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Original Article

Enhanced intestinal lymphocyte homing intervention using Qihuang decoction intestinal instillation in rats after gastrectomy

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Abstract: Intestinal lymphocyte homing is a process in which activated T cells and antibody-secreting cells are targeted to specific gut regions aiming to provide an effective intestinal immune response. In this paper we investigated the effects of Qihuang decoction on intestinal lymphocyte homing after gastrectomy in rats. Model rats were divided into three groups, namely the sham operation group, the enteral nutrition group (EN group), and the Qihuang decoction group (EN+QH group). The amount of homing lymphocyte was significantly increased in the Qihuang decoction group compared with the EN group after gastrectomy according to the counts of ^{51}Cr labelled lymphocyte. The flow cytometry results demonstrated that activated $CD3^+$ T cells were also increased in the EN+QH group with a significance of $P<0.01$. From our immunohistochemistry data, the number of IgA^+ B cells in Peyer's patches (PP) was higher in the EN+QH group than in the EN group ($P<0.05$), indicating a promoted cell proliferation of lymphocytes in PP induced by the Qihuang decoction. Also, the positive molecular expressions and the mRNA expressions of both the lymphocyte homing receptors ($\alpha^4\beta^7$, L-selectin and LFA-1) and the lymphocyte homing ligands (MAdCAM-1 and ICAM-1) were significantly increased in the EN+QH group compared with the EN group. These results suggest an enhanced intestinal lymphocyte homing intervention using Qihuang decoction that works by promoting lymphocyte proliferation and sensitization in the intestines.

Keywords: Qihuang decoction, intestinal lymphocyte homing, intestinal immuno-barrier, gastrectomy

Introduction

The intestinal mucosal barrier is a heterogeneous entity that consists of physical, biochemical, and immune elements complicated by intestinal mucosa, in which the intestinal epithelial layer plays an important role in its dual functions of physical separation and immunological regulation [1]. The intestinal mucosal barrier is of critical importance to the body's immune system, because it not only protects mucosal tissues and the circulatory system, it also isolates pro-inflammatory molecules like cytotoxin and antigens [2, 3]. However, intestinal mucosal barrier dysfunction may occur under different situations such as bromatoxism, celiac disease, bacterial infection, and immunologic inadequacy [4-6]. Clinically, enterogenous infections, sepsis, and diabetes mellitus have been reported in deteriorative cases especially after gastrectomy [7, 8].

Intestinal lymphocyte homing is a process in which activated T cells and antibody-secreting cells are targeted to specialized gut regions in order to provide an effective intestinal immune response, which is of critical importance for the intestinal immune-barrier [9, 10]. The adhesion of the circulating lymphocytes to specific intestinal endothelial cells plays a significant role in the lymphocyte homing process. Lymphocyte homing receptors are cell adhesion molecules (CAMs) that recognize and interact with addressins on the target tissues, which are generally expressed on lymphocyte membranes. Various target-specific adhesions on lymphocyte homing receptors and on vascular addressins are conducive to immune activation

[11]. This mechanism mainly relies on the key interaction between the integrin $\alpha^4\beta^7$ and the addressin MadCAM-1 on the surfaces of certain cells [12-14]. Integrin $\alpha^4\beta^7$ is an important lymphocyte homing receptor that recognizes its counterreceptor MadCAM-1 and is normally expressed in Peyer's patches [15]. Also, $CD3^+$ T cells are also crucial in lymphocyte homing and the immune response, in which $CD3^+$ T cell co-receptors bind signaling molecules aim to promote ligand recognition and initiate the immunologic process. In addition, the majority of activated B cells differentiate into IgA plasma cells, with the gut being the largest immunoglobulin producer. IgA is an important antibody that expresses immunological function on mucosal membranes with secretory IgA protecting against specific antigens and creating a healthy micro-environment [16]. In short, the homing mechanism plays a major role in the maintenance of specialized micro-environments and is crucial for the dispersal and targeting of naive and memory lymphocyte populations that are required for effective immune surveillance [9]. Specifically, intestinal lymphocyte homing provides an effective immune response and makes a great contribution to the intestinal immuno-barrier.

