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ORIGINAL RESEARCH

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Qingre Jiangni formula improves esophageal epithelial barrier function in gastroesophageal reflux rats through male hormones and the p38/MAPK pathway

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Abstract

The incidence rate of gastroesophageal reflux is high. Qingre Jiangni Formula has shown good clinical efficacy. In this study, the therapeutic effect of this formula on inflammation was analyzed in the gastroesophageal reflux model. A rat model of gastroesophageal reflux was constructed using surgical methods. The successful construction of the rat model was confirmed through case testing. The model rats were treated with Qingre herbs, Jiangni herbs, and Qingre + Jiangni herbs for 2 weeks, followed by hematoxylin-eosin staining (HE), Western Blot (WB), enzyme linked immunosorbent assay (Elisa) and polymerase chain reaction (PCR) assays. SPSS 22.0 statistical software was used for analysis. The modeling of gastroesophageal reflux reduced the levels of testosterone and androsterol. The Qingre Jiangni formula helped to improve testosterone and androsterol to normal levels. Treatments in the Qingre group, Jiangni group, and Qingre + Jiangni group can all decrease inflammatory factor levels (interleukin IL-1B, IL-6, IL-8), and lead to a decreasing trend in p38, Claudin-4, Occludin and ZO_S. In addition, the three groups also showed decreased inflammation pathological levels compared with those in the model group ($p < 0.01$). Qingre Jiangni Formula is helpful for male hormones (testosterone and androsterol) and can decrease gastroesophageal reflux disease inflammation, thereby treating gastroesophageal reflux disease.

Keywords

Qingre Jiangni formula; Esophageal epithelial barrier; Gastroesophageal reflux disease; Testosterone; Androsterol

1. Introduction

Gastroesophageal reflux disease (GERD) is a condition that arises due to the backflow of stomach contents, manifesting uncomfortable symptoms and complications, including burning pain in the upper abdomen, acid regurgitation, burning sensation in the chest, difficulty in swallowing, hoarse voice or coughing, and asthma [1]. Epidemiological surveys showed that the overall prevalence rate of GERD was 4.6%, with an increasing trend annually, which adversely impacts the health and quality of life of patients. Proton pump inhibitors (PPIs) are commonly used to treat GERD but fail to provide relief in 10–40% of the patients. Furthermore, the long-term use of PPIs can result in dependence and increase the risk of other conditions such as atrophic gastritis, gastric polyps, and even cancer. Therefore, finding effective ways to alleviate symptoms while minimizing the use of PPIs is currently a major focus in clinical practice [2].

Male hormones are associated with GERD. A previous study has suggested that men are more prone to GERD than women, typically because of their higher levels of testosterone [3]. However, other factors such as diet, weight, and lifestyle can also influence gender-based differences [4]. Fluctuations in male hormone levels may affect the function of the lower esophageal sphincter, a muscle located between the esophagus and the stomach that helps prevent the backflow of stomach contents into the esophagus, thereby affecting the incidence of GERD and the severity of symptoms. Male hormones and weight control share a complex relationship. Obesity, one of the risk factors for GERD, can also affect the metabolism and secretion of male hormones. Obesity may play an intermediary role in the relationship between male hormones and GERD.

The mitogen-activated protein kinase (MAPK) pathway is a well-established signaling pathway that is involved in regulating gene expression, cell proliferation, and apoptosis in the human body [5]. The main branches of this pathway include extracellular signal-regulated kinase, Jun N-terminal kinase, p38/MAPK, and extracellular signal-regulated kinase 5. p38/MAPK is an important signaling molecule within cells that crucially regulates several processes, such as inflammation, cell growth, and apoptosis [6]. It can be activated by various external stimuli, such as Toll-like receptors, tumor necrosis

factor-α, lipopolysaccharides, and hypoxia. p38/MAPK was found to be highly expressed in the tissues of patients with GERD.

