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Basic Study

Effect of moxibustion on expressions of HO-1 and MCP-3 protein in colon of rats with Crohn's disease

艾灸对克罗恩病大鼠结肠 HO-1 和 MCP-3 蛋白表达的影响

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Abstract
Objective: To observe the effect of moxibustion therapy on heme oxygenase-1 (HO-1) and monocyte chemoattractant protein-3 (MCP-3) protein expressions in the colonic mucosa of rats with Crohn's disease (CD), and to explore the intestinal mucosal immune mechanism of moxibustion therapy in treating CD.
Methods: The CD rat model was established using the internationally accepted Morris method. The rats were randomly divided into a model group, a herbal cake-partitioned moxibustion group, a mild moxibustion group, a cigarette moxibustion group and a hot compress group, which were compared with the normal group. Except the normal group and the model group, rats in the other groups accepted different moxibustion therapies on bilateral Tianshu (ST 25). Hematoxylin-eosin (HE) staining was conducted and the pathological changes of the colon were observed under light microscope; the expressions of HO-1 and MCP-3 protein in rat's colonic mucosa were determined by immunohisto-chemistry.
Results: Compared with the normal group, rats in the model group showed mucosal defect, villus destruction or loss, submucosal congestion and edema, glandular destruction or disappearance, reduced goblet cells, ulcer formation, significantly increased positive target area and positive target integral optical density of HO-1 and MCP-3 protein expression (all $P<0.01$). After treatment, compared with the model group, colonic mucosa was significantly improved in the herbal cake-partitioned moxibustion group and the mild moxibustion group, which mainly showed that the intestinal glands were arranged regularly, ulcer surfaces were covered by the neoformative epitheliums, or intestinal ulcers were replaced by the nascent granulation tissue, and submucosal edema was alleviated, with a small amount of inflammatory cell infiltration. The total areas and the integral optical densities of the positive targets for rat's colonic mucosa HO-1 and MCP-3 protein expressions were decreased (all $P<0.01$). Compared with the cigarette moxibustion group and the hot compress group, the total areas and the integral optical densities of the positive targets for rat's colonic mucosa HO-1 and MCP-3 protein expressions were significantly decreased (all $P<0.01$) in the herbal cake-partitioned moxibustion group and the mild moxibustion group.
Conclusion: Herbal cake-partitioned moxibustion and mild moxibustion can significantly improve the inflammatory response of colonic mucosa in CD rats. It can down-regulate the expressions of HO-1 and MCP-3 proteins in the colonic mucosa of CD rats, which may be one of the mechanism in intestinal mucosal immunity caused by moxibustion therapy.
Keywords: Moxibustion Therapy; Moxa Stick Moxibustion; Medicinal Cake-partitioned Moxibustion; Crohn Disease; Heme Oxygenase-1; Chemokine CCL7; Rat

【摘要】目的：通过观察艾灸对克罗恩病(CD)大鼠结肠黏膜血红素氧合酶-1 (HO-1)和趋化因子单核细胞趋化蛋白-3 (MCP-3)蛋白表达的影响，探讨艾灸治疗 CD 的肠黏膜免疫机制。**方法：**采用国际公认的 Morris 方法制备 CD 大鼠模型。将动物随机分为模型组、隔药灸组、温和灸组、烟条灸组和热水灸组，并与正常组大鼠作对照，除正常组和模型组不做治疗外，其他各组大鼠选取双侧天枢进行相应治疗。应用苏木精-伊红(HE)染色，光镜下观察结肠病理学变化；采用免疫组化方法，观察大鼠结肠黏膜 HO-1、MCP-3 蛋白的表达。**结果：**与正常组比较，模型组大鼠可见黏膜缺损，绒毛破坏或缺失，黏膜下层充血水肿，腺体破坏或消失，杯状细胞减少，溃疡形成，大鼠结肠黏膜组织 HO-1、MCP-3 蛋白表达阳性目标总面积和阳性目标积分光密度显著增高(均 $P<0.01$)。治疗后，与模型组比较，隔药灸组和温和灸组大鼠结肠黏膜有明显改善，主要表现为肠腺排列规则，溃疡表面由新生的上皮细胞

