats and increased the expression of tight junction proteins in these rats. The expression of RAGE, HMGB1, TLR4, NF-κBp65, and related downstream inflammatory factors, namely, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β, were significantly decreased after moxibustion treatment. A moxibustion dose of 15 min/day exerted a better therapeutic effect than a dose of 30 min/day. The phosphorylation of NF-κBp65 and IκBa is involved in reducing inflammation by regulating the RAGE signaling pathway. Moxibustion can reduce intestinal mucosal damage and inflammation in 5-Fu-induced IM rats via modulation of the RAGE/TLR4-NF-κBp65 signaling pathways.

Keywords: Inflammation, intestinal epithelium, chemotherapy, RAGE/TLR4-NF-κBp65 axis, moxibustion

Introduction

Chemotherapy, as one of the approaches used for cancer treatment, is accompanied by normal cell damage and thus results in severe adverse effects [1, 2]. Intestinal mucositis (IM) is one of the most common adverse effects of chemotherapy and induces changes in the structure, function and immunological response of the intestine. IM can lead to an increased risk of infection and may cause severe damage to the host's immune system, which is life threatening [3]. This adverse effect is commonly observed in chemotherapy patients [4, 5] and occurs in about 80% of patients administered 5-fluorouracil (5-Fu), a commonly used chemotherapeutic drug [6]. Therefore, exploring strategies for decreasing IM reactions caused by 5-Fu without affecting its anticancer effect is of great significance.

IM is a complex disease involving various mucosal tissues and cells changes [7, 8] and is associated with the environment of the intestinal lumen and systemic inflammation [9]. Previous studies have shown that the activation of nuclear factor kappa-B (NF-κB) by 5-Fu might be an important predisposing factor to IM and that NF-κB is highly activated in the small intestine after the administration of 5-Fu [10]. Activated NF-κB is involved in the regulation of the transcription and expression of inflammatory-related genes, which results in injury to submucosa and epithelial basal layer cells and

thereby leads to apoptosis and tissue damage [11]. Furthermore, the released inflammatory factors, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, can form a negative feedback loop to activate the NF-κB pathway and aggravate mucosal injury. Toll-like receptor (TLR) is a key regulator of innate and adaptive immune responses that can activate NF-κB pathways. The TLR4/NF-κB pathway regulate the gene transcription and protein synthesis of a series of inflammatory cytokines, such as IL-1, IL-6 and IL-8, which worsens the intestinal mucosal damage [12].

Receptor for advanced glycation end products (RAGE) is a pattern recognition receptor that is widely found in many types of cells, including mononuclear macrophages [15, 16], endothelial cells [17], and smooth muscle cells [18]. This receptor induces signaling cascades that activate the NF-κB pathway by binding to a variety of ligands and thus causes inflammation and dysfunction in cells or tissues [19, 20]. A number of studies have recently shown that the RAGE/TLR4-NF-κB signaling pathways play a proinflammatory role in inflammation processes [13, 14]. Accordingly, controlling the activation of the RAGE/TLR4-NF-κB pathway might be a potent strategy for preventing the development of 5-Fu-induced IM.

Moxibustion can effectively regulate the immune function of the human body, improve mucosal lesions and reduce intestinal inflammation. Numerous clinical studies have confirmed that moxibustion can reduce gastrointestinal adverse effects in patients receiving chemotherapy, such as diarrhea and vomiting, and improve their physique [21]. Studies have shown that moxibustion can reduce IM injury in rats by downregulating the expression of genes related to the TLR4- and NF-κBp65-related signaling pathways [22, 23], and moxibustion can regulate RAGE expression in models of central nervous system inflammation [24, 25]. However, whether the RAGE/TLR4-NF-κBp65 pathways play an important role in the action of moxibustion in the treatment of 5-Fu-induced IM remains unknown.

In our current study, a rat model of IM was established for experiments in vivo and the lipopolysaccharide (LPS) induced Caco2 cell model were used in vitro. The aim of this study was to investigate the effects of moxibustion intervention on the RAGE/TLR4-NF-κBp65 pathways and its protective effect on mucosal damage in 5-Fu-induced IM.

Materials and methods

Animals

Forty-eight clean-grade male Sprague-Dawley (SD) rats weighing 140-180 g were used at 4-6 weeks of age. The rats were purchased from Zhejiang Viton Lihua Experimental Animal Technology Co., Ltd., and maintained at the Animal Experimental Center of Shanghai University of Traditional Chinese Medicine. We performed the animal experiments in accordance with the procedures approved by the Animal Experimental Center (Animal Ethics No.: PZSHUTCM191220002) and implemented the relevant provisions from the Guidance Suggestions for the Care and Use of Laboratory Animals formulated by the Ministry of Science and Technology of the People's Republic of China.

Rat model of IM

We used the rat model of chemotherapy-induced IM established as previously described [26], which involves the intraperitoneal injection of a single dose of 5-Fu (150 mg/kg) on the 7th day. The rats in the Control, FPS-ZM1, Moxibustion-15 min/day and Moxibustion-30 min/day groups received an intraperitoneal injection of the same dose of 0.9% sodium chloride solution.

Study procedure

The animal experiment lasted for 17 days. Starting on the 1st day, the rats in each moxibustion intervention group were treated with mild moxibustion at the corresponding dosing schedule. A "pure moxa stick" (Suzhou Moxa Co., Ltd. China) was placed above the acupoints, 1.5-3 cm from the skin of rats. The methods used for locating these points were strictly in accordance with "Experimental Acupuncture and Moxibustion". The Guanyuan (CV 4), Qihai (CV 6) and Bilateral Tianshu (ST 25) acupoints were selected as the mild moxibustion acupoints. The moxibustion treatments were implemented once a day for 16 consecutive days, and on each of these days, the rats in the other groups received the same fixation without moxibustion. In addition, the rats in the

5-Fu+FPS-ZM1 and FPS-ZM1 (a specific inhibitor of RAGE) groups were administered intraperitoneal injections of FPS-ZM1 (1 mg/kg) once a day for 16 consecutive days, and those in the other groups received intraperitoneal injections of 0.9% sodium chloride solution on each of these treatment days to control for the difference caused by nontreatment factors.

On the 7th day after treatment, an intraperitoneal injection of 5-Fu was administered to the rats in the groups that should be received chemotherapy. The physical signs and symptoms (body weight, food consumption and stool condition) of the rats in each of the groups were recorded on the day of modeling (before modeling). One piece of fresh feces from each rat was collected each day for the fecal occult blood test (FOBT). The health status was scored according to the "Disease Activity Index" (DAI) [27] and recorded. Briefly, the DAI scores the health status of rats based on their weight loss, stool consistency and fecal occult blood. Each of these items was given a score in the range of 0 to 4 based on the severity, and the mean value from the three items was considered the DAI score (Table S1). Please see Supplementary Material for details on methods of moxibustion and sacrifice of animals, and preparation of intestinal tissue sections.

Fecal occult blood test

A small amount of feces was dipped on a clean vessel with cotton swabs, and occult blood in feces was assessed using Fecal Occult Blood Test Kit (#JLC14582, Gelatins, China). The faeces samples were mixed with PBS at a ratio of 1:9. After homogenized and centrifuged, the supernatant was taken for detection. Fifty microliters of standard samples at different concentrations (40, 20, 10, 5, 2.5 and 1.25 mg/ml) were added to each standard well. 50 µl of each sample were added to each sample well. Incubated at 37°C for 1 h and developed chromogenic reaction in the dark for 15 min. After termination, the absorbance at 450 nm was measured.

Hematoxylin eosin and immunofluorescence

The whole intestine was removed immediately after sacrifice and opened longitudinally after washed with ice-cold PBS as previously described [28]. For the murine samples, Immunofluorescence was performed to detect total RAGE (anti-mouse RAGE, Abcam) overnight at 4°C. After three washes with PBS, the sections were incubated with secondary antibody, dabbed dry, and incubated with DAPI for 5 min. The cells were then overlaid with coverslips and sealed through the application of nail polish.

Western blotting

After preparation of a separation gel (10%) and a stacking gel, 6 µl of marker and 10 µl of each sample were added with a pipette gun. Polyacrylamide gel electrophoresis was performed at a constant voltage of 100 V until the dye approached the top of the separation gel, and electrophoresis was then performed at a constant voltage of 130 V for approximately 1 h. The gel was placed in Western transfer buffer and assembled in the transfer clamp in the appropriate sequence. The clamp was then inserted into the wet transfer slot, the current was set to 220 mA for 2 h at room temperature, and the samples were transferred to nitrocellulose membranes. After blocking in 5% BSA for 2 h, the nitrocellulose membranes were cut according to the target protein and marker. We used rabbit monoclonal antibodies directed against the tight junction protein occludin (#91131), ZO-1 (#13663), RAGE (#6996), TLR4 (#14358), NF-κBp65 (#8242) and p-NF-κBp65 (#3033) (CST, USA), and the membranes were incubated with these antibodies on a shaker (60 rpm) overnight at 4°C. The cells were then incubated with peroxidase-conjugated goat anti-rabbit IgG (#7074) (CST, USA). A visualizer was then used to observe the stripe, and the grayscale value was calculated using ImageJ.

Quantitative real-time PCR

Tumor tissues were homogenized with 1 mL TRI reagent to extract total RNA. cDNA was synthesized by reverse transcription of total RNA (Epicentre). Quantitative real-time PCR (qRT-PCR) was carried out as previously described [29]. The Oligonucleotide primers for target genes (RAGE, HMGB1, NF-κBp65, IkBα, TLR4, and GAPDH) were shown in Table S2.

Enzyme-linked immunosorbent assay (ELISA)

The expression of serum TNF-α, IL-6 and IL-1β in the samples was determined using ELISA kits (Shinny BIO, Shanghai, China) according to manufacturer's instructions. Fifty microliters of standard proteins at different concentrations

(1000, 500, 250, 100 and 50 pg/ml) were added to each standard well. Ten microliters of each sample were added to each sample well with 40 μl of the diluent. The sealing plate was repeatedly incubated at 37°C for 30 min and washed with distilled water. Fifty microliters of chromogenic agents A and B were added to each well, and the plate was incubated in the dark at 37°C for 15 min. Subsequently, 50 μl of termination fluid was added to each well, and the absorbance at 450 nm was measured.

Cell origin and culture

The human colon cancer cell line Caco2 was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Caco2 cells were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 units/mL streptomycin in a 5% CO_2 humidified atmosphere at 37°C. For each experiment, DMEM medium was used to further dilute 5 mg/ml LPS stock solutions to acquire various required concentrations of LPS, as shown in Table S3.

Cell proliferation and cytotoxicity assay (CCK-8 kit)

Cell proliferation and cytotoxicity were detected using CCK-8 kit. Cells were seeded into 96-well plates at a density of 8×10^4 cell/well (5 duplicate wells for each group) and cultured in incubator for 24 h. After the cells adhered to the wall, change the liquid for starvation treatment for 12 h. Remove the waste solution, DMEM with different concentrations of LPS was added, 100 μL per well and cultured in incubator for 24 h or 48 h. Then DMEM containing 10% CCK-8 was added for continued culture for 1 h, OD value of cells was measured by a microplate reader at the wavelength of 450 nm.

Statistical analysis

The data were analyzed using SPSS 25.0 (IBM Co., Armonk, NY, USA). The measurement data are expressed as the means ± SEMs. For data showing a normal distribution and variance, multiple sample means were compared by one-way ANOVA with the least significant difference method (LSD-t). Dunnett's T3 test was used to compare the data showing variance heterogeneity.

Results

Effects of moxibustion on physical signs and mucosal injury in 5-Fu-induced IM rats

Previously, we showed that moxibustion was sufficient to ameliorate intestinal mucositis in chemotherapy-induced IM rats. In the present study, to investigate the mechanism of moxibustion on chemotherapy-induced intestinal mucositis, 5-Fu was intraperitoneal injected to the rats at first. No significant difference in body weight and variation in food consumption was found among the Control, 5-Fu, 5-Fu+Moxibustion-15 min/day, 5-Fu+Moxibustion-30 min/day, Moxibustion-15 min/day and Moxibustion-30 min/day groups (**Figures 1A**, **1B** and S1A, S1B). After the 5-Fu combined Moxibustion application, the DAI scores (**Figure 1B**) was lessened following the addition of Moxibustion in a dose-dependent manner in combined group, comparing with 5-Fu alone model group (**Figures 1C** and S1C).