Pancreatic enzymes and acid can activate the p38/MAPK pathway, resulting in inflammatory damage to esophageal mucosal epithelial cells and changes in mucosal structure, which compromise the function of the esophageal epithelial barrier [7]. The activation of p38/MAPK upregulates interleukin-1β (IL-1β), IL-6, IL-8 and other inflammation-inducing factors, while downregulating various structural proteins, such as claudins, occludins and Zonula Occludens proteins (ZO_S), which disrupts the mucosal tissue structure [8]. The abnormal expression and distribution of tight junction proteins in the esophageal mucosa of patients with GERD can cause dilated intercellular space between epithelial cells, which, as a morphological feature of impaired mucosal integrity, promotes the penetration of gastric acid into the submucosal layer [9]. Patients with GERD gradually become more sensitive to the acid as it infiltrates the submucosal layer. Additionally, the damage to esophageal epithelial cells is closely related to the inflammatory response. Therefore, the activation of the p38/MAPK signaling pathway is a prominent event responsible for the damage to the esophageal epithelial barrier, which plays a crucial role in the pathogenesis of GERD [10].

The pathogenesis of GERD primarily involves a decrease in esophageal antireflux defense mechanisms and an increase in the erosive effect of refluxed material on the esophageal mucosa. A decrease in defense mechanisms, involving a weakened antireflux barrier, decreased esophageal clearance capacity, decreased tissue resistance, and delayed gastric emptying, is a prerequisite for esophageal epithelial damage. The esophageal surface mucus, non-movable water layer, surface HCO_3^-, stratified squamous epithelium, and abundant blood supply in the submucosa together form a barrier against mucosal damage, resisting the harmful effects of gastric acid, bile salts, and other chemicals [11]. The weakening of the barrier function leads to a decline in esophageal defense mechanisms, which subsequently triggers GERD onset [12, 13].

The approach of clearing heat and mitigating adverse reactions is a traditional Chinese medicine treatment technique extensively employed in the clinical management of gastroesophageal reflux [14]. This method aims to alleviate symptoms associated with gastroesophageal reflux and improve digestive problems by rebalancing the body's heat and restoring normal digestive function. Gastroesophageal reflux often triggers inflammatory reactions that damage the mucosal lining. Herbs used in heat-clearing remedies possess anti-inflammatory, antioxidant, and antibacterial properties, which help reduce inflammation and support tissue repair. Additionally, gastroesophageal reflux involves the backflow of gastric acid and stomach contents into the esophagus, causing discomfort and injury. The heat-clearing and inversion-reducing method suppresses gastric acid secretion, enhances esophageal sphincter function, and reduces the likelihood of acid reflux by enhancing the esophagus' resistance to acid. Moreover, gastrointestinal dysfunction is commonly associated with gastroesophageal reflux. The heat-clearing and inversion-reducing method regulates gastrointestinal peristalsis and digestive fluid secretion, thus enhancing digestive function and minimizing the incidence rate of reflux. Furthermore, certain herbs utilized in this approach have protective effects on the mucosa. They strengthen the integrity of the gastroesophageal mucosal barrier, reduce mucus production, and enhance the mucosa's ability to withstand damage from external factors [15].

Numerous traditional Chinese medicine ingredients possess antioxidant properties, which can inhibit the generation of free radicals and oxidative stress, both of which contribute crucially to inflammation. Some herbs in the Qingre Jiangni Formula exert anti-inflammatory effects [16]. Berberine, the active ingredient in Huanglian, can inhibit the production of inflammatory mediators, such as IL-1β, tumor necrosis factor-α and IL-6, thereby alleviating inflammation [17]. The components in Huanglian can also inhibit the activation and migration of inflammatory cells, such as macrophages and neutrophils, which release inflammatory mediators during inflammation [18, 19]. Baicalin, the active ingredient in *Scutellaria baicalensis*, possesses antioxidant and anti-inflammatory properties. It can suppress the production of inflammatory factors and regulate inflammatory signaling pathways [20, 21].

2. Methods

2.1 Animals

Sixty male Wistar rats, weighing 250 $\pm$ 50 g each and aged 6–7 weeks, were housed in separate cages at room temperature (20 $\pm$ 5 °C) and at a humidity of 50 $\pm$ 10%. They were exposed to 12 h of light per day and provided with sufficient food and water. After 1 week of adaptive feeding, models were established. The rats were randomly categorized using a computer-generated random sequence to ensure unbiased allocation to different groups, thus minimizing potential confounding factors.

