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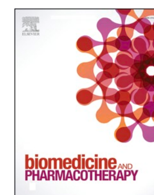

Traditional Chinese Medicine prescription Huang-Qi-Jian-Zhong-Tang ameliorates indomethacin-induced duodenal ulcers in rats by affecting NF-κB and STAT signaling pathways

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A R T I C L E I N F O

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A B S T R A C T

Huang-Qi-Jian-Zhong-Tang (HQJZT) is a well-known traditional Chinese herbal formulation. This study aimed to investigate the duodenoprotective properties of HQJZT against Indomethacin (IND)-induced duodenal ulceration in rats, and the mechanisms involved, particularly through NF-κB and STAT signaling pathways. Our results showed that HQJZT completely protected the duodenal mucosa from ulceration caused by IND, as indicated by improved macroscopic and histological appearances. There was a significant decrease in ulcer index and microscopic score, an increase in villus height and crypt depth, and a normalization of the tissue architecture of the duodenum in rats following HQJZT treatment. Blood flow into the duodenal mucosa was significantly increased after HQJZT administration. HQJZT significantly increased PGE_2 and NO levels in the duodenal mucosa. A significant reduction in the production of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α was observed in the duodenal mucosa under treatment with HQJZT. Mechanistically, the administration of HQJZT significantly lowered the duodenal protein expression of inflammation-related genes, including p-NF-κB and p-IκBβ, compared with the ulcer control group. Furthermore, the STAT signaling pathway-related protein markers p-JAK and p-STAT were significantly reduced in the HQJZT (1.30 and 2.60 g/kg) groups. As a result of these findings, HQJZT alleviates duodenal mucosal ulcers caused by IND. A protective effect of HQJZT on duodenal ulcers is attributed to its ability to improve mucosal blood flow, stimulate the production of cytoprotective mediators, minimize proinflammatory cytokines, and block the activation of NF-κB and STAT signaling pathways.

1. Introduction

Worldwide, duodenal ulcer (DU) is one of the most common digestive conditions. The prevalence of duodenal ulcer peaks between the ages of 45–64 years, and is twice as common in men as in women [1]. The lifetime prevalence of duodenal ulcer disease in the general

Abbreviations: CI, Curative index; ddPCR, droplet digital PCR; DU, Duodenal ulcer; ESO, Esomeprazole; HQJZT, Huang-Qi-Jian-Zhong-Tang; IκB, Inhibitor kappa B; IND, Indomethacin; JAK, Janus kinase; LSCI, Laser speckle contrast imaging; NF-κB, nuclear factor kappa B; NO, Nitric oxide; NSAIDs, Nonsteroidal anti-inflammatory drugs; PGE_2, Prostaglandin E_2; STAT, Signal transducers and activators of transcription; TCM, Traditional Chinese Medicine; UI, Ulcer index.

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population has been estimated at 5–10 %, and the incidence at 0.1–0.3 % per year [2]. Among patients with duodenal ulcer disease, epigastric pain is the most common presenting symptom, which can also include dyspepsia, early satiety, bloating, abdominal fullness, nausea, vomiting, and weight loss [3]. It has been found that smoking, male sex, chronic medical conditions, and poor physiological status have an increased risk of duodenal ulcer disease [4]. It has also been found that duodenal ulcer disease increases with age [3]. Pathologically, duodenal ulcers are the result of a disturbance in the balance of defensive and offensive factors in the gastrointestinal tract. The main aggressive factors are Helicobacter pylori, gastric hydrochloric acid, pepsin, refluxed bile, leukotrienes, reactive free radicals and oxidants, and endothelins. As defensive factors, bicarbonate, gastric mucus barrier, surface active phospholipids, mucosal blood flow, prostaglandins, nitric oxide, as well as enzyme and non-enzyme antioxidant activity are considered [5].

A number of factors can contribute to the development of DU, including Helicobacter pylori infection, nonsteroidal anti-inflammatory drugs (NSAIDs), psychological stress, cigarette smoking, and alcohol consumption [6]. A wide range of medications can be used to treat DU, including antibiotics, histamine-2 receptor antagonists, proton-pump inhibitors, and antacids [7]. However, these drugs are troubled by limited efficacy against gastrohelcosis, high relapse rates, and serious untoward reaction, such as hypoacidity, gynecomastia, impotence, osteoporosis, cardiovascular disease, and hypergastrinemia [8]. Therefore, scientific validation of the efficacy of folkloric medicinal herbs to treat DU may be a way to overcome these drawbacks. It is urgently necessary to discover new drug candidates that provide greater efficacy and lower toxicity for DU prevention and treatment.

A variety of gastrointestinal disorders have been treated with Traditional Chinese Medicine (TCM), which has been systematically developed over thousands of years of continuous refinement and practice [9]. Huang-Qi-Jian-Zhong-Tang (HQJZT) is a famous Chinese herbal formulation first recorded by Zhongjing Zhang, an eminent doctor in Eastern Han dynasty in China, in "Synopsis of Prescriptions of the Golden Chamber (Jingui Yaolüe)" roughly 2000 years ago. HQJZT is formulated with seven herbs, including *Neolitsea cassia* (L.) Kosterm. (family Lauraceae) branch (TCM herb name Guizhi [GZ]), *Astragalus mongholicus* Bunge (family Leguminosae) rhizome (TCM herb name Huangqi [HQ]), *Glycyrrhiza uralensis* Fisch. (family Fabaceae) rhizome (TCM herb name Gancao [GC]), *Cynanchum otophyllum* C.K.Schneid. (family Asclepiadaceae) root (TCM herb name Baishao [BS]), *Zingiber officinale* Roscoe (family Zingerberaceae) rhizome (TCM herb name Shengjiang), *Ziziphus jujuba* Mill. (family Rhamnaceae) fruit (TCM herb name Dazao), and cerealose (TCM herb name Yitang). In terms of TCM theories, HQJZT is most commonly prescribed for the treatment of spleen-stomach vacuity cold syndrome which manifests as chills, fatigue, abdominal distension, abdominal pain, loose stool, nausea, dyspepsia, vomiting, and diarrhea [10]. HQJZT is frequently used in both prevention and treatment of gastrointestinal disorders including gastric ulcer, DU, and chronic atrophic gastritis, and its significant therapeutic effect has been demonstrated in patients [11].

The Janus kinase-signal transducers and activators of transcription (JAK/STAT) pathway plays a critical role in cytokine-mediated inflammatory responses and, as such, has been implicated in the pathogenesis and progression of DU [12]. A high level of inflammatory cytokines and chemokines are produced when the JAK-STAT pathway is activated [13]. Upon cytokine stimulation, the intracellular domains of the relevant receptors recruit JAK to the cell, resulting in phosphorylation of JAK at tyrosine residues and subsequent phosphorylation of STAT. It is important to block JAK or STAT activation to delay the progression of DU since the JAK-STAT pathway plays a major role in the disease [14]. The regulation of DU progression is also greatly influenced by the nuclear factor kappa B (NF-κB) signaling pathway. A multisubunit inhibitor kappa B (IκB) kinase (IKK) complex composed of catalytic (IKKα and IKKβ) and regulatory (IKKγ) subunits activates the NF-κB pathway by phosphorylating IκBα at specific sites. IKK phosphorylates two amino regulatory serine residues on IκB proteins, triggering their ubiquitination and degradation by the proteasome, allowing NF-κB to enter the nucleus and activate stimulus-specific genes [15]. Consequently, the JAK-STAT and NF-κB pathways have emerged as attractive and pivotal therapeutic targets for DU.