In recent years, different therapies have been applied in the treatment of intestinal mucosal barrier dysfunction, such as monoclonal antibody [17], cytokines [18] and enteral alimentation intervention [19]. Fan et al. demonstrated that the intestinal instillation of enteral nutrition in rats can enhance intestinal IgA^+ levels and the proliferation of $CD3^+$ T cells, thus promoting lymphocyte homing and strengthening the intestinal mucosal barrier [20]. The study indicates that intestinal lymphocyte homing can be intervened effectively through the appropriate methods for the recovery of the intestinal barrier. However, studies of traditional Chinese medicine intervention using intestinal instillation combined with an analysis of lymphocyte homing after gastrectomy still remains unexplored. Qihuang is a common traditional Chinese medicine with outstanding medicinal benefits such as immune-boosting [21] and an anti-inflammatory effect [22]. Qihuang has been used for several centuries to regulate immunologic function and enhance disease resistance. Here, we introduce a systematic study of enhanced lymphocyte homing intervened by a Qihuang decoction intestinal instillation after stomach resection in rats, and we analyze the changes in $CD3^+$ T cells, IgA^+ B cells as well as the positive molecular expressions and mRNA expressions of both lymphocyte homing receptors and ligands.

Materials and methods

Materials

The traditional Chinese medicine *Astragalus membranaceus*, *Rheum offcinale*, rhizome of lagehead atractylodes, *Codonopsis pilosula*, Fructus aurantii immaturus, *Mangnolia offcinalis*, *Salvia Miltrorrhiza*, and *Radix Scutellariae* were brought from the TCM pharmacy of the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine. Healthy male Sprague-Dawley (SD) rats of clean grade, weighing (300±10) g, were purchased from the Experimental Animal Center in Anhui University of Traditional Chinese Medicine (Hefei, China) [Certificate of quality no. SCXK (Su) 2014-0007].

Drug preparation and establishment of the rat model

The Qihuang decoction was prepared and examined by HPLC using the same method we described in our previous study [23]. The establishment of the rat model also underwent a similar procedure [23]. A few rats died due to anesthetization or surgical injury. There were 18, 15, and 14 rats that survived in the sham-operated group, the EN group, and the EN+QH group respectively. 10 surviving rats in each group were chosen randomly for further data collection and analysis after the operations. We briefly review here that gastrectomy were performed in the EN group and the EN+QH group. Adequate enteral alimentation was supplemented through intestinal instillation in the EN group after the gastrectomies. The EN+QH group differed from the EN group in that the Qihuang decoction was instilled simultaneously with enteral nutrition in the small intestine. In the sham-operated group, only abdominal incisions and sutures were conducted.

Drainage and reinfusion of lymph

The lymphatic trunk was located above the mesenteric artery and had to be separated from the serosa carefully to avoid the influence

of hemocytes. The white lymph was extracted through a home-made low negative pressure drainage device consisting of a 5 ml injector. The drainage process lasted for 30 minutes and about 0.4-0.6 ml lymph was taken for further examination. Half the lymph was labelled as $Na_2[^{51}Cr]O_4$ and re-injected into the rats' femoral veins until a stable count was detected using a γ counter. One hour later, the amount and distribution of lymph in the intestinal mucosa and intestinal lymphoid tissue were measured using a γ counter. The remaining half was used to measure and analyze the $CD3^+$ T cells and their subset $CD4^+$, $CD8^+$ T cells.

Excision of Peyer's patches and small intestine diffuse lymph nodes

After the lymph drainage, the entire small intestine was eviscerated and cleaned with normal saline and then preserved in a petri dish. Each rat had 6 to 8 Peyer's patches (PP). Those PP were cut out using surgical scissor and processed with 10% formalin for further quantification of the IgA^+ B cells. Half of the PP were used for the γ counter quantification, in which the lymph was squeezed out and filtered, then they were centrifuged at 3000 rpm, cleaned with RPMI-1640 of 5% calf serum in order to form a single cell suspension of PP. The excision of the diffuse lymph nodes in the small intestine underwent a procedure similar to the Peyer's patches.

