Effect of herb-partitioned moxibustion on dopamine levels and dopamine receptor 1 expression in the colon and central nervous system in rats with Crohn's disease

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Abstract

OBJECTIVE: To observe the effect of herb-partitioned moxibustion at the Tianshu (ST 25) and Qihai (CV 6) acupoints in rats with Crohn's disease, and explore the underlying mechanism from dopamine (DA) and dopamine receptor 1 (D1R) in the colon, spinal dorsal horn and hypothalamus.

METHODS: The rats were randomly divided into the normal (CD), herb-partitioned moxibustion (Mox) and mesalazine (Mesa) groups. Damage in the colons was scored and observed by hematoxylin and eosin staining. DA and D1R protein expression in the colonic mucosa were detected by immunohistochemistry. The concentrations of DA and D1R in the spinal dorsal horn and hypothalamus were measured by enzyme-linked immunosorbent assay, and D1R mRNA expression was evaluated by quantitative real-time polymerase chain reaction.

RESULTS: In the colon, compared with the normal group, DA, D1R protein expressions and D1R mRNA expression were significantly higher in the model group, while decreased in the Mox group and the Mesa group. In the spinal dorsal horn and hypothalamus, compared with the normal group, the concentrations of DA and D1R, and the D1R mRNA expressions were significantly higher in the model group, and decreased in the Mox group and the Mesa group.

CONCLUSION: Herb-partitioned moxibustion at the Tianshu (ST 25) and Qihai (CV 6) acupoints relieved ulceration in CD rats, the underlying mechanism maybe relative with the regulation of DA and D1R in the colon, spinal dorsal horn and hypothalamus by moxibustion.

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INTRODUCTION

Crohn’s disease (CD) is a common clinical inflammatory bowel disease (IBD). Currently, IBD is thought to be a pathological intestinal inflammatory response that is the result of interactions among multiple factors, such as the environment, genetic susceptibility and abnormal intestinal anti-microbial immune responses. Presently, commonly used therapeutic drugs for IBD, such as aminosalicylic acid, hormones and immunosuppressive agents, have limited efficacy and severe side effects. Therefore, exploring appropriate treatments for IBD has great importance. Moxibustion, which is an important component of traditional Chinese medicine, has unique advantages and irreplaceable roles. Moxibustion has been applied in treating IBD for years and has shown ideal effects in treating CD in the experiment and clinic. Compared with modern therapy, moxibustion has certain advantages in terms of improving clinical symptoms, preventing complications, improving the CD activity index and quality of patient life. Our team has been conducting basic and clinical researches using acupuncture and moxibustion in treating CD for many years. We induced a rat model of CD with an internationally recognized method of trinitrobenzene sulfonic acid (TNBS) method delivered by rectal enema to study the mechanism of acupuncture and moxibustion in treating CD, and found that acupuncture and/or moxibustion significantly decreased the levels of pathological damage of the intestinal tissue in CD model rats, reduced the abnormal expression of various inflammatory factors in the sera and colonic tissue, including tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, fibroblast growth factor (FGF), connective tissue growth factor (CTGF), insulin-like growth factor (IGF)-1 and its receptor IGF binding protein (IGFBP)-5; alleviated intestinal inflammation; and prevented tissue damage and intestinal fibrosis.

Dopamine (DA) is an effective immunomodulator in the immune system. In various immune-related diseases, changes in the DA concentration and/or dopamine receptor (DR) lead to different immune processes, including IBD. As a neurotransmitter, abnormal elevation of DA is closely related to inflammatory immune responses. Persistent inflammatory stimulation of the intestinal epithelium may lead to changes in intestinal mucosal barrier function and further result in damage to the intestinal mucosal barrier. As such, the mechanisms of IBD are closely related to cytokines and inflammatory mediators. By mediating hippocampal monoamine neurotransmitter activity, inflammation can alter the functional status of the hypothalamic neuroendocrine system, affect the secretion of pituitary hormone and corticosteroids and ultimately affect the reactivity of the hypothalamic-pituitary-adrenal (HPA) axis to injury stimuli. We speculate that the hippocampus-HPA axis is an important neuro-fluid regulation pathway that contributes to the anti-inflammatory immune response of moxibustion by regulating inflammation and the immune response. DA is a neurotransmitter that is widely present in the peripheral tissue. To date, few studies have investigated the regulatory roles of DA during peripheral inflammatory responses. However, studies have shown that DA receptor-mediated signaling pathway in immune cells may be involved in the processes of autoimmune diseases. Additionally, the use of DR inhibitors can improve the symptoms of autoimmune diseases.

