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Original Article

Qihuang decoction promotes the recovery of intestinal immune barrier dysfunction after gastrectomy in rats

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Abstract: Objective: This study aims to observe the effect of Qihuang decoction on small intestinal mucosal barrier after gastrectomy in rats. Materials and methods: A total of 80 Wistar rats were randomly divided into normal group, sham operation group, enteral nutrition group (EN) and Qihuang decoction group (EN+QH), there were 20 rats in each group. Both the EN group and the EN+QH group underwent gastrectomy. Instillation of enteral nutrition in the small intestine was performed after operation in the EN group. Instillation of enteral nutrition and Qihuang decoction in the small intestine was performed after operation in the EN+QH group. Only the abdominal incision and closing was performed in the sham operation group without drug and nutritional intervention. The expression levels of tight junction proteins in intestinal epithelial cells were determined by western blotting method. The sIgA content in different anatomic sites of intestinal mucosa was determined by double antibody-PEG radioimmunoassay technique. The number of IgA+B cells in different anatomic sites of intestinal mucosa was determined by immunohistochemical method. Results: The sIgA content in the sham operated group was significantly lower than that of normal group (P<0.05). The sIgA content and the number of IgA+B cells in Peyer's patches and lamina propria lym-phocytes in the EN+QH group were significantly higher than that of EN group (P<0.01, P<0.05). The expression levels of RhoA, Rac1 and Cdc42 increased in the EN group, and the phosphorylation levels of occludin, claudin-1, claudin-5, ZO-1 and ZO-2 also increased in the EN group, while the expression levels of non-phosphorylated occluding, claudin-1, claudin-5, ZO-1 and ZO-2 proteins decreased in the EN group (P<0.01, P<0.05). After treatment of Qihuang decoction for 7 days, compared with EN group, the expression levels of RhoA, Rac1 and Cdc42, and the phosphorylation levels of occludin, claudin-1, claudin-5, ZO-1 and ZO-2 significantly decreased in the EN+QH group, while the expression levels of non-phosphorylated occluding, claudin-1, claudin-5, ZO-1 and ZO-2 proteins significantly increased in the EN+QH group (P<0.01, P<0.05). Conclusions: Qihuang decoction can promote the proliferation and differentiation of IgA+B lymphocytes and increase the sIgA content in intestinal mucosal immune barrier after gastrectomy in rats, it also can promote the expression of tight junction proteins to improve the permeability of intestinal mucosa and promote the recovery of intestinal immune barrier dysfunction in rats after gastrectomy by inhibiting the tight junction associated proteins' phosphorylation induced by Rho/ROCK signaling pathway.

Keywords: Qihuang decoction, immune barrier, tight junction, occludin protein, claudin1 protein, ZO-1 protein

Introduction

The intestinal immune system can prevent intestinal harmful substances, such as bacteria and toxins, from passing through the intestinal mucosa and entering other tissues, organs, and blood circulation in the body [1, 2]. The barrier of intestinal mucosa contains mechanical barrier, chemical barrier, immune barrier and biological barrier. The intestinal mucosal immune barrier and mechanical barrier are the most important. However, the bacteria and endotoxin in the intestine are shifted after the pathophysiological process such as ischemia and reperfusion in abnormal circumstances, especially in the case of severe stress such as surgery, trauma, burns and infection, which causing enterogenous systemic infections and sepsis, even a systemic inflammatory response syndrome (SIRS) and multiple organ dysfunc-

tion syndrome (MODS), and seriously affect the prognosis of the disease.

The gut is the largest organ of the immune system and the immune barrier is one of the most important barriers. Intestinal mucosal immune barrier consists of the intestinal Peyer's patches (Ps), lamina propria lym-phocytes (LPs), mucosal intraepithelial lymphocytes (IEs) and the intestinal mucosal surface secretory immunoglobulin A (sIgA). Whether its function is normal or not is directly related to the integrity of intestinal barrier [3]. When the organism is stressed, the intestinal mucosal immune cells are damaged. These are directly associated with barrier dysfunction and bacterial translocation [4, 5]. Bacterial translocation was found in the blood after surgical trauma [6-8], which was confirmed by animal experiments [9]. We also found that intestinal mucosal barrier abnormalities and bacterial translocation occurred after gastrectomy in clinical and animal studies [10, 11]. Clinical intervention is often performed in the stress state in order to restore normal mucosal immune function. At present, the main intervention measures include early enteral nutrition, such as glutamine, arginine, insulin-like growth factor, recombinant human growth hormone and so on [12-14]. Traditional Chinese medicine (TCM) has been studied on the protection of intestinal mucosal barrier under stress [15], but little attention has been paid to the protection of intestinal mucosal immune barrier.