In experiments on duodenal ulceration in rats, indomethacin (IND) has been extensively used as a prototypical kind of NSAID [16]. Studies on DU pathophysiology and pharmacology are commonly conducted in rats induced with IND. The pathological characteristics, healing process, and recurrence period of this model closely match those of human peptic ulcers [17]. By using macroscopic and histopathological techniques, HQJZT was examined for its gastroprotective effects in rats suffering from IND-induced duodenal ulcers in the present study. Furthermore, blood flow status, cytoprotective mediators, and inflammatory cytokines were determined. Finally, the mechanism by which HQJZT exerts its efficacy was elucidated by focusing on the STAT and NF-κB signaling pathways.

2. Materials and methods

2.1. Reagents and chemicals

Esomeprazole enteric-coated tablets (Lot. 2110020) were obtained from Jiangxi Shanxiang Pharmaceutical Co., Ltd. (Ganzhou, China). Indomethacin (Lot. H14020771) was purchased from YunPeng Pharmaceutical Group Co., Ltd. (Linfen, China). Pierceable foil heat seal (Lot. 1814040), semi-skirted ddPCR plates (Lot. 64446681), DG8 cartridges for QX200 droplet generator (Lot. 1864008), droplet generation oil for EvaGreen (Lot. 1864006), and droplet generator DG8 gasket (Lot. 1863009) were obtained from Bio-Rad Laboratories, Inc. (California, USA). Eosin solution (Lot. CR2202010), hematoxylin solution (Lot. CR2112051), DAPI solution (Lot. CR2201006), and fluorescent mounting media (Lot. CR2202016) were purchased from Wuhan Sevicebio Biotechnology Co., Ltd. (Wuhan, China). Nitric oxide (NO) ELISA kit (Lot. BC1470) was obtained from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). Rat prostaglandin E_2 (PGE_2) ELISA kit (Lot. ZC-37100) was obtained from Shanghai zcibio technology Co., Ltd. (Shanghai, China). Rabbit anti-p-NF-κB1 (Ser337) antibody (Lot. AD05294512), rabbit anti-p-IκBβ (Ser19) antibody, rabbit anti-JAK1 antibody (Lot. OI10285412), and rabbit anti-STAT1 antibody (Lot. AC04161709) were obtained from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Goat anti-rabbit IgM/Cy3 antibody (Lot. CR2202040) and goat anti-rabbit IgM/FITC antibody (Lot. CR2203121) were purchased from Wuhan Sevicebio Biotechnology Co., Ltd. (Wuhan, China). RNA extraction kit (Lot. A3A2084) and reverse transcription kit (Lot. A3A2044) were purchased from Hunan Accurate Biological Engineering Co., Ltd. (Changsha, China).

2.2. Collection and identification of herbs

All of the herbal materials (HQ, BS, GZ, GC, SJ, DZ, and YT) in HQJZT were obtained from Beijing Tongrentang Co., Ltd. (Changsha, China). All seven herbs were chosen according to the Pharmacopoeia of the People's Republic of China (2020), and authenticated by Dr. Zhi Wang (Hunan University of Chinese Medicine). The authenticated voucher specimens (DT20200530 for HQ, ZS20200426 for BS, BZ20200516 for GZ, BZ20200518 for GC, CS20200512 for SJ, BZ20200501 for DZ, and CS20200526 for YT) were deposited at Hunan Provincial Key Laboratory of Diagnostics Research in Chinese Medicine, Hunan University of Chinese Medicine.

2.3. Preparation of HQJZT extract

With a dry-weight ratio of 6:4:4:4:3:3:3, HQJZT was composed of Huangqi, Baishao, Guizhi, Jiaoyi, Shengjiang, Dazao, and Gancao. HQJZT was prepared as a water extract by using the traditional

decoction method [18]. The air-dried herb samples of HQJZT were separately ground into a very fine powder through an electric grinder. The ingredients were mixed in proportion and macerated in distilled water for 1 h at room temperature, followed by two decoctions for 30 min each (1: 10, W/V). After combining the filtrates, rotary evaporation under reduced pressure was used to concentrate the filtrates to a final concentration of 0.80 g/mL crude drug. A rapid and sensitive LC-MS/MS method was developed for the simultaneous identification of eighteen chemical constituents for quality control of HQJZT (see Supplementary material).

2.4. Test of the acute toxicity of HQJZT

According to the internationally accepted OECD Test Guideline 420 (OECD), HQJZT was tested for acute oral toxicity. Randomly, 12 adult male Sprague-Dawley rats weighing 160–180 g were divided into two groups. HQJZT (26.0 g/kg) was administered to the test group via oral gavage, while 5 % Tween 80 was administered to the control group via oral gavage at 5 mL/kg. Animals were closely observed within the first six hours of dosing, and then every day for 14 days for signs of toxic effects.

2.5. Experimental animals

Male Sprague-Dawley rats (160–180 g) were acquired from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China) and housed in animal cages with a light/dark cycle of 12 h at a temperature of (23 ± 1 °C). The animals were cared for in the animal experimental center of Hunan University of Chinese Medicine. In addition to standard rat chow, rats were provided with unlimited amounts of water. Research Ethics Committee approval was granted (No. LL2021122901) by Hunan University of Chinese Medicine for the study. Protocols involving animals and their care were approved by the Institutional Animal Care and Use Committee of Hunan University of Chinese Medicine and conducted strictly in accordance with NIH guidelines for the Care and Use of Laboratory Animals.

2.6. DU induction and experimental design

Prior to the experiment, 50 rats were allowed to acclimatize for a week. Rats were randomly divided into five groups of ten each. Four of the five animal groups received IND (5 mg/kg) and *Senna alexandrina* Mill. leaf water extract (1 g/kg) for five consecutive days in order to induce duodenal ulcers (TCM syndrome of spleen-stomach vacuity cold), while one group served as normal control group. A 24-hour period after the last treatment with IND, 1 mL distilled water was given to normal control and ulcer control groups, Esomeprazole (ESO) 10 mg/kg was given to the positive control group, and HQJZT extract 1.30 and 2.60 g/kg were given to the two medication groups, respectively. All the treatments were administered orally for three days. 12 h after the last day of treatment, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and sacrificed by decapitation. Samples of blood were collected, centrifuged, and stored at − 20 °C until analysis. Parallel to this, animal duodenums were rapidly removed, opened along the center line. In order to calculate the duodenal ulcer index, duodenal tissue specimens were rinsed gently with physiology saline solution to remove any blood clots. The duodenum was then cut into two equal parts lengthwise, with one part used for histological and immunofluorescent testing and the other for cytoprotective mediators and inflammatory cytokines testing.

2.7. Determination of duodenal ulcer index

With a slight modification, the ulcer index (UI) and curative index (CI) were determined according to Ahmed's method [6]. Duodenums of rats were stretched on a board with their mucosal surfaces facing up and examined macroscopically. Training independent observers who were blinded to the treatment protocol observed and assessed discrete areas of gross mucosal damage under a magnifier. A 0–5 point scale was used to grade macroscopic duodenum lesions based on severity of injury and hemorrhage formation. The scoring system was as follows: 0 indicates no damage, 1 indicates pinpoint erosions, 2 indicates lesions less than 1 mm length, 3 indicates lesions 1–2 mm length, 4 indicates lesions 3–4 mm length, 5 indicates lesions longer than 4 mm. Calculations of UI and CI were performed using the following formulas: UI = Total scores of ulcers in each group/Number of animals in each group. CI (%) = (UI of negative control group − UI of treated group)/UI of negative control group [8].