Images of HE staining indicated that the Control, Moxibustion-15 min/day and Moxibustion-30 min/day groups presented intact and neatly arranged ileal epithelium structures, with no obvious lesions (**Figures 1D** and S1D). The 5-Fu group exhibited severe mucosa damage with incomplete and disorganized epithelial villi, destroyed structures, smaller crypt depths, thinner mucous membranes and separation of the upper cortex and lamina propria, whereas the 5-Fu+Moxibustion-15 min/day group showed separation between the upper cortex and lamina propria, and 5-Fu+Moxibustion-30 min/day groups presented separation between the upper cortex and lamina propria, edema in stroma and mild injury to the ileac epithelium structure (**Figures 1D** and S1D). In addition, we measured the ileal villous height, crypt depth, mucosal thickness, and colonic mucosal thickness (**Figures 1E-H** and S1E-H). All the results suggested that moxibustion treatment significantly improved the physical signs and symptoms of 5-Fu-treated rats, and 15 min/day of moxibustion exerted a better therapeutic effect.

Effects of moxibustion on the tight junction proteins and proinflammatory cytokines in 5-Fu-induced IM rats

Since the expression and distribution of Occludin and ZO-1 were closely related to endo-

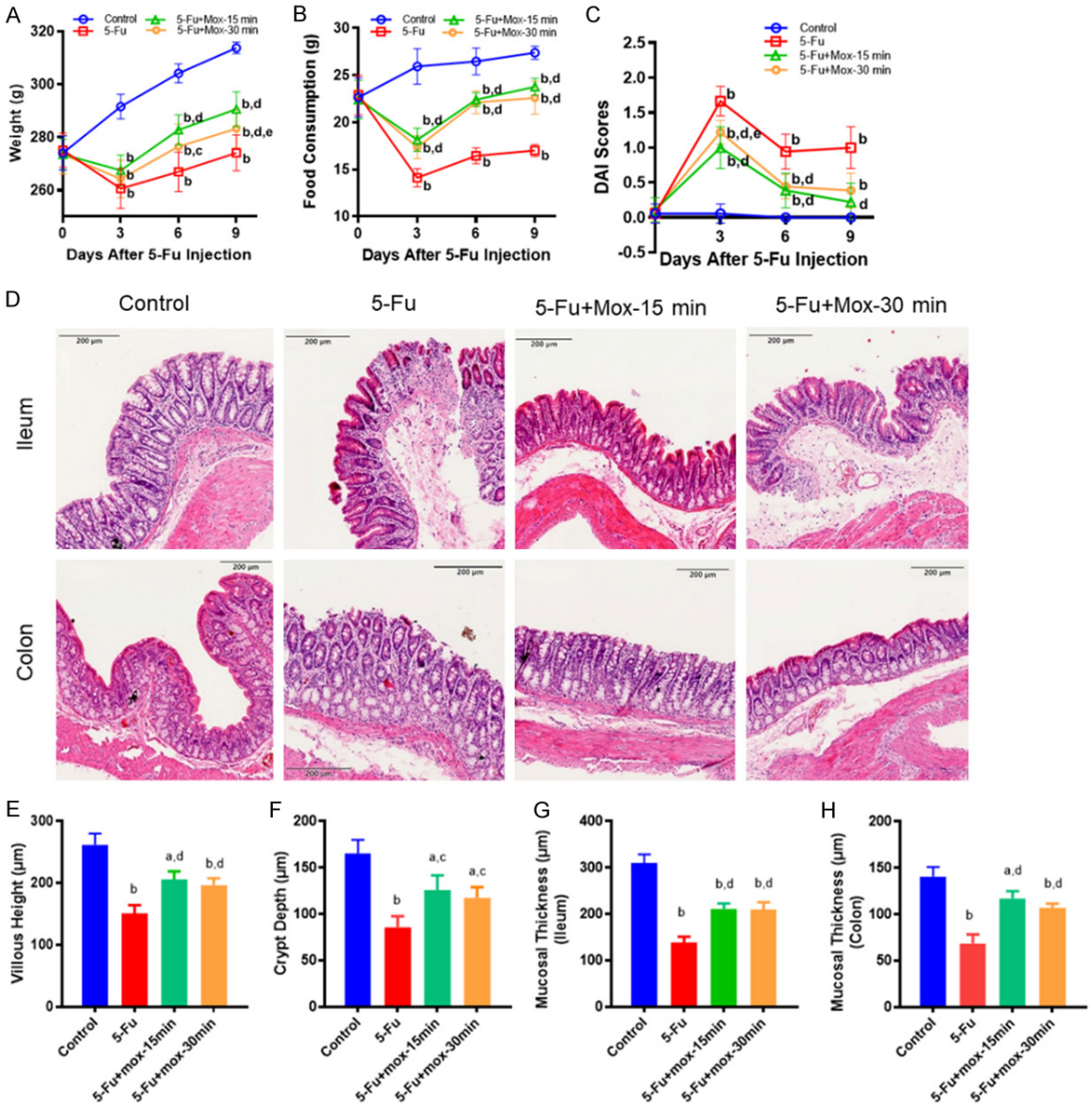


Figure 1. Moxibustion improved the physical signs, reduced the intestinal tissue damage and increased tight junction protein expression in 5-Fu-induced IM rats. (A) Body weights, (B) food consumption and (C) DAI scores of rats in the six groups after 0-9 days of chemotherapy. The points represent the mean ± SEMs (n = 6 per group). (D) Representative HE staining images showing the pathological changes in ileum and colon tissues. Scale bar: 200 μm. A quantitative analysis of (E-G) the ileal villus height, crypt depth, mucosal thickness and (H) colonic mucosal thickness indicated that moxibustion alleviated the intestinal mucosal villus shortening, crypt shallowing and mucosal thinning caused by 5-Fu. The bars represent the means ± SEMs (n = 6 per group). $^{a}P < 0.05$ and $^{b}P < 0.01$ vs the Control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ vs the 5-Fu group; $^{e}P < 0.05$ and $^{f}P < 0.01$ vs the 5-Fu+Moxibustion-15 min/day group.

thelial cell permeability, we examined the localization and expression levels of occludin and ZO-1 by WB assay in the intestinal tissue from the 5-Fu-induced IM rats with or without moxibustion treatment (**Figures 2A** and S2A). The expression of these two proteins in the 5-Fu+Moxibustion-15 min/day and 5-Fu+Moxibustion-30 min/day groups was mostly significantly higher than that in the 5-Fu group and mostly tended to show significant differences compared with the Control group (**Figure 2A**). However, the expression of ZO-1 in the Moxibustion-15 min/day and Moxibustion-30 min/day groups was lower than that in the Control

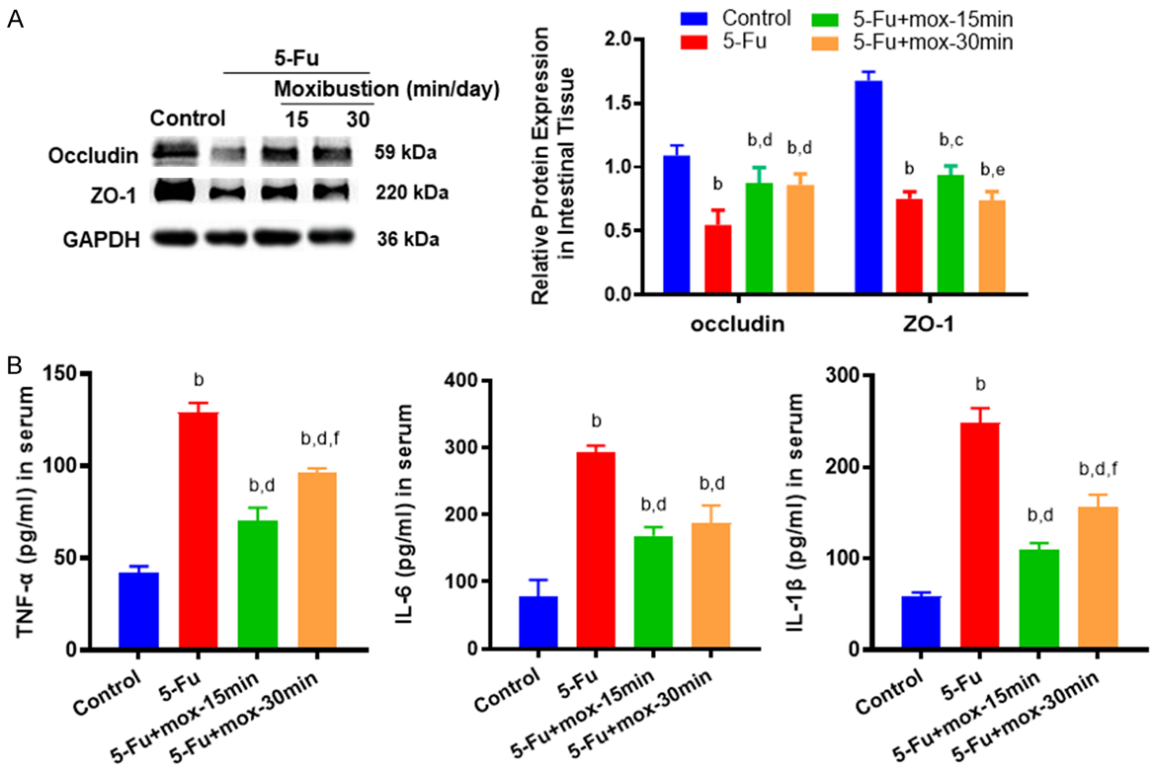


Figure 2. This is a figure. Schemes follow the same formatting. Moxibustion improved the expression of occludin, ZO-1 and proinflammatory cytokines in 5-Fu-induced IM rats. (A) Representative Western blot images showing the protein expression of occludin, ZO-1 and GAPDH (loading control). A quantitative analysis indicated that moxibustion induced recovery of the 5-Fu-mediated reduction in occludin and ZO-1 expression in the ileum, and a moxibustion dose of 15 min/day was more effective in restoring mucosal injury caused by 5-Fu. The bars represent the means ± SEMs (n = 4 per group). $^{a}P < 0.05$ and $^{b}P < 0.01$ vs the Control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ vs the 5-Fu group; $^{e}P < 0.05$ and $^{f}P < 0.01$ vs the Moxibustion-15 min/day group; $^{h}P < 0.01$ vs the Moxibustion-30 min/day group. (B) Moxibustion intervention reduced the 5-Fu-induced expression of TNF-α, IL-6 and IL-1β in serum. The serum expression of TNF-α, IL-6 and IL-1β was evaluated by ELISA. The bars represent the means ± SEMs (n = 6 per group). $^{a}P < 0.05$ and $^{b}P < 0.01$ vs the Control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ vs the 5-Fu group; $^{e}P < 0.05$ and $^{f}P < 0.05$ vs the 5-Fu+Moxibustion-15 min/day group.

group, whereas the expression of occludin in the Moxibustion-15 min/day group was higher than that in the Control group (Figure S2A). These results indicated that moxibustion intervention significantly restored the decreases in tight junction protein expression caused by 5-Fu.

To investigate the mechanism of Moxibustion on intestinal mucositis, we analyzed the serum levels of inflammatory cytokines/chemokines, including TNF-α, IL-6 and IL-1β by ELISA analysis. As shown **Figure 2B**, the expression of all three factors was lower in the 5-Fu+Moxibustion-15 min/day and 5-Fu+Moxibustion-30 min/day groups than in the 5-Fu group, but was still significantly higher than that in the Control group. Moreover, moxibustion intervention at 15 min/day exerted a better effect on reducing the 5-Fu-induced expression of TNF-α and IL-1β than the dose of 30 min/day (**Figures 2B** and S2B).