Lymphocyte homing measurement by γ counter

The amount of lymphocyte homing was determined using a DFM-96 γ counter which is normally applied to probe the radioactivity of cells labeled by radiopharmaceuticals. In the beginning, the lymph cell suspension was labelled using ^{51}Cr using $Na_2[^{51}Cr]O_4$ and preserved at 37°C in a water bath. Centrifugation was applied to remove the dissociative ^{51}Cr at 3000 rpm. Afterwards, the ^{51}Cr labelled lymph suspension was injected into the rats' femoral veins and measured using a γ counter as mentioned above. The lymphocyte homing in the PP, the mesenteric lymph nodes, and the small intestine diffuse lymph nodes were all quantified using a γ counter.

Flow cytometry

A hydrogen ion laser with a wavelength of 488 nm was applied in the flow cytometry. The reception channel was set to side scattering and front scattering. 50 μl draining lymph suspension with cell concentration of 10^6/ml was labeled as CD^3-FIT, CD^4-$PECD^3$-FIT, and CD^8-PE, and then measured using flow cytometry for the T cell examination. Homing receptor $\alpha^4\beta^7$, L-selectin, and LFA-1 were also detected using flow cytometry to count the positive rates of the mononuclear cells in the lymph suspension.

Immunohistochemistry

The lymphocyte homing ligands MAdCAM-1, ICAM-1 and IgA^+ B cells were quantified and analyzed using immunohistochemistry (IHC). The Peyer's patches were dehydrated and packaged using paraffin in order to make PP slices. The positive lymphocyte cytomembrane showed up as claybank under microscopy.

Reverse transcription polymerase chain reaction

RT-PCR is primarily used to measure the amount of a specific RNA. In order to measure the mRNA expression of the lymphocyte homing receptors and ligands in real time, an ABI Prism 7900 RT-PCR instrument was applied in our experiment combined with a relative quantification analysis.

Statistical analysis

SPSS 17.0 was used for the data analysis. All the data values were presented as the mean value ± standard value (SD). Levene's tests were used to examine the homogeneity of variance. A single factor analysis of variance followed by a t test was performed to compare the sample means when a variance was homogeneous. If a non-uniform variance appeared, then nonparametric test was conducted. *P* values <0.05 were considered a significant difference.

Results

$CD3^+$ T cell promotion intervened by Qihuang decoction after gastrectomy

In Peyer's patches, the levels of homing lymphocytes measured by the γ counter in the sham-operated group, the EN group, and the EN+QH group were 4.93±0.50, 2.85±0.37 and 3.24±0.28 respectively. The amount of lymphocyte homing in the EN+QH group was significantly increased compared with the EN

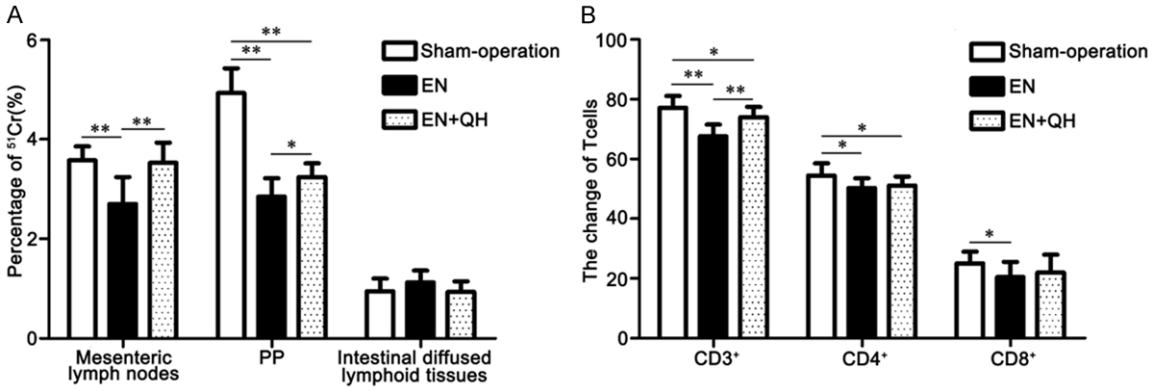

Figure 1. The amount of lymphocyte homing and the activation of T cells. A. The percentage of ^{51}Cr in the PP, mesenteric lymph and intestinal diffused lymphoid tissues of the three groups seven days after the establishment of the rat model, *P<0.05, **P<0.01 (n=10). B. The change in the $CD3^+$ T cells and their subsets $CD4^+$, $CD8^+$ T cells in the three groups, *P<0.05, **P<0.01 (n=10).