Mechanical barrier refers to the complete epithelial structure of intestinal mucosa closely connected with each other, it consists of intestinal epithelial cells (IEC) and their connection. There are many connections between adjacent IEC such as tight junctions (TJ), gap junctions, adhesion junctions [16]. TJ are the core components of mechanical barriers, they are mainly composed of cell membrane proteins (occludin and claudin protein) and cytoplasmic protein (ZO-1). TJ are also called zonula occludens and widely found in vertebrate body surface and between the epithelium of various cavities and glands in the body. TJ make macromolecules difficult to permeate, only allow water molecules and ions to pass through [17-19].

At present, studies on the TJ and molecular structure of intestinal mucosal epithelium have been reported by various methods [20-26]. However, there is no research on Chinese medicine. Qihuang Decoction of Chinese medicine is a prescription that we formulated according to the traditional Chinese prescription for invigorating the spleen and activating the interior. Studies have shown that it could regulate the immune function of B cells and T cells in patients after gastrectomy [27-29]. However, the protective mechanism of Qihuang Decoction on the intestinal mucosal barrier remains unknown. In this study, we explored the effect of Qihuang decoction on small intestinal mucosal barrier after gastrectomy in rats.

Materials and methods

Drug preparation

The traditional Chinese medicine Astragalus membranaceus, rheum officinale, rhizome of lagehead atractylodes, Codonopsis pilosula, Fructus aurantii immaturus, Mangnolia officinalis, Salvia Miltrorrhiza and Radix Scutellariae were mixed according to the mass ratio 20:10:20:20:10:10:15:12 and the total weight of mixed medicine was 234 g, 500 ml H_2O was added and boiled for 30 min according to the references [11, 30]. The Crude Drug Decoction was filtrated and concentrated to 1.0 g/ml, it was preserved at 4°C and re-warmed before administration.

Experimental animals

Eighty healthy male rats of Wistar weight 300±10 g (3 months old) were supplied by Animal Laboratory Center of Anhui Medical University. The rats were placed in a comfortable and quiet room for one week before the initiation of the experimental procedure. A total of 80 Wistar rats were randomly divided into normal group, sham operation group, enteral nutrition group (EN) and Qihuang decoction group (EN+QH), there were 20 rats in each group. The model was established according to the references [11, 30] (**Figure 1**). Both the EN group and the EN+QH group underwent gastrectomy. Instillation of enteral nutrition in the small intestine was performed after operation in the EN group for 7 days. The enteral nutrition was Fresubin and it was dissolve into 830 kJ calories/100 mL, each rat was provided with 120 kJ/(kg·d). Instillation of enteral nutrition and Qihuang decoction in the small intestine was performed after operation in the EN+QH