The aim of this study was to observe the effects of herb-partitioned moxibustion stimulating Tianshu (ST 25) and Qihai (CV 6) acupoints, and to observe the DA and D1R in targeted organs of CD model rats, including the colon, spinal cord dorsal horn and hypothalamus.

MATERIALS AND METHODS

Animals

The Animal Experiment Center of Shanghai University of Traditional Chinese Medicine (SYXK (Shanghai) 2004-2005) provided 40 specific pathogen-free grade 5 week-old Sprague-Dawley male rats weighing (140 ± 10) g. The animals were fed in a light (12 h light: 12 h dark photoperiod) and temperature (22.0 ± 0.5 °C) controlled room with a constant humidity (65%-70%) for 3 d before the experiments.

Groups

Rats were randomly divided into 4 groups: (a) normal group (NG); (b) model group (MG); (c) herb-partitioned moxibustion group (Mox); (d) mesalazine group (Mesa).

Induction of Crohn’s disease rats by TNBS

CD rat models were established using the internationally recognized Morris method with a rectal enema of TNBS in ethanol. The animals were provided drinking water but no food for 24 h before the experiment; then, they received an injection of 1% pentobarbital sodium (30 mg/kg body weight) as abdominal anesthesia. The animals received enema fluid in a 2:1 mixture of 5% (w/v) TNBS and 50% ethanol at 3 mL/kg body weight. The enema fluid was perfused 6-8 cm away from the anus once per week for a total of four times. Seven days after the fourth enema, a rat was randomly chosen from each group, anesthetized and dissected to harvest a 2-cm section of the colon between the pubic symphysis and the cecum. The colonic lesions were observed and stained with hematoxylin and eosin (HE) to verify that the model was established successfully.

Herb-partitioned moxibustion protocol

Acupoints: Bilateral Tianshu (ST 25) and Qihai (CV 6) were located according to Experimental Acupuncture and Moxibustion. The herb cake was made of ac-
onite, cinnamon, salvia, coptis powder and yellow wine into size of 1-cm diameter and 0.5-cm thickness. Methods: Herb-partitioned moxibustion started on day eight. The rats were gently restrained by hand with the abdomen facing up, and the abdominal hair was shaved. The herb cakes were placed on bilateral Tianshu (ST 25) and Qihai (CV 6). The moxa cone, which was approximately 90 mg with 0.6 cm in diameter and 0.6 cm high, was placed on the herb cake for moxibustion. Each acupoint received 2 sessions of moxibustion every day for 10 d (Figure 1).

*Figure 1 Herb-partitioned moxibustion treatment on rat*

**Mesalazine protocol**

The mesalazine solution was administered by gavage. The daily dose was calculated by converting from an adult human dose (70 kg body weight, 4 g/d) to a dose for rats (200 g body weight). The dose for rats was calculated as 0.36 g/kg. The rats received a gavage twice per day for 10 days. At the end of the treatment, the rats with the lightest weight in each group was removed. There were 8 rats in each group for final statistical analysis. Rats in the normal and model groups were used as control groups and received no treatments.