Effects of moxibustion on RAGE and HMGB1 expression in 5-Fu-induced IM rats

Emerging studies have found that the promotion effect of RAGE on intestinal mucositis is mainly related to TLR4-NF-κB signaling pathway [13-15]. To explore underlying mechanisms of RAGE on 5-Fu-induced IM rats, an immunofluorescence analysis was performed to detect the expression of RAGE. As shown **Figure 3A**, the average fluorescence intensity of RAGE in the ileum and colon of the rats in the 5-Fu group was significantly higher than that in the Control

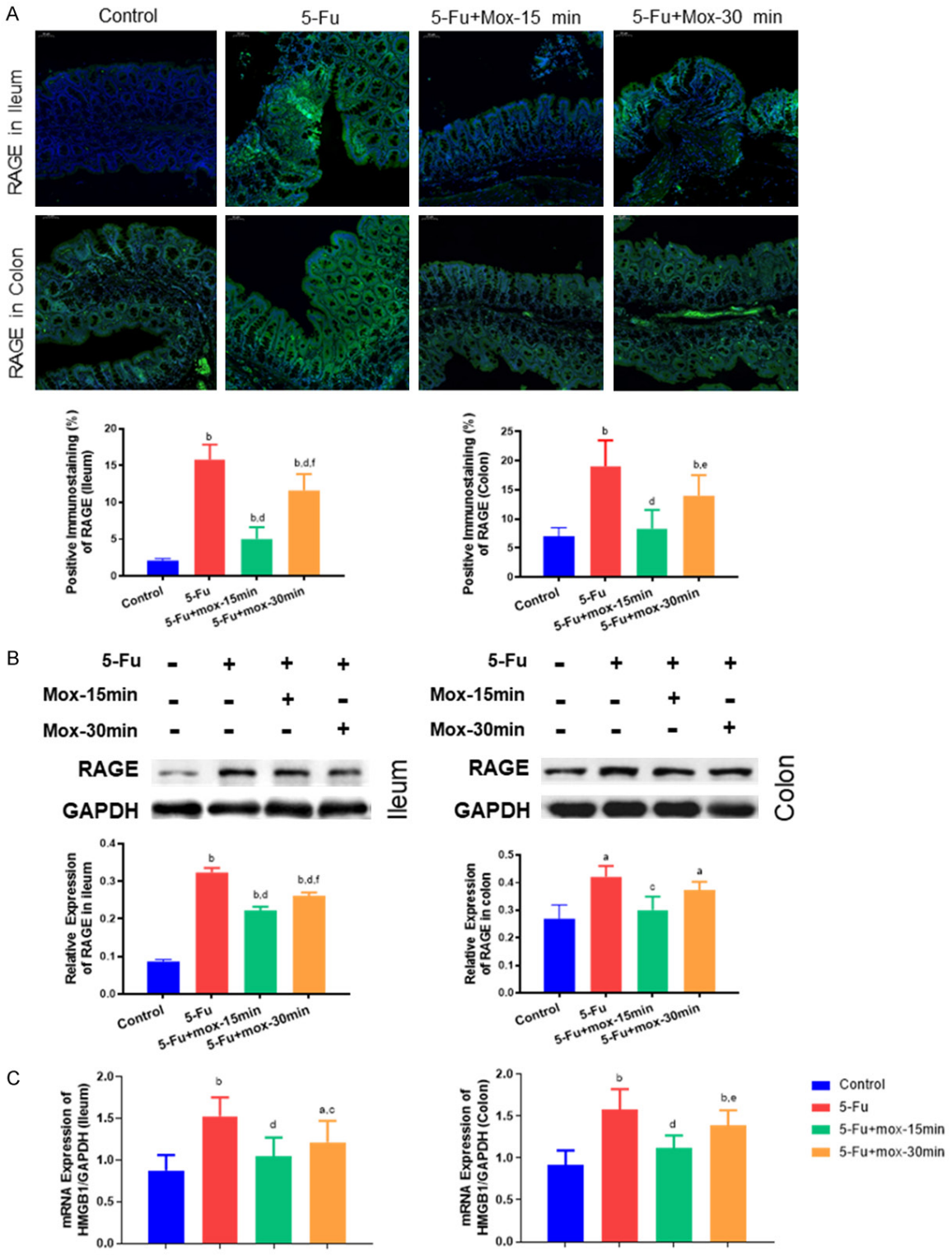


Figure 3. Moxibustion reduced the expression of RAGE and HMGB1 in rat intestinal tissues. A. upper: Representative immunofluorescence images showing the expression of RAGE (green) in the ileum and colon. Scale bar: 50 μm. lower: A quantitative analysis indicated that moxibustion reduced the 5-Fu-induced expression of RAGE in the ileum and colon, and moxibustion intervention at 15 min/day exerted a better inhibitory effect. The intensity of the immunofluorescence intensity of RAGE in four fields of each tissue per rat was quantified using Image J. The bars represent the means ± SEMs (n = 4 per group). $^{b}P < 0.01$ vs the Control group; $^{d}P < 0.01$ vs the 5-Fu group; $^{e}P < 0.05$ and $^{f}P < 0.01$ vs the 5-Fu+Moxibustion-15 min/day group. B. Representative Western blot images showing the

protein expression of RAGE and GAPDH (loading control). A quantitative analysis indicated that moxibustion reduced the 5-Fu-induced expression of RAGE expression in the ileum and colon. Moxibustion intervention at 15 min/day exerted a better inhibitory effect. The bars represent the means ± SEMs (n = 4 per group). $^{a}P < 0.05$ and $^{b}P < 0.01$ vs the Control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ vs the 5-Fu group; $^{f}P < 0.01$ vs the 5-Fu+Moxibustion-15 min/day group. C. The mRNA expression of HMGB1 in the ileum and colon was evaluated by qPCR. The bars represent the means ± SEMs (n = 6 per group). $^{a}P < 0.05$ and $^{b}P < 0.01$ vs the Control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ vs the 5-Fu group; $^{e}P < 0.05$ and $^{f}P < 0.01$ vs the 5-Fu+Moxibustion-15 min/day group.

group. Moreover, moxibustion treatment reduced 5-Fu-induced RAGE expression, and a moxibustion dose of 15 min/day was more effective in reducing RAGE expression than that in a moxibustion dose of 30 min/day (**Figures 3A** and S3A).

Similarly, the protein expression of RAGE was down-regulated in Moxibustion incubated 5-Fu-induced intestinal tissue compared with that not exposed to the Moxibustion treatment (**Figures 3B** and S3B). Consistent with western blot results, the effect of Moxibustion treatment on 5-Fu-induced intestinal tissue was verified by the increased HMGB1 aggregation in our qPCR analysis (**Figures 3C** and S3C).

Effects of moxibustion on TLR4 and NF-κBp65 expression in intestinal tissues of 5-Fu-induced IM rats

We further performed WB and qPCR analyses to detect the protein and mRNA expression of TLR4 and NF-κBp65 in intestinal tissues, and trend of the changes in these proteins was consistent with that found for RAGE. Specifically, the relative expression of TLR4 and NF-κBp65 in ileal and colon tissues was significantly higher in the 5-Fu group than in the Control group (Figure S4A, S4B), and moxibustion intervention reduced the 5-Fu-induced increase in TLR4 and NF-κBp65 expression (**Figure 4A**, **4B**). Consistent with western blot results, qPCR results respectively suggested that the expression of TLR4 in the colon and ileum was lower in the 5-Fu+Moxibustion-15 min/day group than in the 5-Fu+Moxibustion-30 min/day group (**Figures 4C** and S4C).

RAGE inhibition improved mucosal injury and downregulated TLR4 and NF-κBp65 expression in 5-Fu-induced IM rats

To further investigate whether RAGE contributed to active TLR4/NF-κB pathway, we added FPS-ZM1 which is RAGE inhibiter, to test if the activation of RAGE on TLR4/NF-κB pathway in intestinal mucositis of 5-Fu-induced IM rats. As expected, the ileal damage induced by 5-Fu was alleviated by FPS-ZM1 (**Figure 5A**). We further measured the ileal villous height, crypt depth, mucosal thickness, and colonic mucosal thickness. However, no significant difference in these variables with the exception of the ileal villous height was detected between the 5-Fu+FPS-ZM1 and Control groups, whereas the ileal villous height of the 5-Fu+FPS-ZM1 group was significantly improved compared with that of the 5-Fu group (**Figure 5B-D**). These results suggested that the inhibition of RAGE at least partially improved the IM injury caused by 5-Fu.

In addition, it indicated that a significant increases in the TLR4 and NF-κBp65 protein expression level in ileum and colon tissues of 5-Fu group compared with those in the Control group (**Figure 5E**, **5F**). No significant difference in the expression of these proteins with the exception of the expression of NF-κBp65 protein in the colon was found between the FPS-ZM1 and Control groups. The expression of TLR4 and NF-κBp65 proteins in the 5-Fu+FPS-ZM1 group was significantly lower than that in the 5-Fu group but still significantly differ from that in the Control group (**Figure 5E**, **5F**). The above-presented results suggested that the inhibition of RAGE at least partially reduced the 5-Fu-related overexpression of TLR4 and NF-κBp65 in intestinal tissues and that IM injury might be alleviated by downregulating the RAGE/TLR4-NF-κBp65 signaling pathways.

RAGE inhibition reduced LPS-induced proliferation inhibition of Caco2 cells and downregulated RAGE signaling pathway

To explore underlying mechanisms of RAGE on inflammation *in vitro*, we placed Caco2 cells which pretreatment with various concentrations of LPS for 24 h as described in Method. The results showed that intervention with various concentrations of LPS for 24 h did not affect cell proliferation (**Figure 6A**). When the

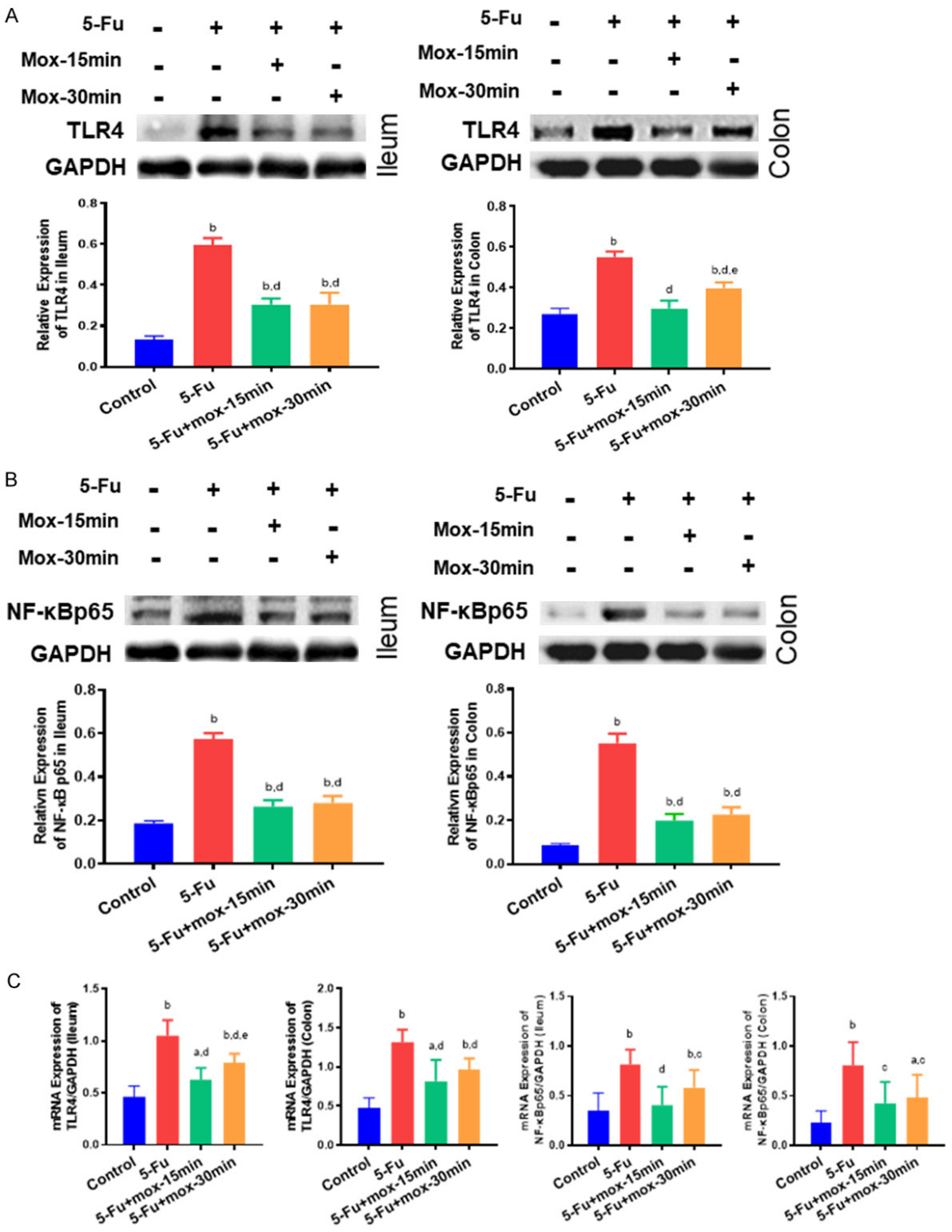


Figure 4. Moxibustion reduced the expression of TLR4 and NF-κBp65 in rat intestinal tissues. A, B. Representative Western blot images showing the protein expression of TLR4, NF-κBp65 and GAPDH (loading control). A quantitative analysis indicated that moxibustion reduced the 5-Fu-induced expression of TLR4 and NF-κBp65 in the ileum and colon. Moxibustion intervention at 15 min/day exerted a better inhibitory effect. The bars represent the means ± SEMs (n = 4 per group). $^{a}P < 0.05$ and $^{b}P < 0.01$ vs the Control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ vs the 5-Fu group; $^{e}P < 0.05$ and $^{f}P < 0.01$ vs the Moxibustion-15 min/day group. C. The mRNA expression of TLR4 and NF-κBp65 in the ileum and colon was evaluated by qPCR. The bars represent the means ± SEMs (n = 6 per group). $^{a}P < 0.05$ and $^{b}P < 0.01$ vs the Control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ vs the 5-Fu group; $^{e}P < 0.05$ vs the Moxibustion-15 min/day group.

