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Acupuncture Research

Electroacupuncture at Sensitized Acupoints Relieves Somatic Referred Pain in Colitis Rats by Inhibiting Sympathetic-Sensory Coupling to Interfere with 5-HT Signaling Pathway*

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ABSTRACT **Objective**: To investigate whether electroacupuncture (EA) at sensitized acupoints could reduce sympathetic-sensory coupling (SSC) and neurogenic inflammatory response by interfering with 5-hydroxytryptamine (5-HT)ergic neural pathways to relieve colitis and somatic referred pain, and explore the underlying mechanisms. **Methods**: Rats were treated with 5% dextran sodium sulfate (DSS) solution for 7 days to establish a colitis model. Twelve rats were randomly divided into the control and model groups according to a random number table (*n*=6). According to the "Research on Rat Acupoint Atlas", sensitized acupoints and non-sensitized acupoints were determined. Rats were randomly divided into the control, model, Zusanli-EA (ST 36), Dachangshu-EA (BL 25), and Xinshu (BL 15) groups (*n*=6), as well as the control, model, EA, and EA + GR113808 (a 5-HT inhibitor) groups (*n*=6). The rats in the control group received no treatment. Acupuncture was administered on 2 days after modeling using the stimulation pavameters: 1 mA, 2 Hz, for 30 min, with sparse and dense waves, for 14 consecutive days. GR113808 was injected into the tail vein at 5 mg/kg before EA for 10 min for 7 consecutive days. Mechanical sensitivity was assessed with von Frey filaments. Body weight and disease activity index (DAI) scores of rats were determined. Hematoxylin and eosin staining was performed to observe colon histopathology. SSC was analyzed by immunofluorescence staining. Immunohistochemical staining was performed to detect 5-HT and substance P (SP) expressions. The calcitonin gene-related peptide (CGRP) in skin tissue and tyrosine hydroxylase (TH) protein levels in DRG were detected by Western blot. The levels of hyaluronic acid (HA), bradykinin (BK), prostaglandin I2 (PGI2) in skin tissue, 5-HT, tryptophan hydroxylase 1 (TPH1), serotonin transporters (SERT), 5-HT 3 receptor (5-HT3R), and 5-HT 4 receptor (5-HT4R) in colon tissue were measured by enzyme-linked immunosorbent assay (ELISA). **Results**: BL 25 and ST 36 acupoints were determined as sensitized acupoints, and BL 15 acupoint was used as a non-sensitized acupoint. EA at sensitized acupoints improved the DAI score, increased mechanical withdrawal thresholds, and alleviated colonic pathological damage of rats. EA at sensitized acupoints reduced SSC structures and decreased TH and CGRP expression levels (P<0.05). Furthermore, EA at sensitized acupoints reduced BK, PGI2, 5-HT, 5-HT3R and TPH1 levels, and increased HA, 5-HT4R and SERT levels in colitis rats (P<0.05). GR113808 treatment diminished the protective effect of EA at sensitized acupoints in colitis rats (P<0.05). **Conclusion**: EA at sensitized acupoints alleviated DSS-induced somatic referred pain in colitis rats by interfering with 5-HTergic neural pathway, and reducing SSC inflammatory response.

KEYWORDS electroacupuncture, sensitized acupoints, sympathetic-sensory coupling, 5-hydroxytryptamine, neurogenic inflammation, somatic referred pain

Inflammatory bowel disease (IBD) is a complex multifactorial disease consisting mainly of ulcerative colitis (UC) and Crohn's disease (CD).[1] However, the pathogenesis of IBD is complex, and the etiology is

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still unclear.[2] Among various models of IBD, dextran sodium sulfate (DSS)-induced colitis model is widely used because of its simplicity and similarity to the clinical features of UC.[3] The main clinical treatments for UC are salicylic acid preparations, glucocorticoids, and immunosuppressive drugs.[4] However, due to the adverse effects of these drugs, there are many limitations in treating UC. Therefore, there is a growing need to develop new, effective, and safe treatments for UC.

