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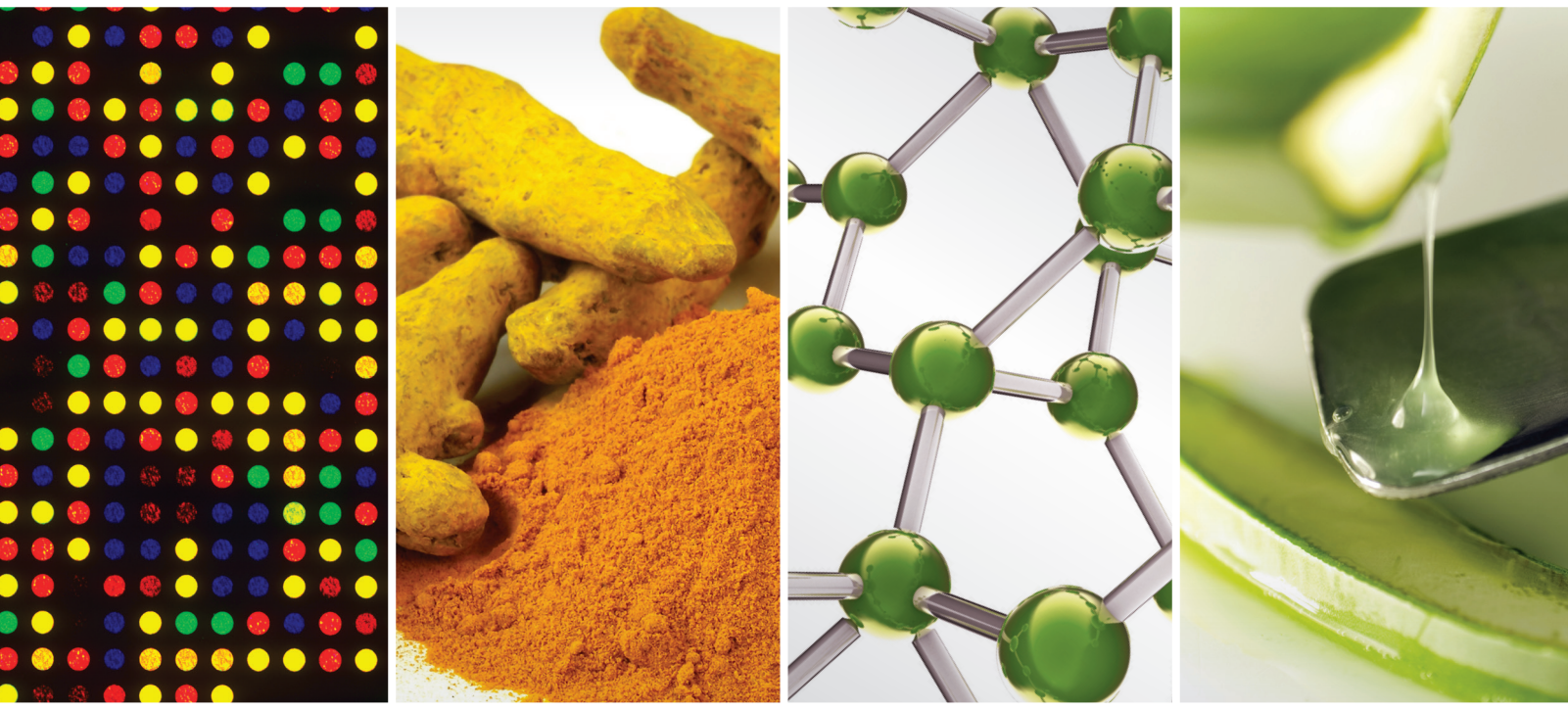
Guest Editors: Fengxia Liang, Edwin L. Cooper, Hua Wang, Xianghong Jing, Juan G. Quispe-Cabanillas, and Tetsuya Kondo



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Editorial Board

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 260620, 2 pages
http://dx.doi.org/10.1155/2015/260620

Editorial

Acupuncture and Immunity

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Received 5 July 2015; Accepted 5 July 2015

As a traditional therapy applied for thousands of years, acupuncture has recently been attracting more and more investigators throughout the world. In the theory of traditional Chinese medicine, it is proposed that acupuncture can strengthen the human body to resist diseases by puncturing needles at certain points. The characteristic that acupuncture enhances resistance is closely related with the immune system, which functions in defense, homeostasis, and surveillance. More and more research has revealed that acupuncture can regulate immunity, for example, to enhance anticancer and antistress immune function and exert anti-inflammation effects. This may be the basis of acupuncture in preventing and treating later diseases. This special issue was developed to stimulate the continuing efforts in promoting the research on acupuncture and immunity.

The acupuncture point ST36 (Zusanli) is widely applied in immune-related diseases. In "Electroacupuncture at Bilateral Zusanli Points (ST36) Protects Intestinal Mucosal Immune Barrier in Sepsis" M. Zhu et al. reported that EA preconditioning at ST36 obviously ameliorated CLP-induced intestinal injury and high permeability and exerted protective effects on intestinal mucosal immune barrier by increasing the concentration of sIgA and the percentage of CD3+, γ/δ, and CD4+ T cells and the ratio of CD4+/CD8+ T cells, which eventually decreased the mortality of sepsis.

"Moxibustion and Acupuncture Ameliorate Crohn's Disease by Regulating the Balance between Th17 and Treg Cells in the Intestinal Mucosa" by C. Zhao et al. provided evidence from a randomized controlled clinical trial that moxibustion and acupuncture regulated the ratio of Th17 and Treg cells in the intestinal mucosa of CD patients and restored the balance between these immune cell subsets, providing the basis for clinical application of treatment for CD.

"Herb-Partitioned Moxibustion Regulates the TLR2/NF-κB Signaling Pathway in a Rat Model of Ulcerative Colitis" by X. Wang et al. assessed the regulation of the TLR2/NF-κB signaling pathway by herb-partitioned moxibustion in the intestinal mucosa of rats with ulcerative colitis (UC). It was reported that herb-partitioned moxibustion modulated the excessive local immune response by inhibiting TLR2 signaling, thereby promoting the repair of damaged colonic mucosa.

"Mediators, Receptors, and Signalling Pathways in the Anti-Inflammatory and Antihyperalgesic Effects of Acupuncture" by J. L. McDonald et al. reviewed both demonstrated and proposed anti-inflammatory effects of acupuncture and analyzed the complex crosstalk between receptors during inflammation to elucidate the mediators and signaling pathways activated by acupuncture. Researches with new advances which demonstrated that acupuncture

activated a novel cholinergic anti-inflammatory pathway and chemokine-mediated proliferation of opioid-containing macrophages in inflamed tissues were also included.

"Immunoregulation on Mice of Low Immunity and Effects on Five Kinds of Human Cancer Cells of *Panax japonicus* Polysaccharide" by Z. Jie et al. investigated the immunoregulative effects of *Panax japonicus* polysaccharide (PJPS) on mice of low immunity. The results indicated that PJPS significantly improved the immune function of mice processed by cyclophosphamide and PJPS did not work on the five kinds of human cancer cells.

In summary, this issue provides different evidence presented by diverse authors covering several topics related to advances in acupuncture for inflammation or immune diseases. As inflammation is the coherent pathophysiologic progress in many kinds of diseases, immune system and response of the human body are influenced in diseases such as cancer; the anti-inflammation effect of acupuncture may be a very important underlying mechanism of acupuncture in treating diseases. Moreover, TCM focuses on the theory that prevention before the onset of the diseases or intervening in an early stage of diseases is much better than treating after the onset. Preconditioning by acupuncture which means stimulation with acupuncture before the onset of diseases has been widely applied from ancient time to present clinical practice, proposing a potential field in preventing diseases. Acupoints selection, combination of the acupoints, or different methods of stimulation of acupuncture may result in quite different effects on immune system. Further research is urgently essential to elucidate the relation between acupuncture and immune responses and what kind of stimulation might induce the best effect.

Of course, the selected topics and papers are not a comprehensive representation of the area of this special issue. Nonetheless, they represent many-faceted evidence that we have the pleasure of sharing with the readers.

Acknowledgments

We would like to express appreciation to the authors for their excellent contributions and patience in assisting us. Finally, the fundamental work of all reviewers on these papers is also very greatly acknowledged.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 938054, 11 pages
http://dx.doi.org/10.1155/2015/938054

Research Article

Moxibustion and Acupuncture Ameliorate Crohn's Disease by Regulating the Balance between Th17 and Treg Cells in the Intestinal Mucosa

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Received 29 December 2014; Accepted 27 April 2015

Academic Editor: Tetsuya Kondo

Previous studies have demonstrated that acupuncture is beneficial to patients with Crohn's disease (CD), but the mechanism underlying its therapeutic effects remains unclear. To identify the mechanism by which acupuncture treats CD, the balance between Th17 and Treg cells was assessed in CD patients. In this study, Ninety-two CD patients were randomly and equally assigned to a treatment group that were treated with herb-partitioned moxibustion and acupuncture or a control group with wheat bran-partitioned moxibustion and superficial acupuncture. The effect of these treatments on Th17 and Treg cells and their related molecular markers in the intestinal mucosa were detected before (week 0) and after (week 12) treatment. The results suggested that the ratio of Th17 and Treg cells was significantly decreased after treatment and that the levels of IL-17 and RORγt in the intestinal mucosa were obviously reduced, while the expression of FOXP3 was increased after treatment in both groups. In the treatment group, the expression of these molecules was more markedly regulated than the control group. In conclusion, moxibustion and acupuncture have been shown to regulate the ratio of Th17 and Treg cells in the intestinal mucosa of CD patients and restore the balance between these immune cell subsets.

1. Introduction

Crohn's disease (CD) is a nonspecific granulomatous inflammatory bowel disease (IBD) with an unknown etiology. The main clinical manifestations are recurrent episodes of abdominal pain, diarrhea, and weight loss and are often complicated with abdominal mass, anal fistula, and intestinal obstruction. The disease is characterized with a long duration and frequent recurrence and cure difficulty. With the disease incidence increasing over years, it has become one of the most common diseases in the intestinal tract [1]. Young adults are the main implicated populations and severely suffer in terms of their health condition and quality of life [2, 3]. Current treatments in modern medicine include aminosalicylates, corticosteroids, and immunomodulators that are effective in the management of the acute symptoms of this disease; however, the side effects accompanied with long periods of usage have limited their long-term use. The biological agent of anti-TNF-α offers an alternative treatment option, but its high price renders it unavailable to many patients. Therefore, new forms of treatment are urgently needed in clinical practice.

Acupuncture and moxibustion are an important component of Traditional Chinese Medicine (TCM) with a history of over 4,000 years and have gradually been accepted by many

countries worldwide. They are widely used in the clinical treatment of various diseases, especially gastrointestinal diseases, such as Crohn's disease [4, 5], ulcerative colitis [6, 7], irritable bowel syndrome [8–10], and functional dyspepsia [11, 12]. Our clinical study has previously demonstrated that moxibustion combined with acupuncture is not only effective in treating CD with an improvement of patients' CDAI scores and their quality of life, and it also reduces CRP levels and increases hemoglobin levels in patients [4]. However, the biological mechanisms underlying the treatment of CD by moxibustion and acupuncture have not been fully elucidated.

Th17 and Treg cells are two recently discovered subsets of T lymphocytes. Research has shown that an imbalance between Th17 and Treg cells is involved in the development of CD [13, 14]. Th17 cells play important roles in mediating inflammatory responses. Treg cells inhibit the proliferation of effector T cells and their reaction to autoantigens, thus controlling the strength of immune responses elicited by effector T cells and limiting the severity of associated tissue damage [15, 16]. Th17 and Treg cells are closely related during differentiation because they share the same precursor population. A balanced production of both cell types is critical to the maintenance of intestinal homeostasis. The purpose of the present study was to determine the anti-inflammatory mechanism of moxibustion and acupuncture in the treatment of CD by assessing the effects of moxibustion and acupuncture on the ratio of Th17 and Treg cells and the expression of key related molecules (forkhead box P3 (FOXP3), retinoid-related orphan receptor gamma (RORγt), and interleukin-17 (IL-17)) in the intestinal mucosa of patients with CD.

2. Materials and Methods

2.1. Study Design

2.1.1. Participants. From January 2010 to April 2013, patients with Crohn's disease (CD) were recruited for this study at the following hospitals: the outpatient clinic for inflammatory bowel disease of Shanghai Institute of Acupuncture and Meridian at Shanghai University of TCM, the Endoscopy Center of Zhongshan Hospital at Fudan University, the Shuguang Hospital at Shanghai University of TCM, and the Yueyang Hospital of Integrated Traditional Chinese and Western Medicine at Shanghai University of TCM. The diagnosis of CD was confirmed in all patients by imaging, endoscopic, and histopathological examinations [4].

Inclusion criteria were as follows: patients with mild to moderate CD (CDAI between 151 and 350), without taking any medication or taking only salicylates and/or prednisone (dose ≤ 15 mg treatment lasting at least one month) and without the use of immunosuppressants or anti-TNF-α biological agents within the past 3 months. Exclusion criteria were as follows: Women who were pregnant or breast-feeding; patients with severe diseases in the heart, brain, liver, kidney, or hematopoietic system; patients with any mental illness.

2.1.2. Interventions. Ninety-two patients with active CD were randomly assigned at a 1 : 1 ratio to a treatment group and a control group. Patients in the treatment group were treated with herb-partitioned moxibustion combined with acupuncture: moxibustion was performed on the Tianshu (ST25, bilateral), Qihai (CV6), and Zhongwan (CV12) acupoints; and acupuncture was performed at the Zusanli (ST36), Shangjuxu (ST37), Sanyinjiao (SP6), Taixi (KI3), Gongsun (SP4), and Taichong (LR3) acupoints. For herb-partitioned moxibustion, the herbal cake contained *Coptis chinensis*, Radix *Aconiti Lateralis*, *Cortex Cinnamomi*, Radix Aucklandiae, *Flos carthami*, *Salvia miltiorrhiza*, and *Angelica sinensis* as the main ingredients. All these herbs were ground into a fine powder, mixed with maltose and warm water to make into a thick paste, and pressed with a mold into herbal cakes with a diameter of 28 mm and thickness of 5 mm. Pure refined moxa sticks that were 16 mm tall with a diameter of 17 mm ("Hanyi," Nanyang, China) were placed on top of the herbal cakes for moxibustion. In each session, two moxa sticks were used per acupoint. Disposable sterile stainless steel needles (0.30 mm in diameter, 40 mm or 25 mm long, "Hwato," Suzhou, China) were used for acupuncture. After local routine disinfection, the needles were directly inserted up to 20–30 mm into the skin to elicit a *de-qi* sensation. The needles were then left in place for 30 minutes. Moxibustion and acupuncture were simultaneously performed once every other day (three times a week) for a total of 36 sessions (12 weeks). Patients in the control group were treated with wheat-bran-partitioned moxibustion combined with superficial needle puncture at nonmeridian, nonacupoint sites. For moxibustion, wheat bran was used instead of herbal powder to make wheat bran cakes of the same size, but the acupoints and the moxibustion method used were the same as the treatment group. The same acupuncture needles were used for superficial needle puncture. Nonmeridian, nonacupoint zones located 1-2 cm away from the acupoints of the treatment group were selected for superficial needle puncture and the same number of sites was inserted with needles. After local routine disinfection, the needles were directly inserted only 1-2 mm into the skin, without eliciting a *de-qi* sensation. These needles were left in place for 30 min. Wheat-bran-partitioned moxibustion and superficial needle puncture were performed at the same time. Total numbers of sessions were the same as in the treatment group.

2.1.3. Sample Collection. Ten patients were randomly selected from each group for the present study. At the time of recruitment (week 0) and at the end of treatment (week 12), these patients received colonoscopic examination and intestinal biopsies were taken from the ileocecal region. Four biopsies were taken each time and stored in formaldehyde or liquid nitrogen for future detection.

2.1.4. Ethical Approval and Trial Registration. This clinical trial was approved by the Ethics Committee at the Yueyang Hospital of Integrated Traditional Chinese and Western Medicine at Shanghai University of TCM. All subjects signed the informed consent forms. This trial was registered at the following website: http://clinicaltrials.gov/ (NCT01697761).

2.2. Experimental Methods

2.2.1. HE Staining. The tissue biopsies were processed in an automated tissue processing machine and then embedded into paraffin. Then 4 μm continuous sections were cut, deparaffinized by two changes of xylene, each lasting 20 min, and then rehydrated through 100%, 95%, 85%, and 75% ethanol, each round lasting 3 min. The sections were stained with hematoxylin for 1 min, differentiated in a 1% hydrochloric acid-ethanol solution (one part of hydrochloric acid with 100 parts of 70% ethanol), and then stained in a 0.5% aqueous solution of eosin for 5 min. The sections were examined and scored under a microscope.

2.2.2. Immunofluorescence Staining. An immunofluorescence double labeling technique was used to determine the ratio of Th17 and Treg cells in the intestinal mucosa. Deparaffinized sections were washed three times in 0.01 M PBS (pH 7.2–7.6) for 5 min each and then heated to 92–98°C for antigen retrieval. Sections were blocked with 10% goat serum for 30 min and then incubated with rabbit anti-human IL-17 and rabbit anti-human FOXP3 (both at 1 : 100, Abcam, U.K.) in a humidified chamber overnight at 4°C. Sections were then incubated with diluted secondary antibodies and mounted with an antifluorescence quenching agent or glycerol: 0.01 M PBS (1 : 1). The sections were photographed under a fluorescent microscope (BX53 Olympus). IL-17 served as the marker for Th17 cells and was labeled with Cy3, and FOXP3 served as the marker for Treg cells and was labeled with FITC. Nuclei were stained by DAPI. Three microscopic fields were randomly chosen from each slide and analyzed with the Image Pro Plus (IPP) analysis system to obtain fluorescence intensity for the positive staining.

2.2.3. Real-Time RT-PCR. Total RNA was extracted from tissues using Trizol (Invitrogen, U.S.) and the RNA content and quality were determined with Nanodrop. Then 4 mg of total RNA was reverse-transcribed into cDNA with a reverse transcription kit (Thermo, U.S.), and 100 ng cDNA was used as template for the real-time PCR reaction. Primers were added at 200 nM into the reactions, and SYBR Green was used for detection. Real-time PCR was run in an ABI7300 instrument (running ABI Prism 7300 SDS Software). The relative expression levels of IL-17, RORγt, and FOXP3 mRNAs are presented as $2^{-\Delta Ct}$ (ΔCt = Ct value of the target gene − Ct reference gene value). Primers are listed in Table 1.

2.2.4. Immunohistochemical Staining. The Envision method was used for immunohistochemistry. Sections were deparaffinized, rehydrated, antigen-retrieved with sodium citrate buffer, and blocked with 20% normal goat serum in a humidified chamber at 37°C for 30 min and then incubated with primary antibodies (mouse anti FOXP3, 1 : 100, Abcam; rabbit anti-IL-17 and rabbit anti-RORγt, 1 : 100, Abcam) at 4°C overnight. Sections were then incubated with HRP-labeled secondary antibodies (rabbit anti-mouse IgG, HRP Conjugated for FOXP3, goat anti-rabbit IgG, HRP Conjugated for IL-17 and RORγt) at 37°C for 60 min, followed by DAB for color development. Sections were then counterstained with hematoxylin, differentiated in a 0.1% hydrochloric acid-ethanol solution, and mounted with a neutral resin. Three microscopic fields were randomly chosen from each slide and analyzed with the IPP image analysis system to obtain integrated optic density (IOD) for the positive staining.

Table 1: Primer sequences used in RT-PCR.

Gene name	Primer	Sequence
IL-17	Forward	5′-TGAAGGCAGGAATCACAATC-3′
	Reverse	5′-CGGTTATGGATGTTCAGGTT-3′
RORγt	Forward	5′-ATGGAGCTCTGCCAGAATGA-3′
	Reverse	5′-TGCGGTTGTCAGCATTGTAG-3′
FOXP3	Forward	5′-CAGCGTGGTTTTTCTTCTCGGTATA-3′
	Reverse	5′-TGGTGAAGTGGACTGACAGAAAAG-3′

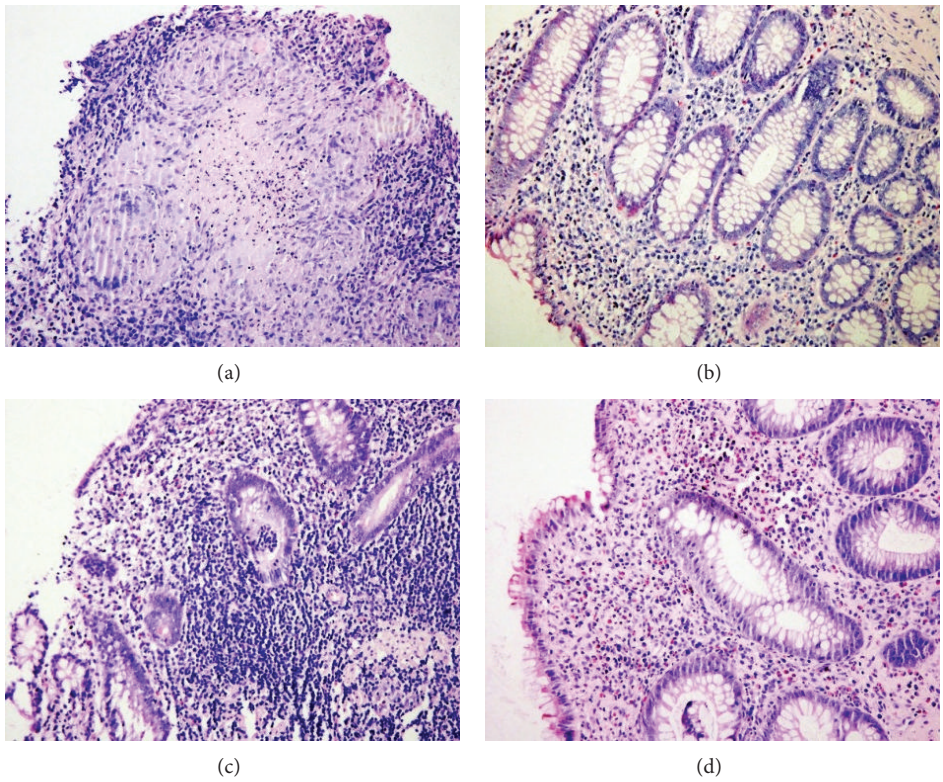
2.3. Statistical Methods.

Statistical analysis was performed using the SPSS13.0 software package. Quantitative data with normal distribution and homogeneous variances are represented as mean ± standard deviation ($\overline{x} \pm s$). Comparisons within groups were conducted with paired t-tests, while comparisons between groups were conducted with two-sample t-tests. Quantitative data that were not normally distributed or showed heterogeneity of variance are represented as median (P25–P75). Comparisons within groups were conducted using paired-sample Wilcoxon signed rank tests, and comparisons between groups were conducted using nonparametric Mann-Whitney U tests. All tests were two sided with significance set to $\alpha = 0.05$, $P < 0.05$ and was considered statistically significant.

3. Results

After the treatments, the CDAI scores of patients in the treatment group ($n = 10$) decreased by 111.15 ± 54.74, while those of patients in the control group ($n = 10$) only decreased by 40.51±31.36. The values decreased between the two groups were significantly different ($P = 0.002$).

3.1. Pathological Changes in the Intestinal Mucosa. Before treatment, the intestinal biopsies from both groups showed pathological characteristics typical of Crohn's disease. A large number of giant, multinucleated cells were present in granulomas, the centers of which contained necrotic cells and infiltrated inflammatory cells. The mucosa epithelium was damaged or missing, and the lamina propria was infiltrated by many lymphocytes. Also visible were structures resembling lymphoid follicles. The intestinal glands were damaged and disorganized, containing necrotic epithelial cells, infiltrated inflammatory cells, and multinucleated giant cells. After the moxibustion and acupuncture treatment, the intestinal mucosal epithelium was intact, the intestinal glands were reorganized, and less inflammatory cells infiltrated. After control treatment, the mucosal epithelium also became intact, but inflammatory cell infiltration was still visible and the glands were less organized than the treatment group (Figure 1).



(a) (b) (c) (d)

Figure 1: HE staining images of the intestinal mucosa from both groups of patients (a) before and (b) after treatment from patients in the treatment group and (c) before and (d) after treatment from patients in the control group (200x).

3.2. Th17 and Treg Cells in the Intestinal Mucosa. Before the treatments, a large number of IL-17 + T cells in the intestinal mucosa infiltrated in both groups, and the IL-17 + T cells were mainly located in the lamina propria. After treatment, there were significantly more FOXP3 + Treg cells in both cases but significantly fewer IL-17 + T cells. Treg cells were localized mainly in the cortex and lamina propria. In both groups of patients, the ratio of Th17/Treg was significantly lower after treatment ($P < 0.01$ and $P < 0.05$). Comparisons between the two groups either before or after treatment showed no significant difference in the Th17/Treg ratio ($P > 0.05$) (Figures 2-3).