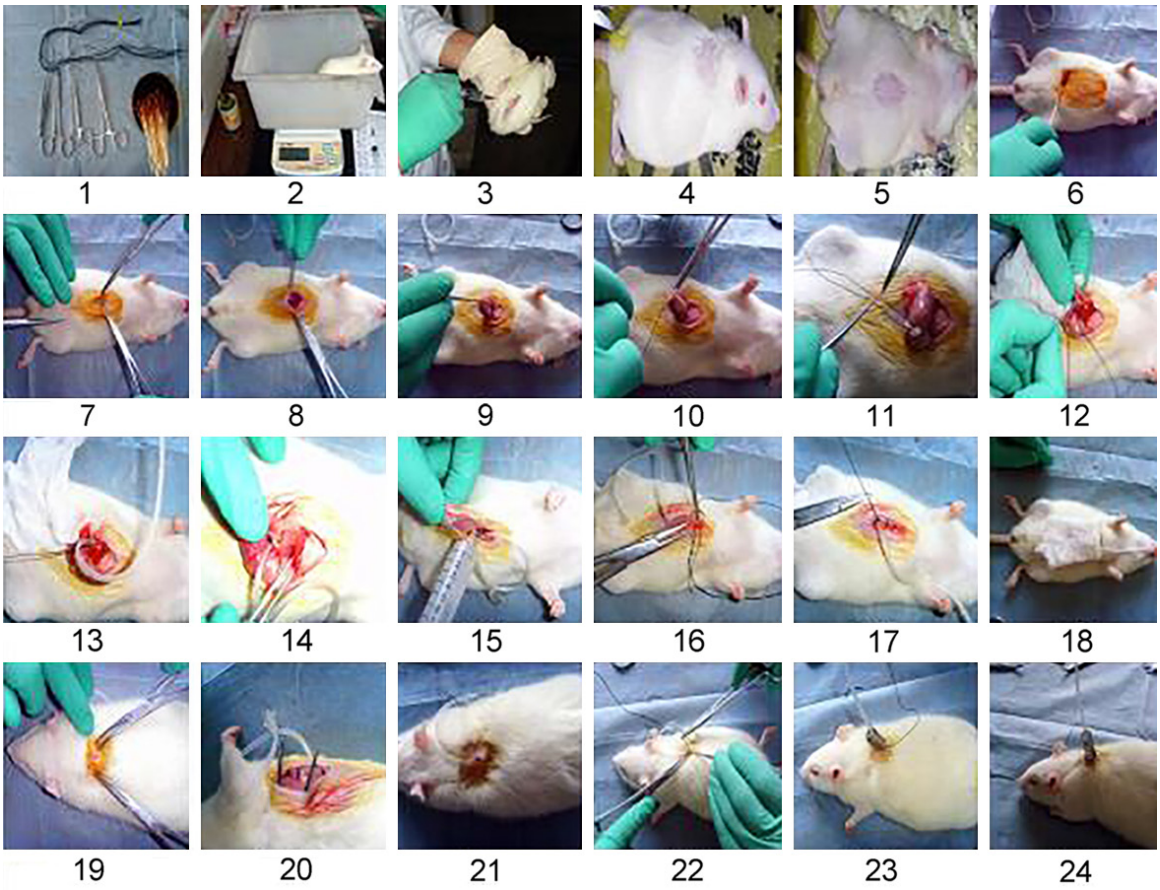

Figure 1. Establishment of model rats. The model rats were successfully established. Both the EN group and the EN+QH group underwent gastrectomy. Only the abdominal incision and closing was performed in the sham operation group without drug and nutritional intervention. Instillation of enteral nutrition in the small intestine was performed after operation in the EN group for 7 days.

group. The volume of Fresubin was the same as that of EN group and 10 g/(kg·d) Qihuang decoction was given to each rat by microinjection pump. Only the abdominal incision and closing was performed in the sham operation group without drug and nutritional intervention.

This study was audited and approved by Animal Ethics Committee of the first affiliated hospital of Anhui University of Chinese Medicine. All experimental procedure and animal care were carried out under the guidance of the Ethics Committee in order to minimize the suffering of animals.

HPLC and fingerprinting analysis

A total of 10 batches (S1 to S10) of compound Qihuang Decoction were prepared. The filtrate was added 4 times the volume of methanol for precipitation. The test solution was filtered with 0.22 μm microporous membrane before test and Agilent 1260 liquid chromatography was used for analysis. Chromatographic column: Welch C18 (4.6 × 250 mm, 5 μm); Speed: 1.0 mL/min; water (containing 0.05% formic acid) as mobile phase A and acetonitrile (containing 0.05% formic acid) as mobile phase B; gradient elution (0~5 min, 10% B→20% B; 5~10 min, 20% B→35% B; 10~30 min; 35% B→36% B; 30~32 min, 36% B→80% B). The column temperature is 30°C, the sample volume is 5 μL and the detection wavelength is 280 nm.

Isolation and detection of sIgA

Rats were anesthetized with urethane after the treatments; laparotomy was performed to extract the full length of the small intestine in each group immediately. Intestinal cavity was cleaned and rinsed the bowel cavity with 10%

acetic acid solution. The lavage fluid was collected and centrifuged at 4°C with 20,000 r/min for 30 min, the supernatant was collected and stored at -70°C, sIgA was detected by double antibody-PEG radioimmunoassay.

IgA+B cells detection in Ps and LPs

The Ps and the small intestine fixed with 10% formalin were removed and dehydrated in the biological tissue automatic dehydrator, they were embedded with paraffin after dehydration and sliced (2 μm). The slices were baked and blocked with normal goat serum and incubated for 20 minutes at room temperature. The excess liquid was discarded and rabbit anti rats IgA (50 μl, 1:300) was added into them and incubated at 37°C for 3 hours. Then they were washed with PBS for three times (3 minutes each time). The HRP labeled Streptavidin secondary antibody was added into them and incubated at 37°C for 15 minutes. They were washed with PBS for three times (3 minutes each time). Then they were conducted with dehydration, transparent, conventional resin sealing and examination under microscope.