2.2 Groups

In the sham surgery group, rats were fasted for 24 h before the operation but had access to water. Anesthesia was induced by injecting pentobarbital intraperitoneally at a dose of 50 mg/kg. A longitudinal incision was made along the midline of the abdomen, and the hepatoduodenal ligament and fascia around the stomach were dissected. The stomach was lifted out, and the left gastric artery was ligated using a 4-0 nylon wire. The stomach was then placed back into the abdominal cavity. Antibiotics and physiological saline were administered to moisten the abdominal cavity and prevent adhesion. Finally, the stomach was closed. After the surgery, the rats in the sham surgery group were individually housed in cages for further observation. They were fasted for 24 h without restriction on water intake. A small amount of food was provided to them 1 day after the surgery. If they exhibited no signs of abdominal bleeding, mental fatigue, or other symptoms within 2–3 days, they were combined into five cages and provided a normal diet.

In the reflux group, rats were fasted for 24 h prior to the surgery without water. Pentobarbital was administered intraperitoneally to induce abdominal anesthesia. A longitudinal incision was made along the midline of the abdomen, and the hepatoduodenal ligament and fascia around the stomach were cut. The stomach was lifted out, exposing the stomach

and pylorus. The pylorus was partially ligated with caution using a 4-0 nylon thread to avoid blood vessels. The left gastric artery was also ligated to prevent bleeding from vessel damage. Starting from the junction of the esophagus and the stomach, the esophagus was carefully separated upwards for approximately 1 cm, ensuring no damage to the esophageal intima. Antibiotics and physiological saline were administered to moisten the abdominal cavity and prevent adhesion. The abdomen was closed once the procedure was completed. Following the surgery, the rats in the reflux group were housed individually in cages and were fasted for 24 h without access to water. A small amount of food was provided to them 1 day after the surgery. If they showed no signs of abdominal bleeding, mental fatigue, or other symptoms within 2–3 days, they were combined into five cages and provided a normal diet [22].

After 21 days of modeling, the rats were euthanized through spinal dislocation, and the throat area was surgically opened. The tissue near the esophagus was stripped until the esophagus was removed. The lower end of the esophageal lumen was gently rinsed with physiological saline. Its overall appearance was assessed and scored before it was fixed in a formaldehyde solution and pathologically examined using hematoxylin and eosin (HE) staining. The remaining 40 rats were successfully modeled as per the method described above. They were randomly divided into four groups: heat-clearing group, anti-inversion group, heat-clearing and anti-inversion group, and model group, with 10 rats in each group. The dosage was determined based on the specific requirements of the study. Rats in the model group received physiological saline twice a day at a rate of 1.5 mL/day for 14 consecutive days.

2.3 Drug composition

The drug formulation provided in this study derives from the experience gained from the long-term treatment of patients in the Digestive Endoscopy Department of Jiangsu Second Traditional Chinese Medicine Hospital. The specific prescription composition was as follows.

The formulation for clearing heat and reducing adverse reactions consisted of 30 g of raw gypsum, 9 g of *Anemarrhena asphodeloides*, 9 g of vinegar bupleurum, 12 g of *S. baicalensis*, 9 g of *Pinellia ternata*, 6 g of *Coptis chinensis*, 3 g of *Tetradium ruticarpum*, 12 g of convoluted flower (decocted), 30 g of ocher substitute, 9 g of loquat leaf and 6 g of dried ginger.

The Qingre formulation consisted of 30 g of raw gypsum, 9 g of *A. asphodeloides*, 6 g of *C. chinensis*, 12 g of *S. baicalensis* and 9 g of Chaihu.

The prescription for reducing adverse reactions included 12 g of *Cynanchum paniculatum* (decocted), 30 g of ochre substitute, 9 g of loquat leaves, 9 g of *P. ternata*, 3 g of *T. ruticarpum* and 6 g of dried ginger.

Traditional Chinese medicine was provided by the Chinese Pharmacy of the Second Traditional Chinese Medicine Hospital of Jiangsu Province.

2.4 Western blotting

Proteins were first extracted from the cells using a radioimmunoprecipitation assay buffer supplemented with protease inhibitors. Their concentrations were determined using the Bradford assay, and they were separated based on their molecular weights on 10% gels using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were subsequently transferred onto a polyvinylidene difluoride membrane, which was blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline containing Tween 20 to prevent non-specific binding, probed overnight at 4 °C with primary antibodies specific to the target protein, washed and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies. The protein bands were visualized using enhanced chemiluminescence captured on a specialized imaging system and analyzed using Image Lab to quantify protein expression and any post-translational modifications.