2.8. Histopathological examination of duodenal mucosa

After treatments, duodenal mucosa samples were preserved in 4 % paraformaldehyde solution for 72 h. Following washing in PBS buffer, they were dehydrated in increasing alcohol concentrations, then embedded in paraffin wax. Hematoxylin and eosin (HE) staining was performed on ultrathin Section (4 μm) prepared using a fully motorized rotary microtome (model RM2016, Leica Inc., Nussloch, Germany). An optical microscope (model BA410E, Motic Group Co., Ltd., Xiamen, China) was used to observe stained stomach slides, which were photographed and then histopathologically analyzed by Motic Images Plus software. Microscopically, duodenal damage was graded on a scale of 0–12, and histological sections were examined for epithelial destruction (0–3), hemorrhagic damage (0–3), inflammatory cell infiltration (0–3), and mucosal edema (0–3) [19].

2.9. Continuous measurement of duodenal mucosal blood flow

In laser speckle contrast imaging (LSCI) system, interference patterns of scattered laser light are blurred by the flow of blood cells, allowing instantaneous visualization of blood perfusion. In brief, an anesthetic of pentobarbital sodium (40 mg/kg) was administered to rats, their heads were fixed in a stereotaxic frame, and they were placed under an RFLSI Pro instrument (RWD Life Technology Co., Ltd, Shenzhen, China). A 784-nm 32-mW laser was used at a 30-deg angle with a beam expander to illuminate the stomach surface. The light intensity was controlled by a polarizer. An LSCI program was used to acquire the images based on the blood flow detected by a CCD camera. A total of 300 frames were acquired at 10 Hz with a time exposure of 10 ms. An assessment of speckle contrast over time was performed by selecting a region of interest (ROI) on the gastric wall at the point where the laser was focused [20].

2.10. Determination of duodenal mucosal nitric oxide (NO) level

Using the Griess reaction [7], nitrite concentration in the duodenum homogenate was measured as an index of NO production. 50 microliters of each sample were mixed with 50 microliters of Griess reagent and incubated at 25 °C for 10 min during which they were protected from light. Nitrite concentration was determined by comparing the sample absorbance to a standard curve for sodium nitrite at 560 nm using a microplate reader.

2.11. Determination of duodenal mucosal prostaglandin E_2 (PGE_2) level

According to the manufacturer's instructions, a PGE_2 assay kit was used to estimate duodenal mucosal PGE_2 production. A 50-microliter sample homogenate, an enzymatic tracer, and the specific antiserum were mixed together in equal volumes. After 1 h of reaction, the mixture was cooled. An absorbance measurement at 412 nm was performed spectrophotometrically.

2.12. Determination of inflammatory cytokine expression levels in duodenal mucosa by droplet digital PCR (ddPCR)

The primers (Table 1) targeted DNA poly region were synthesized following standard procedures by Accurate Biological Engineering Co., Ltd. (Changsha, Hunan, China) before the ddPCR experiment was conducted. As directed by the manufacturer, total RNA was extracted from duodenum tissues using the SteadyPure Universal RNA Extraction Kit (A3A2084, Accurate Biology, Changsha, Hunan, China). In order to estimate the concentrations of total RNA, the absorbance at 260 nm was measured. Total RNA quality measurements were performed using NanoDrop One (Thermo scientific, MA, USA), and only samples with a high quality were used for subsequent analyzes. As instructed by the manufacturer, cDNA was synthesized using the Evo M-MLV RT Premix (A3A2044, Accurate Biology, Changsha, Hunan, China). The synthetic cDNA was quantified after diluting with DNase-free water (1: 10). By using ddPCR, the expression levels of the selected genes, IL-1β, IL-6, and TNF-α, were quantified. β-actin was used as a housekeeping gene. By using the QX200 Droplet Generator, droplets were generated for each sample from a 20 μL reaction system containing ddPCR Supermix, gene specific primers, and cDNA template. After emulsifying the samples, they were transferred to ddPCR plates, where they were amplified using a thermal cycler at 95 °C for 5 min, then 50 cycles at 95 °C for 30 s and 57 °C for 1 min, followed by 90 °C for 5 min. The QX200 Droplet Reader was used to analyze droplets following amplification. QuantaSoft software version 1.7 was used to analyze the data [21].

2.13. Immunofluorescence staining

According to previous reports [8], duodenum slices were examined with immunofluorescence microscopy. After blocking the slices with 5 % BSA in PBS for 30 min, rabbit anti-p-NF-κB (1: 400), rabbit anti-p-IκBβ (1: 400), rabbit anti-p-STAT (1: 400), and rabbit anti-p-JAK (1: 400) primary antibodies were incubated overnight with the slices at 4 °C. Slices were extensively washed with PBS and incubated with Cy3- or FITC-conjugated IgG secondary antibodies for 2 h at room temperature and in the dark after primary antibody incubation. Three PBS rinses were then performed on the slices. The nuclear staining was performed for 10 min using 1 μg/mL DAPI followed by extensive washing in distilled water. The slices were subsequently examined using an inverted fluorescence microscope (model DMi8, Leica, Wetzlar, Germany). With Image-Pro Plus 6.0, each captured picture was analyzed for fluorescence intensity.

2.14. Statistical analysis

All values were expressed as mean ± standard deviation (SD). With SPSS version 22.0, ordinary one-way ANOVA and Dunnett's multiple comparison test were used to compute significant differences between treatment and control groups. A Windows version of Origin Pro 9.0 was used to prepare the analysis and graphs. It was considered significant if the p value was less than 0.05.

Table 1
Primers used in the ddPCR experiment.

Gene	Primer sequence	Product size (bp)
IL-1β	Sense: 5′-AATCTCACAGCAGCATCTCGACAAG-3′	25
	Antisense: 5′-TCCACGGGCAAGACATAGGTAGC-3′	23
IL-6	Sense: 5′-ACTTCCAGCCAGTTGCCTTCTTG-3′	23
	Antisense: 5′-TGGTCTGTTGTGGGTGGTATCCTC-3′	24
TNF-α	Sense: 5′-CACCACGCTCTTCTGTCTACTGAAC-3′	25
	Antisense: 5′-TGGGCTACGGGCTTGTCACTC-3′	21
β-actin	Sense: 5′-GGAGATTACTGCCCTGGCTCCTA-3′	23
	Antisense: 5′-GACTCATCGTACTCCTGCTTGCTG-3′	24

3. Results

3.1. Acute oral toxicity testing of HQJZT

A daily observation of the animals revealed no signs or symptoms of acute toxicities caused by the intragastric gavage of 26.0 g/kg of HQJZT (approximately 60 times human clinical dose). This toxicity study did not result in any changes in animals' behavior. During the first six hours and subsequent hours afterward for two weeks, no mortality was reported. The body weight of rats did not significantly differ between the HQJZT test group and the control group. Based on these findings, HQJZT has a low toxicity profile with a median lethal dose (LD_{50}) of greater than 26 g/kg. In the subsequent experiments, a tenth (2.60 g/kg) and twentieth (1.30 g/kg) of the test dose specified for acute toxicity studies were used to optimize the dose levels.

3.2. Effect of HQJZT on duodenal ulcer index (UI)

Fig. 1 shows the normal appearance of duodenal mucosal epithelium in the normal control group. In rats exposed to IND, there was severe duodenal mucosal congestion and hemorrhage, as well as thinning and ballooning of the duodenal wall with serious ulcerations. In contrast, treatment with HQJZT significantly reduced IND-induced duodenal lesions, moderated congestion, reduced petechial hemorrhage, and decreased UI. As shown in Table 2, oral administration of HQJZT (1.30 and 2.60 g/kg) and ESO (positive control drug) significantly reduced the UI from 30.50 ± 3.83 to 13.67 ± 1.97, 6.17 ± 1.17, and 7.50 ± 1.05, respectively, which corresponds to a curative index (CI) of 55.18 %, 79.78 %, and 75.41 %, respectively.