Western blotting test

The samples were pulverized in liquid nitrogen and lysed with RIPA lysis buffer on ice for 30 min with shaking at 12,000 rpm/min. Total proteins were collected and the concentration was determined by BCA Kit. Proteins (40 μg per lane) were separated using 12% SDS-PAGE electrophoresis. Then it was electrotransferred to the PVDF membrane. The PVDF membrane was rinsed with TBS for 10 to 15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shook at room temperature for one hour. It was incubated at 4°C overnight after added anti-RhoA (abcam, ab54835, 1:1000); anti-Rac1 (abcam, ab33-186, 1:1000); anti-Cdc42 (abcam, ab187643, 1:1000); anti-Claudin-1 (abcam, ab15098, 1:1000); anti-Claudin-5 (abcam, ab131259, 1:1000); anti-Occludin (abcam, ab167161, 1:1000); anti-ZO-1 (Thermo Fisher, RF232767, 1:500); anti-ZO-2 (abcam, ab191133, 1:1000); Anti-Claudin 5 (phospho Y217, abcam, ab17-2968, 1:1000); anti-Occludin (phospho S490, Zymed, 1:250); Anti-ZO-2 (Phospho T1118, Assay Biotechnology, P12-1069, 1:1000) and β-actin (1:1000) monoclonal antibodies (1:500). Then the membrane was washed with TBST for three times (5 minutes each time). The membrane was incubated at 37°C for one hour with Ig G-HRP secondary antibody (1:10000) diluted with TBST containing 0.05% (w/v) skimmed milk powder. It was developed with ECL for 5 minutes. The protein bands were quantified as a ratio to β-actin using Image J software.

The phosphorylation levels of Claudin-1 and ZO-1 were also detected by Western blotting methods. Anti-Claudin-1 (Abcam, ab15098, 1:500) or anti-ZO-1 (Thermo, Fisher, RF232767, 1:500) were added into tissue lysate and incubated at 4°C for 1 h, then protein G-Sepharose beads was added into them and continue to incubate at 4°C for 2 h. Proteins (40 μg per lane) were separated using 12% SDS-PAGE electrophoresis and other procedures were the same as above.

Statistical analysis

All analyses were conducted using SPSS 17.0 software (SPSS Inc., Chicago, USA). The data were expressed as $\bar{x}\pm s$. The homogeneity of variance was tested by Levene method in each group. If the variance is homogeneous, the single factor analysis of variance and t test are used to compare the sample means. The non-parametric test is used if the variance is not uniform. *P* values <0.05 were considered indicative of a significant difference.

Results

The fingerprint of Qihuang decoction

The reference fingerprint of the common mode of Qihuang Decoction was produced by using the similarity evaluation software of chromatographic fingerprint of Chinese herbal medicine, sixteen chromatographic peaks were determined. The similarity evaluation results showed that the similarity between the fingerprints of different batches and the control spectrum was above 0.98, which met the requirements of fingerprint. The number 10 peak (baicalin) with moderate peak area and retention time were selected as reference peaks (S peaks), the relative retention time (RRT) and the relative peak area (RPA) were calculated. The relative standard deviation (RSD) is small, which provides a reference for the quality control of Qihuang Decoction (**Figure 2**).