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覆盖，或肠壁溃疡由新生的肉芽组织代替，黏膜下水肿减轻，有少量炎性细胞浸润，大鼠结肠黏膜组织 HO-1、MCP-3 蛋白表达阳性目标总面积和阳性目标积分光密度降低(均 $P<0.01$)；与烟条灸组和热水灸组相比，隔药灸组及温和灸组大鼠结肠组织 HO-1、MCP-3 蛋白表达阳性目标总面积和阳性目标积分光密度均显著降低(均 $P<0.01$)。**结论**：隔药灸和温和灸能够明显改善 CD 大鼠结肠黏膜组织炎性反应；能够下调 CD 大鼠结肠黏膜 HO-1 和 MCP-3 蛋白的表达，这可能是艾灸治疗 CD 的肠黏膜免疫作用机制之一。

【关键词】灸法；艾条灸；药饼灸疗法；克罗恩病；血红素氧合酶-1；趋化因子 CCL7；大鼠

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Crohn's disease (CD) and ulcerative colitis (UC) are collectively called inflammatory bowel disease (IBD), a group of chronic and nonspecific inflammatory diseases of the intestines. IBD incidence is higher in Western countries; however, recently, it has increased in China year by year[1]. The pathogenesis of CD is not clear, current studies have shown that the pathogenesis may be related to the immune dysfunction of human body and intestinal mucosa[2-3].

In recent years, studies have found that abnormal expressions of chemokines, proinflammatory cytokines and adhesion molecules are closely related to the incidence of IBD[4-5]. Many studies showed that monocyte chemoattractant protein-3 (MCP-3) and heme oxygenase-1 (HO-1) played an important role in the development of IBD[6-10]. We previously found that moxibustion can down-regulate the expressions of MCP-1 and interleukin-8 (IL-8) in colonic mucosa of CD rats[11]. Based on this, we investigated the effect of moxibustion therapy on the expressions of HO-1 and MCP-3 protein in the colonic mucosa of CD rats. The aim of this study was to explore the effects of moxibustion therapy on the immune function of colonic mucosa in CD rats, and its possible mechanisms.

1 Material and Methods

1.1 Experimental animals and groups

A total of 52 clean grade male Sprague-Dawley (SD) rats were provided by the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine, weighing (160 ± 20) g. Randomly selected 10 rats as the normal group, the other 42 rats were used to establish CD models by the Morris method[12].

When the models were established, 2 rats were randomly selected respectively from 42 model rats and the 10 normal rats, and then sacrificed. The colon tissues were separated for hematoxylin-eosin (HE) staining to determine whether the model was successful. After successful model validation, the 40 rat models were randomly divided into a model group, a herbal cake-partitioned moxibustion group, a mild moxibustion group, a cigarette moxibustion group and a hot compress group, with 8 rats in each group.

1.2 Main reagents

Pentobarbital sodium (Shanghai Chemical Reagent Factory, China), 2, 4, 6-three trinitrobenzene sulfonic acid (Sigma, USA), specially made fine moxa stick (Suzhou Dongfang Moxa Factory, China), Huangguoshu Brand cigarettes (Guizhou Zhongyan Industrial Company, China), antibodies against HO-1 and MCP-3 (Sigma, USA) and EnVision reagent (Shanghai Chemical Reagent Factory, China).

1.3 Model preparation

The 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) was used to establish the rat model of Crohn's disease according to the internationally accepted Morris method[12]. The 50% ethanol solution was prepared with 5 mL ethanol and distilled water; TNBS (0.5 mL) and 50% ethanol solution (0.25 mL) were mixed into (2: 1) TNBS enema and sealed for the future use. Intraperitoneal anesthesia was performed with 0.3% pentobarbital sodium. Totally 0.5 mL TNBS enema in 1 mL syringe was injected into the rat rectum via anus in the model group. The rats were maintained up-side down for 10 min, to prevent the outflow of the enema solution. When the models were established, 2 rats were randomly selected respectively from the model rats and the normal rats, and then sacrificed by cervical dislocation. Colonic tissues were collected by clipping from 5 cm above the anus.

To confirm if the modeling was successful, visual observation and HE staining of the colonic mucosal tissues were conducted.

Discoveries from observing the colonic mucosa by naked eyes: The mucosal surface of the model rats showed erosion and bleeding, and the lesions showed thickening bowel wall and stenosis of intestinal lumen, in a jump distribution; significant changes of the paving stones. In normal group, the mucosal surface was smooth and the intestinal wall was normal.