group (P<0.05). In the mesenteric lymph nodes, the percentages were 3.85±0.28, 2.70±0.54, and 3.53±0.40 in the three groups. The EN+QH group also showed a higher value than the EN group, and the difference was significant (P<0.01). In the intestinal diffused lymphoid tissues, there were no significant differences among the three groups (P>0.05). The detailed data are shown in **Figure 1A**. The flow cytometry results showed that the $CD3^+$ T cell levels in the sham-operated group, the EN group, and the EN+QH group were 77.24±3.86, 67.59±3.99, and 74.04±3.40 respectively. The EN+QH group showed greater proliferation of promoted $CD3^+$ T cells than the EN group with a significant difference (P<0.01). The $CD4^+$ T cell levels in the three groups were 54.42±4.09, 50.31±3.22, and 51.11±2.98. The $CD4^+$ T cells in the EN+QH and EN groups were more decreased than the cells in the sham-operated group (P<0.05, P<0.05, respectively). This difference could be attributed to the damage to the immune system caused by the gastrectomies. Similar results can be reproduced for the $CD8^+$ T cells as illustrated in **Figure 1B**. These results indicate that the interventional therapy using the intestinal instillation of Qihuang decoction in rats may enhance the $CD3^+$ T cell levels and contribute to the improvement of intestinal lymphocyte homing after gastrectomy.

Qihuang decoction facilitated the proliferation of IgA^+ B cells after gastrectomy

The IgA^+ B cells in the Peyer's patches decreased to their normal levels after the gastrectomies. The EN group showed more reduced IgA^+ B cell levels in PP than the sham-operated group (P<0.01). The IgA^+ B cell levels in the EN+QH group were also lower than they were in the sham operation group after the gastrectomies, and the difference was significant (P<0.05). The detailed data are shown in **Figure 2**. These results proved that the proliferation and activation of the IgA^+ B cells in the Peyer's patches were seriously damaged after the gastrectomy. Even though the enteral nutrition and the Qihuang decoction intervention were applied in the rat intestines, the IgA^+ B cells were still unable to recover to their normal value. In addition, the IgA^+ B cell levels in the EN+QH group were significantly higher than they were in the EN group (P<0.05), indicating a promoted cell proliferation of the IgA^+ B cells in the Peyer's patches induced by the Qihuang decoction after the gastrectomies.

Promoted positive molecular expressions and the mRNA expressions of the lymphocyte homing receptors

The positive molecular expressions of the lymphocyte homing receptors $\alpha^4\beta^7$, L-selectin, and LFA-1 in the EN+QH and EN groups were more prominently decreased than they were in the sham-operated group with significant differences, according to the flow cytometry results (**Figure 3A**). Furthermore, the lymphocyte homing receptors $\alpha^4\beta^7$ and L-selectin in the EN+QH group were significantly higher than they were in the EN group (P<0.05, P<0.05). These results suggest that the positive expressions of lymphocyte the homing