3.3. Effect of HQJZT on duodenal histopathological changes

In order to confirm the previous macroscopic findings, a histopathology test was conducted. Sections from normal control rats stained with HE showed well-preserved intestinal villi and epithelium, with clear and complete tissue structure and no infiltration of inflammatory cells. Rats of the DU model group, however, suffered hemorrhages, vasodilatation of mucous membranes, infiltration of inflammation cells, as well as villus destruction and crypt abscesses. There was a significant improvement in the histopathological lesions caused by IND after treatment with ESO or HQJZT (1.30 and 2.60 g/kg, respectively). Fig. 2**A** shows that treatment with ESO or HQJZT (1.30 and 2.60 g/kg, respectively) resulted in almost no disruption at the epithelium mucosa, with only minor edema and no hemorrhage, villus destruction, and crypt abscess. Based on Fig. 2**B**, a decreased microscopic score was found when ESO and HQJZT (1.30 and 2.60 g/kg, respectively) were administered, compared to the negative control group, by 82.15 %, 64.20 %, and 79.71 %, respectively. Fig. 2**C and D** illustrate that acute IND exposure significantly reduced villus height and crypt depth in rats from 967.66 ± 25.71 and 546.78 ± 29.09 in the control group to 451.17 ± 65.65 and 191.97 ± 53.03, respectively, in the DU model group. In comparison with the ulcer control group, treatment with 1.30 and 2.60 g/kg HQJZT increased villus height by 63.53 % and 84.74 %, respectively. Additionally, a comparison with the negative control group showed that crypt depth increased by 74.49 % and 135.46 %, respectively, following treatment with 1.30 and 2.60 g/kg HQJZT.

3.4. Effect of HQJZT on duodenal mucosal blood flow

We used a laser-speckle blood flow imaging system to evaluate rats' duodenal mucosal blood flow to determine whether HQJZT affected duodenum microcirculation. The duodenal blood flow of ulcer control rats was significantly reduced by IND when administered orally. Fig. 3 shows that the blood flow in the duodenal mucosa was significantly reduced from 906.67 ± 42.90 in normal control rats to 367.82 ± 39.63 in negative control rats. HQJZT, however, prevented the decrease in

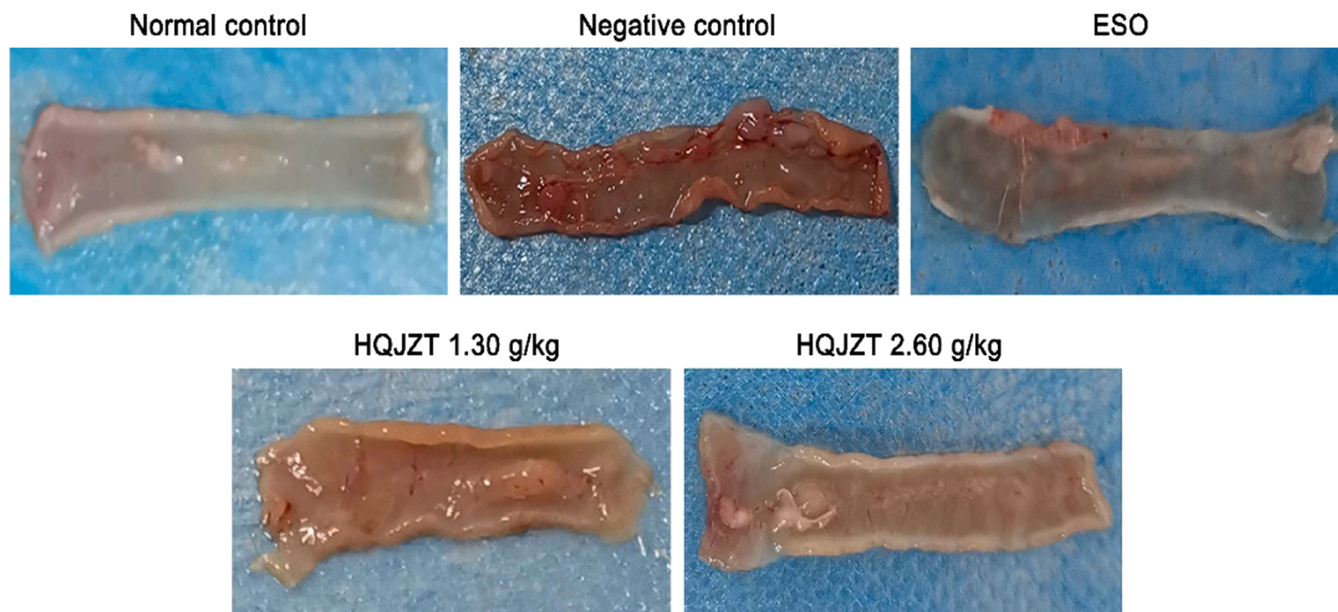

Fig. 1. Effect of different doses of HQJZT on IND-induced duodenal ulcers in rats.

Table 2
Analysis of the therapeutic effect of HQJZT on IND-induced duodenal ulcers in rats using UI and CI.

Groups	Dose	UI	CI (%)
Normal control	–	0.50 ± 0.55	–
IND (Negative control)	10 mg/kg	30.50 ± 3.83**	–
ESO (Positive control)	4 mg/kg	7.50 ± 1.05##	75.41
HQJZT	1.30 g/kg	13.67 ± 1.97##	55.18
HQJZT	2.60 g/kg	6.17 ± 1.17##	79.78

Data are expressed as mean ± SD ($n = 6$).
** $p < 0.01$, compared with normal control.
$p < 0.01$, compared with negative control.

duodenal blood flow caused by IND. Treatment with HQJZT at doses of 1.30 and 2.60 g/kg markedly increased duodenal blood flow by 1.74- and 2.29-fold, respectively, when related to the ulcerative animals. As a result of these data, HQJZT proved to be beneficial to duodenal ulcer models in terms of improving blood flow.

3.5. Effect of HQJZT on duodenal levels of PGE_2 and NO

In the duodenal mucosa, NO and PGE_2 serve as important defensive mediators and maintain the integrity of the mucosa. Fig. 4 shows the effects of HQJZT on PGE_2 and NO levels in the duodenum tissue of IND-challenged rats. As compared to the normal control group (85.87 ± 5.52 and 65.38 ± 5.75, respectively), IND administration significantly reduced PGE_2 and NO concentrations in duodenal tissue (34.23 ± 6.03 and 23.57 ± 3.42, respectively). A significant increase in the duodenal PGE_2 level was observed following HQJZT (1.30 and 2.60 g/kg) treatment after IND administration compared to the negative control group (2.07- and 2.37-fold increase, respectively). The levels of duodenal NO were also significantly increased with HQJZT (1.30 and 2.60 g/kg) treatment after IND administration when compared with the negative control group (2.09- and 2.50-fold increase, respectively).

3.6. Effect of HQJZT on duodenal inflammation

Fig. 5 shows that IND administration to rats evoked a significant increase in IL-1β mRNA level by 160.82 %, IL-6 level by 119.94 %, and TNF-α level by 102.05 %, respectively, as compared to the normal control group. Oral administration of 1.30 g/kg HQJZT after IND elicited a significant decrease in IL-1β mRNA expression by 47.61 %, IL-6 expression by 53.33 %, and TNF-α expression by 55.81 %, respectively, as compared to the ulcer control group. Furthermore, the administration of 2.60 g/kg HQJZT after IND dramatically decreased the expression of IL-1β mRNA by 75.79 %, IL-6 by 79.42 %, and TNF-α by 90.71 %, respectively, compared to the ulcer control group. Based on the results of the present study, HQJZT may have an anti-inflammatory effect on the duodenal mucosa, thereby protecting it from the damaging effects of IND.