3.3. IL-17 Protein and mRNA Expression in the Intestinal Mucosa

3.3.1. IL-17 Protein Expression in the Intestinal Mucosa. IL-17 proteins were mainly found in the lamina propria. IL-17 levels were significantly lower after treatment in both groups ($P < 0.01$ for both). The decrease in IL-17 protein levels was significantly greater in patients in the treatment group ($P < 0.01$) (Figures 4–5(a)).

3.3.2. IL-17 mRNA Expression in the Intestinal Mucosal. IL-17 mRNA levels were significantly lower after treatment in both groups ($P < 0.05$ for both). The decrease in IL-17 mRNA levels was significantly greater in patients in the treatment group ($P < 0.01$) (Figure 5(b)).

3.4. RORγt Protein and mRNA Expression in the Intestinal Mucosa

3.4.1. RORγt Protein Expression in the Intestinal Mucosa. Positive staining for RORγt protein was mainly found in the lamina propria. RORγt protein levels were significantly lower in patients in the treatment group ($P < 0.01$), but these values were not significantly different in patients in the control group ($P > 0.05$). Comparisons between the two groups showed that the treatment group had a significantly greater response in reducing RORγt expression ($P < 0.01$) (Figures 6–7(a)).

3.4.2. RORγt mRNA Expression in the Intestinal Mucosa. In the treatment group, RORγt mRNA levels were significantly decreased after the treatment ($P < 0.05$), but levels were only slightly lower in the control group ($P > 0.05$). Comparisons between the two groups showed that the therapy in the treatment group had a significantly more pronounced effect to downregulate RORγt mRNA expression ($P < 0.01$) (Figures 6–7(a)).

3.5. FOXP3 Protein and mRNA Expression in the Intestinal Mucosa

3.5.1. FOXP3 Protein Expression in the Intestinal Mucosa. FOXP3 protein was mainly detected in the epithelium and lamina propria. In the treatment group, FOXP3 protein levels

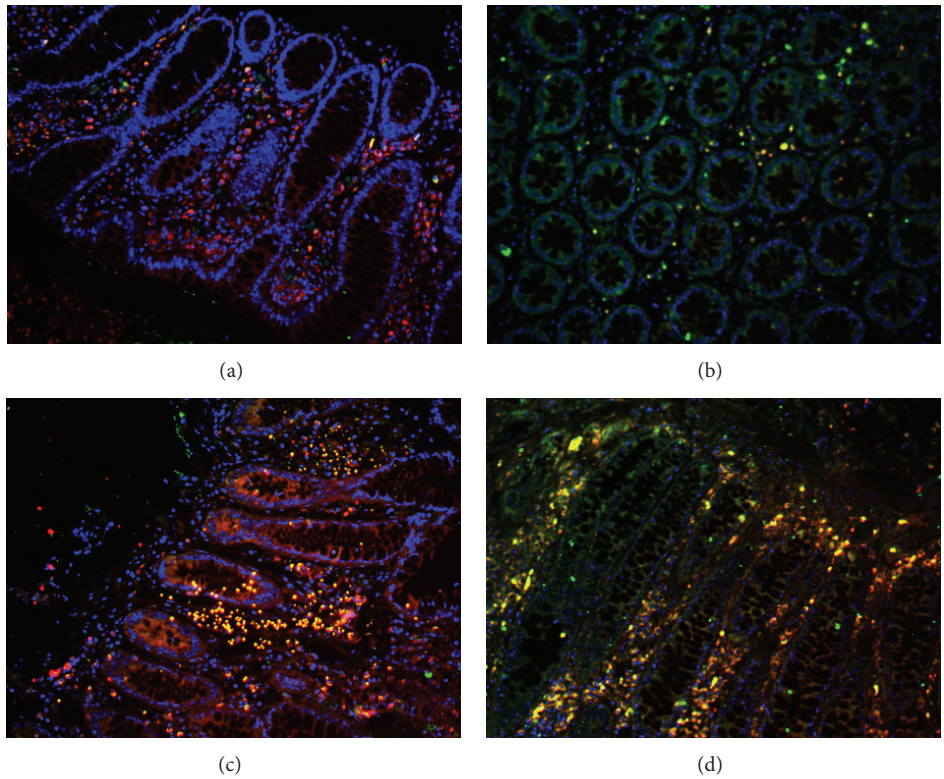


Figure 2: Th17 and Treg cells in the intestinal mucosa. Th17 (red) and Treg (green) cells were detected by immunofluorescence (a) before and (b) after treatment from patients in the treatment group and (c) before and (d) after treatment from patients in the control group (200x).

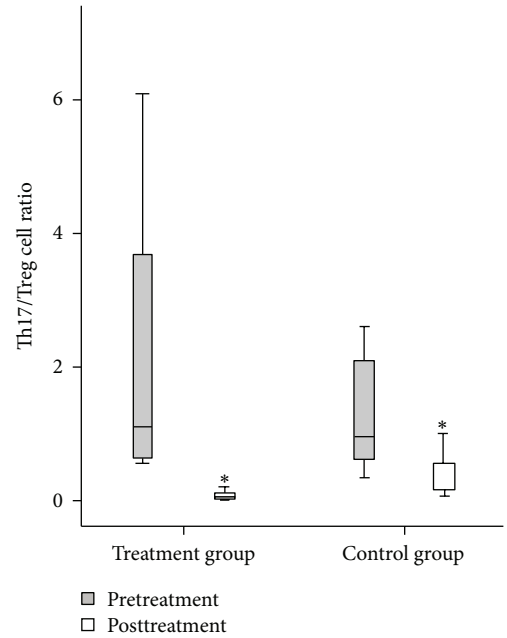


Figure 3: The ratio of Th17/Treg cells in the intestinal mucosa of both groups of patients before and after treatment. Comparison within groups, $^{*}P < 0.05$.

were significantly higher after the treatment ($P < 0.05$) but were not significantly different in the control group ($P > 0.05$). Comparisons between the two groups showed that the therapy in the treatment group has a significantly more pronounced increase in FOXP3 expression ($P < 0.01$) (Figures 8–9(a)).

3.5.2. FOXP3 mRNA Expression in the Intestinal Mucosa. In the treatment group, FOXP3 mRNA levels were significantly higher after the treatment ($P < 0.01$), but the difference was not significant in the control group ($P > 0.05$). Comparisons between the two groups showed that the therapy in the treatment group had a significantly greater effect in upregulating FOXP3 mRNA expression ($P < 0.01$) (Figure 9(b)).

4. Discussion

It has demonstrated that moxibustion and acupuncture are effective and safe in treating CD, but their therapeutic mechanism has not been fully elucidated. The results of the present study showed that moxibustion and acupuncture inhibited the protein and mRNA expression of IL-17 and RORγt but induced the protein and mRNA expression of FOXP3 in the intestinal mucosa of CD patients. Moxibustion and acupuncture restored the balance between intestinal Th17 and Treg cells in CD patients, thus relieving intestinal inflammation in CD patients by regulating the differentiation of these two cell subsets.

The subjects of this study were from a previous randomized, controlled clinical trial [4]. Patients in the treatment group were treated with acupuncture combined with herb-partitioned moxibustion, and patients in the control group

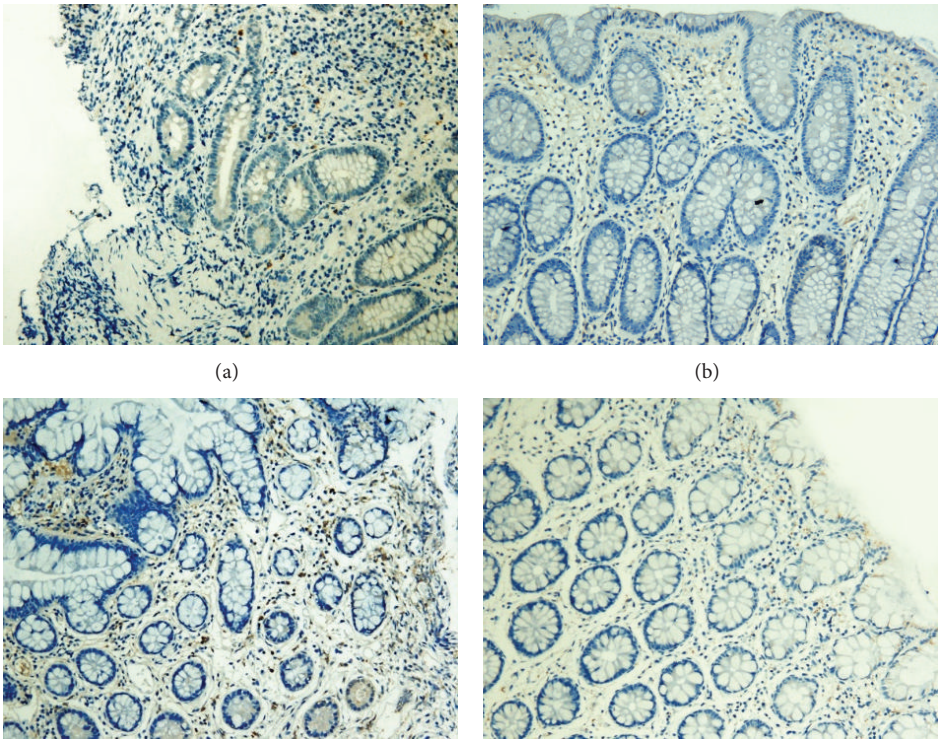


Figure 4: Expression of IL-17 in the intestinal mucosa (a) before and (b) after treatment from patients in the treatment group and (c) before and (d) after treatment from patients in the control group (200x).

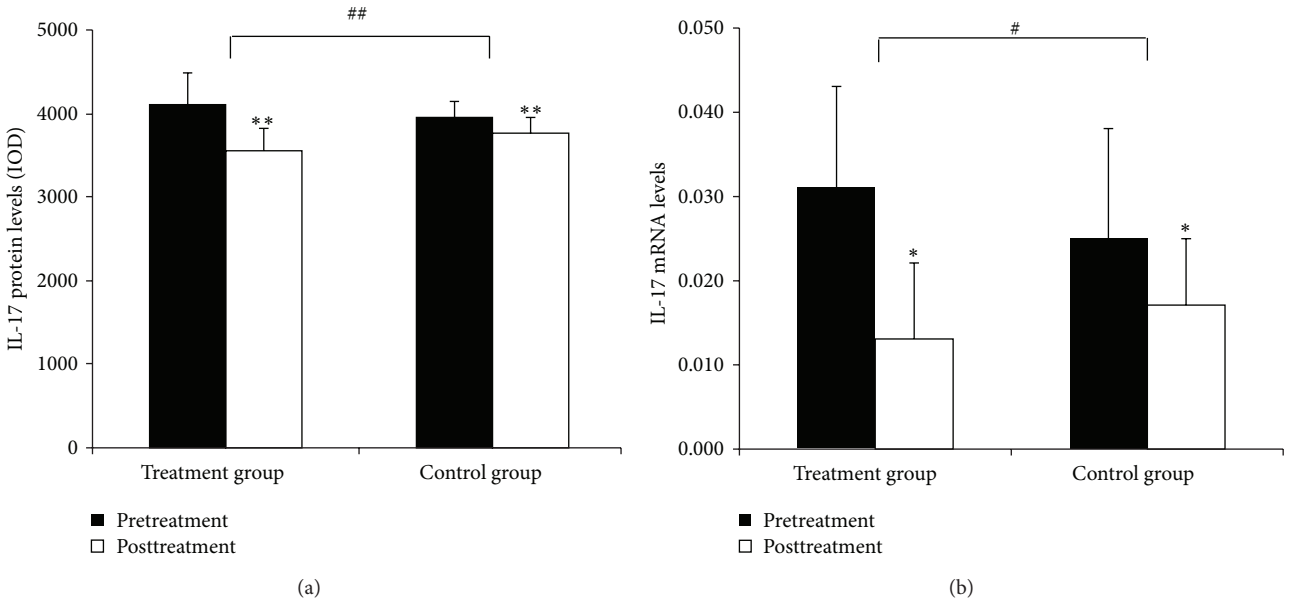


Figure 5: Quantitation of IL-17 mRNA and protein levels in the intestinal mucosa. (a) Levels of IL-17 protein in the intestinal mucosa. (b) Levels of IL-17 mRNA in the intestinal mucosa. Comparison within groups, $^{*}P < 0.05$, $^{**}P < 0.01$; comparison between groups, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

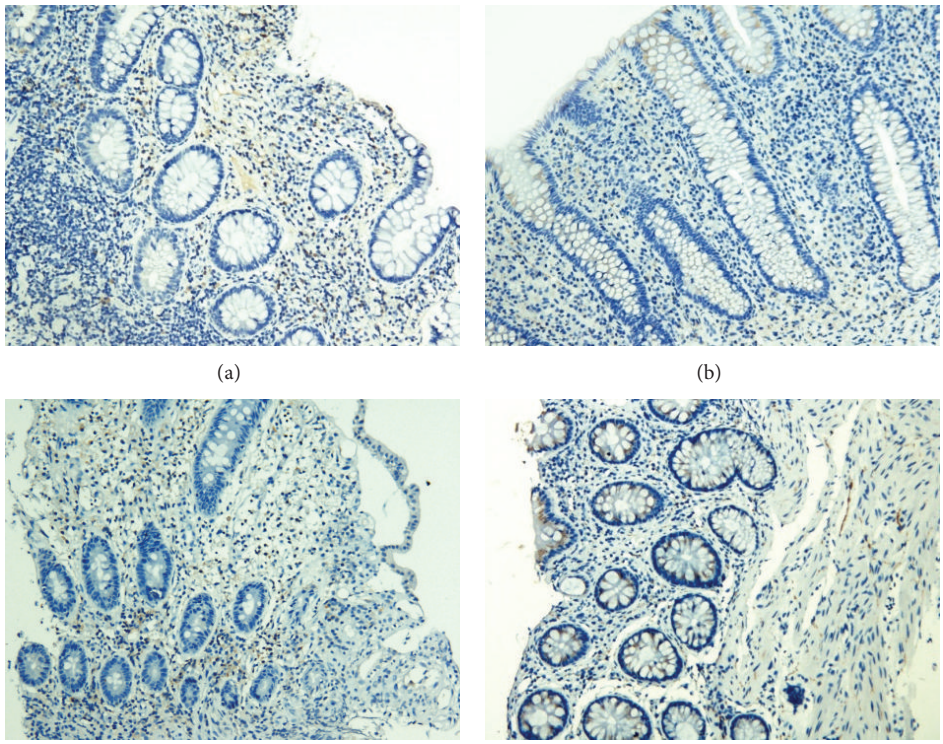


Figure 6: Expression of RORγt in the intestinal mucosa (a) before and (b) after treatment from patients in the treatment group and (c) before and (d) after treatment from patients in the control group (200x).

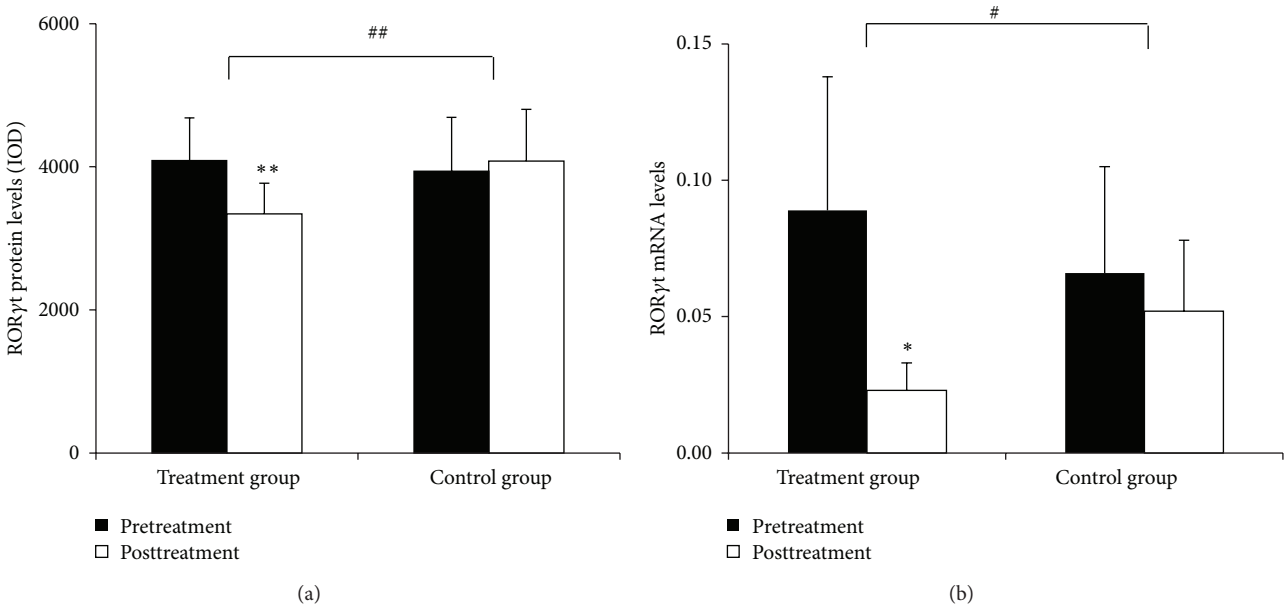


Figure 7: Quantitation of RORγt mRNA and protein levels in the intestinal mucosa. (a) Levels of RORγt protein in the intestinal mucosa. (b) Levels of RORγt mRNA in the intestinal mucosa. Comparison within groups, $^{*}P < 0.05$, $^{**}P < 0.01$; comparison between groups, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

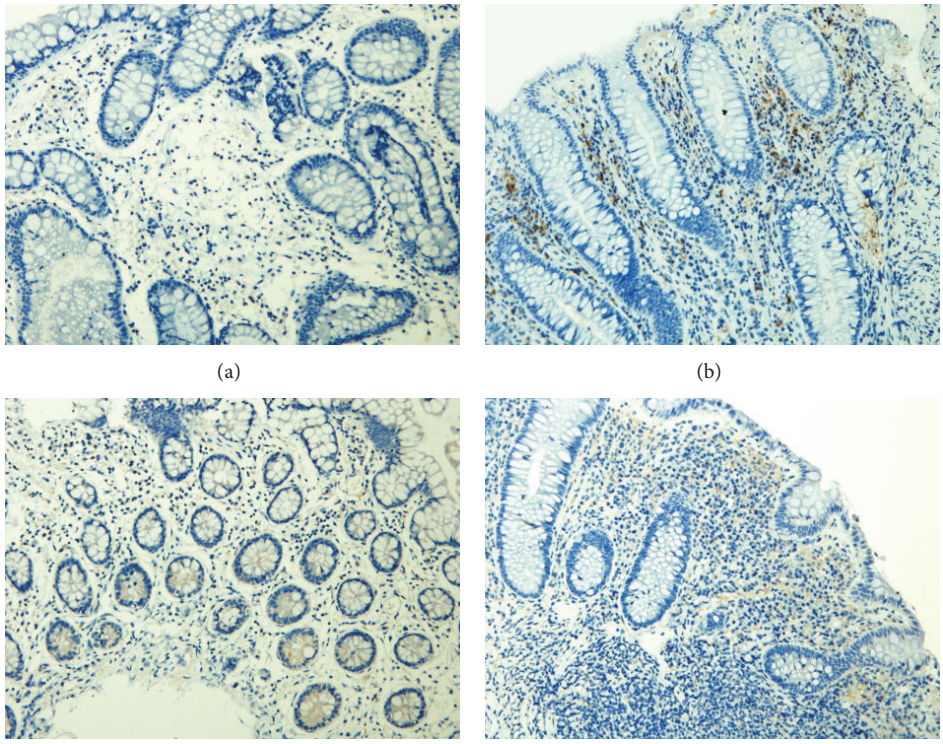


Figure 8: Expression of FOXP3 in the intestinal mucosa (a) before and (b) after treatment from patients in the treatment group and (c) before and (d) after treatment from patients in the control group (200x).

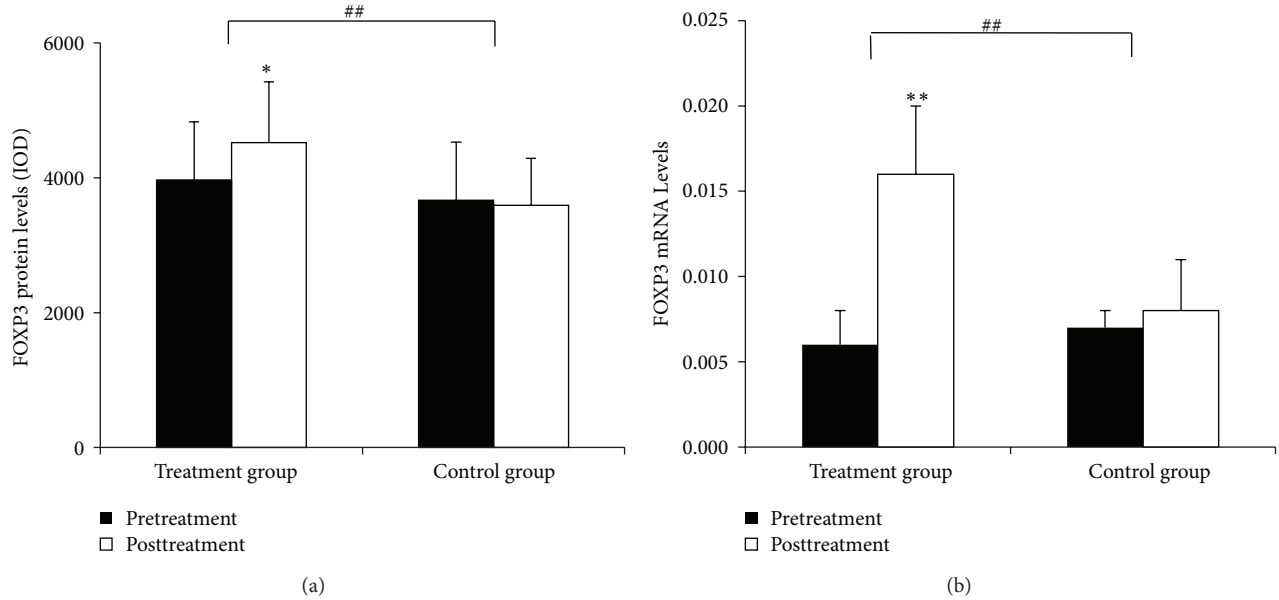


Figure 9: Quantitation of FOXP3 mRNA and protein levels in the intestinal mucosa. (a) Levels of FOXP3 protein in the intestinal mucosa. (b) Levels of FOXP3 mRNA in the intestinal mucosa. Comparison within groups, $^{*}P < 0.05$, $^{**}P < 0.01$; comparison between groups, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

were treated with superficial needle puncture at nonmeridian, nonacupoint sites combined with wheat bran-partitioned moxibustion as the placebo treatment. The results of this trial showed that the moxibustion and acupuncture in the treatment group to be significantly more effective than the control group in relieving the symptoms and intestinal inflammation in CD patients. The moxibustion and acupuncture treatment significantly decreased the CDAI scores of patients and increased their IBDQ scores which is associated with quality of life. In addition, the moxibustion and acupuncture treatment significantly reduced C-reactive protein levels and increased hemoglobin levels of patients. Moxibustion and acupuncture in the treatment group had significantly more pronounced effects than the control group for all these measurements, suggesting that moxibustion and acupuncture have a significant therapeutic effect [4]. Ten patients from each group were randomly selected to undergo colonoscopy and intestinal biopsy, and results showed that the moxibustion and acupuncture treatment outperformed the control treatment in reducing inflammation in the intestinal mucosa. Therefore, it led us to further investigate the mechanism through which moxibustion and acupuncture modulate the inflammation in the intestinal mucosa.

An imbalance between Th17 and Treg cells constituted a key step in the disruption of intestinal homeostasis and is one of the major contributors to the development and progression of CD [17]. The main function of Th17 cells is to secrete IL-17 and other proinflammatory cytokines, which in turn induce the migration of neutrophils towards sites of infection to elicit an inflammatory response. IL-17A can exert a strong proinflammatory effect. It can also enhance cell permeability and promote the production of other proinflammatory cytokines and chemokines. Various types of cells, such as epithelial cells, endothelial cells, and fibroblasts all possess abundant IL-17 receptors (IL-17R) on the cell surface [18, 19]. Research has shown that Th17 cells are the major contributor to inflammation in CD. The intestinal mucosa of CD patients is infiltrated by a large number of Th17 cells. The number of Th17 cells in the intestinal mucosa and the level of IL-17, which is secreted by Th17 cells, in the intestinal mucosa and serum, were significantly higher in patients with CD than in healthy people. They were also higher in patients with acute disease than in patients whose disease is in remission [20, 21]. The level of IL-17A is positively correlated with the severity of CD, while IL-17A deficiency is associated with resistance to colitis development [22], suggesting that IL-17A promotes colitis. RORγt is an important transcription factor regulating Th17 cell differentiation. Th17 cells express inflammatory cytokines, including IL-17A, IL-17F, IL-21, and IL-23, which can initiate innate and adaptive immune responses [17].