2.5 Reverse transcription-PCR (RT-PCR)

A DNA template containing the specific target sequence was prepared and combined with primers designed to match the regions flanking the target sequence. The mixture was initially subjected to denaturation at a high temperature to melt the double-stranded DNA into single strands, followed by cooling to allow the primers to anneal to their respective complementary sequences on the template DNA. After annealing, the temperature was increased to activate the DNA polymerase, which subsequently extended the primers, eventually synthesizing new complementary DNA strands using the provided nucleotides. This cycle of denaturation, annealing, and extension was repeated 30 times to ensure optimal amplification of the target DNA sequence. Once the PCR was completed, the amplified products were analyzed through gel electrophoresis to determine the successful amplification and size of the resulting DNA fragments (Table 1).

TABLE 1. Primer Sequences.

Primer Name	Primer Sequence
IL-1β-F	TTGAGTCTGCACAGTTCCCC
IL-1β-R	TCCTGGGGAAGGCATTAGGA
IL-6-F	CACTTCACAAGTCGGAGGCT
IL-6-R	TCTGACAGTGCATCATCGCT
IL-8-F	CAAAATGATGAACCCCAGCTC
IL-8-R	CATCCTACCATAGCCATTGCAG

IL: interleukin.

2.6 HE staining

HE staining was performed by two researchers. The tissue sample was fixed in formalin to ensure its structural integrity, successively dehydrated using alcohol washes, embedded in paraffin wax for stability during sectioning, sliced into thin sections using a microtome, carefully placed onto a glass slide, deparaffinized with xylene, rehydrated using graded alcohols,

stained with hematoxylin, which imparted a blue color to the cell nuclei, stained with eosin, which imparted a pink color to the cytoplasm and other extracellular components, dehydrated using graded alcohols, cleared with xylene, permanently secured with a coverslip using a mounting medium, and finally examined critically under a microscope by a distinguished veterinary pathologist who evaluated the slides based on specific histological parameters relevant to this study's objectives.

2.7 Enzyme-linked immunosorbent assay

The specific antigen of interest was immobilized onto the surface of microplate wells to provide a foundation for the subsequent binding of the analyte from the sample or the standard. The wells were blocked to prevent non-specific interactions, followed by the addition of the sample or the standard, enabling the analyte to bind to the immobilized antigen. The wells were washed to remove any non-specifically bound components. A tailored primary antibody, specific to the analyte, was added, and the wells were washed to clear any unbound primary antibodies. Next, an enzyme-conjugated secondary antibody was added, and the wells were washed to remove any unbound secondary antibody. Finally, a substrate solution was added, instigating a reaction with the enzyme on the secondary antibody, which manifested as a detectable signal, in our case, a discernible color change. The intensity of this signal was quantified using a spectrophotometer. By juxtaposing these intensity readings with a meticulously prepared standard curve, the concentration of the analyte in our samples was ascertained. The reagents, including IL-1β (SEKR-0002, CYGNUS, NY, US), IL-6 (SEKR-0006, CYGNUS, NY, US) and IL-8 (SEKR-0071, CYGNUS, NY, US), were provided by Solebao Company.

2.8 Statistical processing

Quantitative research on male hormone indicators (testosterone and androsterol) and inflammation indicators (IL-1β, IL-6 and IL-8) was conducted. SPSS 22.0 (IBM, Armonk, NY, USA) was used for analysis, and the data were expressed as mean $\pm$ standard deviation $\bar{x} \pm s$). The *t*-test was used for comparisons between two groups, and one-way analysis of variance was used for comparisons among multiple groups. Any difference was statistically significant when $p < 0.05$.

3. Results

3.1 Construction of a gastroesophageal reflux model

After modeling, the reflux group was observed to display a certain inflammatory reaction compared with the sham group, indicating the successful construction of a gastroesophageal reflux model (Fig. 1).

3.2 Qingre Jiangni Formula helps restore male hormone levels in the gastroesophageal reflux model

The testosterone levels of male rats were affected in the gastroesophageal reflux model group. Therefore, a decrease in testosterone levels may induce gastroesophageal reflux. The testosterone levels in the Qingre + Jiangni group were significantly higher than those in the model group ($p < 0.01$). The androsterol levels were also higher in the Qingre, Jiangni and Qingre + Jiangni groups compared with those in the model group ($p < 0.01$; Fig. 2).