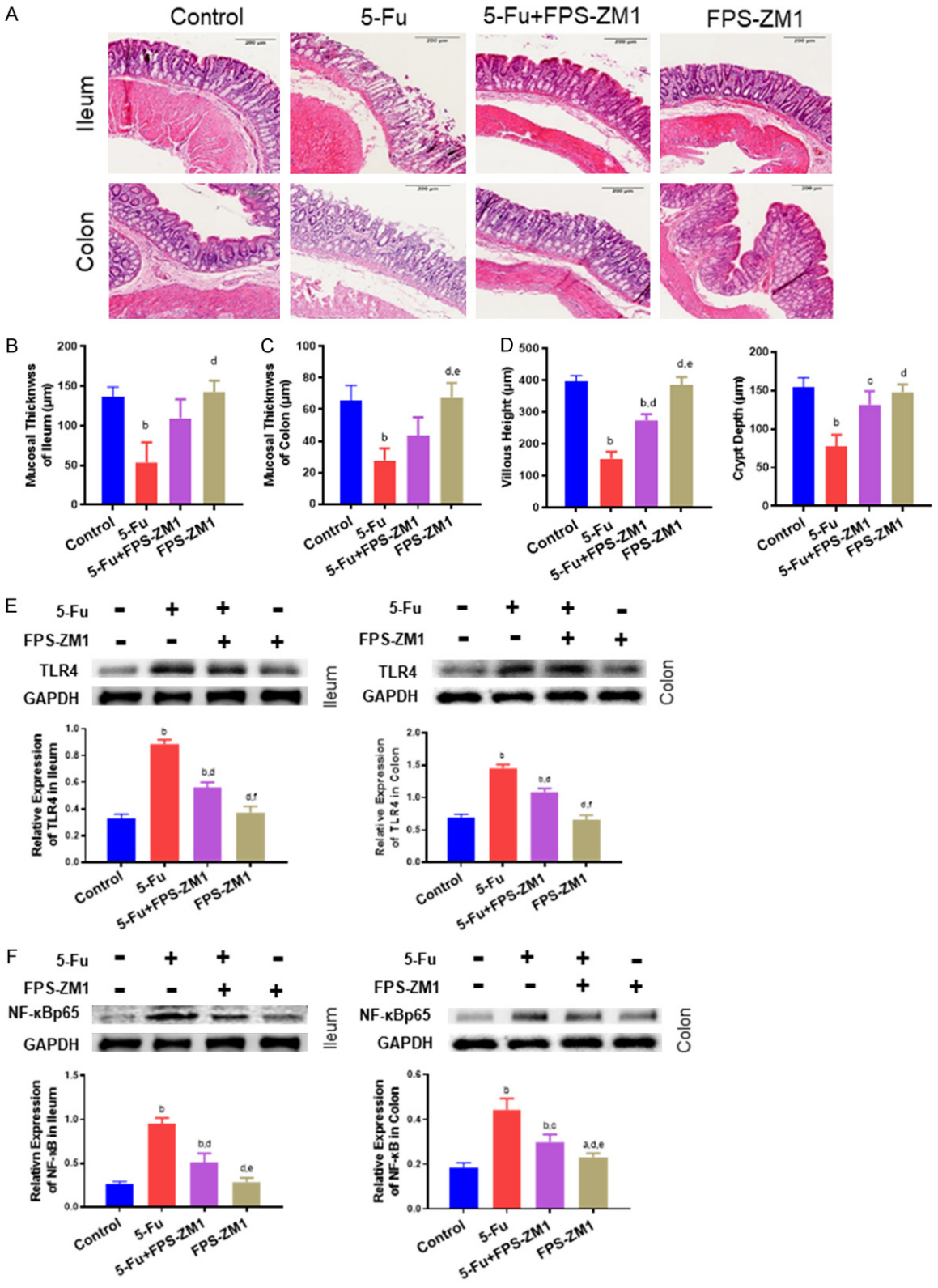


Figure 5. RAGE inhibition improved intestinal tissue injury and downregulated TLR4 and NF-κBp65 expression. (A) Representative HE staining images showing pathological changes in ileum and colon tissues. Scale bar: 200 μm. A quantitative analysis of (B-D) the ileal villous height, crypt depth, and colonic mucosal thickness indicated that the inhibition of RAGE alleviated the intestinal mucosal villus shortening, crypt shallowing and mucosal thinning caused

by 5-Fu. The bars represent the means ± SEMs (n = 6 per group). [b]P < 0.01 vs the Control group; [c]P < 0.05 and [d]P < 0.01 vs the 5-Fu group; [e]P < 0.05 vs the 5-Fu+FPS-ZM1 group. (E, F) Representative Western blot images showing the protein expression of TLR4, NF-κBp65 and GAPDH (loading control). A quantitative analysis indicated that the inhibition of RAGE reduced 5-Fu-related TLR4 and NF-κBp65 expression in the ileum and colon. The bars represent the means ± SEMs (n = 4 per group). [a]P < 0.05 and [b]P < 0.01 vs the Control group; [c]P < 0.05 and [d]P < 0.01 vs the 5-Fu group; [e]P < 0.05 and [f]P < 0.01 vs the 5-Fu+FPS-ZM1 group.

concentration of LPS was ≥ 1 μg/mL and the time was 48 h, it significantly inhibited the proliferation of Caco2, and the inhibitory effect was enhanced with the increase of LPS concentration (data not shown). However, the proliferation of Caco2 cells were decreased after treated with LPS within a certain concentration range (1, 10, 20, 30 μg/mL) and the IC_{50} (50% growth inhibitory values) was 13.6 μg/mL for 48 h (**Figure 6A**).

Then, LPS coculture with Caco2 cells which were pre-treated with FPS-ZM1 (100, 500 and 1000 nM) for 1 h. We found that the secretion levels of the inflammatory cytokines including TNF-α, IL-1β and IL-6 were significantly decreased after FPS-ZM1 added compared with those in LPS group only (**Figure 6B**). These results suggested that RAGE is involved in the inflammatory induced by LPS in Caco2. To further investigate whether RAGE/NF-κBp65 axis contributed to the LPS-induced inflammatory damage, we detected the expression levels of occluding and ZO-1, as well as p-p65 and p-IKBα by WB assay and qPCR. Results showed that with LPS stimulation for 48 h, the expression of occluding and ZO-1 significantly decreased, with RAGE, p-p65 and p-IKBα significantly increased in LPS groups; which were restored by FPS-ZM1. The degree of recovery depends on the concentration of FPS-ZM1 (**Figure 6C-F**). The above-presented results suggested that RAGE/NF-κBp65 axis plays a critical role in LPS-induced pro-inflammatory effect in Caco2 cells.

Discussion

Our results suggested the following: the induction of IM pathogenesis by 5-Fu is mediated by RAGE; the RAGE/TLR4-NF-κBp65 pathways might be involved in the resulting changes in the animal's physical signs, intestinal mechanical barriers and associated inflammatory responses during the development of IM; Moxibustion intervention at the Guanyuan, Tianshu and Qihai points can reduce the pathological changes observed in 5-Fu-induced IM by inhibiting the RAGE/TLR4-NF-κBp65 signaling pathways to improve the symptoms and signs of rats (**Figure 7**).

The 5-Fu-induced IM rat model is widely used because it mimics the clinical manifestations of IM, such as weight loss, gastrointestinal dysmotility and intestinal inflammation [26, 30, 31]. In this study, compared with the control group, 5-Fu caused weight loss, decreased food consumption, increased DAI score and degree of intestinal mucosal damage, and decreased the expression of tight junction protein in IM rats. To assess the role of RAGE in the pathogenesis of IM, we used FPS-ZM1 as a specific inhibitor of RAGE [32, 33]. The results showed that the inhibition of RAGE decreased the expression of TLR4 and NF-κBp65 and improved the degree of intestinal histopathological injury. This finding suggested that RAGE might be an important link in mediating IM pathogenesis, which is consistent with the reported results. Consistent with our study, Costa et al. [34] found that the administration of 5-Fu causes significantly increased expression of RAGE in the intestinal mucosal and villi shortening, crypt loss, inflammatory cell infiltration. In addition, 5-Fu-induced apoptosis has been reported to trigger the release of HMGB1, which stimulates TLR4 and causes the immune response so as to achieve successful treatment [35]. Accumulating data also indicate that RAGE expression was upregulated in the small intestine and colon of patients with inflammatory bowel disease (IBD) [36]. Inhibiting RAGE expression could significantly improve the related symptoms of IM [37, 38].

As a transcription factor known to regulate various inflammatory and immune responses, NF-κBp65 controls many proinflammatory cytokines. The abnormal activation of p65 is closely related to the occurrence of inflammatory bowel injury, and the inhibition of p65 activation has become an important research direction for inflammatory intestinal diseases. During inflammation, a pattern recognition receptor of RAGE can bind to many ligands [19, 20], such