Moreover, studies have suggested that a reduction in the number or function of Treg cells might be a major cause of the pathogenesis of Crohn's disease [23, 24]. Tregs can suppress intestinal mucosal inflammation induced by innate or acquired immunity. FOXP3 and IL-2 promote the differentiation of Treg cells. Reduced numbers of Treg cells and deficiency in their function can cause damage in the intestinal mucosa, resulting in CD [25]. Tregs suppress Th effector cell activity by secreting IL-10 and/or TGF-β1, and IL-10 inhibits the proliferation of Th cells and their production of inflammatory factors. A clinical study showed that the number of Tregs in the peripheral blood and intestinal mucosa was significantly lower in CD patients than in healthy subjects, but the function of Tregs was not altered [26]. FOXP3 is specifically expressed in Treg cells. Its expression was significantly lower in CD patients than in healthy subjects and also lower in patients with acute diseases than those in remission [27]. Although Treg cells can dampen intestinal mucosal inflammation effectively in patients with CD, in the presence of IL-6 and/or IL-23, Tregs can differentiate into Th17 cells, which then accelerate the inflammatory process [28]. It was shown that the ratios of Treg/Th17 cells in the peripheral blood and intestinal mucosa were significantly lower in CD patients [29]. As such, restoring the balance between these cells is essential for the treatment of intestinal inflammation in CD.

The present study showed that moxibustion and acupuncture can regulate and restore the balance between Th17 and Treg cells in intestinal mucosa of patients with CD. The moxibustion and acupuncture treatment reduced the number of Th17 cells and inhibited the expression of Th17-related molecules IL-17 and RORγt in the intestinal mucosa. It also increased the number of Treg cells and the expression of Treg-specific transcription factor FOXP3, thus restoring the ratio of the two cell types. The control treatment also reduced intestinal mucosal inflammation in CD patients to some extent, but it was not as effective as the moxibustion and acupuncture treatment. The regulatory effect of moxibustion and acupuncture on the balance between Th17 and Treg cells has been demonstrated in animal models of autoimmune encephalitis [30, 31]. Therefore, we speculated the following: (1) moxibustion and acupuncture directly regulate the differentiation of naive T cells in intestinal mucosa of patients with CD, which makes T cells differentiate towards the direction of Treg cells, resulting in an increase in the number of Treg cells, whereas the number of Th17 cells is correspondingly reduced; (2) moxibustion and acupuncture inhibit IL-17 and RORγt expression at the protein and mRNA levels and promote the expression of FOXP3 in the intestinal mucosa, so as to regulate native T cell differentiation into Treg cells; (3) moxibustion and acupuncture inhibit Th17 cell proliferation and the immune response and thus weaken the activity of effector T cells and control the intensity of the immune response through the stimulation of the expression of Treg cell and transcription factor FOXP3 in intestinal mucosa. As a result, the ratio of proinflammatory Th17 cells and anti-inflammatory Treg cells and their related molecules in intestinal mucosa tend to be restored and intestinal mucosal inflammation is alleviated.

Previous animal studies of our research group have also indicated that moxibustion and acupuncture can reduce intestinal inflammation in CD rats and promote the recovery of intestinal function. It is reported that moxibustion can decrease the levels of TNF-a and TNFR1, which are abnormally induced in CD rat colons, inhibit excessive apoptosis of colonic epithelial cells, and restore the colonic epithelial barrier [32, 33]. Furthermore, electronic acupuncture can regulate inflammatory signaling pathways in the intestine and immune-related signaling pathways in the spleen in rats with TNBS-induced colitis [34].

One of the limitations of this study is that the sample size is small. These results should be further validated with a larger sample size. Although the patients were followed up for 12 weeks after the treatment to assess their CDAI scores, no intestinal mucosa biopsies were taken to evaluate the long-term effect of moxibustion and acupuncture on the balance between Th17 and Treg cells. Future studies should combine clinical evaluation with microscopic and molecular studies of the intestinal mucosa to further elucidate the long-term efficacy of moxibustion and acupuncture.

5. Conclusion

In conclusion, this study shows that moxibustion and acupuncture can reduce the number of Th17 cells and down-regulate the expression of Th17-related molecules IL-17 and RORγt and increase the number of Treg cells and upregulate the expression of Treg transcription factor FOXP3 in the intestinal mucosa of CD patients. This suggests that moxibustion and acupuncture relieve intestinal inflammation in CD patients by restoring the balance between Th17 and Treg cells, providing the basis for clinical application of treatment for CD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Chen Zhao, Chunhui Bao, and Jing Li contributed equally to this work.

Acknowledgments

The authors thank Dr. Weifeng Chen at Zhongshan Hospital affiliated with Fudan University and Dr. Xin Guan at Shuguang Hospital affiliated with Shanghai University of TCM for their assistant in recruiting patients and thank Dr. Congqi Dai at Fudan University for the advice and paper revision. This work was supported by National Basic Research Program of China (973 program, no. 2009CB522900), The Ministry of Education Program for New Century Excellent Talents (no. NCET-13-0907), Shanghai Municipal Health Bureau (no. 20124028), and Shanghai top clinical medical center of Acupuncture, Moxibustion and Tuina.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 639412, 7 pages
http://dx.doi.org/10.1155/2015/639412

Research Article

Electroacupuncture at Bilateral Zusanli Points (ST36) Protects Intestinal Mucosal Immune Barrier in Sepsis

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Received 1 December 2014; Revised 20 March 2015; Accepted 10 April 2015

Academic Editor: Tetsuya Kondo

Sepsis results in high morbidity and mortality. Immunomodulation strategies could be an adjunctive therapy to treat sepsis. Acupuncture has also been used widely for many years in China to treat sepsis. However, the underlying mechanisms are not well-defined. We demonstrated here that EA preconditioning at ST36 obviously ameliorated CLP-induced intestinal injury and high permeability and reduced the mortality of CLP-induced sepsis rats. Moreover, electroacupuncture (EA) pretreatment exerted protective effects on intestinal mucosal immune barrier by increasing the concentration of sIgA and the percentage of CD3+, γ/δ, and CD4+ T cells and the ratio of CD4+/CD8+ T cells. Although EA at ST36 treatments immediately after closing the abdomen in the CLP procedure with low-frequency or high-frequency could not reduce the mortality of CLP-induced sepsis in rats, these EA treatments could also significantly improve intestinal injury index in rats with sepsis and obviously protected intestinal mucosal immune barrier. In conclusion, our findings demonstrated that EA at ST36 could improve intestinal mucosal immune barrier in sepsis induced by CLP, while the precise mechanism underlying the effects needs to be further elucidated.

1. Introduction

Sepsis frequently occurs after trauma, burns, hemorrhage, or abdominal surgery. It can progress to multiorgan failure (MOF). Although new treatment algorithms focusing on rapid administration of broad spectrum antibiotics and aggressive restoration of tissue oxygen delivery have led to decreases in mortality, the death rate is still high (~30%) [1–4]. Effective and safe treatments for this disease are desperately needed.

Sepsis is a complex immune syndrome characterized by an imbalance between pro- and anti-inflammatory mediators systemically released in high amounts (cytokine storm) in response to an infection, while the precise pathophysiologic mechanisms underlying the development of multiorgan failure (MOF) remain elusive [5, 6]. The gut is often described as the motor of MOF because the loss of its integrity is a critical comorbidity factor for patients after HS, as well as trauma, surgery, sepsis, and burn injuries [7, 8]. Therefore, the intestinal mucosal immune system has recently emerged as a potential target in sepsis treatment.

Traditional Chinese medicine (TCM, including Chinese materia medica, acupuncture, and physiotherapy) always attached importance to gastrointestinal function, believing that the stomach and spleen provide the material basis for the acquired constitution. Acupuncture has also been used widely for many years in China to treat sepsis. Recently, acupuncture has been described as a complementary and alternative medicine (CAM) in which filiform needles are inserted at specific points on the body, called acupoints, which can subsequently be stimulated in various ways, such as electroacupuncture (EA) [9]. It has been reported that in rats with CLP sepsis models EA-ST36 reduces serum TNF levels through VN- and catecholamine-dependent mechanisms. Indeed, the treatment with EA at ST36 can decrease levels of proinflammatory cytokine expression (TNF-α, IL-1, and IL-6) in a lipopolysaccharide-induced model of acute

nephritis, collagen-induced arthritis mouse model, ulcerative colitis rat model, carrageenan-induced mouse model of inflammation, and cerebral ischemia-reperfusion injured rats. Recent studies have shown that the mechanism underlying the effects of this treatment is related to the suppression of the TLR4/NF-κB signaling pathway [10–13].

Although the recent focus has been on the function of EA at ST36, there are a lot of problems to be solved and whether EA at ST36 protects gut barrier is uncertain. The object of this study, based on the rat model of sepsis induced by CLP, was to observe the effects of EA at ST36 on the intestinal mucosal immune barrier.

2. Materials and Methods

2.1. Animals. Male Sprague Dawley (SD) rats (body weight, 180–220 g) were obtained from the Zhejiang province Experimental Animal Center and housed in the laboratory animal center of Zhejiang Chinese Medical University at 22°C with a 12-hour light/dark cycle. All animals used in the study were housed and cared for in accordance with the Chinese Pharmacological Society Guidelines for Animal Use. The work was approved by the Committee on the Ethics of Animal Experiments of the Zhejiang Chinese Medical University (permit number: 2012-0049). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Experimental Sepsis Model by CLP. The rats were subjected to CLP as previously described. Briefly, under aseptic conditions, a 3-cm midline laparotomy was performed to allow the exposure of the cecum and adjoining intestine. The cecum was tightly ligated with a 2.0-silk suture at its base, below the ileocecal valve, and was perforated twice with an 18-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the puncture site. The cecum was then returned to the peritoneal cavity and the laparotomy was closed with 3.0-silk sutures. Sham-operated animals underwent the same surgical procedure although the cecum was neither ligated nor punctured. Saline (3 mL/100 g) was administered to all rats intraperitoneally at the end of the procedure. All animals were returned to their cages with free access to food and water.

2.3. Acupuncture Treatment Procedure. Two pairs of stainless steel needles (diameter, 0.3 mm; length, 30 mm (Suzhou Medical Supplies Co., Jiangsu, China)) were inserted perpendicularly at a depth of 6 mm into the bilateral Zusanli acupoints (ST36), located 5 mm below and lateral to the anterior tubercle of the tibia. EA stimulation was applied at both bilateral ST36 acupoints, and both output leads from the Programmable Electro-Acupuncture Stimulator (HANS, LH202H, Huawei Co., Beijing, China) were connected to the handles of both needles inserted at ST36 acupoints. EA was applied for 30 min, with an intensity of 2 mA and 2–100 Hz.

2.4. Experimental Groups and Protocol. The five groups of animals used in the present study were (1) the sham-operated group (sham, $n = 20$), which underwent a laparotomy; (2) the sepsis group (sepsis, $n = 20$), which underwent CLP; (3) the low-frequency EA group (sepsis + low-frequency EA, $n = 20$), which underwent ST36 acupuncture immediately after closing the abdomen in the CLP procedure and 24 hrs later; (4) the high-frequency EA group (sepsis + high-frequency EA, $n = 20$), which underwent ST36 acupuncture immediately after closing the abdomen in the CLP procedure and 6 hrs, 12 hrs, 18 hrs, and 24 hrs later; and (5) EA preconditioning group (EA + sepsis, $n = 20$), which underwent CLP immediately prior to the application of five days of ST36 acupuncture, once a day.

The rats were kept at a constant environmental temperature of 37°C to maintain body heat following the procedures. At 36 h after the CLP, the rats were reanesthetized, then their abdomens were opened, and ileum was removed for the determination of intestinal mucosal tissue sIgA levels, flow cytometry assay, and histomorphological determination.

2.5. Histological Examination. The fixed intestinal mucosal tissue was cut into 3-mm thickness blocks. The tissue blocks were embedded in paraffin and cut into 4 μm slices. After being deparaffinized using xylene and ethanol dilutions and rehydration, the sections were stained with hematoxylin and eosin (H&E, Bogoo, Shanghai, CN) to examine the tissue structure, inflammatory cell infiltration, and necrosis.

2.6. sIgA Measurement in Intestinal Mucosa and D-Lactose Measurement in Plasma. Intestinal mucosal tissue sIgA level and D-Lactose measurement in plasma were determined by using ELISA Kit (Boster biological Inc., Wuhan, China) following the manufacturer's protocol.

2.7. Flow Cytometry Assay. Follicle-free mucosa destined for mononuclear cells (MC) isolation was cut into smaller pieces of approximately 1 mm^2. These mucosa pieces were incubated separately for 30 min at 37°C in calcium- and magnesium-free Hanks's balanced salt solution, including 1 mM ethylenediamine tetra-acetic acid (Sigma-Aldrich, Shanghai, China) to remove both epithelium and intraepithelial lymphocytes. After washing, the follicle-free mucosal tissue was disrupted mechanically into smaller pieces of approximately 1-2 mm^3 and incubated in RPMI-1640 medium supplemented with 30 mM HEPES, 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamicin, and 2.5 μg/mL amphotericin (all from MCE, Shanghai, China), 0.1% collagenase type IV (Sigma-Aldrich, Shanghai, China), 0.05% DNase I (Roche Diagnostics, Shanghai, China), and 1 μL/mL 2-mercaptoethanol (Sigma-Aldrich, Shanghai, China) at 37°C in humidified 5% CO_2 atmosphere for 1.5~2 hrs with continuous agitation and vigorous vortex every 15 min. Remaining tissue aggregates were removed by a 70 μm nylon cell strainer. The resulting cell suspension was centrifuged at 500 g for 25 min in a 30% isotonic Percoll solution (Borunlaite Sci & Tech Co., Ltd., Beijing, China). The supernatant containing epithelial cells and debris was discarded; the cell pellet was washed and resuspended in RPMI-1640 medium. The MC fraction additionally underwent Ficoll

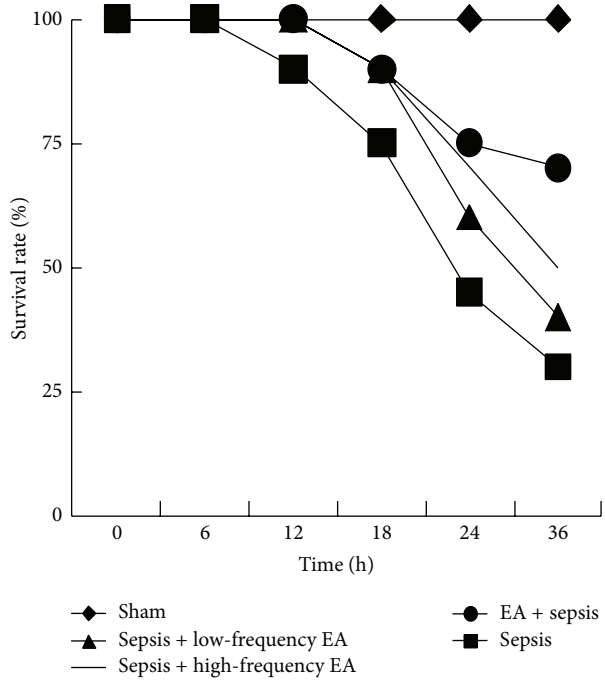

Figure 1: EA preconditioning therapy improved the survival rate in a rat model of sepsis induced by CLP. The percentage survival in 72 hours after surgery is shown.

(Borunlaite Sci & Tech Co., Ltd., Beijing, China) density centrifugation to remove erythrocytes. More than 96% of the isolated cell population was MC.

For cytofluorometric analyses cells were preincubated in PBS and stained for 30 min at 4°C using saturating concentrations of the following antibodies: CD3-FITC+γ/δ T-PE and CD4-FITC+CD8-PE antibody (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Cells were analyzed in a Becton-Dickinson LSRII cytometer using FACS Diva software (Becton Dickinson).

3. Result

3.1. Survival Study. Survival rate of animals was determined. In sepsis, treatment and prevention groups showed significantly lower survival rate compared to sham group. Although low-frequency EA and high-frequency EA treatment groups had more survival rate than sepsis group (50% and 37% versus 26%), the difference between sepsis and these groups was not significant. In EA + sepsis group, EA preconditioning therapy improved the survival rate significantly (70%) (Figure 1).

3.2. EA at ST36 Ameliorates CLP-Induced Intestinal Injury. The degree of intestinal injury sepsis was evaluated in CLP rats treated with or without EA at ST36. As shown in Figure 2(a), histological analysis showed that the ileum from sham mice had the normal architecture of the intestinal epithelium and wall, while CLP induced severe edema and sloughing of the villous tips, as well as infiltration of inflammatory cells into the mucosa. Semiquantitative analysis of histological samples of ileum showed that the intestinal injury score in the septic mice was significantly increased compared with that in the sham group. Administration of EA at ST36 significantly decreased CLP-induced intestinal injury (Figure 2(b)).

We also detected the circulating D-Lactose that can be considered an indirect indication of intestinal permeability. As shown in Figure 2(c), the concentration of circulating D-Lactose was increased significantly in the septic mice. Treatment with high-frequency EA and EA pretreatment could significantly inhibit the increase of circulating D-Lactose induced by CLP.

3.3. Changes of sIgA Content in Intestinal Mucosa Cells. The sIgA concentrations in intestinal mucosa reduced significantly 36 hrs after CLP was developed, while the intestinal mucosal tissue sIgA levels in high-frequency EA group and EA pretreatment group were increased significantly (Figure 3).

3.4. Percentage of CD3+, CD4+, and CD8+ T Lymphocytes in Intestinal Mucosa. As shown in Figure 4, compared with sham group, CD3+, γ/δ, CD4+, and CD4+/CD8+ T lymphocytes were significantly decreased from $(80.75 \pm 10.24)\%$, $(18.64 \pm 7.73)\%$, $(24.59 \pm 6.60)\%$, and (1.89 ± 0.52) to $(38.38 \pm 10.90)\%$, $(7.62 \pm 1.79)\%$, $(7.95 \pm 2.95)\%$, and (0.97 ± 0.67) in the sepsis group. Low-frequency EA could increase the percentage of CD3+, γ/δ, and CD4+ T cells as compared with model group. EA preconditioning significantly increased the percentage of CD3+, γ/δ, and CD4+ T cells and the ratio of

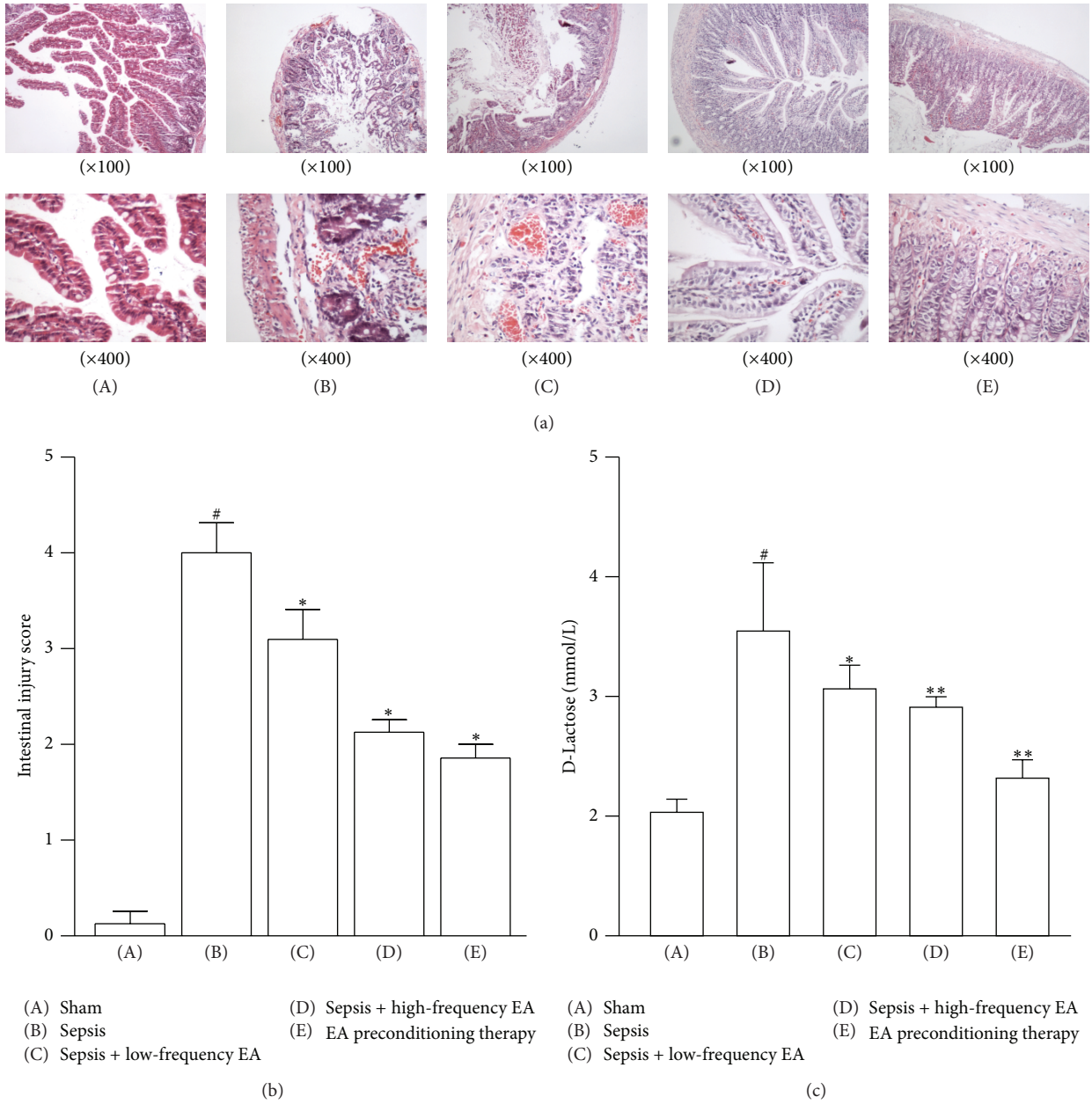

Figure 2: EA at ST36 ameliorates CLP-induced intestinal injury. (a) Ileums were harvested 36 h after CLP for histopathologic examination using H&E staining. Representative images from five animals per group were shown. (b) Semiquantitative analysis of histological samples of ileum showed that EA at ST36 significantly decreased CLP-induced intestinal injury. (c) Effects of EA on the concentration of D-Lactose in the serum. Data were presented as means ± SD ($n = 5$) and $^{\#}p < 0.01$, $^{*}p < 0.05$, and $^{**}p < 0.01$ difference with sham or sepsis group.

CD4+/CD8+ T cells to (77.08 ± 14.43)%, (20.33 ± 4.84)%, (20.14 ± 2.94)%, and (1.43 ± 0.15). However, no difference of the percentage of CD8+ T cells was observed between five groups.

4. Discussion

In the present studies, we showed that the intestinal mucosal immune barrier was seriously damaged in a rat model of sepsis induced by CLP. EA preconditioning at ST36 obviously ameliorated CLP-induced intestinal injury and high permeability and exerted protective effects on intestinal mucosal immune barrier; EA preconditioning significantly increased the percentage of CD3+, γ/δ, and CD4+ T cells and the ratio of CD4+/CD8+ T cells and ultimately reduced the mortality of CLP-induced sepsis in rats. Although EA treatments at ST36 with low-frequency and high-frequency could not reduce the mortality of CLP-induced sepsis in rats, these EA treatments could also significantly improve intestinal injury

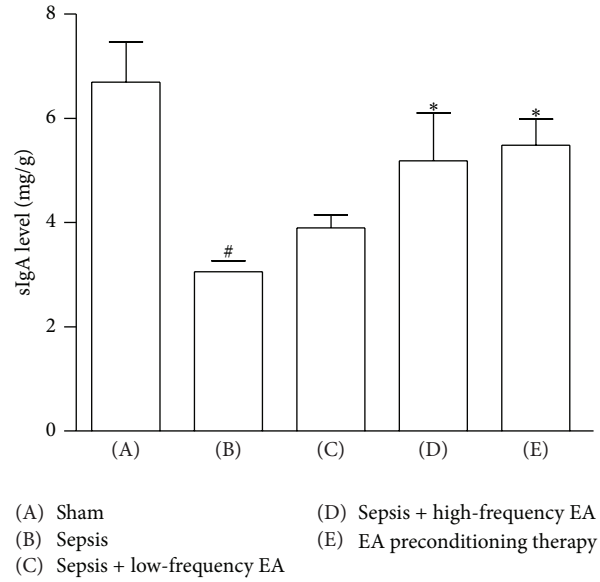

Figure 3: Changes of sIgA content in intestinal mucosa cells. Data were presented as means ± SD ($n = 5$) and $^{\#}p < 0.01$ and $^{*}p < 0.05$ difference with sham or sepsis group.

index in rats with sepsis and obviously protected intestinal mucosal immune barrier.

The gastrointestinal tract is an organ of digestion and absorption. In recent years, the gastrointestinal tract has assumed more importance in the management of the septic patient in the intensive care unit [14–16]. It is now recognized that the small intestine and colon make important contributions to the maintenance of hypermetabolism in sepsis. Owing to the increased intestinal permeability with gut barrier injury, the bacteria and lipopolysaccharide (LPS) can enter the systemic circulation through the portal vein and the mesenteric lymph and result in sepsis and multiple organ dysfunction syndromes (MODS) [17]. Therefore, the intestinal tract is regarded as "initiator" of MODS and supporting general immune function and maintaining the structure and function of the gastrointestinal tract were possible therapeutic strategies for sepsis [14, 18].