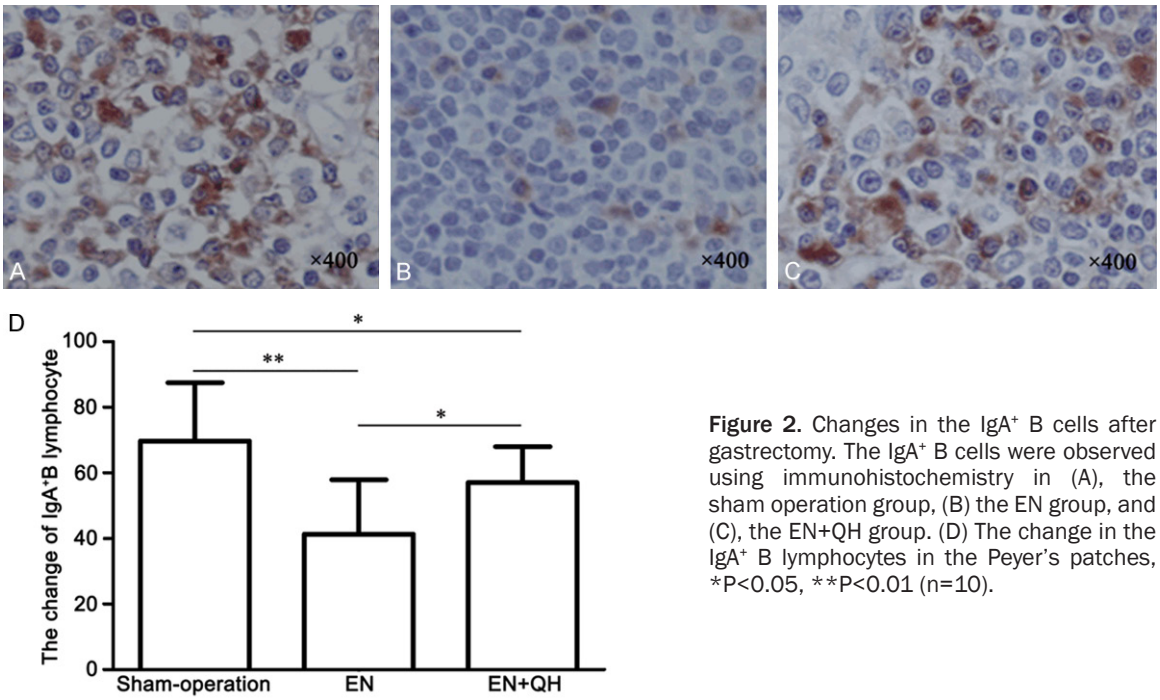

Figure 2. Changes in the IgA$^+$ B cells after gastrectomy. The IgA$^+$ B cells were observed using immunohistochemistry in (A), the sham operation group, (B) the EN group, and (C), the EN+QH group. (D) The change in the IgA$^+$ B lymphocytes in the Peyer's patches, *P<0.05, **P<0.01 (n=10).

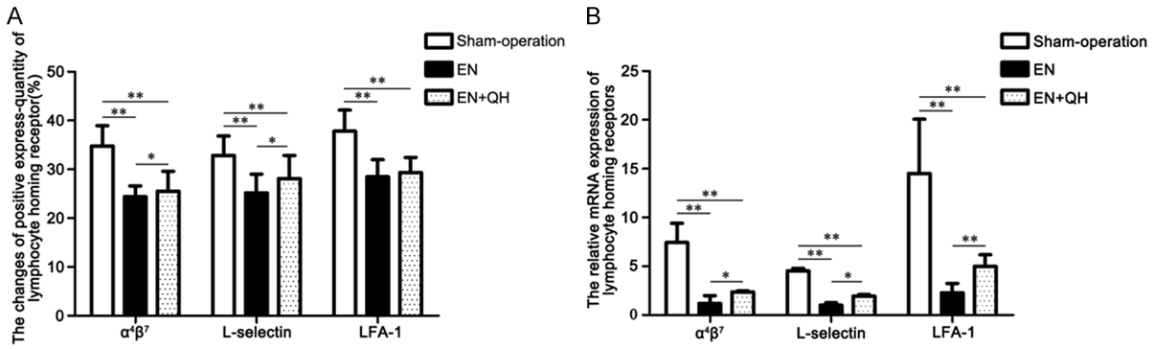

Figure 3. The positive molecular expression and mRNA expressions of the lymphocyte homing receptor, *P<0.05, **P<0.01 (n=10).

receptors $\alpha^4\beta^7$ and L-selectin were promoted by the Qihuang decoction, which can facilitate the recognition and aggregation of lymphocytes after gastrectomy and contribute to the recovery of the intestinal immune-barrier. The RT-PCR data shown in **Figure 3B** clearly demonstrated that the mRNA expressions of lymphocyte homing receptors $\alpha^4\beta^7$, L-selectin, and LFA-1 in the EN+QH and EN groups was prominently lower compared with the sham-operated group (P<0.01), indicating that lymphocyte homing was repressed induced by gastrectomy. In addition, the mRNA expressions of the lymphocyte homing receptors $\alpha^4\beta^7$, L-selectin, and LFA-1 in the EN+QH group were relatively higher than they were in the EN group, with significance levels of P<0.05, P<0.05, and P< 0.01 respectively, which was consistent with our expectation that Qihuang decoction can improve the mRNA expression of lymphocyte homing receptors and consequently promote the recognition and interaction between lymphocyte homing receptors and ligands after gastrectomy.