The levels of inflammatory factors (IL-1β, IL-6, IL-8) were lower in the Qingre, Jiangni and Qingre + Jiangni groups compared with those in the model group ($p < 0.01$), indicating that the Qingre Jiangni method of traditional Chinese medicine can successfully alleviate inflammation (Fig. 3).

3.3 RT-PCR

The mRNA expression of inflammatory factors was detected through RT-PCR. Treatment using the Qingre Jiangni method resulted in a decrease in the expression of IL-1β, IL-6 and IL-8 compared with that in the model group ($p < 0.01$; Fig. 4).

3.4 Qingre Jiangni method ameliorates inflammation

HE staining of samples in the Qingre, Jiangni and Qingre + Jiangni groups revealed a decrease in inflammation (Fig. 5).

3.5 Qingre Jiangni method enhances the protective effect of the esophageal mucosal barrier

Western blotting results indicated that the expression of p38, claudin-4, occludin and ZO_S all exhibited a decreasing trend in the Qingre, Jiangni and Qingre + Jiangni groups compared with that in the model group ($p < 0.01$; Fig. 6).

4. Discussion

In this study, the Qingre Jiangni method can mitigate inflammation and treat GERD. A series of pathological inflammatory changes in the constructed gastroesophageal reflux model was observed using surgical methods. However, treatment with Qingre, Jiangni, and Qingre + Jiangni lowered the levels of inflammatory factors (IL-1β, IL-6, IL-8), reduced the expression of p38, claudin-4, occludin and ZO_S, and significantly alleviated pathological inflammation compared with those in the model group.

GERD occurs when stomach contents flow back into the esophagus due to attenuated esophageal clearance and antireflux barrier mechanisms. The resulting mucosal injury is a result of both direct damage from gastric juice and an immune and inflammatory response, wherein inflammatory cytokines released by the esophageal mucosal epithelium induce neutrophil migration, leading to inflammation [23]. The onset and progression of GERD are multifaceted. Various factors contribute to its pathogenesis, such as heightened sensitivity in the esophagus, inflammation of the esophageal mucosa,

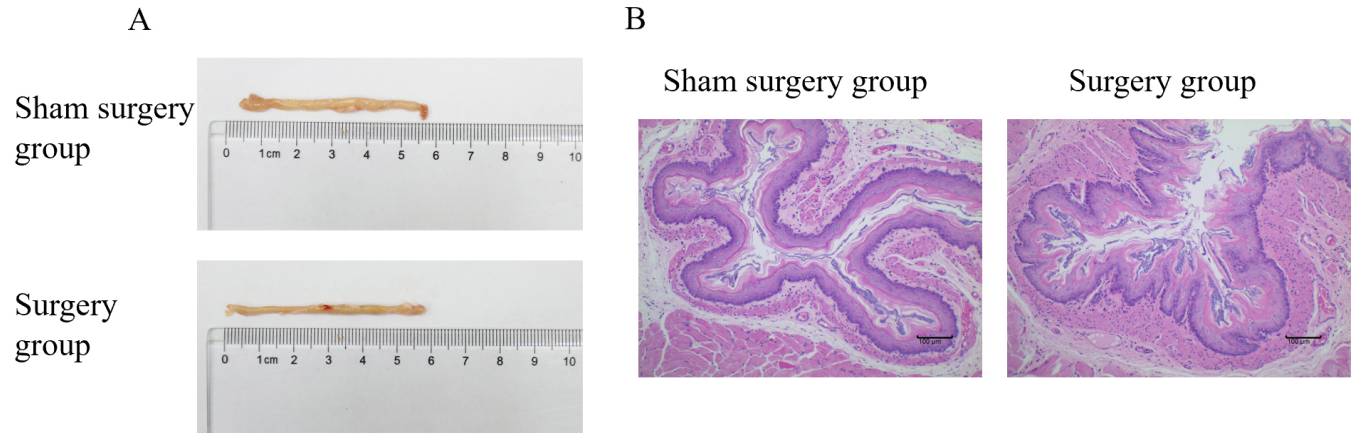

FIGURE 1. HE staining of the model. HE: hematoxylin and eosin. Scales: 100 μm.