Studies have shown that electroacupuncture (EA) could regulate intestinal dysfunction and improve symptoms of colitis, abdominal pain, diarrhea, and purulent stools.[5-7] Acupuncture point sensitization refers to acupuncture points moving from "silent" state to "sensitized" state in the pathological state. Sensitized points not only reflect the functional state of the body, but also play an important role in the diagnosis and treatment of diseases, especially in the treatment of visceral lesions that cause somatic referred pain (SRP), which has obvious efficacy advantages. In addition to abdominal pain, IBD patients might also experience SRP such as back, abdominal muscle, hand, and foot pain. Studies have confirmed that visceral lesions induce SRP and also cause acupuncture points sensitization of body surface, while stimulation of sensitization points can improve visceral function and relieve SRP at the same time.[8-10]

Our previous study found that colitis model rats have severe colonic lesions and somatic hyperalgesia, accompanied by sensitized exudation points in the Zusanli (ST 36) acupoint, and EA stimulation of sensitized acupoint could significantly relieve colon lesions and relieve referred pain.[11,12] In addition, it has demonstrated that the sensitization of SRP associated with visceral lesions is closely related to the sympathetic-sensory coupling (SSC) in the dorsal root ganglion (DRG) and the neurogenic inflammatory response.[13,14] After visceral lesions, SSC occurs in the DRG, accompanied by local sensitization of the skin. This leads to sympathetic-sensory pathological coupling and a neurogenic inflammatory response, which forms an "acupuncture point sensitization area", and abnormal somatosensory afferents occur at the same time.[15,16] However, the underlying mechanisms of SSC and neurogenic inflammatory response in the pathology of visceral lesions triggering and maintaining SRP are not yet clear, and whether the mechanism of acupuncture sensitization points to relieve referred pain remains to be studied. Therefore, on the basis of the previous study, we propose the hypothesis that EA stimulation of sensitized acupoints might relieve colitis and SRP by suppressing the SSC in the DRG and neurogenic inflammatory response.

This study established a DSS-induced colitis model to determine the distribution area of sensitized acupoints. The expression levels of inflammatory and pain-causing substances related to the neurogenic inflammatory response and SSC-related factors were examined before and after the EA stimulation of sensitized acupoints. Furthermore, the interaction between the SSC and the neurogenic inflammatory response was explored in the EA stimulation of sensitized acupoints to relieve SRP.

METHODS

Animals

Sixty-six male Sprague-Dawley (SD) rats (180–200 g) were purchased from Chengdu Dossy Experimental Animals Co., Ltd., China [SCXY (Chuan) 2020-034]. Rats were housed at 22±2 ℃ and 50%±5% relative humidity and subjected to a 7-day light/dark 12-h cycle for acclimatization. Rats were allowed to eat and drink *ad libitum*. All experiments were approved by the Experimental Animal Welfare Ethics Committee of Chengdu Medical College (No. 2023012).

Screening of Sensitized Acupuncture Points in Rats

Twelve rats were randomly divided into the control and model groups according to a random number table (n=6 each group). Rats in the model group were treated with a 5% DSS aqueous solution *ad libitum* for 7 days to establish an acute phase UC model.[17] The rats in the control group drank pure water freely for 7 days. Evans blue (EB, 50 mg/kg) was injected through the tail vein, and the rats were depilated with depilatory cream after 2 h. The sites of EB exudation points were observed and recorded. According to the "Research on rat acupoint atlas",[18] the correlation between EB exudation points and traditional acupoints was compared, and 1–2 sensitized acupoints were determined as therapeutic points.

Evaluation of Therapeutic Effect of EA Stimulation at Sensitized Acupoints in Colitis Rats

Thirty rats were divided into the control, model,

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Zusanli-EA (ST 36), Dachangshu-EA (BL 25), and Xinshu (BL 15) groups (*n*=6). The rats in the control and model groups were only moderately grasped. The ST 36 and BL 25 groups were selected to acupuncture sensitized acupoints, and the rats in the EA non-sensitized acupoints group was selected to acupuncture BL 15 point. Acupuncture was connected to an EA instrument (G6805-2A, Huatuo Brand, Suzhou Medical Appliance Factory, China) with the stimulation parameters: 1 mA, 2 Hz, 30 min, sparse and dense waves. Acupuncture was started on 2 days after modeling for 14 consecutive days. The therapeutic effects of EA for regulating intestinal motility disorders in IBD were further validated by comparing the Disease Activity Index (DAI) score and colon pathological injury.