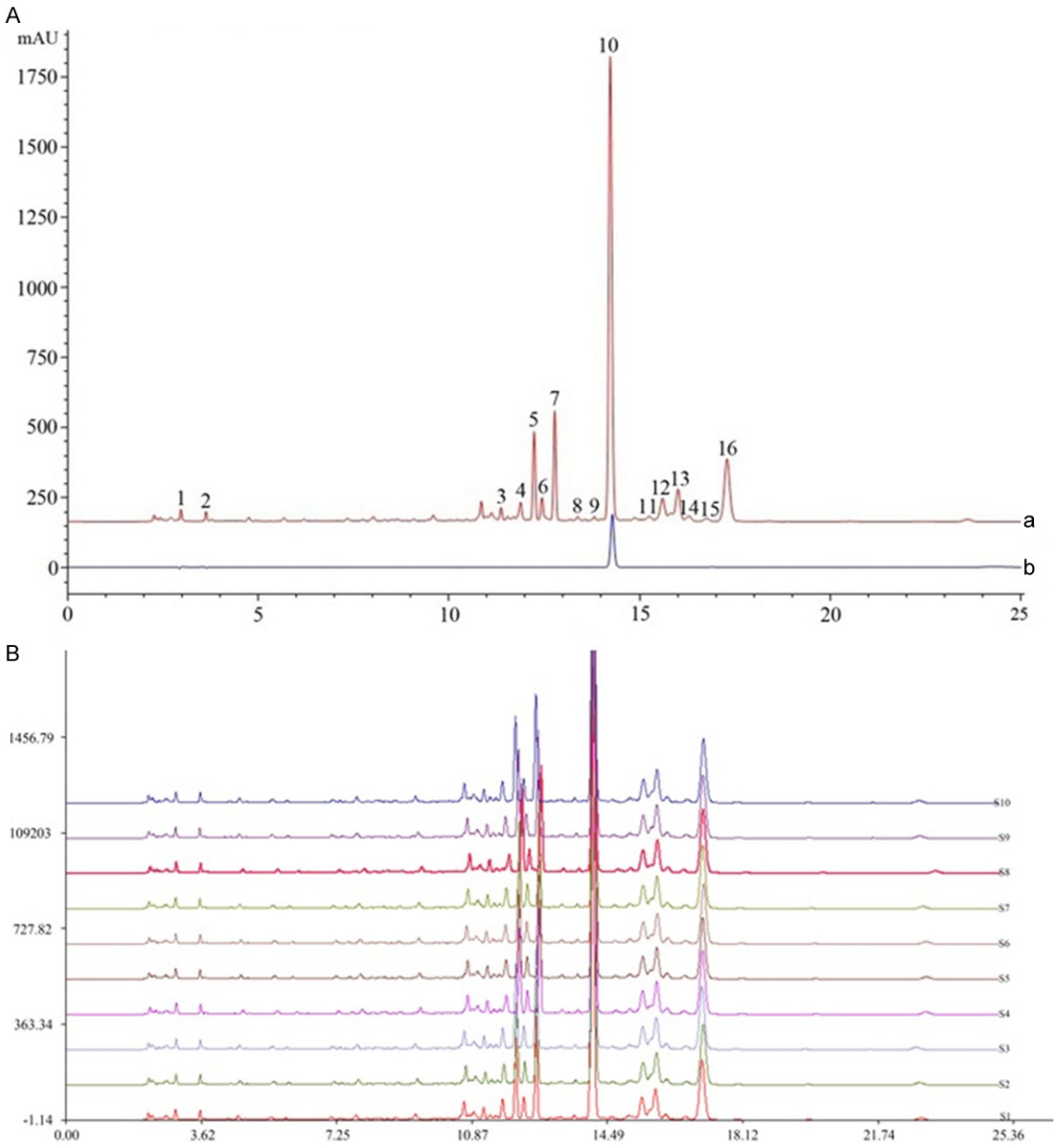

Figure 2. The HPLC fingerprint of Qihuang Decoction. A: The HPLC fingerprint of baicalin and control; a: control; b: baicalin; B: The HPLC fingerprint of Qihuang Decoction samples. The similarity between the fingerprints of different batches and the control spectrum was above 0.98, which met the requirements of fingerprint. The relative standard deviation (RSD) is small, which provides a reference for the quality control of Qihuang Decoction.

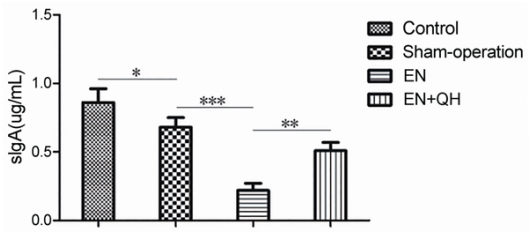

Figure 3. The sIgA content in different groups. The sIgA content in the sham operated group was significantly lower than that of normal group (*P<0.05). The sIgA content in the EN group were significantly lower than that of sham operation group (***P<0.001). The sIgA content in the EN+QH group were significantly higher than that of EN group (**P<0.01). Qihuang decoction can promote the secretion of sIgA on the surface of intestinal mucosa.

The detection of immune indexes

There were some rats died due to the anesthesia, surgery, postoperative management and