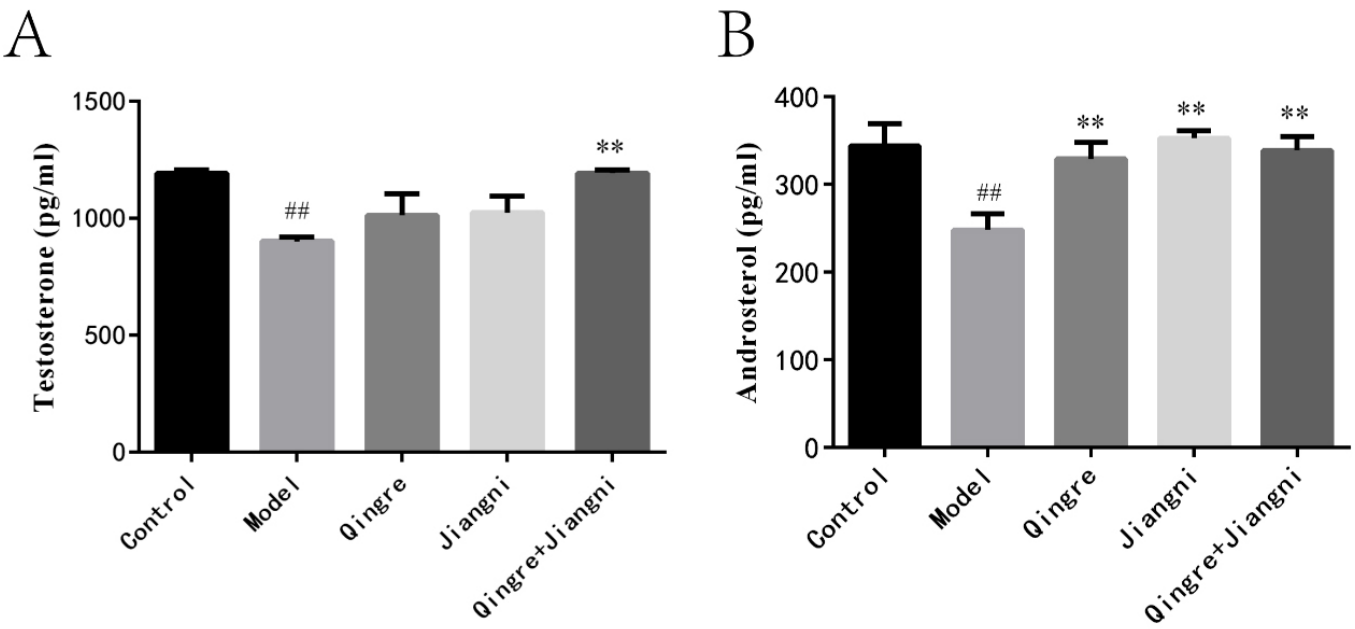

FIGURE 2. Male hormone levels in the gastroesophageal reflux model. ##compared with the control group $p < 0.01$, (A) Testosterone (B) Androsterol. **compared with the model group $p < 0.01$.