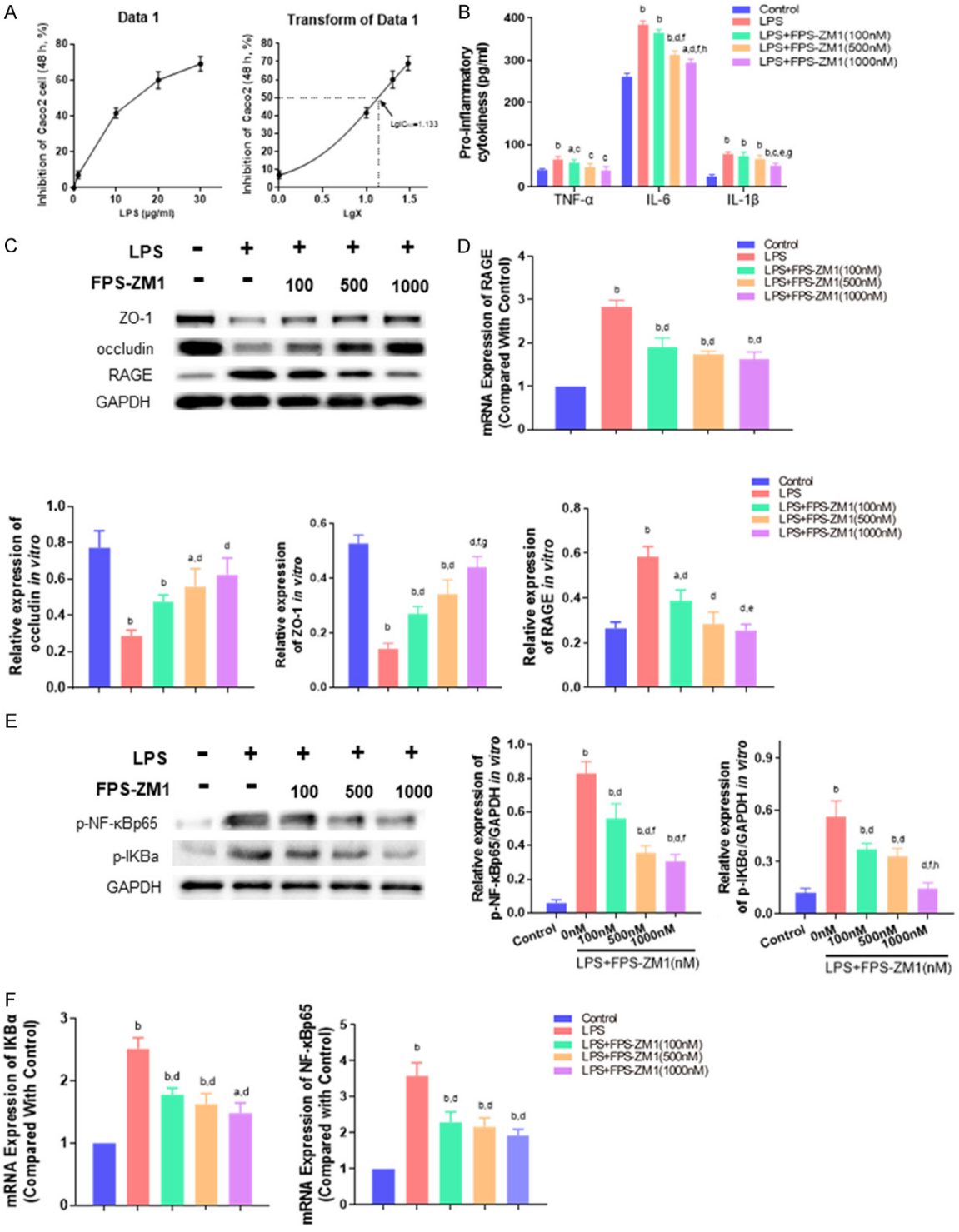


Figure 6. Effect of RAGE inhibitors on RAGE/NF-κBp65 signaling pathway in LPS-induced cell. A. The LPS concentrations and growth inhibition rate curve (n = 3). B. TNF-α, IL-6, and IL-1β levels in supernatant were evaluated using ELISA. The bars represent the means ± SEMs (n = 4 per group). C. Different concentrations of FPS-ZM1 on LPS-induced tight junctions and expression of RAGE protein. D. mRNA expression levels of RAGE in different groups. E. Western blot analysis of p-IKBα and p-p65 in different groups. Quantifications of migrated cells from three independent experiments. F. mRNA expression levels of p-IKBα and p-p65 in different groups. The bars represent the means ± SEMs (n = 3 per group). $^{a}P < 0.05$ and $^{b}P < 0.01$ vs the Control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ vs the LPS group; $^{e}P < 0.05$ and $^{f}P < 0.01$ vs the 500 nM+LPS group; $^{g}P < 0.05$, $^{h}P < 0.01$ vs the 1000 nM + LPS group.

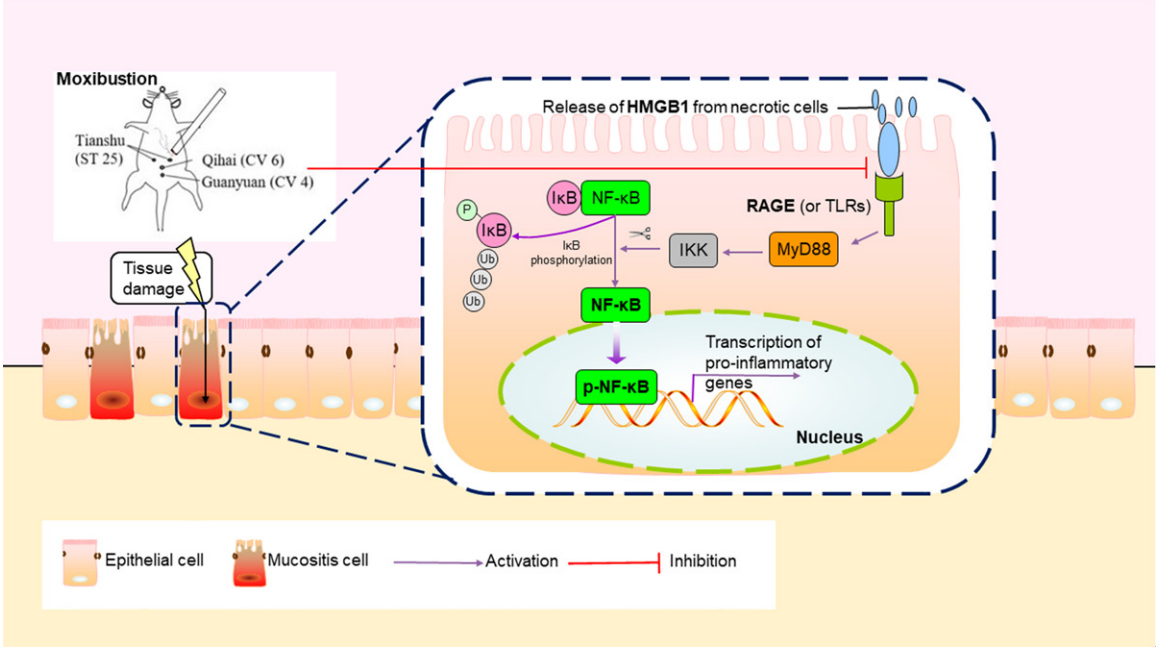


Figure 7. Graphical abstract of factors participated in moxibustion regulation of IM.

as advanced glycation end products (AGEs), S100 proteins, HMGB1, amyloid β-protein (Aβ), modified DNA, and RNA [39], which activate NF-κB signaling via a cascade reaction to cause inflammation or dysfunction in cells or tissues.

The HMGB/TLR axis, which is the upstream regulatory signal of NF-κB, plays a key role in most of inflammatory responses in the body [40, 41]. Previous studies have shown the effect of TLR4 in mediating chemotherapy-induced mucositis [42]. The role of the RAGE/TLR4NFκB pathways in mediating the proinflammatory effect have also been confirmed. Some evidence suggests that AGEs and lipopolysaccharide LPS increase IL-6 secretion via the RAGE/TLR4NFκBROS pathways in the J774 murine macrophage cell line [43]. However, whether the RAGE/TLR4NFκB pathways are related to chemotherapy-induced IM has not yet been reported. In this study, we further studied the role of RAGE/TLR4NFκB signaling pathway in 5-Fu-induced IM, and found that 5-Fu increased the expression of RAGE, TLR4 and NF-κBp65 in the ileum and colon, and the severity of intestinal damage and expression of TLR4 and NF-κBp65 was reduced by the inhibiton of RAGE. These findings suggest that the RAGE/TLR4-NF-κBp65 pathways might be important in improving intestinal injury and the inflammatory in 5-Fu-induced IM.

Previous reports showed that the roles of HMGB1 and RAGE were shown to be mediated by transient phosphorylation and activation of various signaling molecules, such as p38, ERK1/2, IκBα and p65 proteins [44, 45]. To detect the effect of RAGE in intestinal injury and the inflammatory, an LPS-induced cell damage model was established. We found that inhibition of RAGE significantly inhibited tight junction protein decrease and inflammatory response, which is related to the phosphorylation and nuclear translocation of NF-κB, and thus cause the synthesis and release of cytokines.

Several recent studies have confirmed that moxibustion pretreatment at the Tianshu point reduced IM-related damage in UC rats by downregulating the expression of TLR family and the signaling pathway-related molecules [22, 46]. Further research confirms that drug-separated moxibustion can decrease the expression of NF-κBp65 and the downstream proinflammatory factors TNF-α and IL-1β in the colon, which promotes the healing of colonic ulcer tissue, reduces mucosal connective tissue edema, and thereby improves intestinal inflammation [23, 47]. In addition, a previous study has also confirmed that herb-partitioned moxibustion exerts a significant regulatory effect on the TLR4/NF-κB pathway [48] and can causeeffi-

cient therapeutic effects on improving colonic injury and inhibiting colonic inflammation in rats with CD [49]. Our current findings demonstrated that moxibustion inhibited the expression of the RAGE/TLR4-NF-κBp65 signaling pathways in intestinal tissues and reduced 5-Fu-induced IM injury, and the dose of 15 min/day exerted a better therapeutic effect. These findings for the first time demonstrated that moxibustion plays a therapeutic role in 5-Fu-induced IM pathogenesis by regulating the RAGE/TLR4-NF-κBp65 pathways and constitutes the first assessment of the effect of two different moxibustion treatment dosages.

We have confirmed that the RAGE/TLR4-NF-κBp65 pathways might play an important role in the development of 5-Fu-induced IM. Moxibustion improves intestinal tissue damage and the inflammatory response of IM by inhibiting the RAGE/TLR4-NF-κBp65 pathways. Different durations of mild moxibustion treatment exert different therapeutic effects, and moxibustion for 15 min/day offers a better therapeutic effect than 30 min/day.

Conclusion

Our study suggests that the RAGE/TLR4-NF-κBp65 pathways might play a key role in the mechanism of action of moxibustion in 5-Fu-induced IM. The RAGE/TLR4-NF-κBp65 signaling pathways appear to be a valuable research direction for assessing the effects of moxibustion about the adverse effects in the gastrointestinal system induced by chemotherapy.

Acknowledgements

The study was supported by Songjiang Municipal Health Commission; The Third Cycle Talents Discipline Construction and Cooperation Projects on Diseases between Shanghai General Hospital and Songjiang District; TCM Leading Personnel Training Project of Shanghai Municipal Health Commission: ZY (2018-2020)-RCPY-1024; TCM Master Instructing Apprentice in Community of Shanghai Municipal Health Commission; Scientific research project of Shanghai Science and Technology Commission: 22ZR1450600.

Disclosure of conflict of interest

None.

Abbreviations

IM, Intestinal mucositis; 5-Fu, 5-fluorouracil; NF-κB, Nuclear factor kappa-B; TNF, Tumor necrosis factor; IL, Interleukin; TLR, Toll-like receptor; RAGE, the Receptor of Advanced Glycation Endproducts; LPS, Lipopolysaccharide; HE, Hematoxylin-eosin; WB, Western blotting; qPCR, Quantitative real-time PCR; ELISA, Enzyme-linked immunosorbent assay; CCK-8, Cell Counting Kit-8; SD, Sprague-Dawley; FOBT, Fecal occult blood test; DAI, Disease Activity Index; HMGB1, High mobility group protein B1; IBD, Inflammatory bowel disease; AGEs, Advanced glycation end products; Aβ, Amyloid β-protein.

Address correspondence to: Bimeng Zhang, Department of Acupuncture and Moxibustion, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, No. 100, Haining Road, Hongkou District, Shanghai 200080, People's Republic of China. Tel: +86-153-0165-5585; E-mail: pjzhtiger08@aliyun.com; Dr. Hua Sui, Medical Experiment Center, Jiading Branch of Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, No. 800, Huangjiahuayuan Road, Jiading District, Shanghai 201803, People's Republic of China. E-mail: huasui0808@shutcm.edu.cn

References

[1] Prisciandaro LD, Geier MS, Butler RN, Cummins AG and Howarth GS. Evidence supporting the use of probiotics for the prevention and treatment of chemotherapy-induced intestinal mucositis. Crit Rev Food Sci Nutr 2011; 51: 239-47.

[2] Gibson RJ, Keefe DM, Clarke JM, Regester GO, Thompson FM, Goland GJ, Edwards BG and Cummins AG. The effect of keratinocyte growth factor on tumour growth and small intestinal mucositis after chemotherapy in the rat with breast cancer. Cancer Chemother Pharmacol 2002; 50: 53-8.

[3] Abalo R, Uranga JA, Pérez-García I, Andrés R, Girón R, Vera G, López-Pérez AE and Martín-Fontelles MI. May cannabinoids prevent the development of chemotherapy-induced diarrhea and intestinal mucositis? Experimental study in the rat. Neurogastroenterol Motil 2017; 29: 2017.