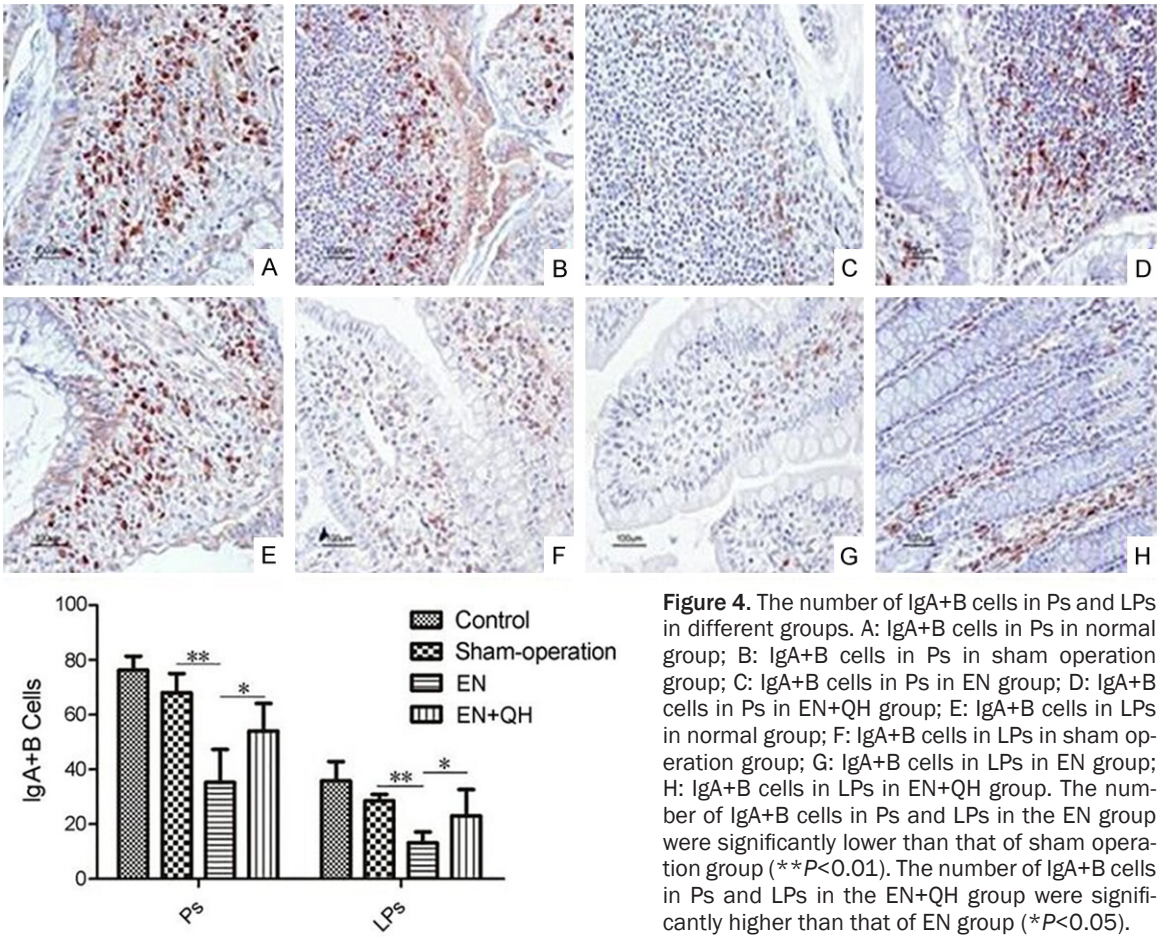

Figure 4. The number of IgA+B cells in Ps and LPs in different groups. A: IgA+B cells in Ps in normal group; B: IgA+B cells in Ps in sham operation group; C: IgA+B cells in Ps in EN group; D: IgA+B cells in Ps in EN+QH group; E: IgA+B cells in LPs in normal group; F: IgA+B cells in LPs in sham operation group; G: IgA+B cells in LPs in EN group; H: IgA+B cells in LPs in EN+QH group. The number of IgA+B cells in Ps and LPs in the EN group were significantly lower than that of sham operation group (**P<0.01). The number of IgA+B cells in Ps and LPs in the EN+QH group were significantly higher than that of EN group (*P<0.05).

other factors. The remaining rats' number in the normal group, sham operation group, enteral nutrition group (EN) and Qihuang decoction group (EN+QH) were 20, 18, 12 and 15 respectively. Ten rats in each group were randomly selected for test.

The sIgA content in the sham operated group was significantly lower than that of normal group (**Figure 3**, P<0.05). The sIgA content and the number of IgA+B cells in Ps and LPs in the EN group were significantly lower than that of sham operation group (**Figures 3**, **4**, P<0.01, P<0.001). The sIgA content and the number of IgA+B cells in Ps and LPs in the EN+QH group were significantly higher than that of EN group (P<0.01, P<0.05).