DAI

On days 0, 7, and 14 of the experiment, the fecal characteristics and fecal occult blood were observed and scored. Scoring for fecal characteristics are as follows: 0, normal stool; 1, semi-formed stool; 2, formed stool; 3, loose stool; and 4, watery stool. Fecal occult blood tests were performed using freshly collected feces with a commercial kit (Nanjing Jiancheng Bioengineering Institute, China). The results were classified into the following 5 levels: no color development within 1 min as 0 point, the emergence of purple-red within 1 min as 1 point, purple-blue color within 10 s as 2 points; a little blood in stool as 3 points; and visible blood in stool as 4 points. DAI score = (fecal characteristics score + fecal occult blood score)/2.[19]

Effect of EA Stimulation at Sensitized Acupoints in Alleviating SRP Induced by Colonic Inflammatory Injury In Rats

Twenty-four rats were randomly divided into the control, model, EA, and EA + GR113808 groups according to a random number table (*n*=6). GR113808 (No. HY-103152, MedChemExpress), a 5-HT inhibitor, was injected into the tail vein at 5 mg/kg before EA for 10 min, for 7 consecutive days.

Measurement of Mechanical Sensitivity

On days 0, 7, and 14 of the experiment, rats underwent a mechanical paw withdrawal threshold (PWT) test. A total of 6 von Frey filaments of 4, 6, 8, 10, 15, and 26 g were selected. Firstly, 4 g von Frey filament was vertically applied to the hind paw for 2 s until it bent. A positive response was considered when the rat quickly retracted or licked its hind paw. If there was no response, the rat was tested with the next high-intensity von Frey filament. The interval between the two tests was 5 min. Until the rats responded, this intensity was the threshold for the PWT test.

Sample Collection

Upon completion of the administration, all rats were subjected to anesthesia using 1% sodium pentobarbital (50 mg/kg) and subsequently euthanized. The colon tissues, serum, ipsilateral lumbar 6 (L6) DRG, and adjacent skin were extracted and preserved at –80 ℃ for subsequent analysis.

Hematoxylin and Eosin Staining

Hematoxylin and eosin (H&E) staining was used to observe the histopathology of the colon in rats. The sections were fixed with 10% formaldehyde, dewaxed to water, stained with hematoxylin for 20 min and eosin for 5 min, and sealed with neutral gum. Finally, the colons were observed under microscopic camera system (BA210Digital, Motic China Group Co., Ltd., China).

Immunofluorescent Staining

Paraffin sections of rat skin tissue (L6 DRG) were dewaxed and hydrated. Sections were incubated in QuickBlock™ closure buffer at room temperature for 30 min. Sections were then incubated with primary antibodies calcitonin gene-related peptide (CGRP, bs-0791R, 1:100, Abcam), tyrosine hydroxylase (TH, ab129991, 1:100, Abcam), and neuronal nuclei (NeuN, ab177487, 1:100. Abcam) overnight at 4 ℃ and washed 3 times with phosphate buffer. Then CY3-labeled goat anti-rabbit IgG (GB21303, 1:100, Servicebio) and FITC-labeled goat anti-mouse IgG (GB22301, 1:100, Servicebio) were added and incubated at 37 ℃ for 30 min. Finally, the staining was observed using an OlyVIA fluorescence microscope (OLYMPUS, Tokyo, Japan), and the fluorescence intensity and area of the images were measured using the Image-J Analysis System (National Institutes of Health, USA). DAPI nuclei are stained blue, TH-stained cells are shown in green, and CGRP-stained and NeuN-stained cells are shown in red.