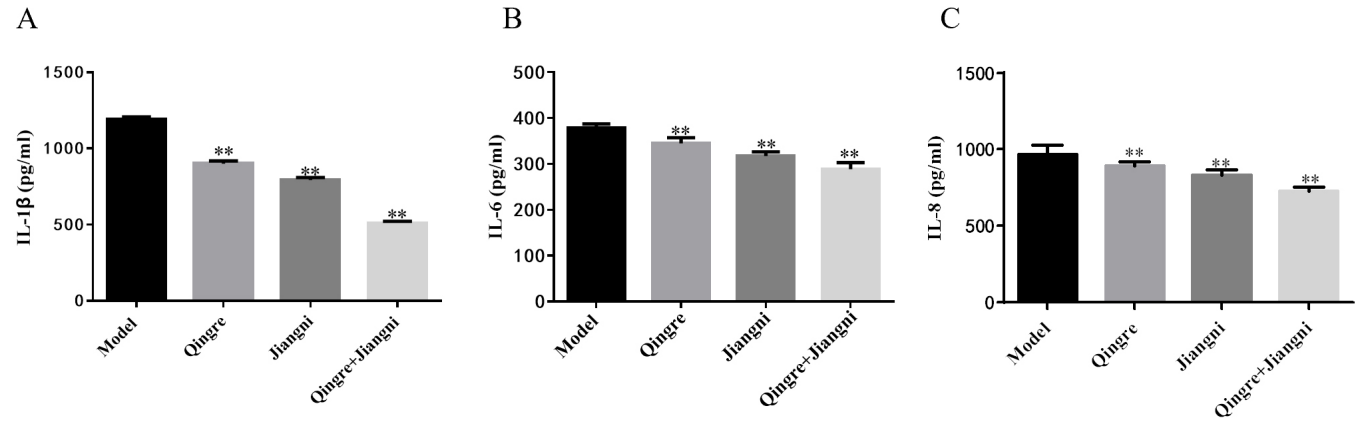

FIGURE 3. Inflammatory factor levels after treatment using the Qingre Jiangni method (A) IL-1β (B) IL-6 (C) IL-8. **$p < 0.01$ compared with the model group. IL-1β: interleukin-1β.

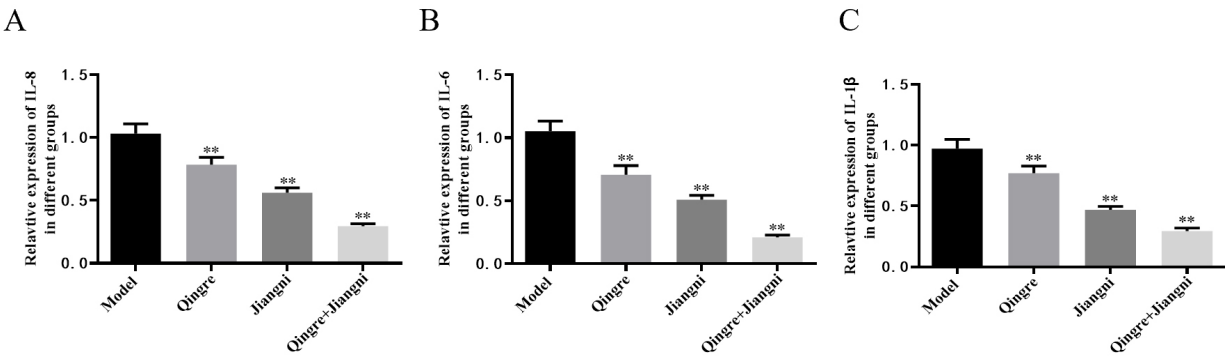

FIGURE 4. RT-PCR to detect the mRNA expression of inflammatory factors (A) IL-1β (B) IL-6 (C) IL-8. $^{**}p < 0.01$ compared with the model group. RT-PCR: reverse transcription-PCR; IL: interleukin.

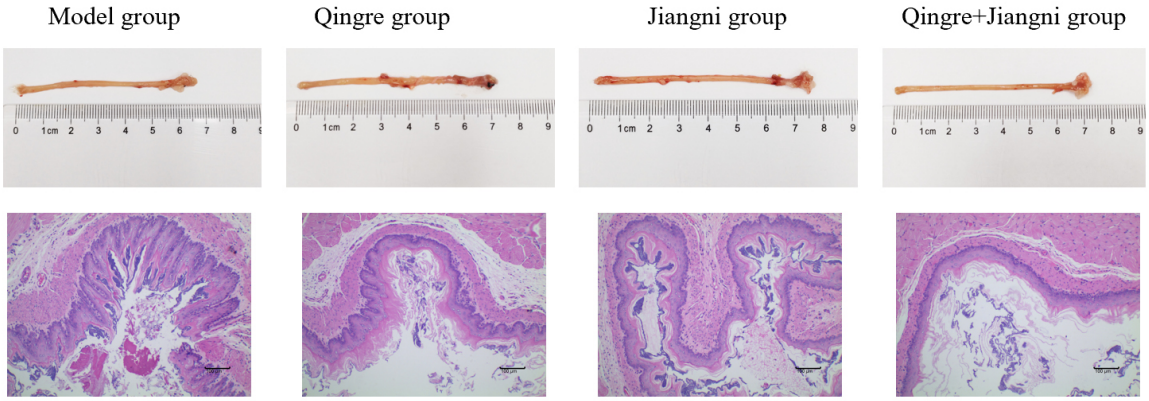

FIGURE 5. HE staining after treatment using the Qingre Jiangni method. Scales: 100 μm.