Proteins expression

Phosphorylation levels and expression changes of Rho-ROCK signaling pathway and TJ related proteins were shown in **Figure 5**. It showed that the phosphorylation levels of occludin, claudin-1, claudin-5, ZO-1, ZO-2 and the expression levels of RhoA, Rac1 and Cdc42 in the EN group was significantly higher than that of normal group (P<0.05), while the expression levels of non-phosphorylated occluding, claudin-1, claudin-5, ZO-1 and ZO-2 proteins in the EN group were significantly lower than that of normal group (P<0.05). The expression levels of RhoA, Rac1 and Cdc42 in the EN+QH group were significantly lower than that of the EN group (P<0.05). The phosphorylation levels of occludin, claudin-1, claudin-5, ZO-1 and ZO-2 significantly decreased in the EN+QH group while the expression levels of non-phosphorylated occluding, claudin-1, claudin-5, ZO-1 and ZO-2 proteins were significantly increased in the EN+QH group (P<0.05).

Discussion

Ps is mainly composed of B cells and T cells. It induces the differentiation and maturation of lymphocytes and plays an important role in the induction of immune response [31]. In this

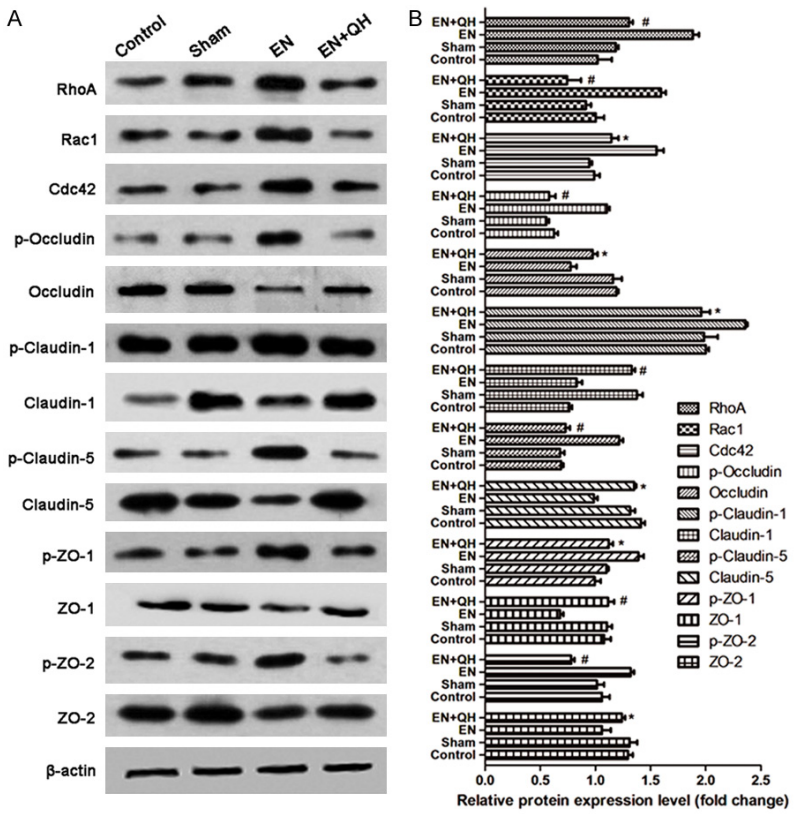

Figure 5. The expression levels of claudin1, occludin and ZO-1 in different groups. **P*<0.05, #*P*<0.01. The expression levels of RhoA, Rac1 and Cdc42 increased in the EN group, and the phosphorylation levels of occludin, claudin-1, claudin-5, ZO-1 and ZO-2 also increased in the EN group, while the expression levels of non-phosphorylated occluding, claudin-1, claudin-5, ZO-1 and ZO-2 proteins decreased in the EN group. After treatment of Qihuang decoction for 7 days, compared with EN group, the expression levels of RhoA, Rac1 and Cdc42, and the phosphorylation levels of occludin, claudin-1, claudin-5, ZO-1 and ZO-2 significantly decreased in the EN+QH group, while the expression levels of non-phosphorylated occluding, claudin-1, claudin-5, ZO-1 and ZO-2 proteins significantly increased in the EN+QH group. These results suggested that the intestinal instillation of Qihuang decoction can protect intestinal mucosal barrier function by inhibiting the tight junction associated protein phosphorylation induced by Rho/ROCK signaling pathway.

study, we found that the sIgA content and the number of IgA+B cells in Ps and LPs in the EN+QH group were significantly higher than that of EN group, which suggested that Qihuang decoction could induce differentiation and maturation of primary lymphocyte in Ps. It lays the foundation for the homing of lymphocytes to the lamina propria of intestinal mucosa and the function of mucosal immune barrier.