Light microscope observation of the colonic mucosa HE staining: The intestinal inflammation of the model rats was significant with inflammatory cell infiltration, epithelial damage, obvious mucosa hyperemia, ulcer, fibroblast proliferation, old hemorrhage, and irregular thickening of the vascular wall, intestinal wall thickening, muscle layer destruction, groups of giant cell reaction and class epithelial reaction, granuloma formation, the typical pathological changes in the intestinal tract of CD, suggesting that the model was successfully established.

1.4 Intervention for different groups

Rats in the normal group and the model group were not treated with moxibustion, but only immobilized as performed in each treatment group.

Herbal cake-partitioned moxibustion group: *Fu Zi* (*Radix Aconiti Lateralis Preparata*), *Rou Gui* (*Cortex Cinnamomi*), *Dan Shen* (*Radix Salviae Miltiorrhizae*), *Hong Hua* (*Flos Carthami*), *Mu Xiang* (*Radix Aucklandiae*), *Huang Lian* (*Rhizoma Coptidis*) and other herbs were ground into fine powder. 2.5 g herbal powder was blended with 3 g yellow rice wine to make a herbal cake of 0.5 cm in diameter and 0.3 cm in thick. Herbal cakes were placed on bilateral Tianshu (ST 25). Acupoint positioning was conducted according to the *Experimental Acupuncture Science*[13]. About 90 mg refined moxa was made into a moxa cone and placed on a herbal cake. Moxibustion therapy was performed for 3 cones on each points (about 10 min), once a day, for a total of 7 times.

Mild moxibustion group: The specially made cigarette type pure moxa stick (0.8 cm in diameter, 11.8 cm in length) was used. One end of the moxa stick was lit to perform mild moxibustion over bilateral Tianshu (ST 25), from 2-3 cm above the skin. Moxibustion therapy was performed for about 10 min over each acupoint each time, once a day, for a total of 7 times.

Cigarette moxibustion group: Huangguoshu Brand cigarette was lit to perform moxibustion over bilateral Tianshu (ST 25), from 2-3 cm above the skin. Moxibustion therapy was performed for about 10 min over each acupoint each time, once a day, with a total of 7 times.

Hot compress group: Glass test tube (about 1 cm in diameter and 10 cm in length) filled with (50±1) ℃ hot water was wrapped by rubber insulation material, including the opening, and fixed. Left the hole unwrapped for inserting the thermometer to monitor the temperature changes. The bottom of the test tube was unwrapped and stuck to the bilateral Tianshu (ST 25). Moxibustion therapy was performed for 10 min over each acupoint each time, once a day, for a total of 7 times.

1.5 Sample collection

At the end of the experiment, the general state of the rats was observed. All the rats were weighed and the sacrificed by cervical dislocation. The abdominal cavity was opened. A piece of 6 cm colon was cut from 5 cm above the anus. After rinsed with tap water, the colon was cut into 2 sections with 3 cm of each. One section was put into liquid nitrogen for cryopreservation; the other section was vertically cut and fixed in 10% formaldehyde solution, labeled and sealed for histopathological and immunohistochemical detection.

1.6 Expression of HO-1 and MCP-3 in colonic mucosa detected by immunohistochemistry

Prepared paraffin sections were subjected to conventional dewaxing to water, then washed with water; washed 3 times with 0.01 mol/L (pH 7.4) PBS for 3 min each time; treated with 1% H_2O_2 for 20 min and washed with PBS for 3 min × 3 times; performed high temperature antigen repair for 10 min × 2 times by microwave, and natural cooling to room temperature; washed 3 min × 3 times with PBS; blocked with 1% normal serum for 20 min at room temperature. Directly added appropriately diluted primary antibody (HO-1, MCP-3, 1:200) and incubated overnight at 4 ℃. Washed 3 min × 3 times with PBS; coloration was performed with 0.04% DAB + 0.03% H_2O_2 for 8 min; washed with water and performed hematoxylin lining dye for 30 s; washed with water, and conducted hydrochloric acid and ethanol differentiation for 2 s; washed with water and performed microwave blue; sealed the slides with resins by conventional method. Randomly selected 3 fields of vision (×200 magnifications) in each slice, and took pictures for the preservation. Total positive target area and positive target integrated optical density were analyzed using MOTIC image analysis system.

1.7 Statistical methods

The data were analyzed by the SPSS 13.0 version statistical software. Measurement data with normal distribution were presented as mean ± standard deviation ($\bar{x} \pm s$). Single factor variance analysis was used to compare among groups (one-way ANOVA). Least significant difference (LSD) was used with homogeneity of variance; Games-Howell test was used for uneven variance. $\alpha=0.05$, $P<0.05$ indicated the difference was of statistical significance.