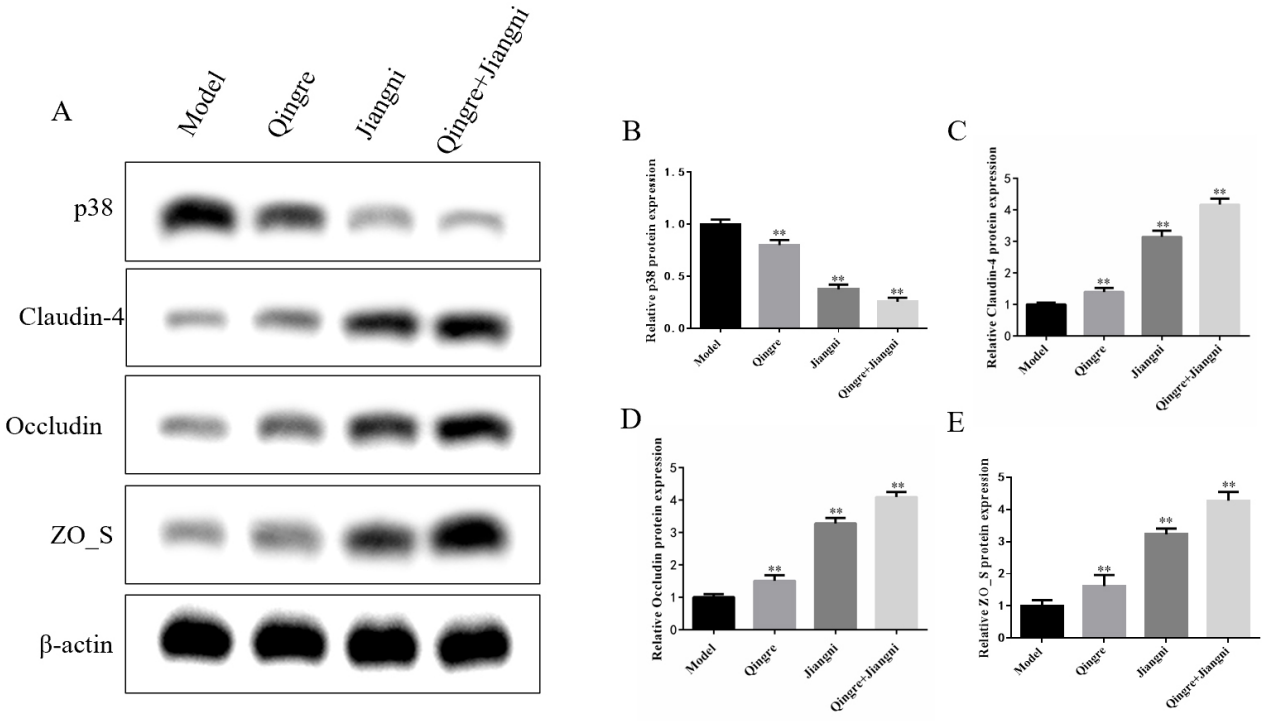

FIGURE 6. Western blotting (A) to detect (B) p38, (C) claudin-4, (D) occludin, and (E) ZO_S. $^{**}p < 0.01$ compared with the model group. ZO_S: Zonula Occludens.

abnormal esophageal muscle contractions, acid reflux, and reduced resistance of the esophageal mucosa [24]. Different symptoms have their predominant mechanisms. Stress can exacerbate heartburn in patients with GERD and trigger inflammation in the esophagus. Additionally, stress and anxiety may amplify the central response to irritation and inflammation in the esophagus [25, 26].

This study has some limitations. Although we surprisingly discovered that gastroesophageal reflux impacted male hormones, whose secretion can be regulated by traditional Chinese medicine, the small sample size and lack of further in-depth research suggest that the role played by these male hormones is still obscure.

5. Conclusions

Qingre Jiangni Formula is helpful for male hormones (testosterone and androsterol) and can decrease gastroesophageal reflux disease inflammation, thereby treating gastroesophageal reflux disease.

AVAILABILITY OF DATA AND MATERIALS

The data are contained within this article.

AUTHOR CONTRIBUTIONS

ZYJ and DQL—designed the research study. ZYJ—performed the research. DQL—analyzed the data. ZYJ, WW and ZJS—wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscripts.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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