3.7. Effect of HQJZT on p-IκBβ and p-NF-κB protein expression and localization

We evaluated the expression of p-IκBβ and p-NF-κB proteins by immunofluorescence in order to gain insights into the mechanisms involved in the healing effects of HQJZT. As shown in Fig. 6, IND administration was associated with increased expression of p-IκBβ and p-NF-κB proteins by 3.7- and 3.9-fold, respectively, as compared to the normal control group. Our study revealed that HQJZT (1.30 g/kg) administration attenuated increased expression of p-IκBβ and p-NF-κB proteins by 3.3- and 3.7-fold, respectively, as compared to the ulcer control group. Also, HQJZT (2.60 g/kg) administration attenuated increased expression of p-IκBβ and p-NF-κB proteins by 3.6- and 3.7-fold, respectively, as compared to the ulcer control group. Our results indicate that p-IκBβ and p-NF-κB proteins are involved in the ulcerogenic effects of IND, and that inhibition of their expression by HQJZT may contribute to the antiulcerogenic action of the drug.

3.8. Effect of HQJZT on p-JAK and p-STAT protein expression and localization

Through immunofluorescence, we further assessed the expression of p-JAK and p-STAT proteins in order to understand the mechanism of HQJZT's healing effect. As shown in Fig. 7, IND-induced ulcer was associated with increased expression of p-JAK and p-STAT proteins by 320.61 % and 316.11 %, respectively, when compared to the normal control group. Administration of HQJZT (1.30 g/kg) significantly decreased the expression of p-JAK and p-STAT proteins by 318.99 % and 187.91 %, respectively, when compared to the ulcer control group. Moreover, administration of HQJZT (2.60 g/kg) significantly decreased

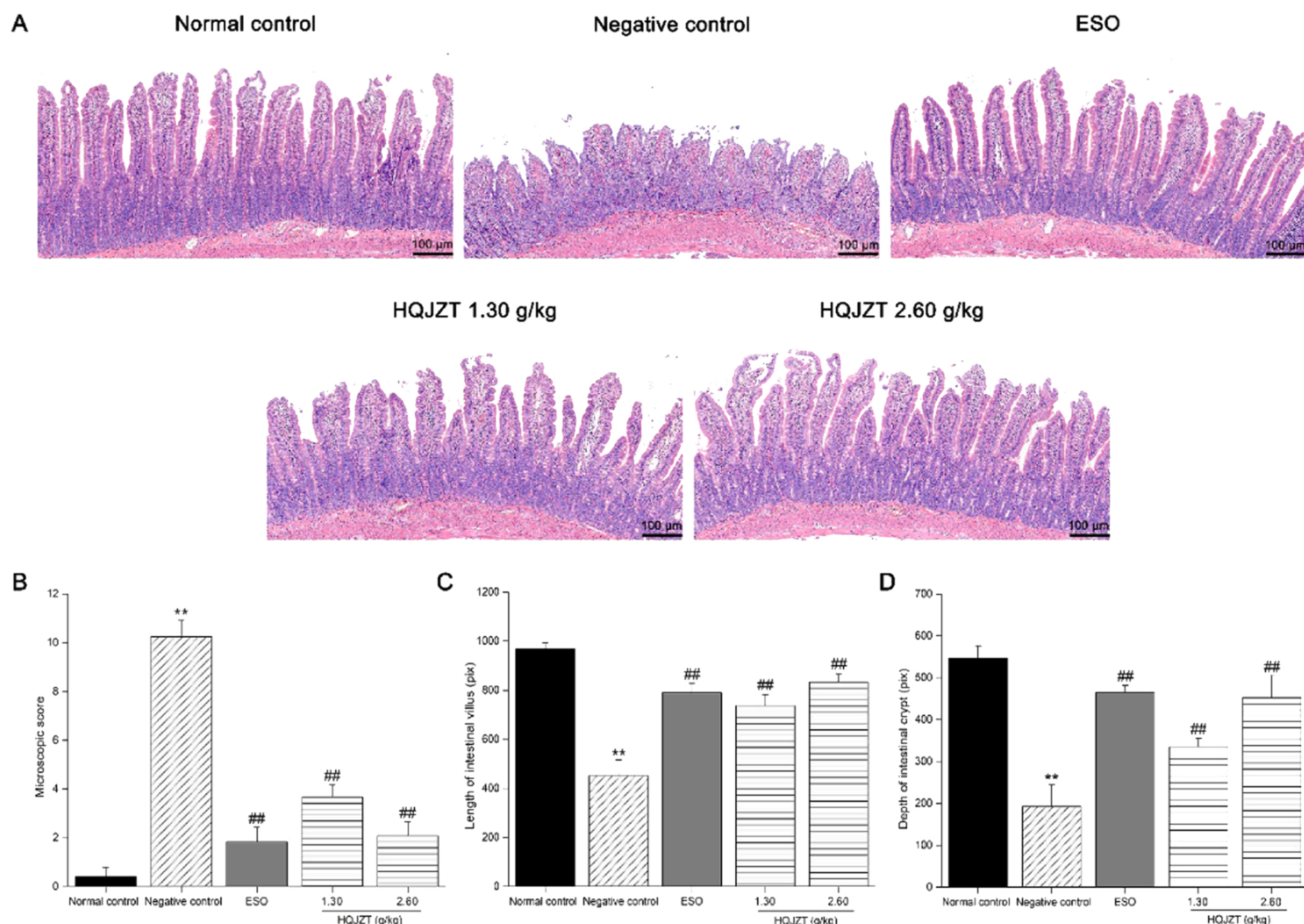

Fig. 2. Effect of HQJZT, in a rat model of DU, on the pathological manifestation of ulceration in duodenum tissue, as observed by HE staining in 150 × magnification. **A.** Representative histological photomicrographs of duodenum sections stained with HE (× 150). **B.** Quantitative analysis of microscopic score of HE staining. **C.** Morphometric analysis of villus height. **D.** Morphometric analysis of crypt depth. Data are expressed as mean ± SD ($n = 6$). $^{**}p < 0.01$, compared with normal control. $^{\#\#}p < 0.01$, compared with negative control.

the expression of p-JAK and p-STAT proteins by 311.75 % and 267.51 %, respectively, when compared to the negative control group. These results indicate that HQJZT may mediate its antiulcerogenic properties by inhibiting the expression of p-JAK and p-STAT proteins.

4. Discussion

The definition of duodenal ulcer disease is a visible deep mucosal break in the duodenum of more than 1 mm in width [22]. Ulcer disease of the duodenum occurs when factors that protect the mucosa of the duodenum are imbalanced with factors that damage it. There may be symptoms such as epigastric or retrosternal pains, bloating, early satiety, belching, nausea, or postprandial distress in patients with duodenal ulcers. The prevalence of duodenal ulcer disease is 1–2 per 1000 people each year in the USA, Europe, and China, according to data from a systematic review [23,24]. In the treatment of duodenal ulcers, Chinese herbal medicine, which has a history of thousands of years, has a definite clinical effect. As a classic TCM prescription, HQJZT was first recorded in synopsis of prescriptions of the golden chamber by Zhongjing Zhang in the late Eastern Han Dynasty. For more than 2000 years, HQJZT has been used in TCM for treating duodenal ulcers associated with syndrome of spleen-stomach vacuity cold [10].