Based on semiquantitative histological examination and the mucosal damage index, we found that the ileum from the mice of sham group had the normal architecture of the intestinal epithelium and wall, while CLP induced severe edema and sloughing of the villous tips, as well as infiltration of inflammatory cells into the mucosa. The intestinal injury score in the septic mice was significantly increased. Administration of EA at ST36 significantly decreased CLP-induced intestinal injury. It suggested that EA at ST36 improved the restitution and mechanical barriers of intestinal mucosa in CLP-induced sepsis. Moreover, plasma D-lactate, produced by intestinal bacteria, was developed as a biomarker of intestinal high permeability [19]. Here, we showed that the concentration of circulating D-Lactose was increased significantly in the septic mice. Treatment with high-frequency EA and EA pretreatment could significantly inhibit the increase of circulating D-Lactose induced by CLP. It indicated that EA could protect the intestinal mucosa epithelial cells and maintain the integrity of gut mucosal barrier.

The gut mucosal barrier comprises both immunological and nonimmunological protective components, with the former being divided into local and systemic components and the latter comprising mechanical and chemical barriers. The sIgA content and percentage of T lymphocytes in intestinal mucosa is important to local intestinal mucosal immune barrier [20]. In this study, both of high-frequency EA treatment and EA preconditioning could increase the intestinal mucosal sIgA concentration. Furthermore, EA preconditioning significantly increased the percentage of CD3+, γ/δ, and CD4+ T cells and the ratio of CD4+/CD8+ T cells.

These results supported that EA treatments at ST36 with low-frequency, high-frequency, and preconditioning can protect the intestinal barrier to different degrees, among which EA preconditioning exerts positive effects on immune barrier and eventually decreases the mortality of sepsis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by Zhejiang Provincial Major Project "Traditional Chinese Medicine in Prevention and Treatment of Major Diseases" (no. 2012ZGG001), Zhejiang Provincial Outstanding Young Talent Program of Traditional Chinese Medicine (no. 2013ZQ013), and Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents.

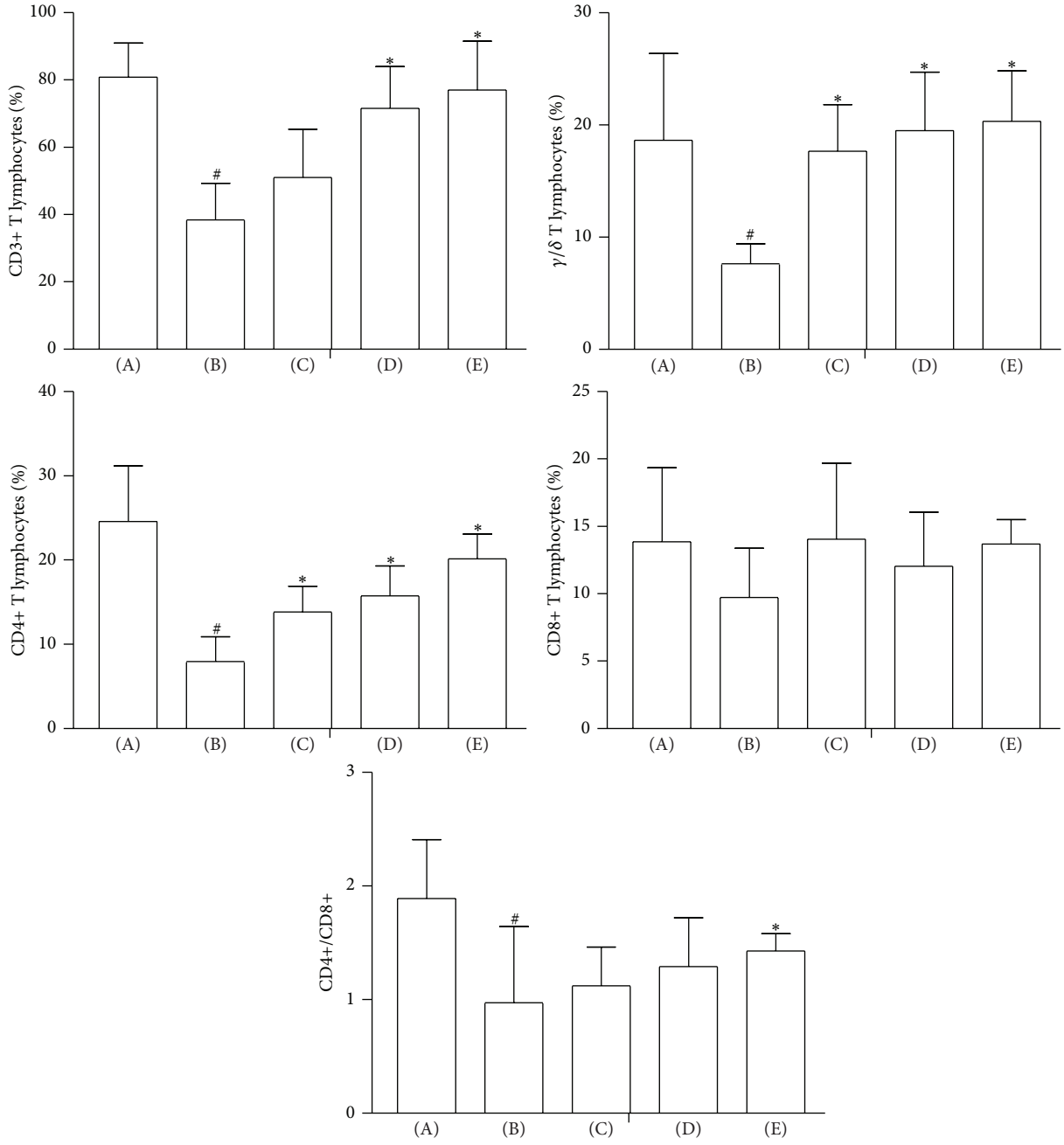

Figure 4: Effects of EA on percentage of CD3+, CD4+, and CD8+ T lymphocytes in intestinal mucosa. Data were presented as means ± SD ($n = 5$) and $^{\#}p < 0.01$ and $^{*}p < 0.05$ difference with sham or sepsis group.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 839697, 9 pages
http://dx.doi.org/10.1155/2015/839697

Research Article

Immunoregulation on Mice of Low Immunity and Effects on Five Kinds of Human Cancer Cells of *Panax japonicus* Polysaccharide

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Received 25 December 2014; Revised 9 April 2015; Accepted 16 April 2015

Academic Editor: Jairo Kennup Bastos

The goal of this study is to investigate the immunoregulative effects of *Panax japonicus* polysaccharide (PJPS) on mice of low immunity. An orthogonal experiment was designed to determine the best extraction process for PJPS. By the tests of macrophages swallow chicken red blood cells, Delayed-type hypersensitivity (DTH), and serum hemolysin value, we studied the immune adjustment ability of PJPS. MTT was employed to detect the effects of different concentrations of PJPS, respectively, in 24 h, 48 h, and 72 h on five kinds of human cancer cells. The results show that the best extraction process for PJPS was as follows: ratio of solvent consumption to raw material 40, extraction temperature 100°C, re-extracted two times, each extraction time 4 hours. PJPS can significantly improve the immune function of mice processed by cyclophosphamide and PJPS did not work on the above five cancer cells.

1. Introduction

Rhizoma Panacis Japonici, a special resource of Tujia nation in Hunan Province, belongs to Araliaceae plant and its dry rootstalk could be used as medicine. It is warm, sweet, and bitter. In Chinese medicine, being a natural plant used as both medicine and food, it has both the nutrition effects of ginseng and can activate blood circulation to dissipate blood stasis [1]. Several studies have indicated its pharmacological effects on the central nervous system [2, 3], digestive system [4], cardiovascular system [5, 6], immune system [7], inflammation [8–10], fatigue [11, 12], tumor [13, 14], and so on.

It contains various saponins, polysaccharides, and some active substances like amino acids, volatile oil [15], and so forth. Saponin is the most abundant material of chemical composition in the *Rhizoma Panacis Japonici*. At present, there are numerous studies concerning the chemical structure and pharmacological activity of saponins. However, the studies of *Panax japonicus* polysaccharide (PJPS) are limited. It may be partly due to the complex chemical structure and ambiguous mechanism of action. Ohtani et al. [16] reported that PJPS extracted from *Rhizoma Panacis Japonici* can activate the reticuloendothelial system, which suggests that PJPS can improve activity of macrophages in the reticuloendothelial system and play a role in immune regulation via this way. Other results suggest that the PJPS can promote immune organ weight index of immunosuppressed mice, significantly improve the spleen lymphocyte proliferation, promote serum IgM level following exposure to chicken red blood cells and QSH reaction, and promote natural killer's cells activity. It has better immune enhancement effect and recovers immune system of immunosuppressed mice [17].

2. Experimental

2.1. Extraction Process of PJPS. Firstly, single factor experiments were studied. *Rhizoma Panacis Japonici* powder, which has been sifted by 50 meshes, was extracted by hot water. The extraction rate of PJPS as index, the number of extractions, solvent ratio, extraction time, and extraction temperature were investigated as the single factors. Secondly, on the basis of single factor experiments, the orthogonal experiment was designed with the extraction rate of PJPS as index to optimize and determine the best extraction process. At last, validated experiment was made.

Selecting anhydrous glucose as the standard, this paper investigated the determination conditions and methodology of anthrone sulfuric acid method [18]. Consider

$$\text{the extraction rate of PJPS (\%)} = \frac{\text{the quality of PJPS extracted from } \textit{Rhizoma Panacis Japonici}}{\text{the quality of PJPS of } \textit{Rhizoma Panacis Japonici}} \times 100\%. \quad (1)$$

2.2. Separations and Refining of PJPS. The aqueous extract, which was acquired according to the optimum extraction method of PJPS, was concentrated to a certain volume; then the concentrate was obtained. The concentrate was further precipitated by adding anhydrous ethanol to its concentration of 80% ethanol. It was refrigerated over 12 h. The sedimentation was separated by being centrifuged for 15 min (5000 rpm). Then, the sedimentation was washed twice with appropriate amount of ethanol and was freeze-dried into constant weight. Through these progresses, the insoluble substance was crude PJPS.

Crude PJPS solution, which was made up at the mass concentration of 1%, was added to 1.2% of papain solution (dynamic unit 400 μ/mg). Under the optimum pH value 6.0 and the optimum enzymatic hydrolysis temperature 40°C, enzymatic hydrolysis was reacted for 2 h. Enzyme deactivation was done at 90°C. This solution was centrifuged for 10 min (5000 rpm) to get rid of denatured protein, then the supernatant was obtained. The supernatant was precipitated for 12 h by the addition of ethanol to a final concentration of 80% (v/v), and centrifuged for 15 min (5000 rpm). The sedimentation was washed twice with appropriate amount of ethanol and was freeze-dried into constant weight. Through these progresses, the insoluble substance was refined PJPS.

The concentration of crude PJPS was 1%, and then 1.2% papain (dynamic unit 400 μ/mg) was added. In its optimum pH value of 6.0, the optimum enzymolysis temperature is 40°C, enzymatic hydrolysis was reacted for 2 h. Enzyme deactivation was done at 90°C. This solution was centrifuged for 10 min (5000 rpm) to get rid of denatured protein, then the supernatant was obtained. The supernatant was precipitated for 12 h by the addition of ethanol to a final concentration of 80% (v/v), and centrifuged for 15 min (5000 rpm). Sedimentation was washed twice with appropriate amount of ethanol and freeze-dried to constant weight. Through these progresses, the insoluble substance was refined PJPS.

2.3. The Immune Regulating Effect of PJPS in Mice with Immunosuppression

2.3.1. Phagocytic Function of Peritoneal Macrophage [19]. According to the different treatment, 90 Kunming mice were randomly divided into normal control group, cyclophosphamide (CY) model control group, CY + lentinan group (positive control group), and CY + low, CY + middle, and CY + high dosage group by weight and gender. Every group has 15 mice. The drug groups were pretreated intragastrically with 100 mg/kg, 200 mg/kg, and 400 mg/kg PJPS. Positive control group was pretreated intragastrically with 150 mg/kg lentinan. Normal control group and cyclophosphamide model group were pretreated with saline at the same volume. All mice were separately administered once every day for 10 days for each group. From 7th day, cyclophosphamide was injected into enterocelia with 100 mg/kg for 3 days. At 11th day, mice were injected by 20% chicken red blood cell with 0.5 mL/only. Mice were executed after 35 minutes and cut open along the middle line of the abdominal wall skin, in which 2 mL physiological saline was injected by peritoneal injection. The experiment kneaded the mice's abdomen for 1 minute. Then abdominal lotion was sucked out, and then 1 drop of abdominal lotion was stilled on the glass slide and was observed by Wright-giemsa stain. Macrophages were counted 100 on each piece in the Oil immersion lens, and then the number of macrophages which swallowed chicken red blood cells and the number of chicken red blood cells which were swallowed were counted. The percentage of phagocytosis and phagocytic index was calculated. Consider

$$\text{the percentage of phagocytosis} = \frac{\text{the number of macrophages which swallow chicken red blood cells}}{\text{100 macrophages}} \times 100\%,$$
$$\text{phagocytic index} = \frac{\text{the number of chicken red blood cells which are swallowed by macrophages}}{\text{100 macrophages}} \times 100\%. \quad (2)$$

At the same time in the count, the degree of chicken red blood cells to be digested determines macrophage phagocytosis and digestion ability and also determines the standards of phagocytosis, which is usually divided into four levels:

Class I: not be digested. Engulfed Chicken red blood cells are complete, cytoplasm is pale red or pale yellow with green, and cell nucleus is light purple.

Class II: mild digestion. Cytoplasm is chartreuse; cell nucleus is pycnosis and purplish blue.

Class III: serious digestion. Cytoplasm is dyed lightly and displays light grey.

Class IV: completely digestion. A physalides similar to the size of chicken red blood cell can be seen in macrophages.

2.3.2. Carbon Granular Clearance Ability. Group and administration were done like Section 2.3.1. On the 10th day after 2 h behind oral administration, each mouse was injected with India ink 0.1 mL/10 g that was diluted 5 times with saline via coccygeal vein. After 5 min and 15 min behind the injection, 60 μL of blood plasma, respectively, was taken from the orbital venous plexus with Vacuum blood tube which was wet by heparin solution in advance in EP tube containing 6 mL 0.1% sodium carbonate solution. The EP tube was shaken well. Then their OD values were measured at 680 nm wavelength with the reagent blank tube zero. At last, mice were dislocated and executed and the immunologic effects were measured by clearance rate K of charcoal particles, phagocytic index α, and thymus and spleen index.

2.3.3. Hemolysin Production Level. Group and administration were done like Section 2.3.1. From the fifth day, all groups were injected with 20% chicken red blood cell (CRBC) suspension of 0.2 mL. After the last treatment, the mice were killed for serum (2000 rpm, 10 min). Serum was diluted 500 times with saline water, to which 10% CRBC suspension of 0.5 mL and diluted guinea pig serum (complement) with 9 times saline water were added. By contrast, blank tube was added to saline 1 mL instead of mice serum liquid. Each tube in 37°C water bath is heated for 10 min and then is inserted in ice water bath for 10 min to terminate the reaction. After cooling, the tube was centrifuged at 2000 rpm for 10 min. Levels of serum hemolytic optical densities were measured in all groups. Results were analyzed by SPSS13.0 to compare differences between OD_{540} values of the groups.

2.3.4. Delayed-Type Hypersensitivity (DTH) Induced by 2,4-Dinitrochlorobenzene (DNCB). Group and administration were like Section 2.3.1. From 4th day, cyclophosphamide was injected into enterocelia with 100 mg/kg once.

(1) Hypersensitive Response. On 5th day, barium sulfide liquid was applied to the abdomen for unhairing. 50 μL of 5% DNCB fluid by pipetting gun was spread on the surface of depilatory parts to cause hypersensitive response.

(2) Checking Allergies. On 10th day, 10 μL of 1% DNCB solution was applied to mice in order to attack both sides of the left ear. At the same time, both sides of the right ear were coated with the same amount of acetone sesame oil as comparison. The mice were killed after 24 hours. The right and left ears were cut, which were punched 8 mm round hole. The wafers were weighed by a scale.

(3) Results Analysis. Comparing the weight of the different groups mice's left auricular and right auricular, experimenter analyzed the average weight difference by SPSS13.0.

2.3.5. MTT Assay. Five kinds of cancer cells were chosen as the research object; they are human lung cancer cells HTB182, human colon cancer cells SW480, human kidney cancer cells HEK293, human nasopharyngeal carcinoma cell 5–8 *F*, and human liver cancer cell HepG2. Briefly, the cancer cells were plated in 96-well plate at a density of 1000–10000 cells/well in their respective medium. The cells were then incubated at 37°C in a 5% CO_2 environment for 24 h. The experiment was divided into normal control group, negative control group, positive control group (DDP), and PJPS different dosage groups. Every group had 6 wells. After 24 hours, the normal control group and the negative control group were joined with the culture medium without drugs and the positive control group was joined with the culture medium containing 5~10 μg/mL DDP (cis-Dichlorodiammineplatinum(II); each cell had its cisplatin sensitivity and actual operation of each cell should choose a suitable concentration). The final concentration of PJPS different dosage groups was, respectively, 50 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL, and 800 μg/mL and was, respectively, incubated at 37°C in a 5% CO_2 environment for 24 h, 48 h, and 72 h. After the designated time period, 20 μL of 3-(4.5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide was added to each well and the plates were incubated at 37°C for additional 4 h. The formazan crystals formed in the wells were dissolved in 150 μL DMSO. The absorbance was measured at 490 nm using ELISA. The OD490 values were analyzed with SPSS13.0 statistical analysis and were compared with the difference.

3. Results

On the basis of the results of single factor experiments, designed L_9 (3^4) orthogonal test of using the extraction rate of PJPS as index and using amount of solvent, extraction time, and extraction times as factors aimed to determine the rational extraction process. Analysis table of orthogonal experiment results is shown in Table 1 and variance analysis is shown in Table 2.

The range in Table 1 shows that the order of the influence factors on extraction rate of PJPS is C (extraction temperature) > A (solvent ratio) > B (time). The results of analysis of variance in Table 2 showed that the solvent ratio and extraction temperature have a statistic significant influence on extraction rate of PJPS while extraction time on the influence of extraction rate of PJPS has no statistical significance. Through K values of other factors and orthogonal experiment result, we can find that the best extraction technology of PJPS is $A_3B_2C_3$. The best extraction process for PJPS is demonstrated as follows: the solvent consumption was 40 times, the steeping time was 40 min, and the extraction temperature was 100°C, re-extracted at two times and each extraction time is 4 hours.

Table 1: Results of orthogonal test for extraction technique of PJPS (%).

Number	A	B	C	D	Extraction rate of PJPS (%)
1	1	1	1	1	65.66
2	1	2	2	2	73.68
3	1	3	3	3	86.55
4	2	1	2	3	75.36
5	2	2	3	1	93.21
6	2	3	1	2	68.23
7	3	1	3	2	97.82
8	3	2	1	3	79.97
9	3	3	2	1	77.44
$K1$	75.297	79.613	71.287	78.757	
$K2$	78.933	82.287	75.480	79.910	
$K3$	85.063	77.393	92.527	80.627	
R	9.766	4.894	21.240	1.870	

Table 2: Analysis of variance.

Factors	Sum of squares	DOF	F	F critical value	Significance
A	146.190	2	27.371	19.000	*
B	36.020	2	6.744	19.000	
C	759.310	2	142.166	19.000	*
Error	5.341	2			

Note. $^{*}P < 0.05$.

Table 3: Results of validation experiments.

Sample	1	2	3	Average	RSD (%)
Extraction rate (%)	97.99	98.35	98.73	98.36	0.38

3.1. Validation Experiments. Table 3 shows that extraction rates of PJPS were 97.99%, 98.35%, and 97.73%, RSD was 0.38%, and the average extraction rate reached 98.36%. It shows that the extraction technology is stable and reliable in gaining high extraction rate.

3.2. Macrophage Cell Swallowed the Chicken Red Blood Cells. The morphological changes of macrophages during the process, in which microphages swallowed chicken erythrocytes, were observed by oil immersion lens. Figures 1, 2, and 3 are several typical cases. The macrophage phagocytic functions in the experiment of peritoneal macrophage phagocytizing chicken red blood cell are showed in Table 4.

In comparison with the control group, phagocytic rate and phagocytic index in CY group were significantly reduced. The results showed that CY could effectively decrease the mouse's peritoneal macrophage phagocytosis. Compared with CY group, phagocytic rate and phagocytic index in positive drug group were significantly enhanced ($P < 0.01$). The phagocytic rate and phagocytic index in middle (200 mg·kg^{-1}) and high dose (400 mg·kg^{-1}) of PJPS groups were promoted greatly compared to the CY group ($P < 0.05$), which indicated that the middle and high dose of PJPS could recover phagocytosis function of immunosuppression mice caused by CY. Lentinan group had no significant differences compared to the high dose group of PJPS.

Table 4: The influence of PJPS on peritoneal macrophage phagocytizing chicken red blood cell.

Groups	Phagocytic rate (%)	Phagocytic index (%)
Normal group	16.36 ± 0.81	19.64 ± 0.93
CY group	$9.00 \pm 0.49^{**}$	$10.31 \pm 0.44^{**}$
Lentinan group	$14.31 \pm 0.66^{*\#\#}$	$16.92 \pm 0.64^{**\#\#}$
PJPS low dosage group	$9.07 \pm 0.50^{**\triangle\triangle}$	$10.54 \pm 0.49^{**\triangle\triangle}$
PJPS middle dosage group	$10.85 \pm 0.63^{**\#\triangle\triangle}$	$12.38 \pm 0.79^{**\#\triangle\triangle}$
PJPS high dosage group	$13.76 \pm 0.74^{**\#\#}$	$15.92 \pm 0.74^{**\#\#}$

Note: values represent mean ± SEM; $n = 15$; significance as per Student's t-test compared with normal control group; $^{*}P < 0.05$; $^{**}P < 0.01$, compared with CY group; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$, compared with lentinan group; $^{\triangle\triangle}P < 0.01$.

3.3. Carbon Clearance Test. In comparison with the normal group, carbon clearance index K and Phagocytic index α in CY group were significantly reduced. The results showed that CY could effectively decrease the mouse's peritoneal macrophage phagocytosis. In addition, high dose

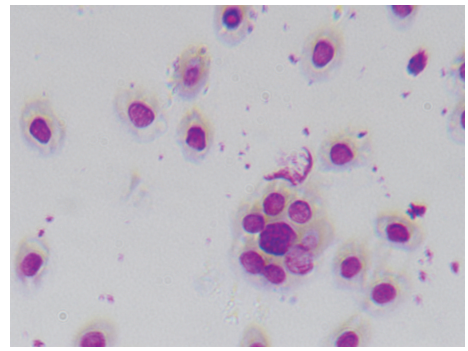

Figure 1: Macrophages adsorbed multiple chicken red blood cells.

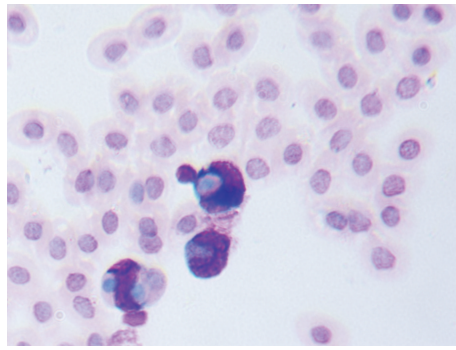

Figure 2: Macrophages swallowed multiple chicken red blood cells.

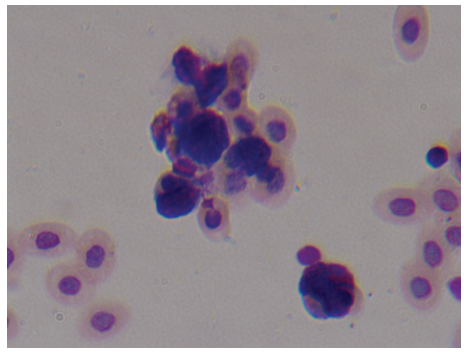

Figure 3: Macrophages adsorbed and swallowed multiple chicken red blood cells.

($400\,mg{\cdot}kg^{-1}$) of PJPS group had no significance compared with normal group, which indicated that PJPS could make the mouse's phagocytosis function return back to normal. Compared with CY group, carbon clearance index K in positive drug group and high dose ($400\,mg{\cdot}kg^{-1}$) of PJPS groups were significantly enhanced ($P < 0.05$). Lentinan group ($P < 0.01$) and middle ($200\,mg{\cdot}kg^{-1}$) ($P < 0.05$) and high dose ($400\,mg{\cdot}kg^{-1}$) PJPS groups ($P < 0.01$) were promoted greatly compared to the CY group, which indicated that lentinan group and the middle and high dose of PJPS could recover the phagocytosis function of immunosuppression mice caused by CY. The influences of PJPS on the carbon clearance index $K^{1/2}$ and Phagocytic index α were showed in the Table 5.

Table 5: The influence of PJPS on the carbon clearance index K and Phagocytic index α.