**Morphology observation and assessment**

After the intervention treatment, colon sections were cut between the pubic symphysis and the cecum. The colon sections were longitudinally cut along the mesenteric membrane and observed under a magnifying glass. A routine histopathological procedure was performed. The tissues were embedded in paraffin blocks, cut into 5 μm sections by a microtome (Leica, Heidelberg, Germany). The sections were rinsed twice with 0.01 mol/L PBS for 5 min, stained with hematoxylin for 1 min, differentiated in 1% hydrochloric acid alcohol for 3 s, stained in eosin for 30 s, dehydrated and sealed with neutral resin. Assessment of colon inflammation induced by TNBS was determined by a histological grading scale (0-12 score). Colonic gross damage scores were recorded according to the severity of changes: 0, no change; 1, mild; 2, moderate; 3, severe. The parameters were damage/necrosis, inflammatory cell infiltration, submucosal edema, and hemorrhage of mucosa.

**Immunohistochemistry**

The slides were examined using the immunohistochemical method. The paraffin sections were subjected to microwave heating for antigen retrieval (0.01 mol/L citrate buffer, pH 6.0) and rinsed 3 times in 0.01 mol/L PBS (pH 7.4) for 5 min. Endogenous peroxidase activity was deactivated with 0.3% H2O2. Then, the slides were rinsed 3 times in 0.01 mol/L PBS (pH 7.4) for 5 min. Rabbit anti-mouse DA and D1R primary antibodies were diluted 1: 500 and added to the slides dropwise. The slides were incubated at 37 °C for 2 h and then rinsed 3 times in 0.01 mol/L PBS (pH 7.4) for 5 min. The secondary antibodies (1:500) were added, incubated at 37 °C for 2 h and then rinsed 3 times in 0.01 mol/L PBS (pH 7.4) for 5 min once again. The diaminobenzidine (DAB) was added (antibodies and DAB were from EnVision, Shanghai Gene Technology Co., Ltd., Shanghai, China), and the termination time was controlled by observation under a microscope. The integral optical density (IOD) was analyzed with a Motic image analysis system.

**ELISA**

Fresh hypothalamus and spinal dorsal horn samples were blended and centrifuged, and the supernatants were collected. Then, 100 μL of the standards and 100 μL of the diluted supernatants were added to the wells, mixed with 100 μL of rabbit anti-mouse DA and incubated for 20 min, followed by washes. Next, 100 μL of 1x horseradish peroxidase (HRP) was added to the wells, followed by washes. Then, 100 μL of 3, 3’, 5, 5’-tetramethylbenzidine (TMB) substrate was added to each well and gently mixed for 10 s. Finally, 100 μL of stop buffer was added to each well and gently mixed for 30 s (reagents were from EnVision, Shanghai Gene Technology Co., Ltd., Shanghai, China). The OD values were recorded at 450 nm after 15 min. DA (ng/mL) = concentration on the standard curve x dilution factor of the samples.

**Real-time PCR**

Total RNA was extracted from the colon, hypothalamus and spinal dorsal horn tissues, and cDNA was synthesized using reverse transcriptase (Shanghai Kilton Biotechnology Co., Ltd., Shanghai, China). The relative gene expression levels were calculated using the ΔΔCT method with GAPDH as a reference. The D1R primer sets were designed and synthesized by Primer Express.

D1R Primer F: 5’ TGCTGCTGGCTCCCTTTCTTC 3’;
were mostly intact in the Mox group and Mesa group. No obvious receptor was observed in the colonic tissues from the Mox group and Mesa group. Compared with the normal group, the model group showed inflammation, mucosal ulcers, loss of the local mucosal epithelium and fissure ulcers. Edema was observed in the intrauterine interstitial layer and submucosal connective tissue. We also observed obvious eosinophil and lymphocyte infiltration. Part of the submucosal layer showed nodular granulomatous granulomatosis coupled with fibrous hyperplasia. The local mucosal lamellar layer showed goblet cell hyperplasia. The glands appeared to be disordered. These symptoms were consistent with the colonic pathological manifestations of CD. After herb-partitioned moxibustion treatment, the rats in the Mox group showed improved ulcers, reduced congestion and edema, a thickened lamina propria and goblet cell hyperplasia. After mesalazine treatment, the colonic tissues showed similar improvements compared to the colons in the Mox group (Figure 3).