A potent NSAID widely used in clinical practice, IND has a higher ulcerogenic potency than other NSAIDs, making it suitable for the induction of duodenal ulcers in animals. There is a large body of literature related to IND-induced duodenal ulcers and it is well described [25,26]. TCM pharmacological evaluation requires the establishment of whole animal models that reflect certain functional states or certain syndromes. In the present study, the model of spleen-stomach vacuity cold syndrome was established by oral administration of *Senna alexandrina* Mill. leaf water extract. The current study also found that IND induced duodenal ulcers and mucosal damage by increasing the ulcer index, similar to previous studies [19,25,26]. Low ulcer index values showed that HQJZT significantly inhibited IND-induced duodenal injuries, and the effect of HQJZT was comparable to that of ESO.

HQJZT was then tested on histopathological findings to determine whether it could reverse IND-induced duodenal structural changes. Our results showed that sections of the IND group suffered hemorrhages, mucous membrane vasodilation, and inflammation cell infiltration, as well as villus destruction and crypt abscesses. In the HQJZT treatment groups, the duodenal mucosa was significantly improved with well-preserved villi and epithelium, and tissue structure was complete and clear. The findings are consistent with those of a previous study that showed Li-Zhong decoction, a TCM formula can warm the center and fortify the spleen like HQJZT, mitigated IND-induced duodenal histological changes in rats [19]. The published reports indicate that duodenal ulcer disease is heavily influenced by vascular structural changes and microvascular dysfunction [27]. Through Laser Speckle Contrast Imaging, we measured blood flow velocity and blood perfusion area in the duodenal mucosa of rats. As we predicted, DU model rats showed significantly reduced blood flow velocity and blood perfusion area compared with normal control rats. In ulcerative animals,

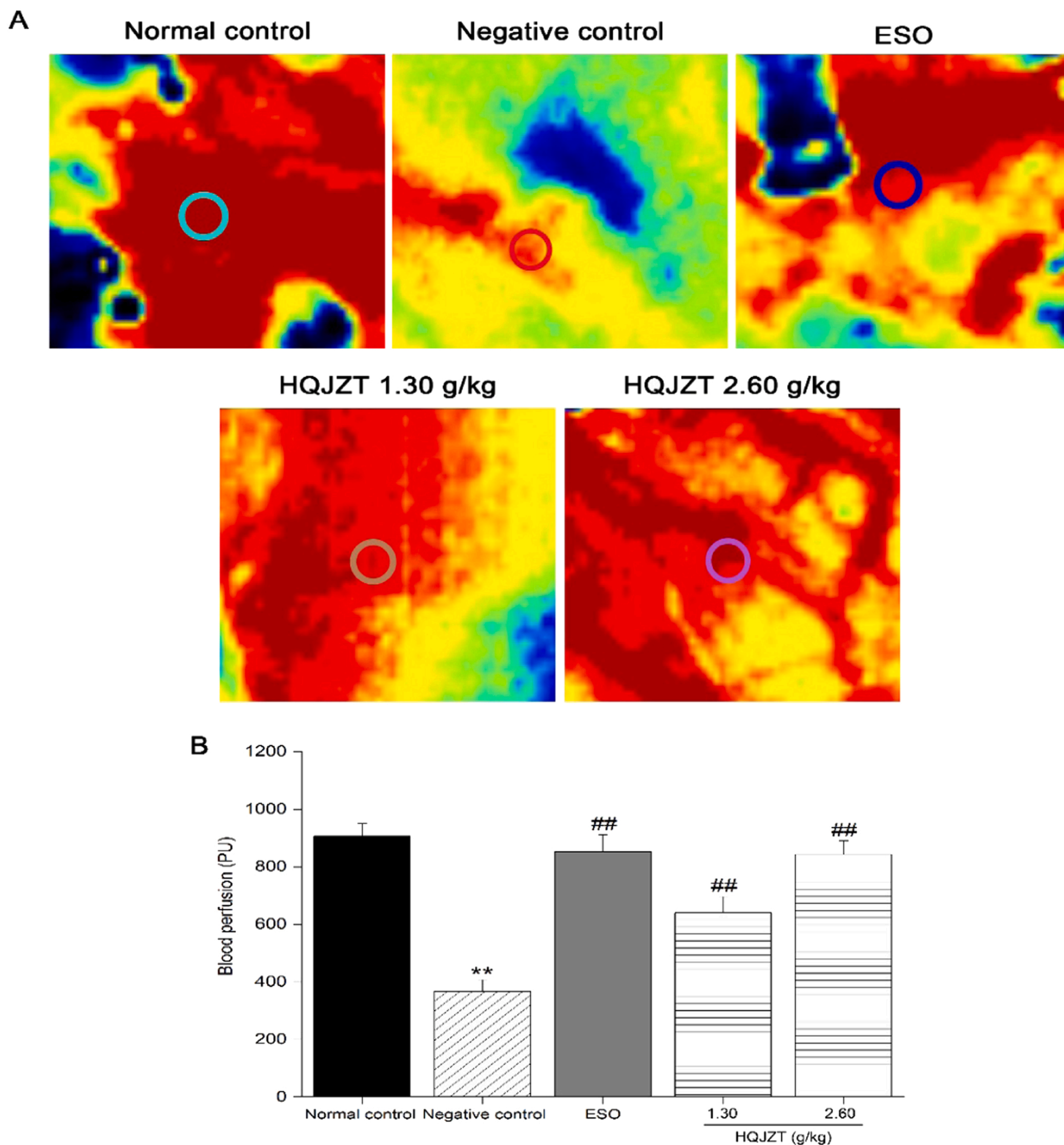

Fig. 3. HQJZT treatment improved duodenal mucosal blood flow. **A.** Representative images of the regional blood flow in the duodenums of rats in different groups. Blue denotes low blood flow, red denotes high blood flow, and yellow or green denotes intermediate blood flow. **B.** Quantitative analysis of blood flow in the duodenal mucosa. Data are expressed as mean ± SD ($n = 6$). $^{**}p < 0.01$, compared with normal control. $^{\#\#}p < 0.01$, compared with negative control.

treatment with ESO or HQJZT at both dose levels (1.30 and 2.60 g/kg) markedly increased blood flow velocity and blood perfusion area. These results suggest that HQJZT induced duodenal protection partly by improving blood flow in the duodenal mucosa.

It has been documented that PGE_2, which acts as an anti-ulcer agent, can inhibit gastric acid secretion, strengthen mucosal barriers, increase blood circulation, and enhance mucus and bicarbonate excretion [28]. It is paradoxical that NO exerts its action on duodenal mucosa. Chemically, NO is produced from L-arginine by either cytotoxic-inducible NO synthase or cytoprotective constitutive NO synthase [29]. One of the aims of this study was to examine whether HQJZT affects PGE_2 and NO contents and its role in duodenal protection. The present study revealed that IND-treated rats exhibited significant decrease in PGE_2 and NO levels when compared to the normal control rats. Treatment with HQJZT markedly increased PGE_2 and NO levels when related to the ulcerative animals. According to the present study, the increased production of PGE_2 and NO by HQJZT may account for the observed protective effects of HQJZT on the duodenum.