Groups	Carbon clearance index $K^{1/2}$	Phagocytic index α
Normal group	0.146 ± 0.026	4.170 ± 0.464
CY group	$0.059 \pm 0.013^{**}$	$2.071 \pm 0.334^{**}$
Lentinan group	$0.099 \pm 0.011^{*\#}$	$3.264 \pm 0.219^{*\#\#}$
PJPS low dosage group	$0.084 \pm 0.013^{**}$	$2.817 \pm 0.306^{**}$
PJPS middle dosage group	$0.096 \pm 0.006^{*}$	$3.111 \pm 0.145^{*\#}$
PJPS high dosage group	$0.101 \pm 0.008^{*\#}$	$3.379 \pm 0.221^{\#\#}$

Note: values represent mean ± SEM; $n = 15$; significance as per Student's t-test compared with normal control group; $^{*}P < 0.05$; $^{**}P < 0.01$, compared with CY group; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

3.4. Delayed-Type Hypersensitivity (DTH) Induced by DNCB. In comparison with the normal group, auricle swelling degree in CY group and all dosage groups of PJPS were significantly enhanced. Compared with CY group, lentinan group has statistical significance ($P < 0.01$). In comparison with the normal group, the spleen index and thymus index were decreased significantly in CY group ($P < 0.01$). The influences of PJPS on the auricle swelling degree and visceral index were showed in the Table 6. It showed that the immune suppression model that were induced via intraperitoneal injection of CY was established successfully. Compared with CY group, the spleen index in lentinan group and PJPS high dosage group were increased significantly ($P < 0.05$). The thymus index in both lentinan group ($P < 0.01$) and high dose group of PJPS ($P < 0.05$) was increased significantly compared to the CY group. Lentinan group had no significant differences compared to the high dose group of PJPS.

3.5. Hemolysin Production Level. By comparing the difference of each OD_{540} values in Table 7, it can be found that OD_{540} values of groups were significantly lower than

Table 6: The influence of PJPS on the auricle swelling degree and visceral index.

Groups	Auricle swelling degree (mg)	Thymus index (mg/g)	Spleen index (mg/g)
Normal group	3.08 ± 0.47	3.20 ± 0.21	6.04 ± 0.44
CY group	$5.31 \pm 0.66^{**}$	$1.77 \pm 0.13^{**}$	$3.67 \pm 0.35^{**}$
Lentinan group	$3.08 \pm 0.42^{\#\#}$	$2.54 \pm 0.11^{**\#\#}$	$5.15 \pm 0.34^{*\#\#}$
PJPS low dosage group	$5.07 \pm 0.47^{*\triangle}$	$2.06 \pm 0.23^{**\triangle}$	$3.86 \pm 0.38^{**\triangle}$
PJPS middle dosage group	$4.62 \pm 0.46^{*\triangle}$	$2.17 \pm 0.18^{**}$	$4.09 \pm 0.36^{**}$
PJPS high dosage group	$4.46 \pm 0.66^{*\triangle}$	$2.28 \pm 0.15^{**\#}$	$4.72 \pm 0.31^{**\#}$

Note: values represent mean ± SEM; $n = 15$; significance as per Student's t-test compared with normal control group; $^{*}P < 0.05$; $^{**}P < 0.01$, compared with CY group; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$, compared with lentinan group; $^{\triangle}P < 0.05$.

Table 7: The influence of PJPS on the OD_{540} value.

Groups	OD_{540} value
Normal group	0.108 ± 0.001
CY group	$0.090 \pm 0.001^{**}$
Lentinan group	$0.099 \pm 0.003^{**\#\#}$
PJPS low dosage group	$0.098 \pm 0.003^{**\#\#}$
PJPS middle dosage group	$0.098 \pm 0.001^{**\#\#}$
PJPS high dosage group	$0.103 \pm 0.001^{**\#\#\triangle}$

Note: values represent mean ± SEM; $n = 15$; significance as per Student's t-test compared with normal control group; $^{*}P < 0.05$; $^{**}P < 0.01$, compared with CY group; $^{\#\#}P < 0.01$, compared with lentinan group; $^{\triangle}P < 0.05$.

normal control group. CY group, Lentinan group, low dose group, and middle dose group compared with normal control group also had extremely significant difference ($P < 0.01$). Compared with CY group, OD_{540} values of PJPS different dosage groups were significantly higher than the CY group ($P < 0.01$), and with the increase of the dose of PJPS, OD_{540} values had a tendency to increase but had no statistical difference between PJPS different dosage groups. OD_{540} value of the high dose group was obviously higher than that of lentinan group ($P < 0.05$).

3.6. The Influence of PJPS on Proliferation of the Five Kinds of Human Cancer Cells in Tables 8, 9, 10, 11, and 12. The results showed that the OD value of DDP group in 24 h, 48 h, and 72 h was lower than that of negative control group ($P < 0.05$), but all different doses of PJPS groups have no significant differences compared with negative control group. The result showed that PJPS had no obvious proliferation inhibition for five kinds of cancer cells.

4. Discussions

The orthogonal test was used to optimize the extraction conditions of polysaccharides from the rhizomes of *Panax japonicus* and elevate the extraction rate of PJPS. In the result, the best extraction process of PJPS is demonstrated as follows: the solvent consumption was 40 times, the steeping time was 40 min, the extraction temperature was 100°C, re-extraction at two times and each extraction time is 4 hours. All of PJPS were heteropolysaccharide, and polysaccharides with different ethanol concentration consist of different components. Polysaccharides contain pyranose ring, and the sugar composition analysis showed that PJPS are composed of arabinose, glucose, and galactose and the content of arabinose increased with the increasing of ethanol concentration [20, 21]. In activity analysis, polysaccharides with different ethanol concentration display different levels in activity, and polysaccharides with higher ethanol concentration have stronger recovery effect on DNA impaired [22].

As is well known, chemotherapy treatments for malignant tumour have strong adverse reactions; meanwhile myelosuppression and immunosuppression are especially severe and common. These toxic and side effects cause numerous patients to get infected and die of serious infectious diseases [23]. Polysaccharides have activity in regulation of body's immunity, which draw the attentions of domestic and international scholars, and some polysaccharides or extracts mainly containing polysaccharides have been further taken to clinical assessment in humans, such as polysaccharides from mushrooms. At present, the anticancer properties of polysaccharides have been shown to be primarily mediated via three approaches of direct cytotoxicity, immunoenhancement, and synergistic effects in combination with anticancer drugs. Meanwhile, the synergistic effects are known to be mediated by enhancing the sensitivity of tumour and elevating immune response to treatments [24]. Our experiments indicate that PJPS have effects on resisting immunosuppression caused by CY, such as improving macrophage phagocytosis and boosting the level of IgM in plasma. Some researches indicated that PJPS have the haematopoietic effect, repair impaired DNA, and have antioxidant activities [20, 22, 23, 25, 26]. Even though MTT assay indicated that PJPS do not have directly obvious effects on proliferation of lung cancer cells HTB182, colon cancer cells SW480, kidney cancer cells HEK293, nasopharyngeal carcinoma cella (NPC) 5–8 *F*, and human liver cancer cells HepG2, we can utilize haematopoietic effect and immunoenhancement of PJPS to resist myelosuppression and immunosuppression of chemotherapy; in other words, we can use PJPS in combination with anticancer drugs in clinic. Through this way, the risk of infection and myelosuppression may be decreased, as well as adverse effects, psychological and economical decline of using antibiotics; consequently PJPS in combination with chemotherapy may reduce mortality of cancer patients. However, this hypothesis needs to be proved by more studies. The mechanisms of PJPS

Table 8: The influence of PJPS on human nasopharyngeal carcinoma cell 5-8F proliferation in different time.

Groups	Drug concentration (μg/mL)	24 h OD_{490} value	48 h OD_{490} value	72 h OD_{490} value
Negative group	—	0.494 ± 0.019	0.598 ± 0.016	0.675 ± 0.017
Different dosage groups of PJPS	50	0.478 ± 0.017	0.580 ± 0.020	0.694 ± 0.013
	100	0.492 ± 0.018	0.575 ± 0.018	0.676 ± 0.013
	200	0.504 ± 0.024	0.584 ± 0.012	0.680 ± 0.018
	400	0.508 ± 0.025	0.590 ± 0.021	0.683 ± 0.010
	800	0.484 ± 0.035	0.597 ± 0.019	0.687 ± 0.018
DDP group	5	0.401 ± 0.016**	0.404 ± 0.023**	0.325 ± 0.027**

Note: values represent mean ± SEM; $n = 6$; significance as per Student's t-test compared with negative group; $^{**}P < 0.01$.

Table 9: The influence of PJPS on lung cancer cells HTB182 proliferation in different time.

Groups	Drug concentration (μg/mL)	24 h OD_{490} values	48 h OD_{490} values	72 h OD_{490} values
Negative group	—	0.474 ± 0.015	0.536 ± 0.015	0.590 ± 0.014
Different dosage groups of PJPS	50	0.496 ± 0.018	0.522 ± 0.018	0.594 ± 0.015
	100	0.482 ± 0.016	0.513 ± 0.013	0.589 ± 0.015
	200	0.500 ± 0.015	0.510 ± 0.015	0.611 ± 0.021
	400	0.484 ± 0.011	0.516 ± 0.019	0.601 ± 0.014
	800	0.495 ± 0.011	0.514 ± 0.018	0.584 ± 0.024
DDP group	10	0.376 ± 0.015**	0.405 ± 0.016**	0.396 ± 0.018**

Note: values represent mean ± SEM; $n = 6$; significance as per Student's t-test compared with negative group; $^{**}P < 0.01$.

Table 10: The influence of PJPS on kidney cancer cells HEK293 proliferation in different time.

Groups	Drug concentration (μg/mL)	24 h OD_{490} values	48 h OD_{490} values	72 h OD_{490} values
Negative group	—	0.376 ± 0.021	0.543 ± 0.018	0.662 ± 0.012
Different dosage groups of PJPS	50	0.402 ± 0.015	0.581 ± 0.009	0.666 ± 0.011
	100	0.403 ± 0.011	0.592 ± 0.012	0.662 ± 0.015
	200	0.375 ± 0.016	0.594 ± 0.017	0.669 ± 0.014
	400	0.399 ± 0.013	0.595 ± 0.014	0.664 ± 0.007
	800	0.377 ± 0.011	0.588 ± 0.013	0.662 ± 0.011
DDP group	10	0.266 ± 0.016**	0.363 ± 0.019**	0.438 ± 0.007**

Note: values represent mean ± SEM; $n = 6$; significance as per Student's t-test compared with negative group; $^{**}P < 0.01$.

Table 11: The influence of PJPS on colon cancer cells SW480 proliferation in different time.

Groups	Drug concentration (μg/mL)	24 h OD_{490} values	48 h OD_{490} values	72 h OD_{490} values
Negative group	—	0.494 ± 0.030	0.490 ± 0.019	0.601 ± 0.013
Different dosage groups of PJPS	50	0.440 ± 0.020	0.491 ± 0.011	0.600 ± 0.011
	100	0.418 ± 0.017	0.500 ± 0.01	0.599 ± 0.009
	200	0.431 ± 0.023	0.496 ± 0.01	0.595 ± 0.011
	400	0.436 ± 0.033	0.497 ± 0.014	0.599 ± 0.013
	800	0.428 ± 0.037	0.495 ± 0.011	0.610 ± 0.012
DDP Value	20	0.307 ± 0.023**	0.403 ± 0.018**	0.467 ± 0.017**

Note: values represent mean ± SEM; $n = 6$; significance as per Student's t-test compared with negative group; $^{**}P < 0.01$.

Table 12: The influence of PJPS on human liver cancer cells HepG2 proliferation in different time.

Groups	Drug concentration (μg/mL)	24 h OD_{490} values	48 h OD_{490} values	72 h OD_{490} values
Negative group	—	0.440 ± 0.023	0.588 ± 0.017	0.689 ± 0.022
Different dosage groups of PJPS	50	0.473 ± 0.019	0.608 ± 0.019	0.678 ± 0.022
	100	0.471 ± 0.017	0.601 ± 0.019	0.702 ± 0.023
	200	0.461 ± 0.028	0.613 ± 0.020	0.689 ± 0.026
	400	0.488 ± 0.021	0.605 ± 0.013	0.685 ± 0.017
	800	0.385 ± 0.015	0.564 ± 0.015	0.698 ± 0.023
DDP Group	10	0.354 ± 0.018**	0.351 ± 0.019**	0.347 ± 0.017**

Note: values represent mean ± SEM; $n = 6$; significance as per Student's t-test compared with negative group; ** $P < 0.01$.

resisting myelosuppression and immunosuppression are also supposed to be further confirmed.

5. Conclusion

PJPS can significantly improve the immune function of mice processed by cyclophosphamide.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

The authors are grateful to the school of pharmaceutical sciences of central south university, for providing research facilities and encouragement.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 949065, 12 pages
http://dx.doi.org/10.1155/2015/949065

Research Article

Herb-Partitioned Moxibustion Regulates the TLR2/NF-κB Signaling Pathway in a Rat Model of Ulcerative Colitis

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Received 31 December 2014; Accepted 2 June 2015

Academic Editor: Tetsuya Kondo

The TLR2/NF-κB signaling pathway plays an important role in the pathomechanism of ulcerative colitis (UC); acupuncture and moxibustion can improve the damage in colonic tissues of UC, but the regulatory mechanism remains unknown. This study observed the effect of moxibustion on the TLR2/NF-κB signaling pathway at the Tianshu (ST25) and Qihai (CV6) acupuncture points in the UC rat. The result shows that TLR2, IRAK1, and IKK-b mRNA and protein levels in the colonic mucosa were significantly higher in the UC rats than in the control rats. Herb-partitioned moxibustion reduced the expression of TLR2, IRAK1, and IKK-b mRNA and proteins in the UC rats. Similarly, the expression of NF-κB was significantly increased and IFN-β and IL-10 were significantly decreased in the colonic mucosa of UC rats, but herb-partitioned moxibustion reduced the expression of IFN-β and upregulating the expression of IFN-β and IL-10 significantly. It indicates that herb-partitioned moxibustion can inhibit the expression of multiple signaling molecules of the TLR2 pathway effectively, and it may modulate the excessive local immune response by inhibiting TLR2 signaling, thereby promoting the repair of damaged colonic mucosa.

1. Introduction

Ulcerative colitis (UC), also known as nonspecific ulcerative colitis, is an inflammatory bowel disease (IBD) of unknown etiology. The incidence of IBD in China has shown a clear upward trend in recent years [1]. Although its pathogenesis is still largely unknown, progress in the study of the toll-like receptors (TLRs) and nuclear factor-κB (NF-κB) has improved understanding of the pathogenic process of UC. Under normal physiological conditions, intestinal epithelial cells can tolerate the commensal bacteria in the gut and maintain homeostasis. Studies have demonstrated that "tolerance" and "nontolerance" of the intestinal mucosa towards intestinal bacteria are dependent on TLR-mediated signaling pathways [2]. TLR pathways also play important roles in maintaining tissue integrity and repairing damaged tissues [2]. TLRs play an immunoregulatory role in the colonic mucosa, since they activate downstream signaling pathways upon recognition of pathogen-associated molecular patterns (PAMPs) on pathogenic microbes [3–7]. TLRs are pattern recognition receptors that recognize common antigens on pathogenic microorganisms and they play key roles in the innate immune response [8]. Growing knowledge of the TLR/NF-κB pathway provides a new opportunity in the research of UC pathogenesis. Inhibiting key molecules of this pathway to block excessive inflammation may be a new direction in the treatment of UC [9].

Many clinical and experimental studies have clearly demonstrated that acupuncture has a protective effect on the intestinal mucosa [10, 11]. Long-term clinical and experimental studies have shown that herb-partitioned moxibustion is effective in the treatment of UC. It has been reported that TLR2 mRNA and protein are either not expressed or expressed at very low levels in the normal colonic mucosa of healthy human subjects but are upregulated in the colonic

mucosa of patients with UC [12, 13]. Furthermore, the expression of TLR2 increases with the severity of the disease, as indicated by clinical and endoscopic examinations [12, 13]. In addition, there is more NF-κB DNA binding activity in the nuclei of cells of the colonic mucosa of patients with UC than in those of other individuals [14, 15]. This suggests that activation of the TLR/NF-κB signaling pathways is closely associated with the development of UC. Based on previous studies, the activity of the TLR2/NF-κB pathway was here analyzed in UC rat colons to determine the therapeutic mechanisms of herb-partitioned moxibustion in UC. Upstream molecules TLR2, IRAK1, IKK-β, and NF-κB and downstream molecules IFN-β and IL-10 served as markers.

2. Materials and Methods

2.1. Animals. Forty male Sprague-Dawley rats (specific-pathogen-free), weighing 120 ± 20 g, were purchased from the Experimental Animal Center of the Fudan University School of Medicine. The animals were housed for seven days to allow them to adapt to the new environment before experimentation, and all animals were in good health when experimentation began.

2.2. Generation of the Ulcerative Colitis (UC) Model and Intervention. The 40 SD rats were randomly divided into control, UC, UC with herb-partitioned moxibustion treatment, and UC with salicylazosulfapyridine (SASP) treatment groups. Except for rats in the control group, UC was induced in all rats using an immunological method combined with local irritation [16]. Adjuvant mixture (containing protein antigens released from UC colon patients) was injected into the front footpad, hind footpad, dorsa, inguinal, and abdominal cavities on days 0, 10, 17, 24, and 31, respectively. On day 38, rats were administered 3 mL of 3% formalin and a 2 mL enema of antigen fluid.

Rats in the control and UC groups did not receive any intervention, but they were sham-handled in the same way as rats in the intervention groups. Intervention was initiated immediately after UC generation. For herb-partitioned moxibustion, premade medicinal cakes (diameter 0.5 cm, height 0.3 cm) were placed on the Tianshu (ST25) and Qihai (CV6) acupuncture points [17], and moxa cones (Nanyang Hanye Moxa Plant, Henan, China) about 90 mg in size were placed on the medicinal cakes. The moxa cones were ignited and allowed to burn out. Two cones were used for each acupuncture point once daily for a total of 14 times. For SASP treatment, salicylazosulfapyridine (SASP) (Lot# 201007C30, Sunve Pharmaceutical Ltd., Shanghai, China) solution was administered by gavage. The dose was determined with the ratio of an adult human (70 kg body weight) per rat (200 g body weight) at 1 : 0.018 (*Pharmacological Experimental Methodology*). The SASP solution was administered twice daily, 3 mL each time, for a total of 14 days.

2.3. Tissue Collection. At the end of the intervention, animals were killed by cervical dislocation and their abdomens were opened. The colon was quickly removed (about 8 cm from the anus toward the proximal end) and dissected free of the surrounding connective tissues and fat. It was then cut open lengthwise, washed in saline, and laid flat in a dish mucosa side up, and the gross morphology of the mucosa was examined by eye. The colon was then divided in half. One piece was fixed in 10% neutral-buffered formalin, paraffin-embedded, and cut into sections. The other piece was immediately frozen in liquid nitrogen and stored at −80°C for RNA preparation and qRT-PCR analysis.

2.4. UC Disease Monitor and Marker Analysis

2.4.1. Monitoring of General Health Conditions of Rats. Rats were monitored for their demeanor, food and water intake, body weight, appearance of fur, responsiveness, activity, appearance of feces, and other body conditions.

2.4.2. Morphological and Histopathological Examination of Rat Colon Mucosa. The colon tissue from the anus to the ileocecal region was dissected and its gross morphology was examined by eye. Tissue sections were stained by H + E and observed under an optical microscope to further examine the morphology of rat colonic mucosa.

2.4.3. Fluorescence Quantitative RT-PCR (qRT-PCR) Analysis. (1) RNA extraction: rat colon tissues were removed from liquid nitrogen, and 1 mL TRIzol Reagent was added to every 100 mg of tissue. The tissue was homogenized twice for 15 s each, with a 10 s interval. 0.2 mL chloroform was added per 1 mL TRIzol Reagent, and then this mixture was vigorously shaken for 15 s, cooled on ice for 5 min, and centrifuged at 12,000 rpm and 4°C for 15 min in a microcentrifuge (Thermo, US). The upper aqueous phase was taken into another microcentrifuge tube, and an equal volume of prechilled isopropanol was added. The two solutions were slowly mixed, cooled on ice for 15–20 min, and then centrifuged at 12,000 rpm, 4°C for 10 min. The pellet was washed with 75% ethanol (DEPC-water : ethanol = 1 : 3) and centrifuged again at 7,500 rpm, 4°C for 8 min. The supernatant was removed, and the tubes were blotted dry on sterilized filter paper. The pellet was washed again, the ethanol was removed, and the pellet was air-dried. After the pellet was dry; it was dissolved in DEPC-water. Then 1 mL of total RNA solution was used to determine RNA concentration and purity using NanoDrop. (2) Primer design and synthesis: primer 5 software was used to design qRT-PCR primers for the genes TLR2, IRAK1, IKK-β, and β-actin (Table 1). Primers were synthesized and purified by Sangon Biotech (Shanghai, China). (3) cDNA synthesis by one-step reverse transcription: here, 16.0 μL 5x iScript reaction mix, 4.0 μL iScript reverse transcriptase, 4 μg total RNA (volume depends on the concentration), and 60.0 μL DEPC-H_2O 60.0 μL were added to each 0.5 mL RNase-free centrifuge tube to a total reaction volume of 80 μL. The reaction program was as follows: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. (4) qPCR reaction: here, 1.0 μL iQ SYBR Green Supermix, 1.0 μL cDNA, 1.0 μL each sense and antisense primer, and 8 μL ddH_2O were added to each well of 0.2 mL 96-well PCR plates. The plates were centrifuged briefly and placed into a PCR machine (LC96 PCR system, Roche, Switzerland) for amplification. The PCR

Table 1: The gene sequences of the primers.

ID	Primer name	Sequence (5′ to 3′)	Base number
gi|392350511|ref|XM_003750630.1|	TLR2-domain	CCCAAGCACACTCACTCAACT	20
gi|189011593|ref|NM_001127555.1|	IRAK-1	CAAGGAGGCACTACCAGAGAAT	22
gi|158508715|ref|NM_053355.2|	IKK-β	CCAAGAGACCAAAGGACAGAAG	22

program was (1) 95°C for 5 min and (2) 40 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 10 s.

2.4.4. Immunohistochemical Detection of TLR2, IRAK1, IKK-β, NF-κB, IFN-β, and IL-10 Proteins in Colonic Mucosa. Tissue sections were deparaffinized with the following solutions: three changes of xylene, each lasting for 10 min: 100%, 95%, and 85%; 75% ethanol for 2 min and then placing in water. Sections were washed three times with 0.01 M PBS (pH 7.4) for 3 min each and then incubated with 1% H_2O_2 for 20 min. Then they were washed three times in PBS for 3 min each and three distilled water washes, 3 min each. Sections were incubated with PBS for 5 min and then subjected to antigen retrieval. For antigen retrieval, the sections were placed in 0.01 M citrate buffer (pH 6.0) and boiled three times in a microwave at setting III (98°C) for 2.5 min, 1.5 min, and 1 min, respectively. Sections were kept in the hot buffer for 15 min each between rounds of boiling. Sections were let to cool at room temperature and then washed twice with distilled water, followed by three washes with PBS, 3 min each. Sections were blocked in 5% goat serum at room temperature for 20 min and then incubated with diluted primary antibodies (30–50 μL) at 4°C overnight. The next day, sections were washed three times with PBS for 3 min each and then incubated with biotinylated secondary antibody-EnVision reagent (30–50 μL) for 30 min at 37°C, followed by three washes with PBS, 3 min each. To allow visualization of bound antibodies, sections were incubated with 0.04% DAB + 0.03% H_2O_2 for 8 min for color development. Sections were washed in water, counterstained with hematoxylin for 30 s, washed again with water, dipped in a bluing solution containing hydrochloric acid and ethanol for 2 s, washed in water, and mounted with resin.

Stained sections were examined under a light microscope. Positive staining was brown, and counterstained nuclei were blue. Three randomly chosen microscopic fields were analyzed with the MOTIC image analysis system. Total area and integrated optical density of positive signals were measured for each field, and the mean values from the three fields of each sample were used for statistical analysis.

2.5. Statistical Analysis. Quantitative data are here presented as mean ± standard deviation ($\overline{x} \pm s$), and statistical analysis was performed with the PASS 13.0 software. Comparisons between groups were conducted using one-way ANOVA with the following methods: LSD/SNK-q method was used when pairwise tests showed that the variances of different groups were equal, but Dunnett's T3 method was used when the variances were not equal. The level of significance was set to $\alpha = 0.05$ and $P < 0.05$.