2 Results

2.1 Morphological observation of rat colon under light microscope

The colonic mucosal epithelia of the normal rats were intact and no inflammatory cells were infiltrated. The monolayer columnar epithelium, lamina propria and colon intestinal glands of mucosal muscular layer were orderly and regularly arranged with clear structure. In the model group, mucosal defects, chorionic villi destruction or loss, and congestion and edema of submucosal layer, destruction or disappearance of the glands, reduction of goblet cells, and formation of ulcer were observed; a small amount of eosinophils and inflammatory cells (mainly neutrophils and lymphocytes) infiltration were found in the lamina propria of the mucosa; part of the mucosal glands appeared atypical

hyperplasia, as well as cryptitis and crypt abscess. Compared with the model group, colonic mucosa showed no significant improvement in the cigarette moxibustion group and the hot compress group. Colonic mucosa was significantly improved in the mild moxibustion group and the herbal cake-partitioned moxibustion group versus the model group. This mainly showed that colonic glands were orderly arranged; the ulcer surfaces were covered by new epithelium; or intestinal ulcers were replaced by new granulation tissues; submucosal edema was reduced with a small amount of inflammatory cell infiltration.

Morphological comparison of colonic mucosa among the groups was shown in Figure 1.

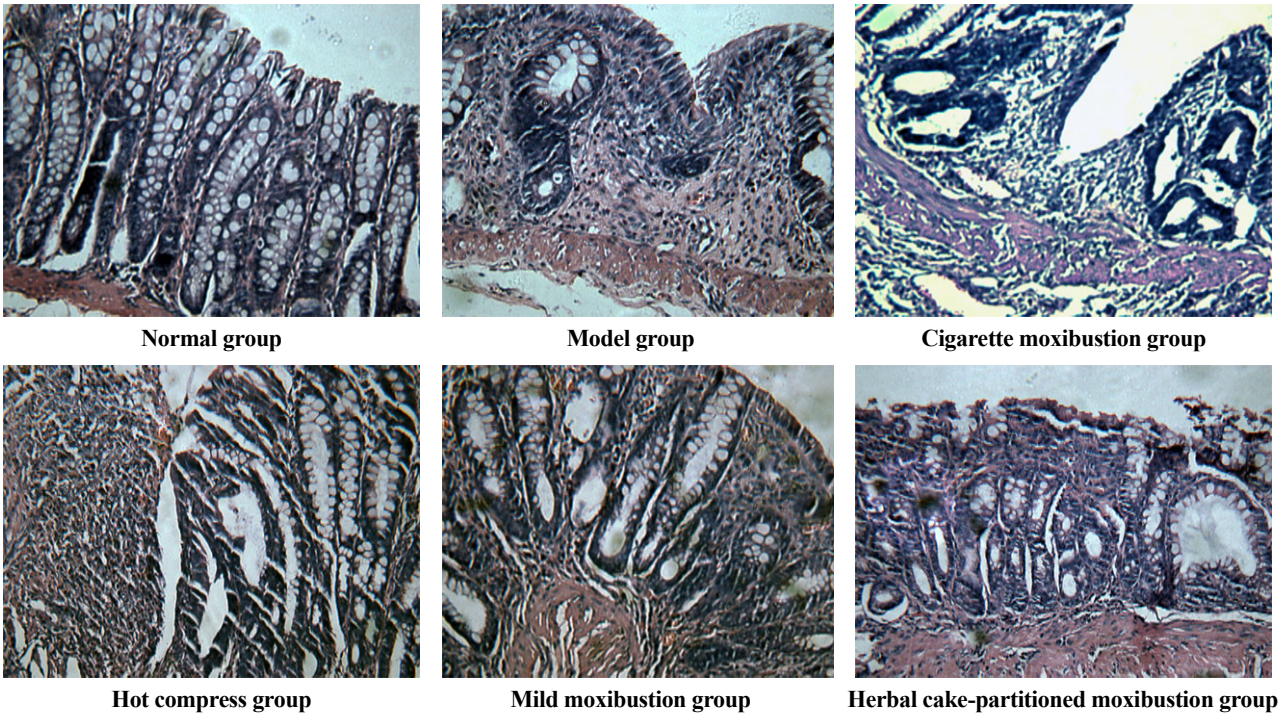

Figure 1. Morphological comparison of colonic mucosa in each group (HE staining, ×200)