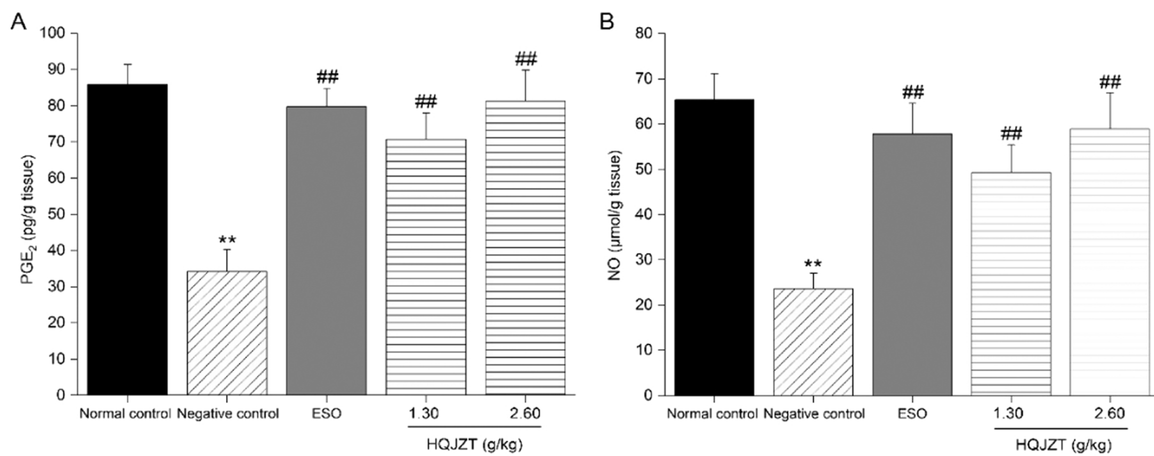

Fig. 4. Effect of HQJZT on duodenal mucosal PGE_2 (**A**) and NO (**B**) contents determined in IND-induced duodenal ulceration model. Data are expressed as mean ± SD ($n = 6$). $^{**}p < 0.01$, compared with normal control. $^{\#\#}p < 0.01$, compared with negative control.

Fig. 5. Effect of HQJZT on IND-induced duodenal inflammation as revealed by ddPCR analysis. **A**. ddPCR analysis of mRNA expression of IL-1β, IL-6, and TNF-α in duodenal mucosa of rats submitted to duodenal ulcer induced by IND. In the case of a positive droplet, the fluorescence amplitude value was greater than the pink line value. **B–D**. Quantitative analysis of mRNA expression in the duodenum tissue of proinflammatory cytokines IL-1β (**B**), IL-6 (**C**), and TNF-α (**D**). Data are expressed as mean ± SD ($n = 3$). $^{**}p < 0.01$, compared with normal control. $^{\#\#}p < 0.01$, compared with negative control.

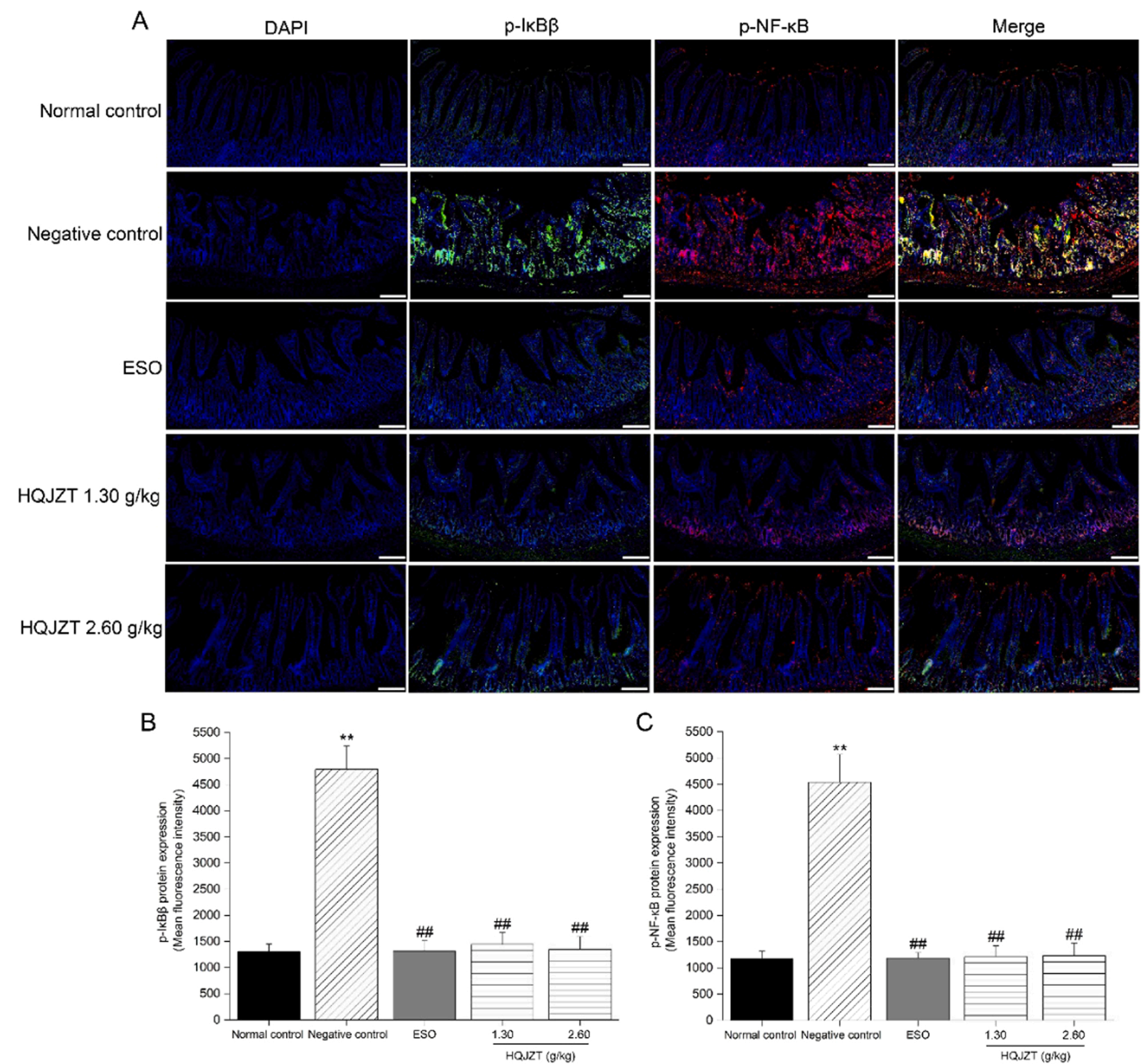

Fig. 6. HQJZT suppressed the expression of phosphorylated IκBβ and NF-κB proteins in duodenal mucosa of IND-induced DU rats. **A.** Immunofluorescence double staining for p-IκBβ (green) and p-NF-κB (red) expression and localization. Sections were counterstained with DAPI (blue). Magnifications × 200, scale bars = 40 μm. **B.** Quantitative analysis of p-IκBβ immunofluorescence. **C.** Quantitative analysis of p-NF-κB immunofluorescence. Data are expressed as mean ± SD ($n = 3$). $^{**}p < 0.01$, compared with normal control. $^{\#\#}p < 0.01$, compared with negative control.

There are numerous inflammatory variables that influence duodenal ulcer induction and healing. In an earlier study, we found that IND administration significantly elevated IL-6 levels, and significantly reduced the levels of IL-4 and IL-10 in duodenal tissues [19]. Proinflammatory cytokines like IL-1β, TNF-α, and IL-6 play a crucial role in ulceration and inflammation in the duodenum [30]. The production of TNF-α results in the production of additional inflammatory factors, like IL-1β and IL-6, which activate neutrophils, obstruct mucosal blood microcirculation, release oxygen-free radicals, and injure the mucosa of the duodenum [31]. In the current study, IND-induced duodenal injury rats also showed significantly elevated levels of the inflammatory factors IL-1β, TNF-α, and IL-6. A significant decrease in inflammatory factors was observed after HQJZT treatment in rats, indicating that HQJZT effectively relieved duodenal inflammation.