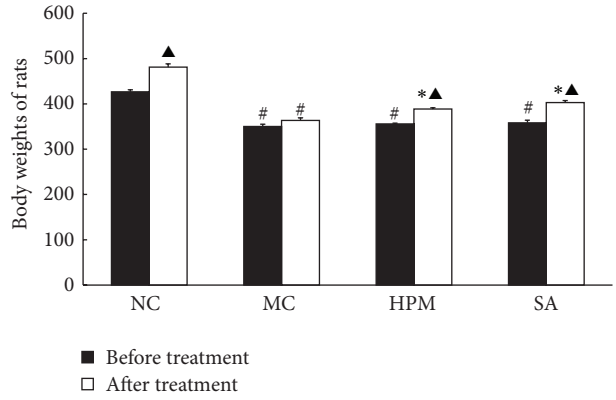

Figure 1: The body weights of rats in each group. NC: normal control; MC: ulcerative colitis; HPM: UC with herb-partitioned moxibustion; SA: UC with salicylazosulfapyridine. $^{\#}P < 0.01$ versus NC; $^{*}P < 0.01$ versus MC; $^{\blacktriangle}P < 0.01$ versus before treatment.

3. Results

3.1. General Health Conditions of Rats in the Experimental Groups. Rats in the control group had normal food and water intake, were active, and had dense and shiny pelts. UC rats showed debility and anorexia, their food intake was reduced, and they had a hunched posture. They showed reduced activity levels and were easily startled. Their feces appeared normal and their perianal skin remained clean. Their coats appeared rough and less shiny. They had increased stool frequency, bloody mucus was seen in the feces, and the perianal skin was dirty with feces. The toes injected with antigen were bruised and swollen. One rat in this group died during the experimental period. Rats in the herb-partitioned moxibustion and SASP treatment groups displayed better food intake, responsiveness, activity levels, and appearance of the fur coat than rats in the UC group.

As shown in Figure 1, rats in the control group gained significantly weight by the end of the experimental period ($P < 0.01$). The body weights of UC rats were significantly lower than those of the control rats ($P < 0.01$). The body weights of rats in the herb-partitioned moxibustion and SASP treatment groups were greater than those of rats in the UC group ($P < 0.01$).

3.2. Macro- and Microscopic Morphology of Rat Colonic Mucosa. Observed by the naked eye, the colonic mucosa of control rats had a smooth surface with small amounts of mucus. The blood vessels underneath the mucosal folds were visible, and there was no erosion or ulceration. The colonic mucosa of UC rats had severe hyperemia, edema, and

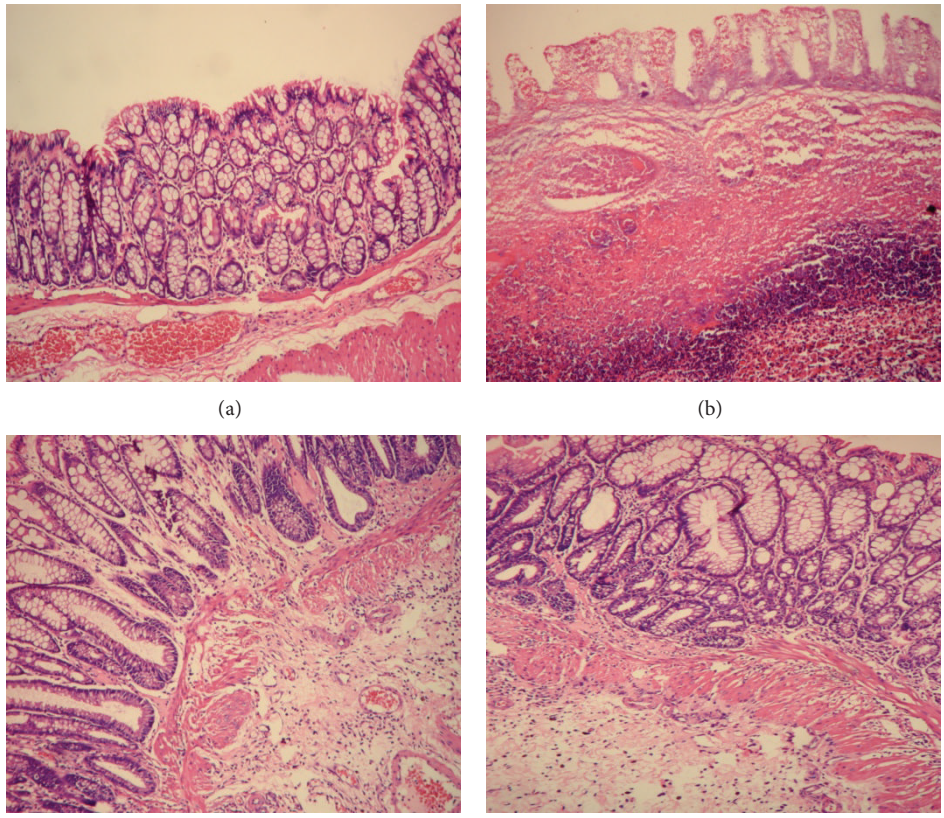

Figure 2: The histological observation of rats in each group. Hematoxylin-Eosin staining method, ×200. (a) Normal control; (b) ulcerative colitis; (c) UC with herb-partitioned moxibustion; (d) UC with salicylazosulfapyridine.

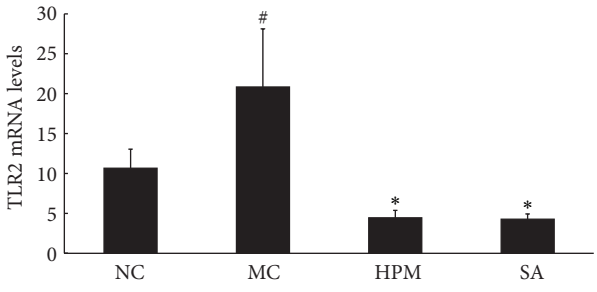

Figure 3: The TLR2 mRNA levels in the rat colon of each group. NC: normal control; MC: ulcerative colitis; HPM: UC with herb-partitioned moxibustion; SA: UC with salicylazosulfapyridine. $^{\#}P < 0.01$ versus NC; $^{*}P < 0.01$ versus MC.

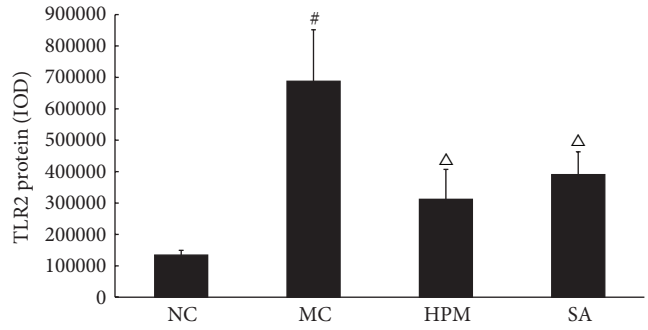

Figure 4: Expression of TLR2 protein in the colonic mucosa of rat. NC: normal control; MC: ulcerative colitis; HPM: UC with herb-partitioned moxibustion; SA: UC with salicylazosulfapyridine. $^{\#}P < 0.01$ versus NC; $^{\triangle}P < 0.05$ versus MC.

erosion, and ulcers were observed. In contrast, the appearance of colonic mucosa from rats in the herb-partitioned moxibustion and SASP treatment groups was markedly better than those of other groups. Even though the mucosal surface was not as smooth as in normal mucosa and there was some visible edema, the muscles under the mucosal folds were visible and the extent of hyperemia, edema, and erosion of the mucosa was significantly less pronounced than in UC rats.

As shown in Figure 2, under a light microscope, normal colonic mucosa had a well-organized structure with intestinal glands arranged in rows and an intact colonic epithelium. Capillaries and scattered lymphocytes were visible in the lamina propria, but there was no significant inflammatory cell infiltration. In the colonic mucosa of UC rats, the crypts were shorter and the epithelium was not intact. The mucosa and submucosa had been infiltrated by a large number of

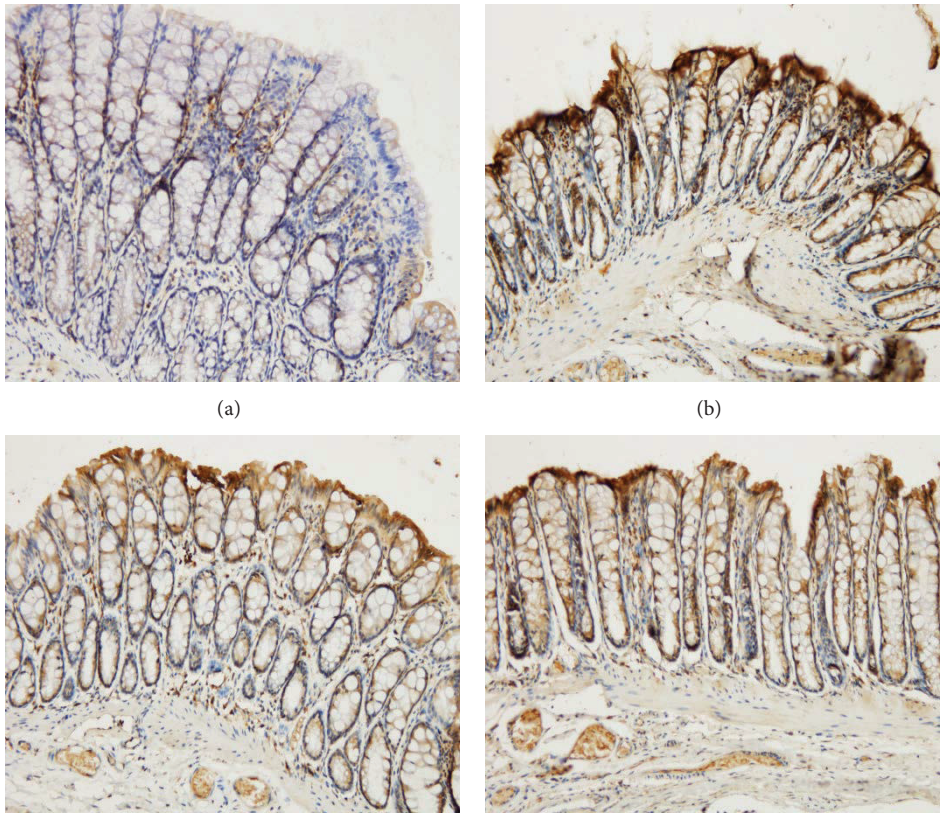

Figure 5: The integral optical density (IOD) of TLR2 in each rat group. EnVision Plus method, ×200. (a) Normal control; (b) ulcerative colitis; (c) UC with herb-partitioned moxibustion; (d) UC with salicylazosulfapyridine.

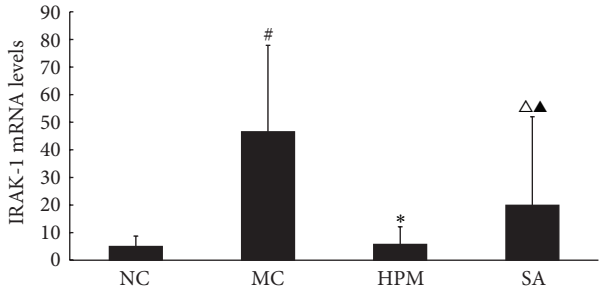

Figure 6: The IRAK-1 mRNA levels in the rat colon of each group. NC: normal control; MC: ulcerative colitis; HPM: UC with herb-partitioned moxibustion; SA: UC with salicylazosulfapyridine. $^{\#}P < 0.01$ versus NC; $^{*}P < 0.01$, $^{\triangle}P < 0.05$ versus MC; $^{\blacktriangle}P < 0.01$ versus HPM.

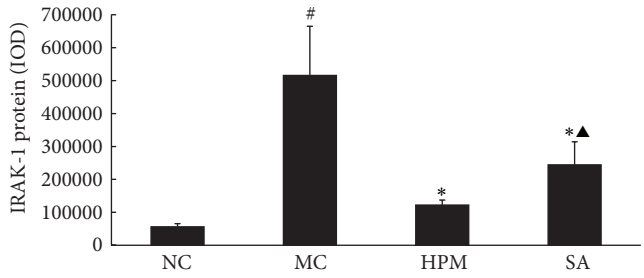

Figure 7: Expression of IRAK-1 protein in the colonic mucosa of rat. NC: normal control; MC: ulcerative colitis; HPM: UC with herb-partitioned moxibustion; SA: UC with salicylazosulfapyridine. $^{\#}P < 0.01$ versus NC; $^{*}P < 0.01$ versus MC; $^{\blacktriangle}P < 0.01$ versus HPM.

inflammatory cells, focal hyperemia and edema were visible, and the structures of the glands were disorganized, indicating that ulcers had formed. Both herb-partitioned moxibustion and SASP treatments greatly improved the morphology of colonic mucosa. Intestinal glands were more organized than those in UC rats. Ulceration was less severe, and the mucosa surface was covered by epithelial cells. Edema and inflammatory cell infiltration in submucosa were still present, but they were less severe than in UC rats.

3.3. TLR2 mRNA and Protein Expression in Rat Colonic Mucosa. As shown in Figure 3, TLR2 mRNA level in the colonic mucosa was significantly higher in the UC rats than in the control rats ($P < 0.01$). Both herb-partitioned moxibustion and SASP treatments significantly reduced TLR2 mRNA expression in the colonic mucosa of UC rats ($P < 0.01$). This inhibitory effect was slightly greater with SASP treatment than with herb-partitioned moxibustion, but the difference was not statistically significant ($P > 0.05$).

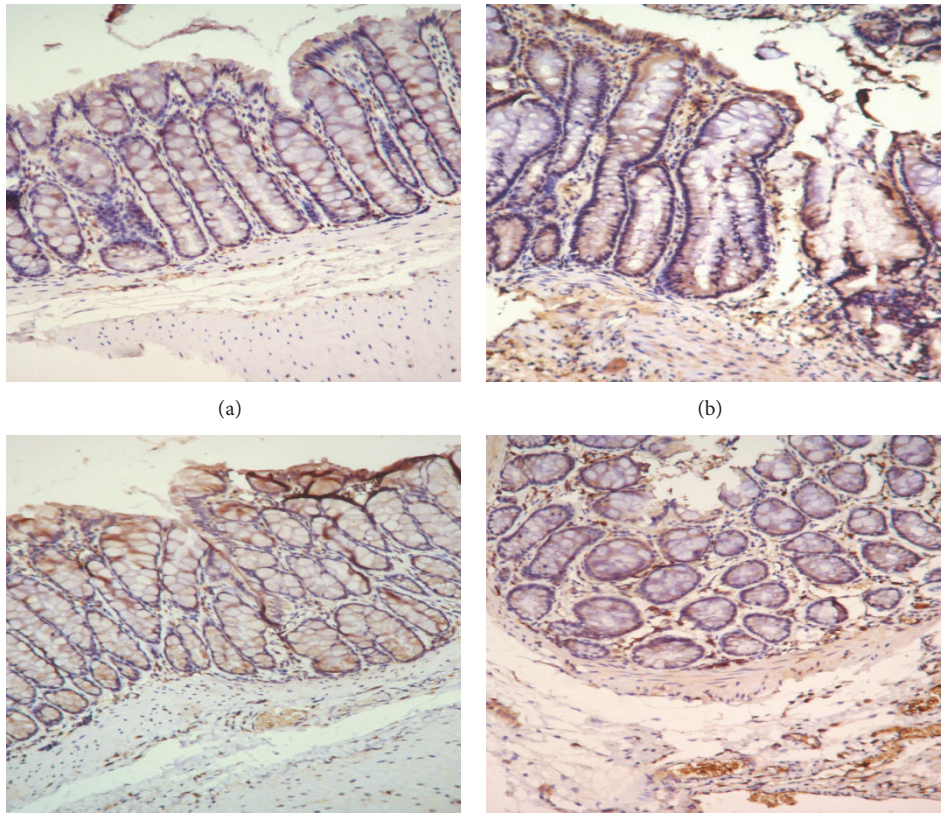

Figure 8: The integral optical density (IOD) of IRAK-1 in each rat group. EnVision Plus method, ×200. (a) Normal control; (b) ulcerative colitis; (c) UC with herb-partitioned moxibustion; (d) UC with salicylazosulfapyridine.

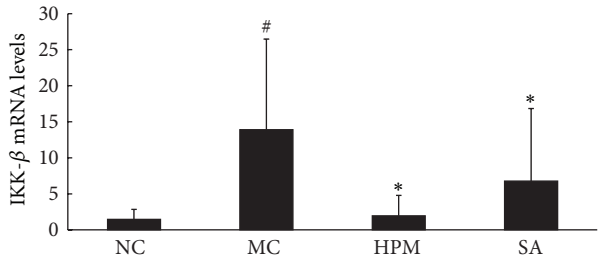

Figure 9: The IKK-β mRNA levels in the rat colon of each group. NC: normal control; MC: ulcerative colitis; HPM: UC with herb-partitioned moxibustion; SA: UC with salicylazosulfapyridine. $^{\#}P < 0.01$ versus NC; $^{*}P < 0.01$ versus MC.

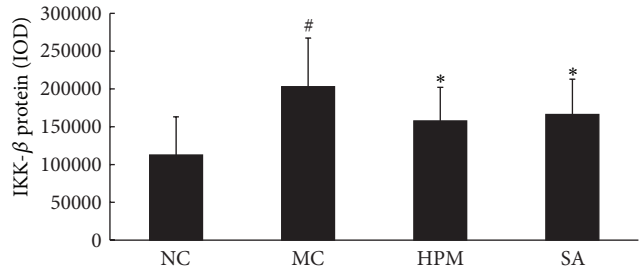

Figure 10: Expression of IKK-β protein in the colonic mucosa of rat. NC: normal control; MC: ulcerative colitis; HPM: UC with herb-partitioned moxibustion; SA: UC with salicylazosulfapyridine. $^{\#}P < 0.01$ versus NC; $^{*}P < 0.01$ versus MC.

TLR2 protein expression was determined using immunohistochemistry and quantified by the total area of staining and the integrated optical density. As shown in Figures 4 and 5, TLR2 protein levels in the colonic mucosa were higher in UC rats than in control rats, as indicated by both of these measurements ($P < 0.01$). The total area of TLR2 staining was significantly lower in both the herb-partitioned moxibustion and SASP treated groups than in the UC group ($P < 0.01$, $P < 0.05$). The integrated optical density was also significantly lower in both treatment groups ($P < 0.05$). TLR2 protein levels were slightly lower in the herb-partitioned moxibustion group than in the SASP group, but the difference was not statistically significant ($P > 0.05$).

3.4. IRAK1 mRNA and Protein Expression in Rat Colonic Mucosa. As shown in Figure 6, rats in the UC group expressed significantly higher levels of IRAK1 mRNA in the colonic mucosa than rats in the control group ($P < 0.01$). Both herb-partitioned moxibustion and SASP treatments significantly reduced IRAK-1 mRNA expression in the colonic

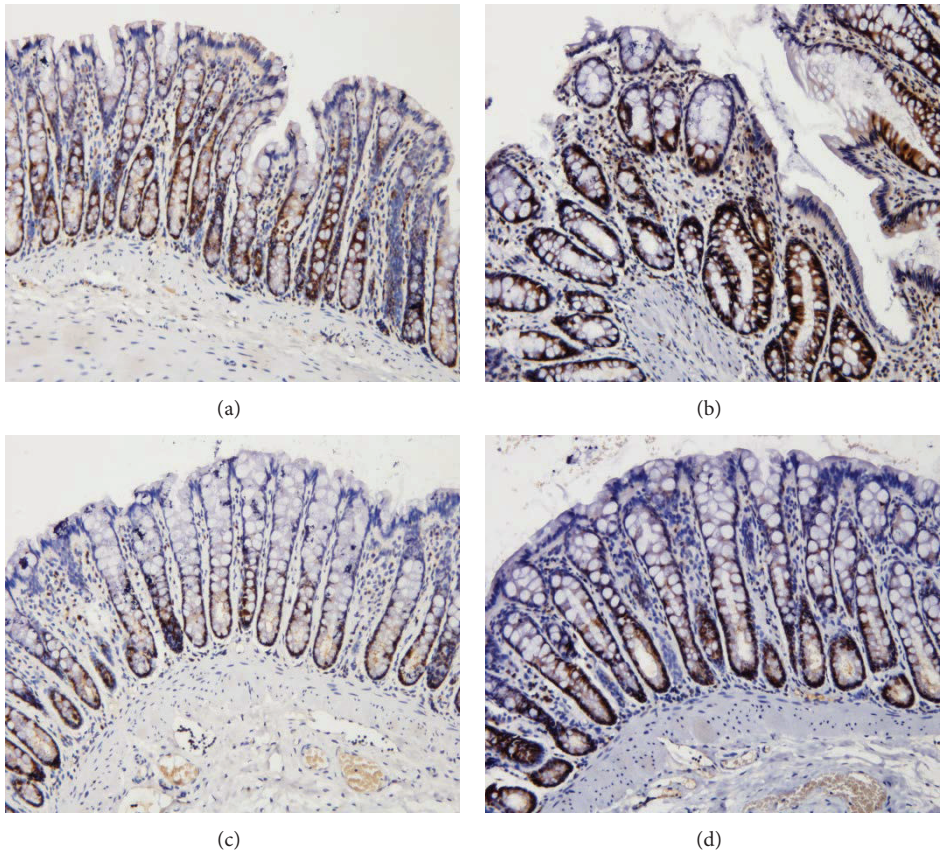

Figure 11: The integral optical density (IOD) of IKK-β in each rat group. EnVision Plus method, ×200. (a) Normal control; (b) ulcerative colitis; (c) UC with herb-partitioned moxibustion; (d) UC with salicylazosulfapyridine.

mucosa of UC rats ($P < 0.01$, $P < 0.05$). Between the two treatment groups, IRAK1 mRNA expression in the colonic mucosa was significantly higher with SASP than with herb-partitioned moxibustion treatment ($P < 0.01$).

As shown in Figures 7 and 8, IRAK1 protein levels in the colonic mucosa were significantly higher in UC rats than in the control rats, as measured by both the total area of staining and the integrated optical density ($P < 0.01$ in both cases). Both herb-partitioned moxibustion and SASP treatments significantly reduced IRAK1 protein levels in the colonic mucosa of UC rats, as indicated by both of these measurements ($P < 0.01$ in all cases). IRAK1 protein levels in the colonic mucosa were significantly lower in the herb-partitioned moxibustion than in the SASP treatment by both of these measurements ($P < 0.01$ in both cases).

3.5. IKK-β mRNA and Protein Expression in Rat Colonic Mucosa. As shown in Figure 9, rats in the UC group expressed significantly higher levels of IKK-β mRNA in the colonic mucosa than rats in the control group ($P < 0.01$). Both herb-partitioned moxibustion and SASP treatments significantly reduced IKK-β mRNA expression in the colonic mucosa of UC rats ($P < 0.01$).

As shown in Figures 10 and 11, IKK-β protein levels in the colonic mucosa were significantly higher in UC rats than in the control rats, as measured by both the total area of staining and the integrated optical density ($P < 0.05$, $P < 0.01$). Both herb-partitioned moxibustion and SASP treatments significantly reduced IKK-β protein levels in the colonic mucosa of UC rats, as indicated by both of these measurements ($P < 0.05$).

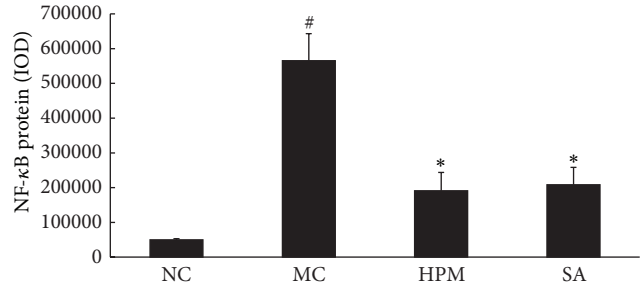

Figure 12: Expression of NF-κB protein in the colonic mucosa of rat. NC: normal control; MC: ulcerative colitis; HPM: UC with herb-partitioned moxibustion; SA: UC with salicylazosulfapyridine. $^{\#}P < 0.01$ versus NC; $^{*}P < 0.01$ versus MC.