[4] Touchefeu Y, Montassier E, Nieman K, Gastinne T, Potel G, Varannes SB, Vacon FL and Cochetière MF. Systematic review: the role of the gut microbiota in chemotherapy- or radiation-induced gastrointestinal mucositis -

current evidence and potential clinical applications. Aliment Pharmacol Ther 2014; 40: 409-21.
[5] Vera-Llonch M, Oster G, Ford CM, Lu J and Sonis S. Oral mucositis and outcomes of allogeneic hematopoietic stem-cell transplantation in patients with hematologic malignancies. Support Care Cancer 2007; 15: 491-6.
[6] Sonis ST, Elting LS, Keefe D, Peterson DE, Schubert M, Hauer-Jensen M, Bekele BN, Raber-Durlacher J, Donnelly JP and Rubenstein EB. Perspectives on cancer therapy-induced mucosal injury: pathogenesis, measurement, epidemiology, and consequences for patients. Cancer 2004; 100: 1995-2025.
[7] Lockhart PB and Sonis ST. Alterations in the oral mucosa caused by chemotherapeutic agents. A histologic study. J Dermatol Surg Oncol 1981; 7: 1019-25.
[8] Sonis ST, Elting LS, Keefe D, Peterson DE, Schubert M, Hauer-Jensen M, Bekele BN, Raber-Durlacher J, Donnelly JP and Rubenstein EB. Perspectives on cancer therapy-induced mucosal injury: pathogenesis, measurement, epidemiology, and consequences for patients. Cancer 2004; 100: 1995-2025.
[9] van der Velden WJ, Herbers AH, Netea MG and Blijlevens NM. Mucosal barrier injury, fever and infection in neutropenic patients with cancer: introducing the paradigm febrile mucositis. Br J Haematol 2014; 167: 441-52.
[10] Chang CT, Ho TY, Lin H, Liang JA, Huang HC, Li CC, Lo HY, Wu SL, Huang YF and Hsiang CY. 5-fluorouracil induced intestinal mucositis via nuclear factor-κB activation by transcriptomic analysis and in vivo bioluminescence imaging. PLoS One 2012; 7: e31808.
[11] Bamba S, Andoh A, Yasui H, Araki Y, Bamba T and Fujiyama Y. Matrix metalloproteinase-3 secretion from human colonic subepithelial myofibroblasts: role of interleukin-17. J Gastroenterol 2003; 38: 548-54.
[12] Yuan DD, Chi XJ, Jin Y, Li X, Ge M, Gao WL, Guan JQ, Zhang AL and Hei ZQ. Intestinal injury following liver transplantation was mediated by TLR4/NF-κB activation-induced cell apoptosis. Mol Med Rep 2016; 13: 1525-32.
[13] Ohashi K, Takahashi HK, Mori S, Liu K, Wake H, Sadamori H, Matsuda H, Yagi T, Yoshino T, Nishibori M and Tanaka N. Advanced glycation end products enhance monocyte activation during human mixed lymphocyte reaction. Clin Immunol 2010; 134: 345-53.
[14] Wang Y, Wang H, Piper MG, McMaken S, Mo X, Opalek J, Schmidt AM and Marsh CB. sRAGE induces human monocyte survival and differentiation. J Immunol 2010; 185: 1822-35.
[15] Pollreisz A, Hudson BI, Chang JS, Qu W, Cheng B, Papapanou PN, Schmidt AM and Lalla E. Receptor for advanced glycation endproducts mediates pro-atherogenic responses to periodontal infection in vascular endothelial cells. Atherosclerosis 2010; 212: 451-6.
[16] Zhang F, Kent KC, Yamanouchi D, Zhang Y, Kato K, Tsai S, Nowygrod R, Schmidt AM and Liu B. Anti-receptor for advanced glycation end products therapies as novel treatment for abdominal aortic aneurysm. Ann Surg 2009; 250: 416-23.
[17] Chavakis T, Bierhaus A, Al-Fakhri N, Schneider D, Witte S, Linn T, Nagashima M, Morser J. Arnold B, Preiiner KT and Nawroth PP. The pattern recognition receptor (RAGE) is a counterreceptor for leukocyte integrins: a novel pathway for inflammatory cell recruitment. J Exp Med 2003; 198: 1507-15.
[18] Yan SF, Ramasamy R, Naka Y and Schmidt AM. Glycation, inflammation, and RAGE: a scaffold for the macrovascular complications of diabetes and beyond. Circ Res 2003; 93: 1159-69.
[19] Wang L, Zhang X, Liu L, Cui L, Yang R, Li M and Du W. Tanshinone II A down-regulates HMGB1, RAGE, TLR4, NF-kappaB expression, ameliorates BBB permeability and endothelial cell function, and protects rat brains against focal ischemia. Brain Res 2010; 1321: 143-51.
[20] Wang L, Zhang X, Liu L, Yang R, Cui L and Li M. Atorvastatin protects rat brains against permanent focal ischemia and downregulates HMGB1, HMGB1 receptors (RAGE and TLR4), NF-kappaB expression. Neurosci Lett 2010; 471: 152-6.
[21] Xu YL, Du YH, Xu XM and He TY. Observation on therapeutic effect of herb-partitioned spread moxibustion for treatment of chronic nonspecific ulcerative colitis. Zhongguo Zhen Jiu 2010; 30: 289-91.
[22] Yang L, Huang Y, Huang R, Ma X, Ma Z, Liu Y, Zheng H, Zhu Y, Liu H and Wang Z. Study on the regulating effect of moxibustion pretreatment at Tianshu (ST25) on TLR4/TRIF signaling pathway in UC rats. Shanghai Journal of Acupuncture and Moxibustion 2019; 38: 819-826.
[23] Wei K, Zhang D, Dou C, Ma X, Yang L, Wu H,Hong Y, Zhu Y, Zhang C, Liu J, et al. Study on the regulating effect of moxibustion on NF-κB p65, TNF-α, and IL-1β in colons of CD rats. World Chinese Medicine 2013; 8: 862-866.
[24] Jiang Y, Zhao C, Wei R, Huang Y and Lu M. Effect of moxibustion to GV4 on AGEs and RAGE of brain tissue in aging rats. JCAM 2020; 36: 56-60.
[25] Wang JJ, Wu SB, Cheng HL, Yang C, Chen Y and Yang J. Effect of moxibustion on expression of RAGE and LRP-1 and neuronal ultrastructure of frontal cortex and hippocampus in vascular

dementia rats. Acupuncture Research 2020; 45: 33-9.
[26] Carneiro-Filho BA, Oriá RB, Wood Rea K, Brito GA, Fujii J, Obrig T, Lima AA and Guerrant RL. Alanyl-glutamine hastens morphologic recovery from 5-fluorouracil-induced mucositis in mice. Nutrition 2004; 20: 934-41.
[27] Murano M, Maemura K, Hirata I, Toshina K, Nishikawa T, Hamamoto N, Sasaki S, Saitoh O and Katsu K. Therapeutic effect of intracolonically administered nuclear factor kappa B (p65) antisense oligonucleotide on mouse dextran sulphate sodium (DSS)-induced colitis. Clin Exp Immunol 2000; 120: 51-8.
[28] Li BR, Shao SY, Yuan L, Jia R, Sun J, Ji Q, Sui H, Zhou LH, Zhang Y, Liu H, Li Q, Wang Y and Zhang BM. Effects of mild moxibustion on intestinal microbiome and NLRP3 inflammasome in rats with 5-fluorouracil-induced intestinal mucositis. J Integr Med 2021; 19: 144-157.
[29] Fritz M, Klawonn AM, Zhao Q, Sullivan EV, Zahr NM and Pfefferbaum A. Structural and biochemical imaging reveals systemic LPS-induced changes in the rat brain. J Neuroimmunol 2020; 348: 577367.
[30] Lu H, Liu H, Wang J, Shen J, Weng S, Han L, Sun T, Qian L, Wu M, Zhu S, Yu Y, Han W and Zhu J. The chemokine CXCL9 exacerbates chemotherapy-induced acute intestinal damage through inhibition of mucosal restitution. J Cancer Res Clin Oncol 2015; 141: 983-92.
[31] Justino PF, Melo LF, Nogueira AF, Costa JV, Silva LM, Santos CM, Mendes WO, Costa MR, Franco AX, Lima AA, Ribeiro RA, Souza MH and Soares PM. Treatment with Saccharomyces boulardii reduces the inflammation and dysfunction of the gastrointestinal tract in 5-fluorouracil-induced intestinal mucositis in mice. Br J Nutr 2014; 111: 1611-21.
[32] Shen C, Ma Y, Zeng Z, Yin Q, Hong Y, Hou X and Liu X. RAGE-specific inhibitor FPS-ZM1 attenuates AGEs-induced neuroinflammation and oxidative stress in rat primary microglia. Neurochem Res 2017; 42: 2902-2911.
[33] Kwak T, Drews-Elger K, Ergonul A, Miller PC, Braley A, Hwang GH, Zhao D, Besser A, Yamamoto Y, Yamamoto H, El-Ashry D, Slingerland JM, Lippman ME and Hudson BI. Targeting of RAGE-ligand signaling impairs breast cancer cell invasion and metastasis. Oncogene 2017; 36: 1559-1572.
[34] Costa DVS, Bon-Frauches AC, Silva A, Lima-Júnior RCP, Martins CS, Leitão RFC, Freitas GB, Castelucci P, Bolick DT, Guerrant RL, Warren CA, Moura-Neto V and Brito GAC. 5-fluorouracil induces enteric neuron death and glial activation during intestinal mucositis via a S100B-RAGE-NFκB-dependent pathway. Sci Rep 2019; 9: 665.
[35] Zitvogel L, Apetoh L, Ghiringhelli F, André F, Tesniere A and Kroemer G. The anticancer immune response: indispensable for therapeutic success? J Clin Invest 2018; 11: 1991-2001.
[36] Body-Malapel M, Djouina M, Waxin C, Langlois A, Gower-Rousseau C, Zerbib P, Schmidt AM, Desreumaux P, Boulanger E and Vignal C. The RAGE signaling pathway is involved in intestinal inflammation and represents a promising therapeutic target for Inflammatory Bowel Diseases. Mucosal Immunol 2019; 12: 468-478.
[37] Ciccocioppo R, Vanoli A, Klersy C, Imbesi V, Boccaccio V, Manca R, Betti E, Cangemi GC, Strada E, Besio R, Rossi A, Falcone C, Ardizzone S, Fociani P, Danelli P and Corazza GR. Role of the advanced glycation end products receptor in Crohn's disease inflammation. World J Gastroenterol 2013; 19: 8269-81.
[38] Hampe J, Kucharzik T, Lügering A, Broeckel U, Schreiber S, Spieker T, Stoll M and Foell D. The functional -374T/A polymorphism of the receptor for advanced glycation end products may modulate Crohn's disease. Am J Physiol Gastrointest Liver Physiol 2011; 300: G823-32.
[39] Ott C, Jacobs K, Haucke E, Navarrete SA, Grune T and Simm A. Role of advanced glycation end products in cellular signaling. Redox Biol 2014; 2: 411-29.
[40] Yang Y, Wang J, Yang Q, Wu S, Yang Z, Zhu H, Zheng M, Liu W, Wu W, He J and Chen Z. Shikonin inhibits the lipopolysaccharide-induced release of HMGB1 in RAW264.7 cells via IFN and NF-κB signaling pathways. Int Immunopharmacol 2014; 19: 81-7.
[41] Antón M, Alén F, Gómez de Heras R, Serrano A, Pavón FJ, Leza JC, García-Bueno B, Rodríguez de Fonseca F and Orio L. Oleoylethanolamide prevents neuroimmune HMGB1/TLR4/NF-kB danger signaling in rat frontal cortex and depressive-like behavior induced by ethanol binge administration. Addict Biol 2017; 22: 724-741.
[42] Khan S, Wardill HR and Bowen JM. Role of toll-like receptor 4 (TLR4)-mediated interleukin-6 (IL-6) production in chemotherapy-induced mucositis. Cancer Chemother Pharmacol 2018; 82: 31-37.
[43] Ohtsu A, Shibutani Y, Seno K, Iwata H, Kuwayama T and Shirasuna K. Advanced glycation end products and lipopolysaccharides stimulate interleukin-6 secretion via the RAGE/TLR4-NF-κB-ROS pathways and resveratrol attenuates these inflammatory responses in mouse macrophages. Exp Ther Med 2017; 14: 4363-4370.