Promoted positive molecular expressions and the mRNA expressions of the lymphocyte homing ligands

The positive molecular expressions of the lymphocyte homing ligands MAdCAM-1 and ICAM-1 in PP were measured using immunohis-

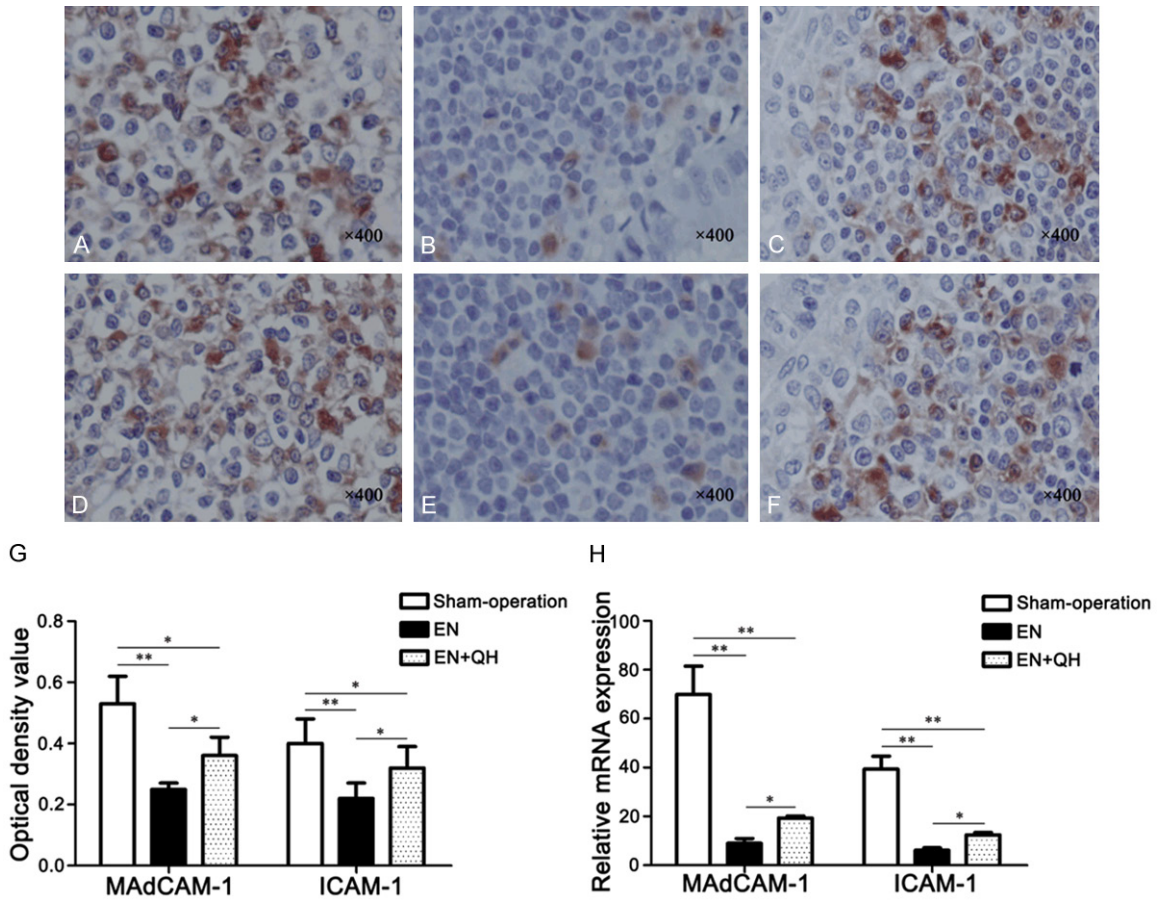

Figure 4. A-C. The MAdCAM-1 expression levels of the sham operation group, the EN group, and the EN+QH group, D-F. The ICAM-1 expression levels of the sham operation group, the EN group, and the EN+QH group. G. The optical density value. H. mRNA expression of lymphocyte homing ligands, *P<0.05, **P<0.01 (n=10).