3.6. NF-κB Expression in Rat Colonic Mucosa. As shown in Figures 12 and 13, NF-κB protein levels in the colonic mucosa were significantly higher in UC rats than in the control

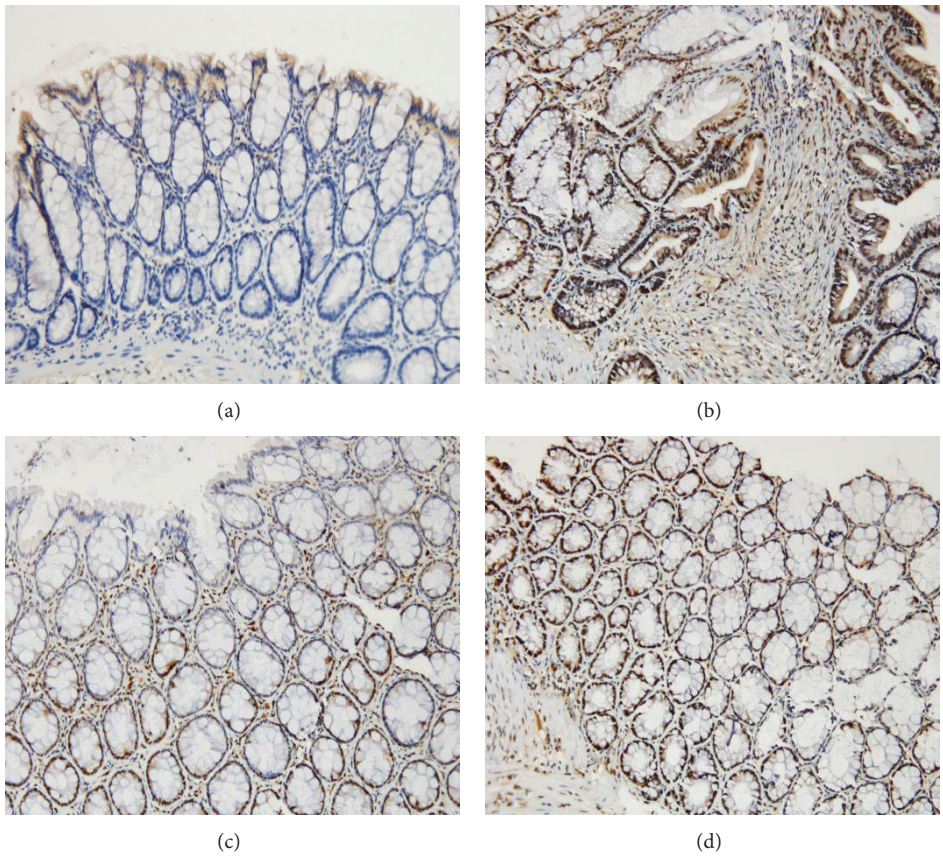

Figure 13: The integral optical density (IOD) of NF-κB in each rat group. EnVision Plus method, ×200. (a) Normal control; (b) ulcerative colitis; (c) UC with herb-partitioned moxibustion; (d) UC with salicylazosulfapyridine.

rats, as measured by both the total area of staining and the integrated optical density ($P < 0.01$). Both herb-partitioned moxibustion and SASP treatments were associated with lower NF-κB protein levels in the colonic mucosa of UC rats as indicated by the total area of staining ($P < 0.01$). NF-κB protein levels were slightly lower in the herb-partitioned moxibustion group than in the SASP group, but the difference was not statistically significant ($P > 0.05$).

3.7. IFN-β Expression in Rat Colonic Mucosa. As shown in Figures 14 and 15, IFN-β protein levels in the colonic mucosa were significantly lower in UC rats than in the control rats, as indicated by both the total area of staining and the integrated optical density ($P < 0.01$ in both cases). Both herb-partitioned moxibustion and SASP treatments significantly increased IFN-β protein levels in the colonic mucosa of UC rats by both of these measurements ($P < 0.01$ in both cases). Between the two treatment groups, IFN-β protein levels in the colonic mucosa were significantly higher with herb-partitioned moxibustion than with SASP treatment as indicated by both of these measurements ($P < 0.05$ in both cases).

3.8. IL-10 Expression in Rat Colonic Mucosa. As shown in Figures 16 and 17, IL-10 protein levels in the colonic mucosa were significantly lower in UC rats than in the control

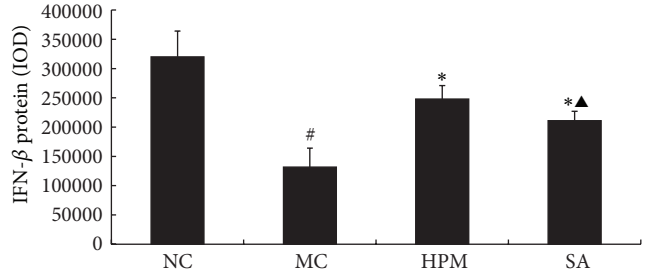

Figure 14: Expression of IFN-β protein in the colonic mucosa of rat. NC: normal control; MC: ulcerative colitis; HPM: UC with herb-partitioned moxibustion; SA: UC with salicylazosulfapyridine. $^{\#}P < 0.01$ versus NC; $^{*}P < 0.01$ versus MC; $^{\blacktriangle}P < 0.01$ versus HPM.

rats, as indicated by both the total area of staining and the integrated optical density ($P < 0.05$ in both cases). Both herb-partitioned moxibustion and SASP treatments significantly increased IL-10 protein levels in the colonic mucosa of UC rats by both of these measurements ($P < 0.05$ in all cases). The total area of IL-10 staining in the colonic mucosa was significantly higher in the herb-partitioned moxibustion group than in the SASP group ($P < 0.05$) but the integrated optical density did not reach statistical significance, although

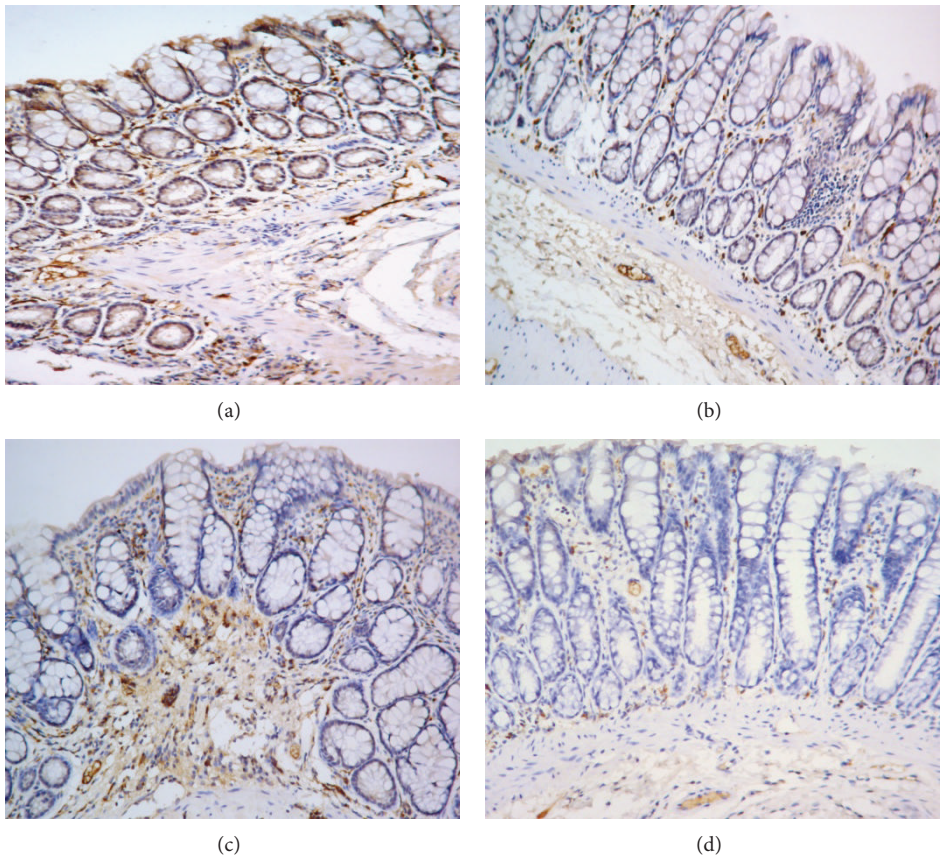

Figure 15: The integral optical density (IOD) of IFN-β in each rat group. EnVision Plus method, ×200. (a) Normal control; (b) ulcerative colitis; (c) UC with herb-partitioned moxibustion; (d) UC with salicylazosulfapyridine.

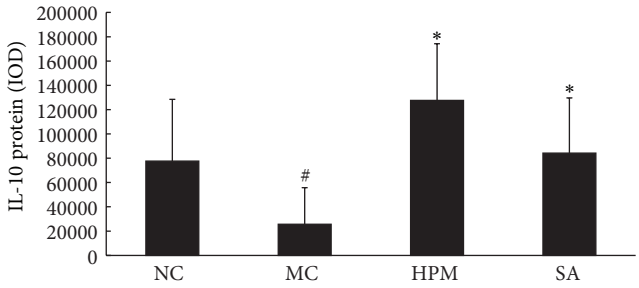

Figure 16: Expression of IL-10 protein in the colonic mucosa of rat. NC: normal control; MC: ulcerative colitis; HPM: UC with herb-partitioned moxibustion; SA: UC with salicylazosulfapyridine. $^{\#}P < 0.01$ versus NC; $^{*}P < 0.01$ versus MC.

it was higher in the herb-partitioned moxibustion group than in the SASP group ($P > 0.05$).

4. Discussion

According to its clinical manifestations in the traditional Chinese medicine, ulcerative colitis belongs to scope of "diarrhea" and "abdominal pain." It occurs in the large intestine, but the basic pathogenesis is spleen deficient and hyperactivity of damp. Tianshu (ST25) acupoint, located at 2 inches next to navel of the stomach channel of foot-yangming, is the Mu point of large intestine, it is a key point regulating the ascending and descending Qi and is usually used to treat the abdominal pain and diarrhea [18], and it was recorded in ancient medical books, such as "Qianjin Yaofang." Qihai (CV6) acupoint is located at 1.5 inches below navel of Ren meridian and the place gathering original Qi; it can regulate the ascending and descending Qi and complement original Qi of the human body. Therefore, moxibustion at ST25 and CV6 can improve the symptoms of diarrhea and abdominal pain in patient with ulcerative colitis, not only regulating Qi and reinforcing spleen and transporting dampness, but also complementing original Qi to promote recovery of patient.

Ulcerative colitis is associated with multiple aspects of immune dysfunction, including autoimmunity and dysregulated humoral and cellular immunity. It has been recognized that immune dysfunction plays an important role in the pathogenesis of UC [19–22].

The activation of TLR signaling pathways is involved in the development of UC. TLR2 activates NF-κB through the MyD88-dependent pathway [23]. Activated NF-κB induces the expression of TLR2 transcriptionally, resulting in a TLR2-NF-κB-TLR2 positive feedback loop that propagates the inflammatory response [24]. The IRAK1 family protein acts

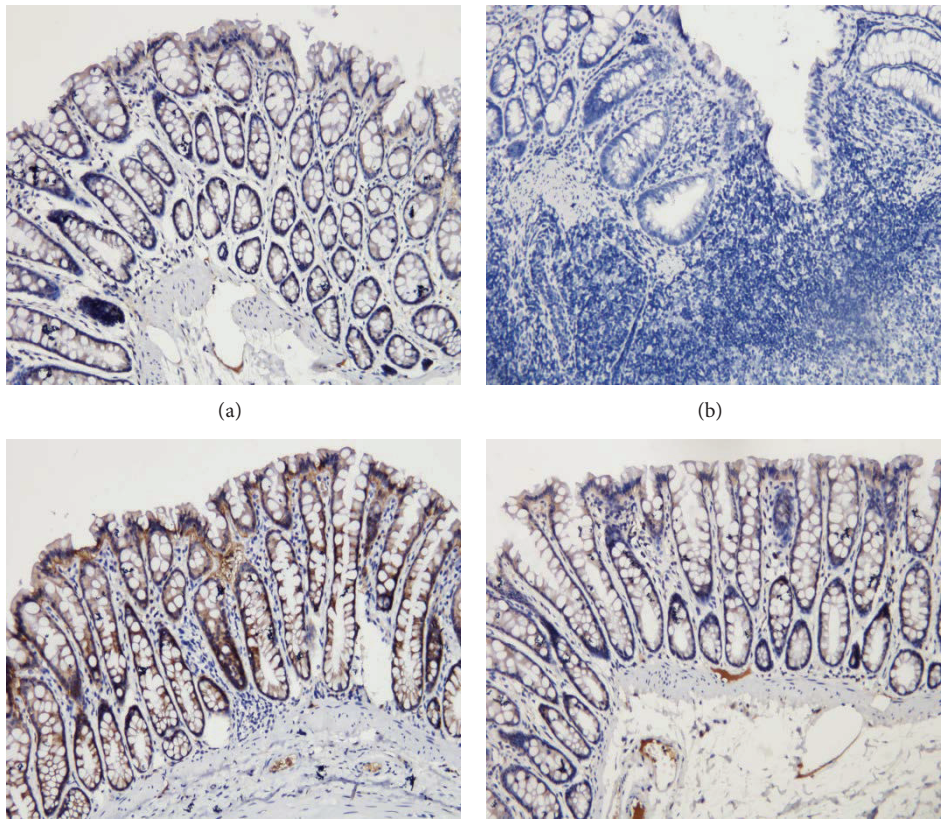

Figure 17: The integral optical density (IOD) of IL-10 in each rat group. EnVision Plus method, ×200. (a) Normal control; (b) ulcerative colitis; (c) UC with herb-partitioned moxibustion; (d) UC with salicylazosulfapyridine.

as an adaptor protein and mediates TLR-induced production of proinflammatory cytokines. Degradation of IRAK1 comprises an important negative feedback mechanism that prevents excessive inflammation [25, 26]. The IKKβ/NF-κB signaling pathway plays key roles in inducing the immune response downstream of TLRs. IKK-β is an essential catalytic subunit of the protein complex that activates NF-κB in response to inflammatory cytokines, such as TNF-α, IL-1, and lipopolysaccharide. Studies have shown that TLR2 expression is low in epithelial cells of normal intestinal mucosa but becomes high during inflammation [27]. Abnormally high expression of TLR2 was observed in the colonic mucosa of patients with UC and in mouse models of UC [28, 29]. In addition, the DNA binding activity of NF-κB in cell nucleus was found to be higher in the colonic mucosa of patients with UC [30, 31]. These discoveries suggest that the activation of the TLR2/NF-κB signaling pathway is closely associated with the development of UC.

Proinflammatory cytokines TNF-α and IL-12 and anti-inflammatory cytokines IL-10 and IFN-β play important roles in the pathogenesis of UC. In the presence of IL-6 and TNF-α causes microvascular dysfunction in the intestinal mucosa, damaging its barrier function. TNF-α also acts synergistically with IFN-γ to alter the structure and the barrier function of the intestinal mucosal epithelium, leading to increased permeability of the colonic mucosa and apoptosis of epithelial cells [32]. IL-12 stimulates the differentiation of T cells into IFN-γ-secreting T-helper type 1 (Th1) cells, which promotes inflammation by activating macrophages and neutrophils [24]. IL-10 is mainly produced by Th2 cells, activated B cells, monocytes, and macrophages. It dampens immune responses by antagonizing inflammatory mediators. IFN-β is a cytokine produced by fibroblasts and white blood cells. It can inhibit the proliferation of fibroblasts, epithelial cells, endothelial cells, and hematopoietic cells [33]. IFN-β plays an immunoregulatory role by promoting the secretion of IL-10 and T lymphocytes [34]. Studies have shown that the levels of IL-12 and TNF-α are significantly increased in the peripheral blood of patients with IBD [25, 26, 35]. The expression of IL-12 mRNA in the colonic mucosa of patients with UC is significantly higher during the active phase of the disease, and the degree of the increase correlates with the severity of the disease [36, 37]. Genomewide association studies have identified an association between polymorphisms of IL-10 and increased susceptibility to IBD [38–40]. The function of anti-inflammatory macrophages is altered in IL-10R-deficient patients with IBD [41]. IL-10 expression in colonic epithelial cells is elevated in patients with UC [42]. A study showed that IFN-β suppressed inflammation induced by high levels of IL-13 in patients with UC [43]. Another study showed that natural IFN-β was effective in treating patients with active, steroid refractory UC [44]. In this way, inhibiting

the TLR2/NF-κB signaling pathway by modulating the key mediators of this pathway, such as TLR2, IRAK1, IKK-β, and NF-κB, and downstream effector cytokines, such as TNF-α, IL-12, IL-10, and IFN-β, may provide effective treatments for UC.

Herb-partitioned moxibustion is beneficial for patients with UC [45]. This treatment can reduce the expression of IL-8 and ICAM-1 mRNA and proteins in the colonic mucosa of patients with UC [46]. Previous studies have found that IL -12 and TNF-α expressions are significantly higher in UC rat colons, but herb-partitioned moxibustion reduces their expression [47]. Herb-partitioned moxibustion can also reduce the concentrations of IL-1β, IL-6, and TNF-α from induced peripheral blood mononuclear cells (PBMCs) cultured supernatants of UC rats, relieving intestinal inflammation [48]. In the present study, results showed that, in UC rat colons, the normal mucosa structure was damaged and the levels of TLR2, IRAK1, and IKK-β mRNA and proteins were significantly higher, and the levels of IL-10 and IFN-β proteins were significantly lower than those of the control rat colon. With herb-partitioned moxibustion treatment, the levels of TLR2, IRAK1, and IKK-β mRNA and proteins were significantly lower, and the levels of IL-10 and IFN-β proteins were significantly higher. These data suggest that the TLR2/NF-κB signaling pathway is involved in the development of UC, and herb-partitioned moxibustion can reverse the dysregulated expression of signaling molecules in the TLR2 pathway effectively, thereby inhibiting TLR2/NF-κB pathway activation, modulating the local immune response in the colonic mucosa, protecting the mucosa, and promoting repair of injured mucosa.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Xiaomei Wang, Yanan Liu, and Hongsheng Dong contributed equally to this paper.

Acknowledgments

The authors thank the support of Shanghai Health System of Outstanding Young Talent Cultivation Program (no. XYQ2011068), the National Natural Science Foundation of China (no. 81001549 and no. 81473758), National Basic Research Program of China (973 program, no. 2009CB522900), and Innovation Program of Shanghai Municipal Education Commission (no. 2014YZ052).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 975632, 10 pages
http://dx.doi.org/10.1155/2015/975632

Review Article

Mediators, Receptors, and Signalling Pathways in the Anti-Inflammatory and Antihyperalgesic Effects of Acupuncture

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Received 11 December 2014; Accepted 30 March 2015

Academic Editor: Xianghong Jing

Acupuncture has been used for millennia to treat allergic diseases including both intermittent rhinitis and persistent rhinitis. Besides the research on the efficacy and safety of acupuncture treatment for allergic rhinitis, research has also investigated how acupuncture might modulate immune function to exert anti-inflammatory effects. A proposed model has previously hypothesized that acupuncture might downregulate proinflammatory neuropeptides, proinflammatory cytokines, and neurotrophins, modulating transient receptor potential vallinoid (TRPV1), a G-protein coupled receptor which plays a central role in allergic rhinitis. Recent research has been largely supportive of this model. New advances in research include the discovery of a novel cholinergic anti-inflammatory pathway activated by acupuncture. A chemokine-mediated proliferation of opioid-containing macrophages in inflamed tissues, in response to acupuncture, has also been demonstrated for the first time. Further research on the complex cross talk between receptors during inflammation is also helping to elucidate the mediators and signalling pathways activated by acupuncture.

1. Introduction

In our previous publication, research on the anti-inflammatory effects of acupuncture was reviewed and a model was proposed to guide further research [1]. This review included both demonstrated and proposed effects of acupuncture and drew from both animal and human studies. Anti-inflammatory effects of acupuncture in contexts other than allergic rhinitis were explored for their potential relevance to allergic rhinitis, especially research involving proinflammatory neuropeptides and neurotrophins and modulation of the transient receptor potential vallinoid 1 (TRPV1), a central receptor in allergic inflammatory response.

New research has expanded and clarified the understanding of inflammatory response and how acupuncture might modulate it, and hence a revised model for the anti-inflammatory effects of acupuncture is proposed.

2. The Previously Proposed Model (2013 Model)

Our 2013 model proposed that, in allergic rhinitis, acupuncture downregulated proinflammatory neuropeptides, substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP); downregulated neurotrophins, nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF); and downregulated Th2 and proinflammatory cytokines and possibly upregulated Th1 cytokines, thereby shifting Th1/Th2 balance away from Th2 dominance [1]. Acupuncture-induced modulation of TRPV1 (both demonstrated and hypothesised) was also explored.

3. Effects of Acupuncture on Inflammatory Oedema

Inflammatory oedema has been significantly reduced by acupuncture in rodent and murine models of induced hind paw inflammation [2, 3]. This antioedema effect is mediated via the HPA axis as various disruptions of the HPA axis obliterate or significantly attenuate the effect [2, 3]. Many of the pathways, mediators, and receptors involved in acupuncture attenuation of inflammatory hyperalgesia appear to have either no effect or delayed effects on inflammatory oedema [4, 5]. For example, opioid pathways are implicated in acupuncture effects on inflammatory hyperalgesia but have

generally been shown to have no influence on inflammatory oedema as opioid antagonists did not diminish the antioedema effect [4, 6–8]. One study, however, has shown slight reduction in inflammatory oedema which was blocked by opiate antagonists naloxone and natrindole [9].

A single acupuncture treatment was previously reported to produce a significant but short-lived (less than 15 minutes) improvement in the patency of the nasal airway [28]. However, subjective sensations of nasal congestion have consistently been reported to decrease significantly for much longer periods following acupuncture for allergic rhinitis in adults [29–33].

Swelling of the nasal mucosa in allergic rhinitis has been linked to proinflammatory neuropeptides, proinflammatory cytokines, neurotrophins, and chemokines [34].

3.1. Neuropeptides. Downregulation of proinflammatory neuropeptides, SP, and VIP, after EA, has previously been reported in adults with persistent allergic rhinitis [35]. SP was also found to decrease sharply (by 83.2%) 18 to 24 hours after the first manual acupuncture treatment in adults with persistent allergic rhinitis in a study conducted by the authors (unpublished data). Substance P expression was also inhibited in the nasal mucosa of mice with experimentally induced allergic rhinitis following acupuncture (associated with inhibition of signal transducer and activator of transcription 6 (STAT 6), nuclear factor kappa B (NFκB), and inducible nitric oxide synthase (iNOS)) [36]. While CGRP downregulation after acupuncture has been reported in migraine, menopausal hot flushes, and spinal nerve lesion studies, to date no studies have shown a downregulation of CGRP in allergic rhinitis.

3.2. Cytokines. STAT 6 and NFκB are transcription factors which play an essential role in Th2 cell differentiation [36]. Inhibition of STAT 6 and NFκB would therefore suppress Th2 cell differentiation, shifting the Th1/Th2 balance away from Th2 dominance, which characterizes atopy [36]. Significant decreases in STAT 6 and NFκB, along with the vasodilator iNOS were reported in the mouse study mentioned in the previous paragraph [36]. A recent human study comparing acupuncture with Loratadine for perennial allergic rhinitis reported an increase in mean values for interleukin-10 (IL-10), but the increase was not statistically significant, possibly due the study being underpowered [37]. The same study also found no change in allergen-specific immunoglobulin E (IgE) for house dust mite, total IgE, interleukin-4 (IL-4), or interferon gamma (IFN-γ) [37]. A small number of earlier human studies on the treatment of allergic rhinitis with acupuncture showed that a trend towards Th2 cytokines os being downregulated, but very little evidence of Th1 cytokines is being upregulated [31, 38–40].

3.3. Neurotrophins. Neurotrophins have been shown to be selectively upregulated or downregulated by acupuncture depending on the condition. Recent research on neurotrophins has mainly focused on cerebral ischaemia, spinal cord injury, and depression [13, 41–44]. No research has yet been published on the possible modulation of neurotrophins by acupuncture in allergic rhinitis. It is hypothesised, however, that the abundance of VIP+ parasympathetic nerves around blood vessels in the lamina propria of the nasal mucosa (which has been observed in allergic rhinitis) may be the result of upregulation of neurotrophins, and this neuronal abundance would contribute to exacerbating inflammatory oedema in the nasal mucosa [45–47].

3.4. Chemokines. Chemokines have received little attention from acupuncture researchers until very recently. A new study has reported that EA upregulated chemokine CXCL10 in a rodent model of complete Freund's adjuvant- (CFA-) induced hind paw inflammation [16]. EA increased the production and release of both IFN-γ and CXCL10 which in turn increased the number of infiltrating opioid peptide-containing CXCR3+ macrophages [16]. This study establishes an important novel link between immune system mediators such as cytokines, chemokines, and macrophages and the nervous system's opioid pathways.

The effects of acupuncture on chemokines and chemokine receptors appear to be selective. The upregulation of CXCL10 increased the production and release of CXCR3+ macrophages which contain opioids [16]. Activation of opioid receptors, in turn, will desensitize chemokine receptors thereby inhibiting the production and release of proinflammatory cytokines as well as the chemokines CCL2, CCL3, and CXCL8 [48]. In a rat model of embryo implantation failure, acupuncture increased expression of CCL2, CXCL8, and the subset of uterine natural killer (uNK) cells in the endometrium and reduced embryo implantation failure [49].

4. Complex Cross Talk between Receptors

Chemokine receptors, opioid receptors and TRPV1 receptors have been reported to participate in complex cross talk, not unlike that between neuropeptides, cytokines, and neurotrophins [48] Chemokine receptors and opioid receptors can inhibit each other by a process known as heterologous desensitization [48]. Adenosine, by activating A2a receptors, can also desensitize chemokine receptors [48] (see Figure 1). Chemokine receptors sensitize TRPV1; hence, any desensitization of chemokine receptors (by adenosine or opioid receptors) would inhibit the sensitization of TRPV1 (via a phospholipase C β/protein kinase C [PLCβ/PKC] pathway) [48]. Interactions between cannabinoid receptors and opioid receptors after acupuncture have been reported. Cannabinoid receptor CB2 has been shown to participate in the EA-induced increase of opioid expression in keratinocytes in inflamed skin (in a CFA rat hind paw inflammation model) [12].

Cannabinoid receptor CB1 has been shown to mediate phosphorylation of both STAT3 and glycogen synthase kinase-3β after acupuncture in rodent cerebral ischaemia studies [50, 51].