[44] Tropea M, Gerstenberger E, Shelhamer JH and Suffredini AF. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. Blood 2003; 101: 2652-60.
[45] Arieli D, Nahmany G, Casap N, Ad-El D and Samuni Y. The effect of a nitroxide antioxidant on ischemia-reperfusion injury in the rat in vivo hind limb model. Free Radic Res 2018; 42: 114-23.
[46] Wang X, Liu Y, Dong H, Wu L, Feng X, Zhou Z, Zhao C, Liu H and Wu H. Herb-partitioned moxibustion regulates the TLR2/NF-κB signaling pathway in a rat model of ulcerative colitis. Evid Based Complement Alternat Med 2015; 2015: 949065.
[47] Ma TM, Han Y, Ma XD, Zeng XX and Ge W. Influence of moxibustion with different duration on colonic epithelial structure, serum inflammatory cytokines, and intestinal mucosa inflammatory cell signal transduction pathways. Acupuncture Research 2014; 39: 20-6.
[48] Yoon HM, Jang KJ, Han MS, Jeong JW, Kim GY, Lee JH and Choi YH. Ganoderma lucidum ethanol extract inhibits the inflammatory response by suppressing the NF-κB and toll-like receptor pathways in lipopolysaccharide-stimulated BV2 microglial cells. Exp Ther Med 2013; 5: 957-963.
[49] Hong J, Zhang C, Wu L, Liu J, Zhu Y, Yang L, Wei K and Yan H. Efficacy of herb-partitioned moxibustion at Qihai (CV 6) and bilateral Tianshu (ST 25) on colonic damage and the TLR4/NF-I masculineB signaling pathway in rats with Crohn's disease. J Tradit Chin Med 2018; 38: 218-226.

Supplementary material

Animals treatment with moxibustion

After adaptive feeding for 1 week, the rats were randomly divided into eight groups: Control (healthy rats that received only saline solution), Moxibustion-15 min/day (healthy rats that received moxibustion for 15 min once per day), and Moxibustion-30 min/day (healthy rats that received moxibustion for 30 min once per day). Each group consisted of six rats.

Sacrifice of rats

On the 16th day after treatment, each group was fasted for 24 h, and on the 17th day, the rats were sacrificed for sample collecting. Briefly, the rats were anesthetized by intraabdominal injection with 3% pentobarbital sodium (2 ml/kg), and blood samples were collected from the rat hearts through the space at the left between the third and fourth ribs using a 5-ml syringe with a 0.21-mm internal diameter-needle. The samples were stored overnight at 4°C, centrifuged at 3000 rpm and 4°C for 15 min to separate the serum, and then stored at -80°C. After blood sample collecting, the abdominal cavity was opened to separate the intestinal tissues. Ileum and colon samples were separated 1 cm from the upper and lower ileocecal section, and samples consisting of 6-8 cm of each section were collected. After removal, the intestine was cut longitudinally along the mesenteric line, rinsed in normal saline and divided into two parts: 3 cm of each sample was fixed in 4% paraformaldehyde and stored at 4°C, and the rest of the sample was stored in liquid nitrogen.

Preparation of intestinal tissue sections

The intestinal tissues fixed in 4% paraformaldehyde were dehydrated in a dehydration box and then sequentially immersed in 30% alcohol, 50% alcohol, 70% alcohol, 80% alcohol and 90% alcohol (40 min in each solution). Subsequently, 95% alcohol and anhydrous ethanol were used to remove the water in the tissues (incubated in each solution for 20 min). The tissues were then treated with xylene I and II (20 min with each solution), embedded in paraffin and sectioned into 4-μm slices.

Table S1. Disease activity index

Items \ Score	0	1	2	3	4
Weight loss (%)	(-)	1-5	5-10	11-15	> 15
Stool consistency	Normal		Loose		Diarrhea
Occult/gross bleeding	Normal		Guaiac (+)		Gross bleeding
DAI	(Combined score from the weight loss, stool consistency and bleeding)/3				

Table S2. PCR primers

gene	Forward primer	Reverse primer
RAGE	CTGCCTCTGAACTCACAGCCAAT	TCCTGGTCTCCTCCTTCACAACTG
HMGB1	CCTCCTTCGGCCTTCTTCTTGTTC	TCATCCGCAGCAGTGTTGTTCC
NF-κBp65 [1]	GACCTGGCATCTGTGGACAAC	TCCGCAATGGAGGAGAAGTCT
IkBα	ATGGCCAAGTGCAGGAAC	GTCAAGGAGCTGCAGGAGAT
TLR4	TTGCTGCCAACATCATCCAGGAAG	CAGAGCGGCTACTCAGAAACTGC
GAPDH	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT

Table S3. Grouping of cells for differential treatment

Groups	Treatment
Control	-
LPS pre-experimental	Treated with 0.1 μg/mL, 1 μg/mL, 10 μg/mL, 20 μg/mL and 30 μg/mL LPS for 24 h and 48 h, respectively
LPS	Treated with 13.6 μg/ml LPS for 48 h
100 nM+LPS	Treated with 100 nM FPS-ZM1 for 1 h then 13.6 μg/ml LPS for 48 h
500 nM+LPS	Treated with 500 nM FPS-ZM1 for 1 h then 13.6 μg/ml LPS for 48 h
1000 nM+LPS	Treated with 1000 nM FPS-ZM1 for 1 h then 13.6 μg/ml LPS for 48 h

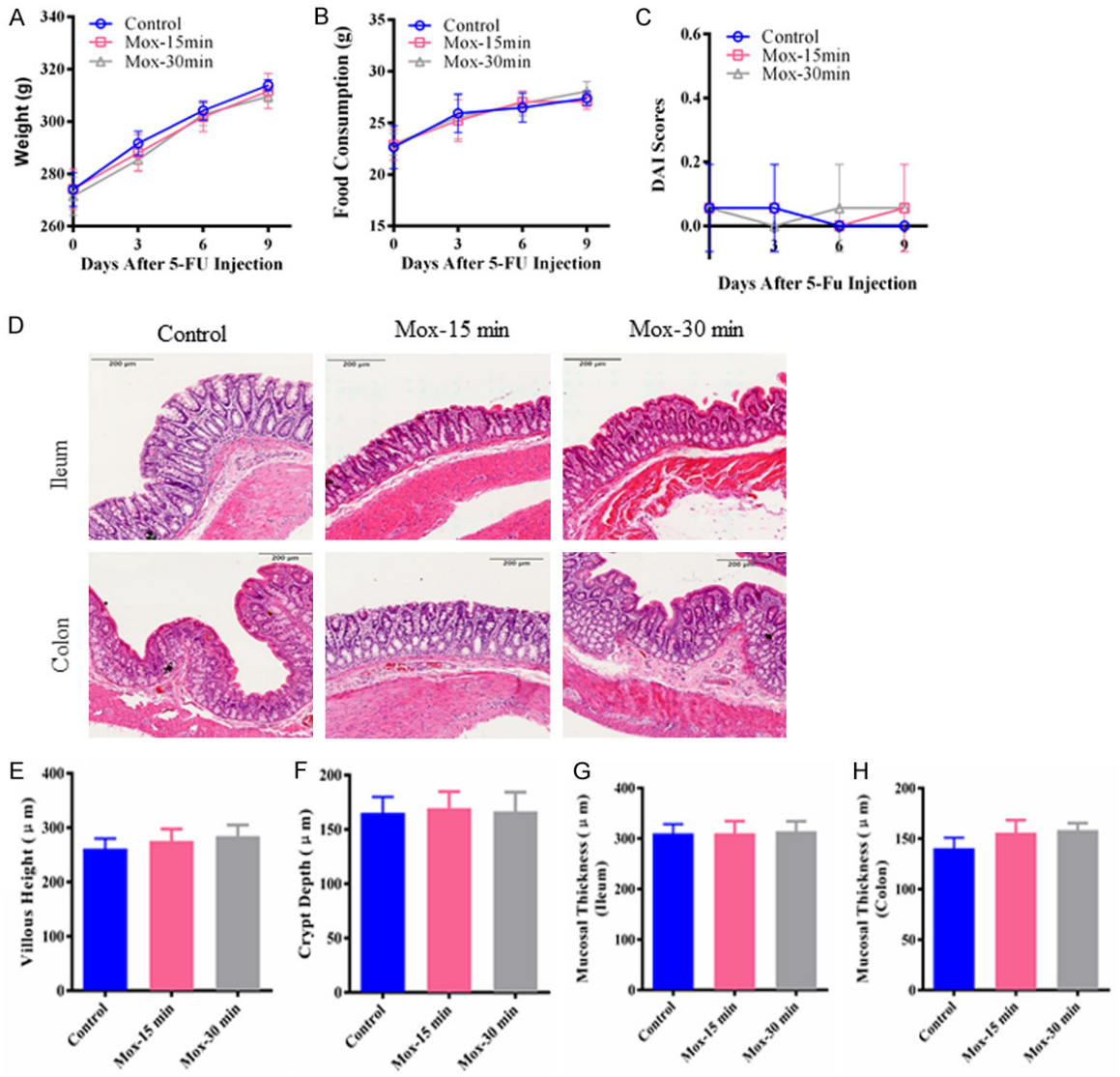


Figure S1. Effect of Moxibustion on intestinal tissue damage. (A) Body weights, (B) food consumption and (C) DAI scores of rats in the six groups after 0-9 days of chemotherapy. (D) Representative HE staining images showing the pathological changes in ileum and colon tissues. Scale bar: 200 μm. A quantitative analysis of (E-H) the ileal villus height, crypt depth, and colonic mucosal thickness. The bars represent the means ± SEMs (n = 6 per group). A quantitative analysis indicated that there is no significant difference between moxibustion group and Control group.