**RESULTS**

**Gross damage scores of the colon**

The colons from the CD rats were dark and obviously sticky. We found local intestinal dilatation thickening coupled with fecal obstruction and severe fibrosis. The colonic mucosa was less smooth and was coupled with congestion, edema and erosion. We also observed multiple ulcers. These ulcers had scattered distributions, with some located deep in the muscle layer. These distributions were representative of the pathological manifestations of CD. After herb-partitioned moxibustion intervention, the colons of the rats had a soft pink color with improved tissue adhesion. The mucosal surfaces were smoother in the Mox group than those in the model group. The congestion and edema severity were also reduced in the Mox group compared with that in the model group. Furthermore, the colonic damage scores of the Mox group and Mesa group were significantly decreased compared with those of the model group (Figure 2).

**Histopathological changes in colon tissues**

The colonic structure of the normal group was intact. No edema and hyperplasia were observed in the lamina propria and its lower layers. The muscular and serosal layers were intact. The colons of the model group showed inflammation, mucosal ulcers, loss of the local mucosal epithelium and fissure ulcers. Edema was observed in the intrauterine interstitial layer and submucosal connective tissue. We also observed obvious eosinophil and lymphocyte infiltration. Part of the submucosal layer showed nodular granulomatous granulomatosis coupled with fibrous hyperplasia. The local mucosal lamellar layer showed goblet cell hyperplasia. The glands appeared to be disordered. These symptoms were consistent with the colonic pathological manifestations of CD. After herb-partitioned moxibustion treatment, the rats in the Mox group showed improved ulcers, reduced congestion and edema, a thickened lamina propria and goblet cell hyperplasia. After mesalazine treatment, the colonic tissues showed similar improvements compared to the colons in the Mox group (Figure 3).

**DA and D1R protein expressions in the colon of CD rats**

We observed DA and D1R protein expressions in every layer of the colonic mucosa of rats. In the normal group, a small amount of positive DA and D1R signals was observed at the edges of the normal colonic epithelial cells and at the top of the cell membrane. Compared with the normal group, the model group showed significant increase in DA and D1R protein expressions in the colonic epithelium, the lamina propria and the lower layers. The staining was generally dark. Positive or strongly positive reactions were observed in regions of eosinophil and lymphocyte infiltration, especially in the submucosa. The intestinal epithelium were mostly intact in the Mox group and Mesa group. No obvious receptor was observed in the colonic tissues from the Mox group and Mesa group (Figure 4).

**DA and D1R concentrations in the hypothalamus and dorsal horn of the spinal cord**

We used ELISA to study DA and D1R concentrations in the hypothalamus. Compared with the normal group, DA and D1R concentrations in the hypothalamus were significantly higher in the model group (P < 0.01). Compared with the model group, DA and D1R concentrations in the hypothalamus were significantly decreased in the Mox group and Mesa group (P < 0.05) (Figure 5).

Compared with the normal group, DA and D1R concentrations in the spinal dorsal horn were significantly higher in the model group (P < 0.01). Compared with the model group, DA and D1R concentrations in the spinal dorsal horn were significantly decreased in the Mox and Mesa group (P < 0.05) (Figure 6).

**D1R mRNA expressions in the rat colonic mucosa, hypothalamus and dorsal horn of the spinal cord**

We examined D1R mRNA expression in the colonic
tissues, hypothalamus and spinal dorsal horns using real-time PCR. The D1R mRNA expressions in the 3 sites presented the same results. Compared with the normal group, D1R mRNA expressions were significantly higher in the model group ($P < 0.01$). Compared with the model group, D1R mRNA expressions were significantly lower in the Mox group and Mesa group ($P < 0.01$) (Figure 7).
of the protective effect of DA on the intestinal tract. The acute inflammatory phase could be a manifestation of IBD. Based on our results, increased DA synthesis in the colonic mucosa of CD model rats. Herb-partitioned moxibustion had obvious regulatory effects on the DA content in the colonic mucosa and promoted the repair process of colonic inflammatory disease. The D2R agonist quinpirole inhibited the progression of colonic inflammation in IL-10 knockout mice and significantly decreased the colonic damage score in UC model rats. These findings provide important parallel evidence for the important roles of the dopamine system in the pathogenesis of IBD.