Upregulation of CB1 expression after EA also produced upregulation of dopamine receptors D1 and D2 in the striatum (in a CFA rat hind paw inflammation model) [14].

Since acupuncture has already been shown to be capable of activating adenosine and opioid receptors and now has

been shown to selectively modulate chemokines, there are a number of possible pathways via which TRPV1 might be downregulated by acupuncture.

EA has previously been reported to downregulate TRPV1 by inhibiting phosphorylation of PI3K in the dorsal horn of rats (in a carrageenan hind paw inflammation model) [19]. NGF activates the tyrosine kinase A/phosphatidylinositol 3-kinase/phosphatidylinositol phosphate 3/protein kinase Akt (trkA/PI3K/PIP3/Akt) signalling pathway which increases the sensitivity and expression of TRPV1, so blocking this pathway would prevent this sensitization of TRPV1 by NGF [19]. EA also activated p38 mitogen activated protein kinase/activating transcription factor 2/transient receptor potential vallinoid (p38 MAPK/ATF-2/TRPV1) signalling pathway thereby downregulating TRPV1 expression in the dorsal horn of rats; however, cyclooxygenase 2 (COX-2) was found to play no role (in a CFA hind paw inflammation model) [5]. In the same study, hind paw oedema was unchanged until day 14 after CFA injection. However, the reasons for this delayed action are unclear [5].

5. Effects of Acupuncture on Thermal Hyperalgesia and Mechanical Allodynia in Inflammatory and Neuropathic Pain

According to the ancient Roman doctor Celsius, inflammation was characterised by redness, heat, swelling, and pain [52]. In research using animal inflammatory pain models, pain threshold is generally measured as a response to mechanical pressure (allodynia) or to changes in temperature, usually heat (thermal hyperalgesia). In reducing inflammatory hyperalgesia, low frequency EA (typically 1 to 4 Hz) has been consistently reported to be effective (see Table 1). However, there is conflicting data on the effectiveness of high frequency EA (100–150 Hz) with some researchers reporting that 120 Hz EA failed to alleviate thermal hyperalgesia and mechanical allodynia in diabetic neuropathic pain in rats, while 2 Hz EA was effective [22]. Other studies have reported effective attenuation of inflammatory hind paw hyperalgesia in rats using 100 Hz EA [11, 14, 16].

Low frequency EA (2 Hz) effectively relieved mechanical allodynia in a rat spinal nerve ligation model [23]. This improvement in allodynia was associated with a reduction of both TRPV1 and CGRP in the ipsilateral spinal dorsal horn immediately above and below the lesioned level (L5) [23]. Since TRPV1 is capable of producing and releasing both CGRP and SP, the decrease in CGRP may be a direct result of the reduced expression of TRPV1 [53]. Additionally, since CGRP and SP interpromote each other, the CGRP downregulation could be secondary to a downregulation of SP production and release by TRPV1 [54].

EA effects on thermal hyperalgesia were blunted, but not obliterated, in TRPV1 knock-out mice [55]. TRPV1 knock-out mice required a stronger EA stimulation than wild type (C 57 BL/6) mice to achieve an analgesic effect [55]. This suggests that while TRPV1 downregulation contributes to EA effects on thermal hyperalgesia, TRPV1 is not solely responsible, as TRPV1 knock-out did not obliterate the effect. In contrast, histamine-induced itch is completely extinguished in TRPV1 knock-out mice [56].

Low frequency EA (2 Hz) has also been shown to decrease mechanical hyperalgesia in both CFA and carrageenan-induced CD1 mouse hind paw inflammation, accompanied by a significant downregulation of acid sensing ion channel 3 (ASIC3) overexpression in dorsal root ganglion neurons [17]. In another study, also using CFA and carrageenan-induced mouse hind paw inflammation (this time in ICR mice), 2 Hz EA reduced mechanical and thermal hyperalgesia accompanied with decreased expression of sodium voltage-gated (Nav) channels Nav1.7 and Nav1.8 (but not Nav1.9) in dorsal root ganglion neurons [18].

In another CFA hind paw inflammation study with rats, EA alternating between 2 Hz and 100 Hz alleviated mechanical allodynia and blocked activation of the extracellular-regulated protein kinase 1/2-cyclooxygenase-2 (ERK1/2-COX-2) and extracellular-regulated protein kinase 1/2-cAMP response element binding protein- neurokinin-1 receptor (ERK1/2-CREB-NK-1) pathways, but had no effect on Ets-like kinase 1 (Elk1) (a downstream nuclear substrate of ERK1/2) in the spinal dorsal horn [15]. Since SP is the endogenous ligand for NK-1 receptor, blocking NK-1 would downregulate the proinflammatory effects of SP, as has been previously reported [57].

In a spinal cord injury neuropathic pain model in rats, manual acupuncture significantly relieved thermal hyperalgesia and mechanical allodynia [24]. These acupuncture analgesic effects were associated with Jun-N-terminal kinase (JNK) inhibition in astrocytes in lamina I-II of the dorsal horn and inhibited phosphorylation of JNK downstream substrate, c-Jun [24]. Acupuncture also inhibited JNK-dependent expression of chemokines monocyte chemotactic protein 1 (MCP-1) (also known as CCL2), macrophage inflammatory protein 1β (MIP-1β) (also known as CCL4), and macrophage inflammatory protein 3α (MIP-3α) (also known as CCL20) in spinal astrocytes [24]. In a rat CFA hind paw study, EA alleviated mechanical allodynia (alternating between 2 Hz and 100 Hz) but not paw oedema by suppressing the spinal JNK1/2 pathway [4]. In this study, TRPV1 expression in the spinal dorsal horn was unaffected [4].

Further research is needed to investigate whether these modulations of TRPV1, produced by acupuncture in the spinal dorsal horn of rats or mice, can also be found in the trigeminal ganglia or nasal mucosa of humans with allergic rhinitis.

6. A Novel Cholinergic Anti-Inflammatory Pathway

It has been suggested that acupuncture may activate a cholinergic anti-inflammatory pathway involving acetylcholine release from vagus nerves binding to α7-nicotinic receptors (α7-nAChRs) on macrophages, thereby inhibiting the release of proinflammatory cytokines [58]. A recent study by Torres-Rosas et al. has demonstrated that there is in fact an anti-inflammatory pathway activated by EA which involves the vagus and sciatic nerves and is mediated by dopamine [59].

Table 1: Effects of electroacupuncture and manual acupuncture on inflammatory signalling pathways and receptors.

Author and year	Model	EA/acup	Acupuncture points	Effect	Thermal hyperalgesia	Mechanical allodynia	Oedema
Huang et al. 2004 [10]	CFA hind paw FSD rats	EA 100 Hz	(B) ST 36, SP 6		No effect	Reduced	
Huang et al. 2008 [11]	CFA hind paw FSD rats	EA 100 Hz	(B) ST 36, SP 6	Reduced thermal hyperalgesia and increased EA tolerance EA effects abolished by high dose Naloxone suggesting dynorphin mediation	Reduced		
Su et al. 2011 [12]	CFA hind paw MSD rats	EA 2 Hz	(I) GB 30, GB 34	Increased POMC and β-END expression in keratinocytes, macrophages, and T-lymphocytes in inflamed skin via activation of CB2 cannabinoid receptors	Reduced	Reduced	
Zhang et al. 2012 [13]	CFA hind paw rats	EA 10 Hz	GB 30	Suppressed spinal IL-17 and p-NR1	Reduced	Reduced	
Shou et al. 2013 [14]	CFA hind paw MSD rats	EA 2 Hz EA 100 Hz	ST 36, BL 60	Increased CB1 expression with upregulation of D1 and D2 expression in striatum	Reduced		
Fang et al. 2013 [5]	CFA hind paw MSD rats	EA 2/100 Hz alt	(B) ST 36, BL 60	Inhibition of p38/MAPK/ATF-2/TRPV1 pathway producing downregulation of TRPV1 in spinal dorsal horn		Reduced	Reduced only at day 14
Fang et al. 2014 [15]	CFA hind paw MSD rats	EA 2/100 Hz alt	(B) ST 36, BL 60	Inhibited ERK1/2-COX-2 and ERK1/2-CREB-NK-1 pathway Downregulated NK-1 hence inhibiting SP		Reduced	
Wang et al. 2013 [9]	CFA hind paw M Wistar rats	EA 100 Hz	(B) GB 30	EA effects suppressed by opiate antagonists naloxone and natrindole	Reduced	Reduced	Reduced slightly
Wang et al. 2014 [16]	CFA hind paw M Wistar rats	EA 100 Hz	(B) GB 30	TNF-α and IL-1β downregulated, IL-13 upregulated, and IL-1α and IL-4 unchanged IFN-γ up-regulated stimulating CXCL10 increasing CXCR3+ macrophages	Reduced	Reduced	
Du et al. 2014 [4]	CFA hind paw MSD rats	EA 2/100 Hz alt	(B) ST 36, BL 60	Inhibited JNK1/2 and COX-2 but not TRPV1		Reduced	No effect
Chen et al. 2011 [17]	Carrageenan & CFA hind paw CDI mice	EA 2 Hz	ST 36	Decreased overexpression of ASIC3 in DRG		Reduced	
Huang et al. 2013 [18]	Carrageenan & CFA hind paw F ICR mice	EA 2 Hz	ST 36	Decreased expression of sodium voltage-gated channels Nav 1.7 and Nav 1.8 but not Nav 1.9 in DRG	Reduced	Reduced	
Kim et al. 2012 [19]	Carrageenan hind paw MSD rats	EA 2/100 Hz alt	(B) ST 36, SP 6	Inhibition of p-PI3K blocked trkA/PI3K/PIP3/Akt pathway	Reduced	Reduced	
da Silva et al. 2015 [20]	Carrageenan gastrocnemius C57BL/6 mice	Manual acup	SP 6	Increased IL-10 in inflamed muscle Induced a phenotypic switch from M1 to M2 macrophages in inflamed muscle	Reduced	Reduced	Reduced
Kim et al. 2009 [21]	Capsaicin hind paw MSD rats	EA 2 Hz	(I) SI 3, TE 8 (GB 30, GB 34; BL 40, BL 60; GV 2, GV 6; LI 3, LI 6)	SI 3 & TE 8 effective, but other point combinations were not Secondary (but nor primary) hyperalgesia is mediated by MOR and DOR but not KOR or adrenergic receptors		Reduced secondary but not primary hyperalgesia	

Table 1: Continued.

Author and year	Model	EA/acup	Acupuncture points	Effect	Thermal hyperalgesia	Mechanical allodynia	Oedema
Hwang et al. 2011 [22]	Exp. diabetic neuropathic pain MSD rats	EA 2 Hz EA 120 Hz	(B) SP 9 or ST 36	Decreased cleavage of p35 to p25 hence inhibited p35/p25/Cdk5/MAPK and/or p35/p25/Cdk5/NMDA pathways	EA 2 Hz reduced; EA 120 Hz no effect	EA 2 Hz reduced; EA 120 Hz no effect	
Jiang et al. 2013 [23]	SNL MSD rats	EA 2 Hz	(I) ST 36, BL 60	Downregulated TRPV1 in spinal dorsal horn and reduced CGRP		Reduced	
Lee et al. 2013 [24]	SCI MSD rats	Manual acup	GV 26, (B) GB 34	Inhibited JNK/p-c-Jun in spinal astrocytes Decreased chemokines MCP-1, MIP-1β, and MIP-3α	Reduced	Reduced	
Yu et al. 2013 [25]	CCI SD rats	EA 2 Hz EA 15 Hz	ST 36, GB 34	EA reduced ERK1/2 phosphorlylation and P2X3 expression in spinal cord	Reduced EA 2 Hz > 15 Hz	Reduced EA 2 Hz > 15 Hz	
Hsu et al. 2014 [26]	CCI MSD rats	EA 2 Hz EA 15 Hz	(R) ST 36, ST 37	EA increased cerebral TRPV4 but not TRPV1 No change in spinal TRPV4 or TRPV1	Reduced	Reduced	
Wang et al. 2009 [27]	Surgical trauma MSD rats	EA 2/60 Hz alt	(B) ST 36, Lanwei (M-LE-13)	Suppressed lymphocyte proliferation Reduced splenic T cells production of Th1 cytokines (IL-2 and IFN-γ) increased Th2 cytokines (IL-4, IL-10) Suppressed activity of ERK1/2, p38, NF-κB, and AP-1	Reduced	Reduced	

EA: electroacupuncture, acup: acupuncture, CFA hind paw: model of inflammation induced by injection of complete Freund's adjuvant into rats' hind paws, FSD rats: female Sprague Dawley rats, MSD: male Sprague Dawley rats, alt: alternating, (B): bilateral, (I): ipsilateral, CB: cannabinoid receptor, D: dopamine receptor, TRPV: transient receptor potential vallinoid, proopiomelanocortin, β-END: beta endorphin, p-NR1: phosphorylation of NR1, SP: substance P, CGRP: calcitonin gene-related peptide, NK-1: neurokinin 1 receptor, DRG: dorsal root ganglion, MAPK: mitogen activated protein kinase, ATF-2: activating transcription factor 2, ERK: extracellular-regulated protein kinase, CREB: cAMP response element binding protein, NK-1: neurokinin 1 receptor, JNK: c-jun N terminal kinase, ASIC3: acid sensing ion channel 3, Nav: sodium voltage-gated channels, trkA: tyrosine kinase A, PI3K/PIP3: phosphatidylinositol 3 kinase/phosphatidylinositol phosphate 3 pathway, MOR: Mu opioid receptor, DOR: delta opioid receptor, KOR: kappa opioid receptor, AP-1: activator protein 1, Cdk5: cyclin-dependent kinase 5, and NMDA: N-methyl-D-aspartate.

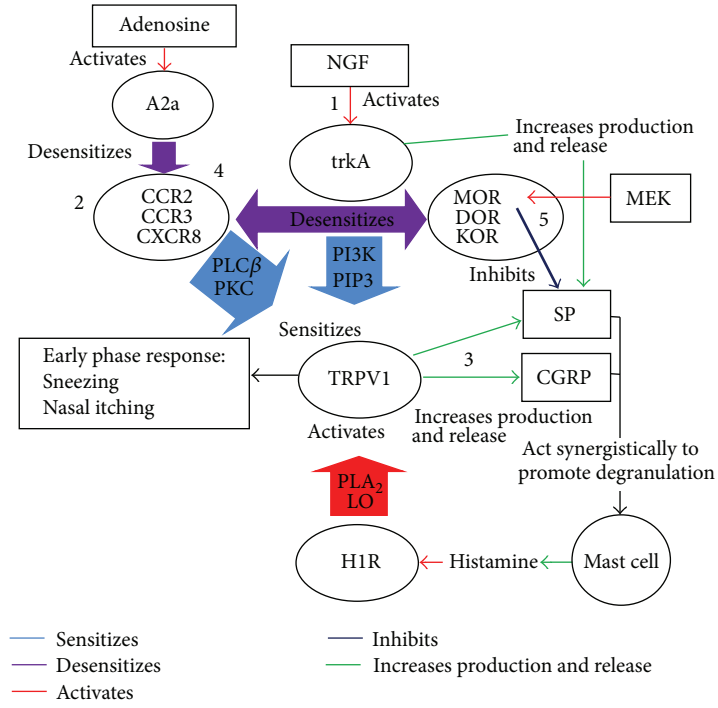

Figure 1: Proposed model for the complex cross talk between various receptors and mediators in early phase response in allergic rhinitis. 1: nerve growth factor (NGF) activates tyrosine kinase A (TrkA) receptor which in turn increases production and release of substance P (SP). Activation of TrkA receptor also initiates signalling via the PI3K/PIP3 pathway to increase expression and sensitivity of transient receptor potential vallinoid (TRPV1) receptor. 2: chemokine receptors (CCR2, CCR3, and CXCR8) sensitize TRPV1 receptor via a PLCβ/PKC pathway. 3: TRPV1 receptor increases production and release of proinflammatory neuropeptides SP and CGRP which act synergistically to promote degranulation of primed mast cells. Histamine released by mast cells activates histamine 1 receptor (H1R) producing signalling via the phospholipase A_2/lipoxygenase pathway to activate TRPV1, triggering early phase allergic inflammatory response. 4: chemokine receptors are heterologously desensitized by both adenosine (A2a) receptors and opioid receptors (MOR, DOR, and KOR). 5: Substance P is inhibited by met-enkephalin via Mu opioid receptors (MOR). A2a: adenosine 2a receptor, CCR2, CCR3: CC chemokine receptors 2 & 3, CXCR8: CXC chemokine receptor 8, PLCβ: phospholipase C β, PKC: protein kinase C, NGF: nerve growth factor, TRPV1: transient receptor potential vallinoid 1, TrkA: tyrosine kinase A receptor, H1R: histamine 1 receptor, SP: substance P, CGRP: calcitonin gene-related peptide, PI3K/PIP3: phosphatidylinositol 3 kinase/phosphatidylinositol phosphate 3 pathway, PLA_2/LO: phospholipase A_2/lipoxygenase pathway, MOR: Mu opioid receptor, DOR: delta opioid receptor, KOR: kappa opioid receptor, and MEK: met-enkephalin.

However, this study also found that α7-nAChRs were not involved in this anti-inflammatory pathway [59]. In a mouse model of induced sepsis, EA rescued mice from polymicrobial peritonitis and controlled systemic inflammation [59]. EA stimulation of the sciatic nerve induced release of DOPA decarboxylase from the vagus nerve leading to dopamine production in the adrenal medulla [59]. Of the dopamine receptor family, D1 receptors were shown to be essential to EA anti-inflammatory effects, but D2 receptors appeared to have little involvement [59]. Vagal stimulation was also associated with reduced serum levels of the cytokines tumour necrosis factor (TNF), MCP-1/CCL2, IL-6, and IFN-γ [59]. TRPV1 agonist, capsaicin, abolished the anti-inflammatory effects of EA [59]. This underlines the importance of TRPV1 role in the anti-inflammatory effects of acupuncture.

7. Adenosine

Adenosine triphosphate (ATP) released locally at acupuncture points, in response to needle stimulation is metabolized into adenosine, which activates adenosine A1 receptors, creating an analgesic effect in inflammatory pain [60, 61]. Adenosine, via A2a receptors, has also been shown to desensitize chemokine receptors, hence blocking the capacity of chemokine receptors to both sensitize TRPV1 receptors via a PLCβ/PKC pathway and to desensitize opioid receptors [48]. Adenosine has previously been reported to inhibit TRPV1 activation, and this action may be mediated via the PLCβ/PKC pathway [62]. In addition, adenosine, via A3 receptor activation has recently been shown to be effective in relieving persistent neuropathic pain [63]. Further investigation is needed to determine whether or not adenosine modulates TRPV1 (and possibly opioid receptors) in the nasal mucosa or trigeminal ganglia.

8. A Revised Model for the Effects of Acupuncture in Allergic Rhinitis

The central importance of TRPV1 in allergic rhinitis raises the question of how acupuncture might modulate TRPV1

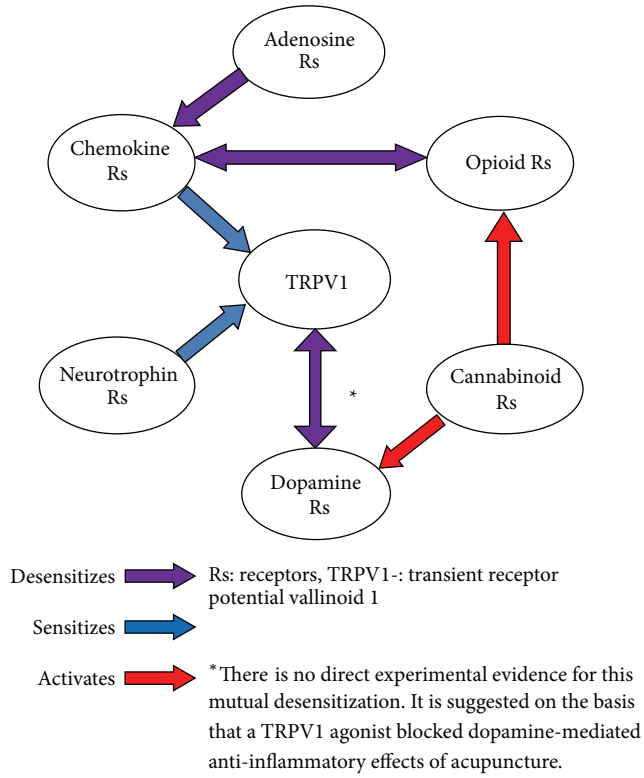

Figure 2: Receptor interactions potentially involved in the anti-inflammatory effects of acupuncture.

either directly or indirectly. In our previous proposed model, it was suggested that acupuncture might modulate TRPV1 by blocking neurotrophic sensitization (by blocking phosphorylation of PI3K, as seen in EA for neuropathic pain), by downregulating SP and CGRP (with consequent suppression of degranulation of primed mast cells) and possibly through some pathway associated with adenosine. It now appears that this adenosine-induced downregulation of TRPV1 may occur through desensitization of chemokines receptors (which sensitize TRPV1 via a PLCβ/PKC pathway) [48] (see Figure 1).

Downregulation of SP by met-enkephalin has previously been reported [64, 65]. New research showing that acupuncture can stimulate production of CXCR3+ macrophages (which contain met-enkephalin) clarifies the source of the opioid stimulation. Opioid receptor desensitization of chemokine receptors, with consequent inhibition of both cytokine production and sensitization of TRPV1, provides another pathway via which acupuncture-induced opioid stimulation could generate anti-inflammatory effects.

TRPV1 interactions with both cannabinoid and dopamine receptors have also now been demonstrated in murine and rodent studies of inflammatory hyperalgesia (see Table 1). Further studies are needed to determine whether these effects of acupuncture in the spinal dorsal root ganglia during inflammatory hyperalgesia are relevant to the trigeminal ganglia in allergic rhinitis.

Recent research into the effects of acupuncture on signalling pathways and receptors in inflammatory pain has focused mainly on mechanical allodynia and thermal hyperalgesia, but little new work has been done on inflammatory oedema. Since TRPV1 appears to play a central role in inflammatory oedema, it is possible that signalling pathways activated by acupuncture which are independent of TRPV1 may be associated with reduction of thermal and mechanical hyperalgesia but not inflammatory oedema [66]. Since nasal swelling is more significant clinically in allergic rhinitis than hyperalgesia, signalling pathways identified in hyperalgesia studies which do not involve TRPV1 may be less relevant to acupuncture's effects on allergic rhinitis.

The effects of acupuncture on chemokines and chemokine receptors appear to be selective. While desensitization of chemokine receptors by adenosine and by interaction with opioid receptors may reduce sensitivity of TRPV1, it is also clear that acupuncture can increase chemokines such as CXCL10, CCL2, and CXCL8.

Interactions between receptor types are now being reported with an increasing frequency. Acupuncture has been shown to influence the interactions between opioid receptors and chemokine receptors, as well as cannabinoid receptors (see Figure 2). Interactions between cannabinoid receptors and dopamine receptors have also been shown to be modulated by acupuncture [14].

9. Conclusion

Our 2013 model proposed that acupuncture downregulates proinflammatory neuropeptides and neurotrophins, alters Th1/Th2 cytokine balance, and modulates TRPV1.

Acupuncture has already been shown to downregulate SP and VIP in allergic rhinitis and may also downregulate

CGRP. This reduction in SP and VIP has been associated with improvements in clinical signs and symptoms.

Acupuncture may downregulate neurotrophins NGF and BDNF in allergic rhinitis, but evidence is currently lacking.

Acupuncture has been shown to alter Th1/Th2 cytokine balance away from Th2 dominance in a variety of clinical contexts including allergic rhinitis; however, more robust evidence is still needed. The role of transcription factors involved in Th2 differentiation (such as STAT 6 and NFκB) merits further investigation.

While numerous new signalling pathways have been identified in the anti-inflammatory actions of acupuncture in animal model studies on induced hind paw inflammation, spinal cord injury, and chronic constriction injury, it is unclear how much relevance these studies might have to allergic rhinitis (see Table 1). Frequently, the main outcome measures for these studies have been measures of inflammatory pain, thermal and mechanical hyperalgesia, as opposed to inflammatory oedema, which would be more likely to be relevant to allergic rhinitis.

Recent research has also shown that acupuncture can modulate chemokines and that interactions between chemokine and opioid receptors may have relevance to reducing the expression and sensitivity of TRPV1. Further research on the effects of acupuncture on chemokines is needed.

A novel cholinergic anti-inflammatory pathway involving vagal stimulation and dopamine mediation has now been demonstrated [59]. This pathway is neither parasympathetic nor sympathetic but involves both. In acupuncture analgesia research, dopamine receptors have been shown to act in a selective manner. D1 receptors in the brain inhibited acupuncture analgesia, while D2 receptors in the spine enhanced analgesic effects [27]. Torres-Rosas et al. demonstrated that D1 receptors appeared to play a significant role in the anti-inflammatory effects of acupuncture while D2 receptors did not [59]. The role of dopamine receptors and their interactions with cannabinoid receptors, and possibly TRPV1, in the anti-inflammatory effects of acupuncture have only just begun to be explored. Further exploration of receptor interactions and their possible involvement in acupuncture effects would be valuable.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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