2.2 Expression of HO-1 protein in the colonic mucosa of rats in each group

Compared with the normal group, the total positive target area and the positive target integral optical density of HO-1 protein expression were significantly increased in the model group ($P<0.01$). Compared with the model group, the total positive target area and the positive target integral optical density of HO-1 protein expression were decreased in the mild moxibustion group and the herbal cake-partitioned moxibustion group ($P < 0.01$). Compared with the cigarette moxibustion group and the hot compress group, the total positive target area and the positive target integral optical density of HO-1 protein expression were significantly decreased in the herbal cake-partitioned moxibustion group and the mild moxibustion group ($P<0.01$), (Table 1).

Table 1. Comparing total positive target area and positive target integral optical density of HO-1 ($\bar{x}$ ±*s*)

Group	*n*	Total positive target area	Positive target integral optical density
Normal group	8	3377.792±1320.658	1736.264±739.758
Model group	8	32347.620±11085.290[1)]	16491.153±6662.400[1)]
Cigarette moxibustion group	8	32505.625±6900.412	18263.291±3268.755
Hot compress group	8	26206.667±8333.321	14963.558±6007.232
Mild moxibustion group	8	4691.458±2986.881[2)3)]	2140.817±1393.632[2)3)]
Herbal cake-partitioned moxibustion group	8	5766.583±4215.376[2)3)]	3107.455±2657.531[2)3)]

Note: Compared with the normal group, 1) $P<0.01$; compared with the model group, 2) $P<0.01$; compared with the cigarette moxibustion group and the hot compress group, 3) $P<0.01$

2.3 Expression of MCP-3 protein in colonic mucosa of rats in each group

Compared with the normal group, the total positive target area and the positive target integral optical density of MCP-3 protein expression in rat's colonic mucosa were significantly increased in the model group ($P<0.01$). Compared with the model group, the total positive target area and the positive target integral optical density of MCP-3 protein expression in rat's colonic mucosa were all decreased ($P<0.01$) in the herbal cake-partitioned moxibustion group and the mild moxibustion group. Compared with the cigarette moxibustion group and the hot compress group, the total positive target area and the positive target integral optical density of MCP-3 protein expression in rat's colonic mucosa were all significantly decreased ($P<0.01$) in the herbal cake-partitioned moxibustion group and the mild moxibustion group (Table 2).

Table 2. Comparing total positive target area and positive target integral optical density of MCP-3 expression ($\bar{x}$ ±s)

Group	*n*	Total positive target area	Positive target integral optical density
Normal group	8	2271.333±1908.270	887.003±541.610
Model group	8	38365.790±10487.800[1)]	15623.401±4197.702[1)]
Cigarette moxibustion group	8	37827.167±4997.774	14523.345±2059.824
Hot compress group	8	34054.917±8797.707	14991.441±4957.430
Mild moxibustion group	8	5878.583±3540.137[2)3)]	2661.170±1343.754[2)3)]
Herbal cake-partitioned moxibustion group	8	4206.083±3112.483[2)3)]	2031.032±1527.505[2)3)]

Note: Compared with the normal group, 1) $P<0.01$; compared with the model group, 2) $P<0.01$; compared with the cigarette moxibustion group and the hot compress group, 3) $P<0.01$

3 Discussion

According to the etiology and pathogenesis, and the clinical symptoms, CD is classified under the scopes of bloody stools (*Chang Pi*), chronic dysentery, hematochezia, tenesmus diarrhea and abdominal pain in traditional Chinese medicine. We previously found that herbal cake-partitioned moxibustion at Tianshu (ST 25) and Qihai (CV 6) could treat mild to moderate CD, the short-term effective rate was 72.7%[14]. Based on this, we carried out a series of basic researches to explore the mechanisms of acupuncture treatment of CD. This study was to investigate the effect of moxibustion therapy on the expressions of HO-1 and MCP-3 protein in the mucosa of CD rats, and to explore the mechanisms of moxibustion on intestinal mucosal immunity.

Recent studies have found that induced HO-1 and chemokine MCP-3 played an important role in the pathogenesis of IBD[9,15]. HO-1 has anti-inflammatory, anti-apoptosis and anti-proliferative effects[15]. Studies have shown that HO-1 had protective effect on the animal's colon epithelium[10]. When gastrointestinal lesion/injury occurs, a large amount of HO-1 will be induced to repair the lesion/injury. In the colitis animal models, HO-1 expression is significantly increased and the inflammatory response is recovered quickly[16-17]. Recent studies have shown that HO-1 may inhibit the inflammatory response through promoting the secretion of IL-10[18].