Studies have shown that the HPA axis can induce a systemic response in the inflammatory bowel disease process. We studied the efficacy and mechanism of herb-partitioned moxibustion using a CD model rat. The target organs include the colon, spinal cord and hypothalamus of the HPA axis. We previously observed the protein expressions of DA and its receptor in the colonic mucosa of CD model rats. Herb-partitioned moxibustion had obvious regulatory effects on the DA content in the colonic mucosa and promoted the repair process of colonic inflammatory disease. The release of cytokines in an inflammatory site can stimulate and influence the cytokine levels in the hypothalamus as well as the pituitary and adrenal glands. Enhancing HPA axis activity is important to ensure both

**DISCUSSION**

Our study found that the colonic tissue, which is a peripheral target organ, showed induced inflammatory changes following a rectal enema with TNBS in ethanol solution. Additionally, a significant increase of DA was observed in every layer of the colonic mucosa of the CD model rats. The staining was generally dark, and positive or strongly positive staining was observed particularly in the submucosal regions with eosinophil and lymphocyte infiltration. Herb-partitioned moxibustion improved the pathological changes of colitis, resulting in improvement of the ulcer, reduced congestion and edema and thickening of the mucosal lamina propria. Additionally, herb-partitioned moxibustion decreased DA protein expressions in the colon. D1R mRNA expressions in the colonic tissues were evaluated by real-time PCR, and were higher in the model group than those in the normal group. Herb-partitioned moxibustion had an inhibitory effect on the elevated D1R and D1R mRNA expressions in the colon. Damage to the dopamine system is one of the pathological features of IBD. DA plays a protective role in IBD. Based on our results, increased DA synthesis in the acute inflammatory phase could be a manifestation of the protective effect of DA on the intestinal tract.
inflammatory and anti-inflammatory immune responses. Inflammation can change the functional status of the hypothalamic neuroendocrine system by adjusting the pituitary hormone level and affecting corticosteroid secretion. These effects ultimately alter the response to injury of the HPA system. Therefore, we examined the concentrations of DA and its receptor D1R in the hypothalamus within the HPA axis. This approach was undertaken to explore a potential 'neurotransmitter-humoral regulatory pathway' in the anti-inflammatory effects of herb-partitioned moxibustion in CD model rats. The results showed that the DA, D1R concentrations and D1R mRNA expressions in the hypothalamus were different among the 4 groups. TNBS up-regulated DA and D1R concentrations as well as D1R mRNA expressions in the hypothalamus. Herb-partitioned moxibustion alleviated inflammation in the colon and regulated DA and D1R concentrations as well as D1R mRNA expressions in the hypothalamus. This result may be due to the reduced release of inflammatory cytokines in the colon, resulting in changes in the functional status of the hypothalamic neuroendocrine response. This phenomenon can explain the down regulation of DA and D1R concentrations as well as D1R mRNA expressions in the hypothalamus of the Mox group. Studies have also shown that the receptor-mediated signaling pathways affected by DA in immune cells may be involved in autoimmune diseases, and the use of DA receptor inhibitors improves the symptoms of autoimmune diseases.

In conclusion, our findings suggest that TNBS also up-regulated DA and D1R concentrations as well as D1R mRNA expression in the dorsal horn of the spinal cord and that herb-partitioned moxibustion reduced this trend. DA D2 receptor knockout mice developed inflammatory responses in the central nervous system, indicating that DA and its downstream signals had anti-inflammatory effects. In our future experiments, the roles of sympathetic nerves in the anti-inflammatory effect of herb-partitioned moxibustion will be addressed.

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