The NF-κB pathway has traditionally been considered as a classical pro-inflammatory signaling pathway since it regulates the expression of proinflammatory genes. An inhibitor of p65/p50 heterodimer (IκBs) is phosphorylated and degraded by IKKs in response to stimuli such as TNF-α or LPS. This results in p65/p50 nuclear translocation. Based on our results, HQJZT suppressed activation of NF-κB signaling triggered by IND, decreased phosphorylation of IκBβ, and prevented phosphorylation and nuclear translocation of NF-κB. The results are consistent with the findings of a previous study by Piao et al. who reported overexpression of NF-κB p65 in IND-treated mice. By reducing NF-κB p65 expression, 1-Deoxynojirimycin inhibited inflammation levels in GU mice [32]. The JAK/STAT signaling pathway is primarily regulated by cytokines and plays a crucial role in initiating the innate immune response, controlling the adaptive immune system, and finally restricting inflammatory responses. JAK/STAT pathway interference was found to be effective in preventing ulcers [33]. A target for treating duodenal ulcers is probably

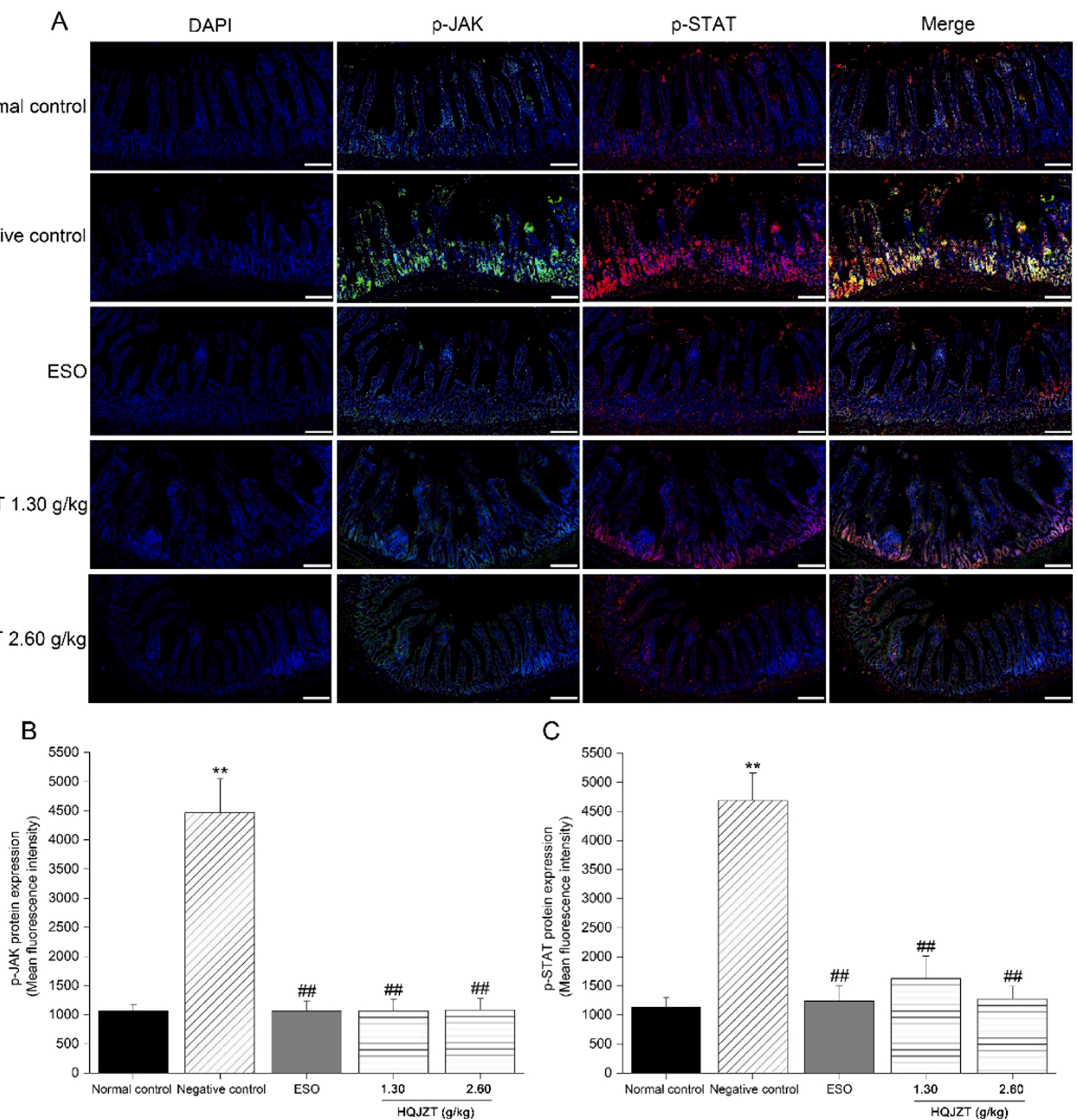

Fig. 7. HQJZT suppressed the expression of phosphorylated JAK and STAT proteins in duodenal mucosa of IND-induced DU rats. **A.** Immunofluorescence double staining for p-JAK (green) and p-STAT (red) expression and localization. Sections were counterstained with DAPI (blue). Magnifications × 200, scale bars = 40 μm. **B.** Quantitative analysis of p-JAK immunofluorescence. **C.** Quantitative analysis of p-STAT immunofluorescence. Data are expressed as mean ± SD ($n = 3$). $^{**}p < 0.01$, compared with normal control. $^{\#\#}p < 0.01$, compared with negative control.

the JAK/STAT signaling pathway. Here we showed that the IND administration is associated with increased p-JAK and p-STAT expression. Liu et al. [33] also reported that the ulcer control group overexpresses p-JAK and p-STAT, which is consistent with the results of this study. In addition, our results showed that HQJZT significantly attenuated increased levels of p-JAK and p-STAT expression. The present study demonstrated that NF-κB and STAT signaling pathways play a crucial role in the ulcerogenic effect of IND, and inhibition of their activation by HQJZT and ESO may mediate their antiulcerogenic effects.

5. Conclusion

In conclusion, this study demonstrated that treatment of rats with HQJZT prevents indomethacin from damaging the duodenal mucosa and accelerates ulcer healing. It is possible to explain at least a part of the protective and therapeutic effects of HQJZT by its ability to maintain PGE_2 production and NO formation, increase blood flow, and exert anti-inflammatory effects. In addition, we demonstrate for the first time that HQJZT exerts duodenoprotective effects by inhibiting the activation of NF-κB and STAT signaling pathways. In the clinical setting, these results support the use of HQJZT as a general TCM prescription for the relief of drug-induced duodenal ulcers.

CRediT authorship contribution statement

Houpan Song, Meiyan Zeng, and Qinghua Peng conceived and

designed the experiments. Houpan Song, Jingyue Qiu, Chang Yu, Meng Xiong, and Chen Ou conducted the experiments. Baoping Ren and Meiqi Zhong analyzed the data. Houpan Song drafted the manuscript. Qinghua Peng and Meiyan Zeng revised and edited the final version of the manuscript. The final version of the manuscript was read and approved by all authors.

Declaration of Competing Interest

The authors declare that there are no potential conflicts of interest regarding the research, authorship, and/or publication of this paper.

Data Availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113866.

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