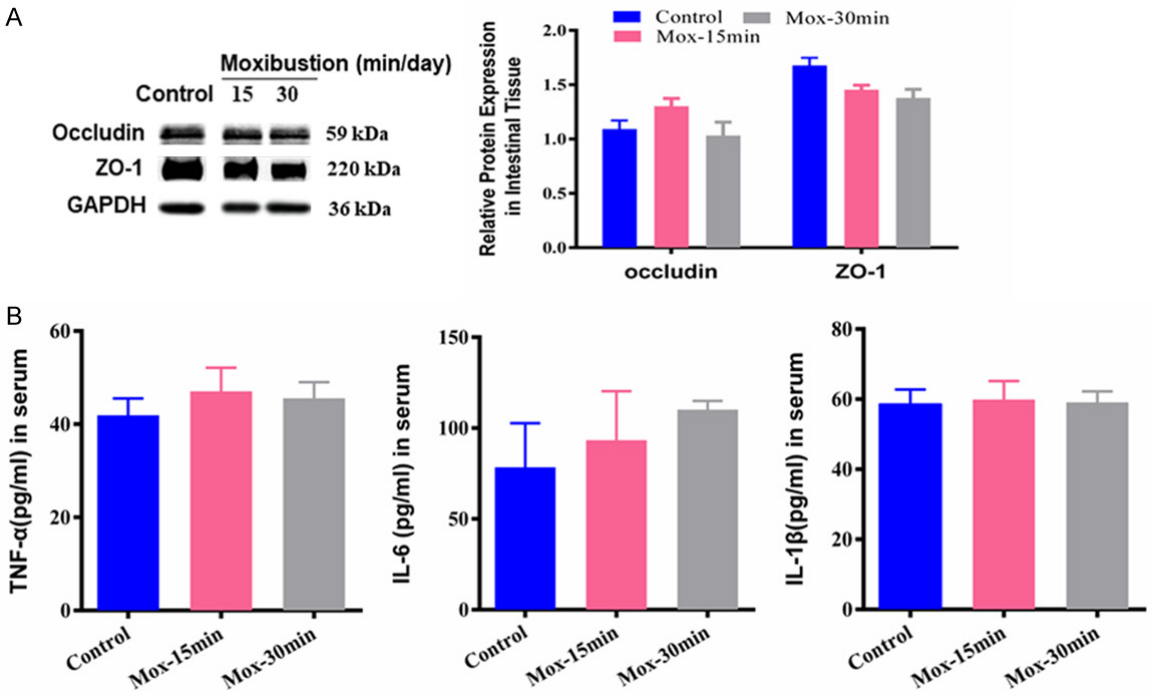
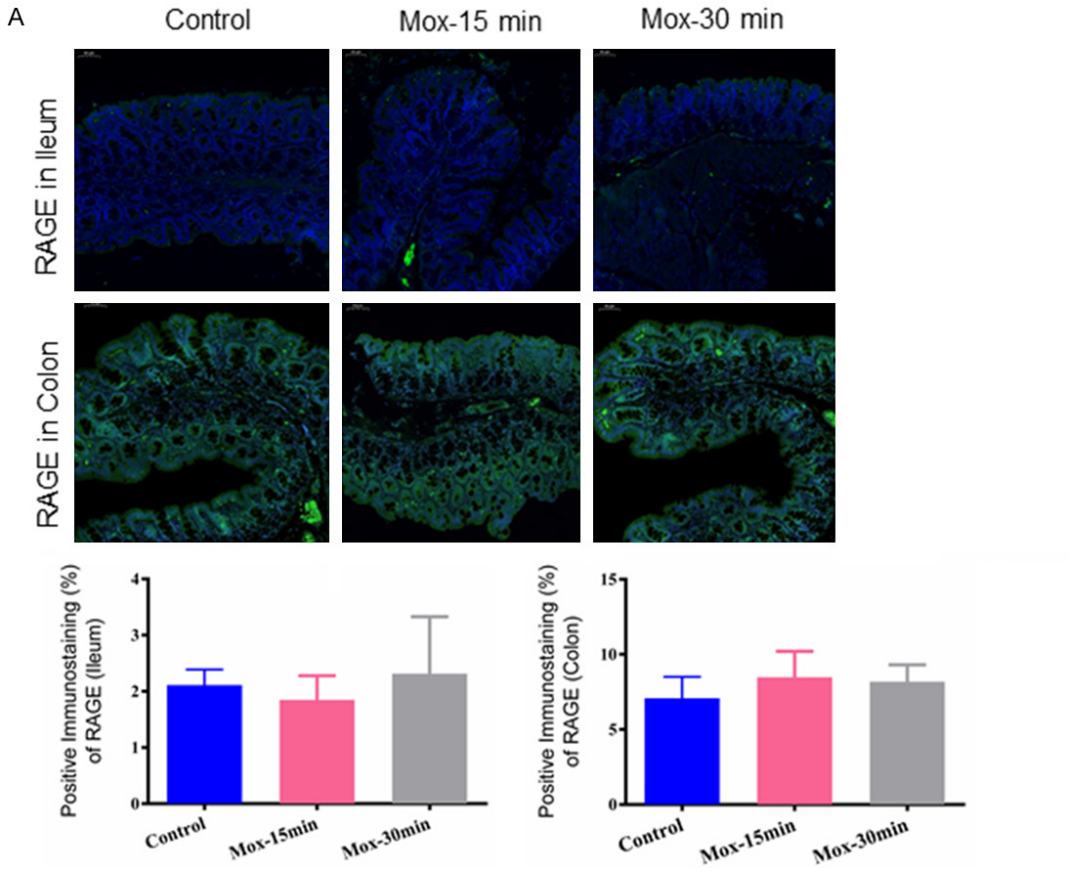


Figure S2. Effect of Moxibustion on tight junction proteins and proinflammatory cytokines *in vivo*. (A) Western blotting analysis of occluding and ZO-1 levels in intestinal tissue after mice treatment with or without Moxibustion. GAPDH is the loading control. The bars represent the means ± SEMs (n = 6 per group). (B) The serum expression of TNF-α, IL-6 and IL-1β was evaluated by ELISA. The bars represent the means ± SEMs (n = 6 per group). A quantitative analysis indicated that there is no significant difference between moxibustion group and Control group.



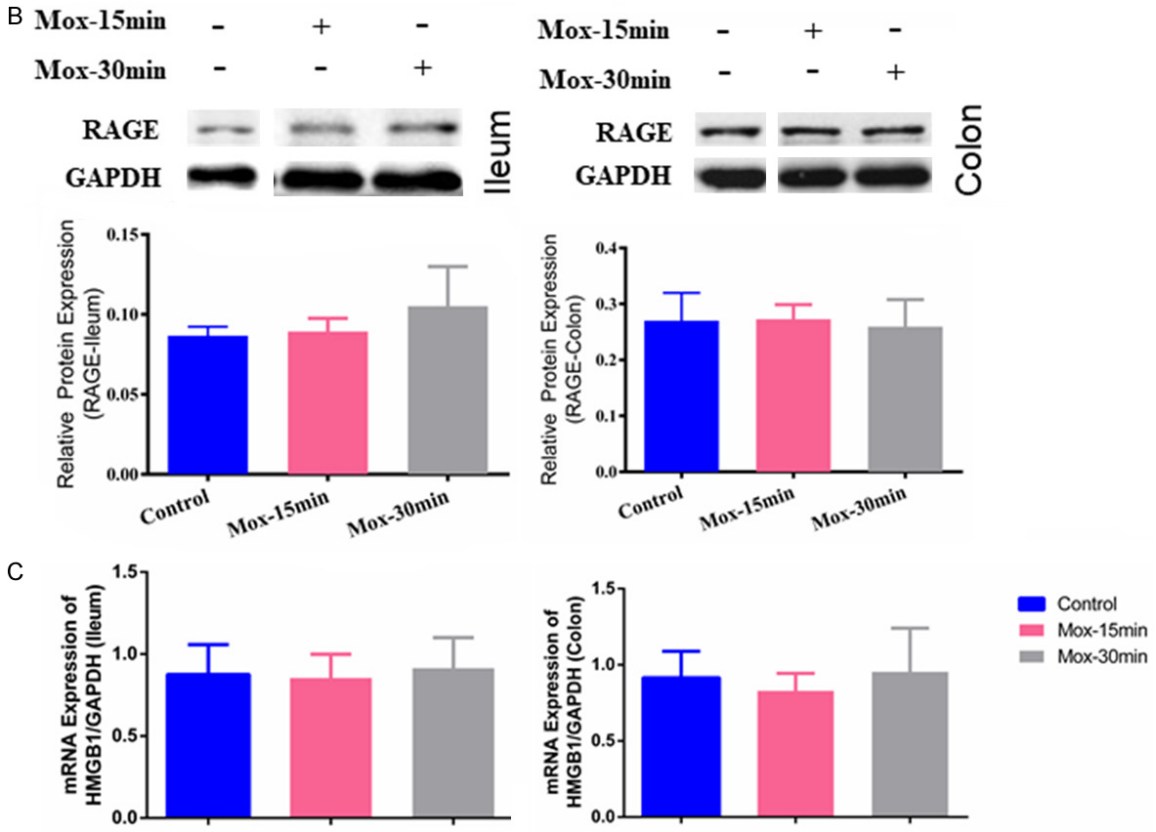


Figure S3. Effect of Moxibustion on the expression of RAGE and HMGB1 *in vivo*. A. Immunofluorescence images showing the expression of RAGE (green) in the ileum and colon. Scale bar: 50 μm. A quantitative analysis indicated that level of expression of RAGE in the ileum and colon. B. Representative Western blot images showing the protein expression of RAGE and GAPDH (loading control). A quantitative analysis indicated that there is no significant difference between moxibustion group and Control group. C. The mRNA expression of HMGB1 in colon was evaluated by qPCR. The bars represent the means ± SEMs (n = 6 per group). A quantitative analysis indicated that there is no significant difference between moxibustion groups and Control group.

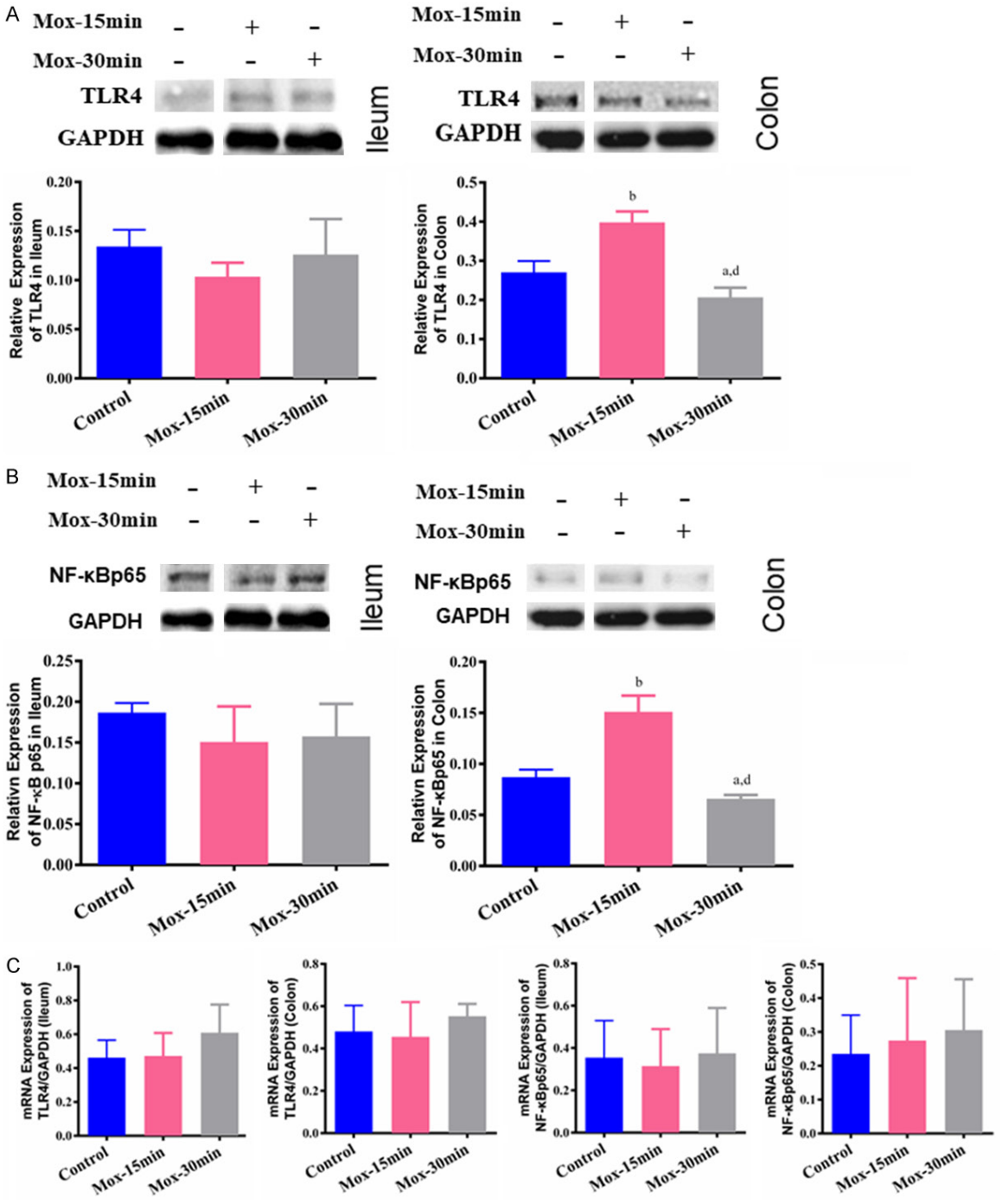


Figure S4. Effect of Moxibustion on the expression of TLR4 and NF-κBp65 *in vivo*. A, B. Western blotting analysis of TLR4 and NF-κBp65 levels in intestinal tissue after mice treatment with or without Moxibustion. GAPDH is the loading control. $^{a}P < 0.05$ and $^{b}P < 0.01$ vs the Control group; $^{d}P < 0.01$ vs the Mox-15 min group. C. The mRNA expression of TLR4 and NF-κBp65 in the ileum and colon was evaluated by qPCR. The bars represent the means ± SEMs (n = 6 per group). A quantitative analysis indicated that there is no significant difference between moxibustion group and Control group.