tochemistry as shown in **Figure 4A-G**. The EN+QH and EN groups showed a significant decrease compared with the sham-operated group. Meanwhile, the positive expressions of the homing ligands MAdCAM-1 and ICAM-1 in the EN+QH group were higher than they were in the EN group, and the difference was significant (P<0.05, P<0.05). As for the mRNA expression of homing ligands MAdCAM-1 and ICAM-1, the RT-PCR data showed that these two values in the EN+QH group were also more increased than they were in the EN group (P<0.05, P<0.05 respectively) as illustrated in **Figure 4H**. These results suggest that both the positive molecular expression and the mRNA expression of the lymphocyte homing ligands MAdCAM-1 and ICAM-1 were promoted by the Qihuang decoction, contributing to the recognition between lymphocyte homing receptors and ligands, which is beneficial for the recovery of the intestinal mucosal barrier.

Discussion

The intestinal tract is the largest immune organ of the human body. The intestinal mucosa behave like a barrier to isolate pathogenic bacteria or cytotoxins from the blood and inner tissue, which is the so-called intestinal mucosal barrier [24, 25]. Lymphocytes in the intestinal mucosa and epithelia undertake the most basic responsibility for the intestinal mucosal barrier [26, 27]. However, naive lymphocytes produced in intestine are not able to exert an immune function. These naive lymphocytes can travel to the Peyer's patches for sensitization [9]. Further development and differentiation in the thymus is important for the immunity activation of the lymphocytes. Therefore, functional lymphocytes in the intestine need to travel from the blood to the specific intestinal mucosa area to provide an effective intestinal immune response [10, 28, 29].

Lymphocyte binding to the endothelium is a requisite first step for the expression of immunity [9]. Indeed, the precise recognition in lymphocyte binding is mainly controlled by the lymphocyte homing receptors ($\alpha^4\beta^7$, L-selectin, and LFA-1) [15, 30, 31] and the lymphocyte homing ligands (MAdCAM-1, ICAM-1) [32-34].

Serious situations like trauma, infection, and surgery may cause the dysfunction of the intestinal mucosal barrier, resulting in enterogenous infections and even systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) [7, 8, 34]. Various methods such as monoclonal antibodies, cytokines, and enteral nutrition intervention have been applied to investigate the molecular mechanisms of lymphocyte homing in order to intervene in immune disorders [17-19]. Nevertheless, traditional Chinese medicine intervention through intestinal instillation combined with a lymphocyte homing analysis after gastrectomy is hardly ever reported to the best of our knowledge. Here, we demonstrate a delicate experiment consisting of a sham operation group, an enteral nutrition group, and a Qihuang decoction group to investigate the influence of Qihuang decoction on lymphocyte homing and intestinal immune-barrier after gastrectomy in rats. Flow cytometry, γ counter measurements, and the immunohistochemistry data showed that the intervention of Qihuang decoction intestinal instillation can remarkably promote the proliferation and activation of homing lymphocytes. The RT-PCR data can also verify that Qihuang decoction is able to facilitate the binding between the lymphocyte homing receptors and ligands by increasing the mRNA expression levels of $\alpha^4\beta^7$, L-selectin, LFA-1, and MAdCAM-1, ICAM-1.

Conclusion

We conclude that the intestinal instillation of Qihuang decoction can increase the homing lymphocyte levels after gastrectomy and promote the proliferation and differentiation of $CD3^+$ T cells and IgA^+ B cells. Meanwhile, the positive molecular expressions and mRNA expressions of both lymphocyte homing receptors ($\alpha^4\beta^7$, L-selectin) and lymphocyte homing ligands (MAdCAM-1, ICAM-1) are also enhanced, thus promoting the sensitization of the lymphocytes and consequently contributing to the improvement of the intestinal immune-barrier after gastrectomy. Our work may provide a good foundation for the intervention and treatment of intestinal mucosal barrier dysfunction based on promoted lymphocyte homing intervened by Qihuang decoction after gastrectomy.

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Disclosure of conflict of interest

None.

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