MCP-3 is one of the inflammatory chemokines involved in the regulation of immune function and plays an important role in the development of IBD and other diseases[9]. In the experiments to prevent colitis in mice, gene expressions of MCP-3 and its receptor were all lower in colitis mice[19]. As one of the eosinophil chemotactic factors, MCP-3 can promote the aggregation of immune cells to the inflammatory sites[20]. Studies on 8 ulcerative colitis patients with rectal anastomosis and 14 patients with acute colonic haustra inflammation found that, the expressions of IL-8 and MCP-3 in colonic haustra inflammation sites were significantly higher than that in normal colonic haustra. After anti-inflammatory therapy, IL-8 and MCP-3 expressions were significantly decreased[21].

The results of the current studies have suggested that, under the light microscope, mucosal defect, villus destruction or loss, submucosal congestion and edema, glandular destruction or disappearance, reduction of goblet cells and ulcer formation could be seen in the colonic mucosa of the model group; a small amount of eosinophilic granulocytes and inflammatory cell infiltration (mainly neutrophils and lymphocytes) could be seen in the mucosal lamina propria; atypical hyperplasia of a part of mucosal glands, crypt epithelial hyperplasia, cryptitis and crypt abscess were also observed. The protein expressions of HO-1 and MCP-3 in the colonic mucosa of the model group were significantly higher than that of the normal control group, suggesting that a large amount of HO-1 was induced in the colitis animal model and played an anti-inflammatory effect to protect and repair the injured colonic mucosa epithelium; MCP-3 is one of the chemokines of eosinophils, which can accumulate the immune cells to the inflammatory sites and play an anti-inflammatory role through the immunoregulatory

pathway, which is beneficial to the repair of the injured tissue.

After treatment with mild moxibustion and herbal cake-partitioned moxibustion, the inflammatory response in the colonic tissues of CD model rats was significantly improved, and the protein expressions of HO-1 and MCP-3 were significantly decreased. This indicated that moxibustion therapy could support the healthy energy and resist the external pathogen, and played a role in the promoting and adjusting the CD rat's immune system, and reduced the inflammatory response, and repaired the rat's colon mucosal damage. At the same time, we found that the protein expressions of HO-1 and MCP-3 in the colonic mucosa of CD rats showed no significant changes in the cigarette moxibustion and the hot compress groups, and significantly higher than that in the mild moxibustion and the herbal cake-partitioned moxibustion groups. This suggested that moxa played an important role in the efficacy of moxibustion therapy on regulating the immune function of colonic mucosa and reducing the inflammatory reaction in CD rats. Moxa, as a conventional material for moxibustion, has been used for thousands of years. Materials and methods for moxibustion were continuously developed and accumulated in the clinical practice in history. However, moxa has been used all the time. Wu HG and his colleagues confirmed that moxa is the best moxibustion material[22]. Heat, light, smoke, and other substances generated during moxibustion therapy all are effective treatment elements.

Tianshu (ST 25) is the Front-Mu point of the large intestine. It acts to strengthen the spleen, resolve dampness, harmonize the stomach, circulate qi, move blood, and thus stop bleeding and relieve diarrha[23-26]. Moxibustion at Tianshu (ST 25) has a good effect on abdominal pain, diarrhea, and bloody purulent stool[27-30].

The studies of our group showed that the herbal cake-partitioned moxibustion and mild moxibustion could significantly improve the inflammatory response of colonic mucosa in CD rats. It could down-regulate the expressions of HO-1 and MCP-3 protein in the colonic mucosa of CD rats. Promoting and regulating the immune system of CD rats to reduce the inflammatory reaction may be the intestinal mucosal immunity mechanisms in moxibustion treatment of CD. Cigarette moxibustion and hot compress showed no effect on the damage of colonic mucosa in CD rats, suggesting that moxa played an important role in reducing inflammatory reaction of colonic mucosa in CD rats. The regulation of moxibustion therapy on CD rat's colon mucosal immune function is beyond the effect of thermal stimulation, which needs further interpretations and studies.

Conflict of Interest

The authors declared that there was no potential conflict of interest in this article.

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Statement of Human and Animal Rights

The treatment of animals conformed to the ethical criteria in this experiment.

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