LPs is located under the basement membrane of mucosal epithelial cells. The number of B lymphocytes accounts for the absolute predominance of LPs, and these B cells are mostly differentiated mature IgA+B cells, which have the secretory function of IgA and are called plasma cells. The IgA bound to J chains to form a dimer or polymers, and then bound to the (secretory, component, SC) secreted by mucous epithelial cells form sIgA, which plays the first defense role of mucosal immune barrier. sIgA exerts immune effect mainly through two pathways. First, it adheres and includes the invading pathogens of mucosal epithelial cells to form antigen antibody complexes and removes them. Second, sIgA binds to an invaded pathogen and carries it from the cell to the mucosal cavity, thereby avoiding damage to mucosal epithelial cells. T cells in LPs are mainly CD4+ αβ TCR T cells accounting for 60% to 70%. It helps IgA+B cells homing from Ps to differentiate into IgA+ plasma cells by secreting multiple cytokines [32]. In this study, we found that Qihuang decoction could promote and induce the proliferation, differentiation and maturation of lymphocytes in LPs. We hypothesized that this may be related to the promotion of lymphocyte homing, which was crucial for the eventual development of the immune barrier of the intestinal mucosa. It was found that the rheum officinale in Qihuang decoction could adjust the immune function of the organism and enhance local immunity of intestinal tract [33]. The increase of sIgA levels on the surface of midgut mucosa in this study is also the embodiment of this effect.

The intestinal mechanical barrier consists of mucus layer, intestinal epithelial cells and their TJ, lamina propria and so on. The TJ have the function of “barrier” and “fence”. Studies have shown that occludin, claudin and ZO-1 proteins were closely connected to the role of TJ [34].

Claudin-1 protein plays an important role in maintaining the integrity of intercellular TJ and the normal functioning of epithelial barrier function. Abnormal expression of Claudin-1 protein results in barrier and TJ dysfunction, and increased tissue permeability. It was reported that inflammation can cause excessive expression of claudin1 [35]. In this study, we found that postoperative trauma combined with inflammatory response and infection may result in overexpression of claudin1, this may be due to the postoperative self repair of intestinal tract function. The Qihuang decoction could further promote the expression of claudin1, thereby protecting the intestinal mucosal barrier. Occludin is a transmembrane protein that plays a role in maintaining TJ stability and barrier function. ZO-1 is located in the zonula occludens of epithelial cells and endothelial cells. Occludin is linked to the cytoskeleton by ZO-1 [36-38]. The transmembrane protein and cytoskeleton are connected together after the interaction of Occludin and ZO-1, which regulating the intracellular and extracellular signal transduction pathways and the permeability. In this study, we found that surgical trauma resulted in a decrease in the expression of Occludin and ZO-1, the Qihuang decoction could increase the expression of Occludin and ZO-1 and improve the permeability of intestinal mucosa.

Phosphorylation is an important way to regulate TJ related proteins, it can reduce the binding ability of Occludin and claudin, ZO-1 and ZO-2. Rho/ROCK signaling pathway is an important signaling pathway to regulate the phosphorylation of TJ related proteins. In this study, we found that Qihuang decoction treatment could down-regulate the expression levels of RhoA, Rac1 and Cdc42 and the phosphorylation levels of occludin, claudin-1, claudin-5, ZO-1 and ZO-2, increase the expression levels of non-phosphorylated occluding, claudin-1, claudin-5, ZO-1 and ZO-2 proteins. These results suggested that the intestinal instillation of Qihuang decoction can protect intestinal mucosal barrier function by inhibiting the TJ associated protein phosphorylation induced by Rho/ROCK signaling pathway.

Conclusions

Surgical trauma can cause intestinal mucosal immune abnormalities and barrier dysfunction, resulting in bacterial translocation, SIRS and MODS, and seriously affecting the prognosis and prognosis of the disease. Qihuang decoction has the function of regulating the immune function and protecting the intestinal mucosa barrier function. Qihuang decoction can promote the secretion of sIgA on the surface of intestinal mucosa, promote the proliferation, differentiation and maturation of Ps and LPs, promote the expression of TJ proteins to improve the permeability of intestinal mucosa and promote the recovery of intestinal immune barrier dysfunction in rats after gastrectomy by inhibiting the TJ associated protein phosphorylation induced by Rho/ROCK signaling pathway. It lays a foundation for the protection of intestinal mucosal barrier function and the prevention and treatment of critical diseases by traditional Chinese Medicine. Its mechanism is not clear and will be our further study.

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Disclosure of conflict of interest

None.

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