Immunohistochemistry Staining

Paraffin sections of rat skin tissues were dewaxed to water. 5-hydroxytryptamine (5-HT) and substance P (SP) expression were detected according to the instructions. Antigen repair was

performed using microwave heating, and sections were placed in 3% hydrogen peroxide and reacted at room temperature for 10 min to block endogenous peroxidase. Primary antibodies (5-HT, BS-1126R, 1:100, Beijing Boiss Biotechnology Co., Ltd., China; SP, PA5-106934, 1:50, Thermo Fisher Scientific, USA) was added, incubated overnight at 4 ℃. The secondary antibody [HRP-conjugated goat anti-rabbit IgG (H+L), GB23303, 1:100, Servicebio, China] were then added and incubated for 30 min at 37 ℃. The color was developed using DAB and then re-stained with hematoxylin for 3 min. Finally, images was acquired using a BA400Digital microscope camera system (Mike Audi Industrial Group Co., Ltd., China). Image quantification was performed using the Halo Data Analysis System (Indica Labs, USA). The nucleus stained with hematoxylin appears blue, and the positive expression of DAB is brownish yellow.

Western Blot

Western blot was used to detect the expression levels of CGRP in skin tissue and tyrosine hydroxylase (TH) levels in DRG. Protein concentrations were determined using a bicinchoninic acid (BCA) kit (P0009, Beyotime, China). Total proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes at 200 mA for 2 h, and PVDF membranes were incubated in 5% skim milk for 2 h. The PVDF membranes were then placed in primary antibodies (CGRP, A5542, 1:1,000, Abclonal, China; TH, A5079, 1:1,000, Abclonal, China; β-actin, AC026, 1:20,000, Abclonal, China) and incubated overnight at 4 ℃. Then, the membranes were washed with phosphate buffered saline with Tween-20 (TBST) and incubated with HRP-conjugated goat anti-rabbit IgG (H+L, GB23303, 1:100, Servicebio, China) for 90 min. Bands were displayed using the electrochemiluminescence (ECL) system, and β-actin was used as an internal control. Finally, the bands were scanned and photographed using a chemiluminescent gel imager (Tanon, Shanghai, China).

Enzyme-Linked Immunosorbent Assay

The expressions of hyaluronic acid (HA), bradykinin (BK), prostaglandin I2 (PGI2) in skin tissue and 5-HT, tryptophan hydroxylase 1 (TPH1), serotonin transporters (SERT), 5-HT 3 receptor (5-HT3R), and 5-HT 4 receptor (5-HT4R) in colon tissue were measured by enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. Rat HA ELISA kit (ZC-37269), rat BK ELISA kit (ZC-36714), rat PGI2 ELISA kit (ZC-37097), rat 5-HT ELISA kit (ZC-35959), rat TPH1 ELISA kit (ZC-37146), rat SERT ELISA kit (ZC-35694), rat 5-HT3R ELISA kit (ZC-35961), and rat 5-HT4R ELISA kit (ZC-35962) were purchased from ZCIBIO Technology Co., Ltd., Shanghai, China. The absorbance (OD) of each well was measured at 450 nm using an enzyme marker (SpectraMAX Plus384, Shanghai Molecular Devices Co., Ltd., China), and the sample concentrations were calculated.

Statistical Analysis

SPSS 17.0 statistical software (SPSS Inc, USA) was used for statistical analysis. Data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and the one-way ANOVA test was used for comparison between multiple sample means, the LSD test for data with homogeneity of variance, and Tamhane's t2 test for data with unequal variance. The results were considered significant when the difference between groups was $P<0.05$.

RESULTS

Determination of Sensitized Acupoints and Effect of EA at Sensitized Acupoints on DAI Scores and Mechanical Sensitivity in Colitis Rats

The correlation between EB exudation points and traditional acupuncture points was compared with the "acupoint atlas of Xingbang Hua". The results showed that the exudation points on the back of the rats were highly correlated with BL 25 and ST 36 (Figure 1A). Few EB exudate points were located in the chest segment of the back, so BL 15 in the chest segment were used as treatment points for the non-sensitized acupoint group. BL 25 and ST 36 were selected as the treatment acupoints, and BL 15 acupoint was used as the treatment point in the non-sensitized acupoint group in the following experiment (Figure 1B).

As shown in Figure 1C, DSS caused a significant decrease in body weight in UC rats ($P<0.05$ or $P<0.01$), however, the EA acupoints treatment did not cause a significant difference in body weight ($P>0.05$). The results showed that the DAI scores of rats increased significantly at 7 days of modeling compared with the control group ($P<0.01$). DAI scores of the ST 36 and BL 25 groups decreased at 14 days

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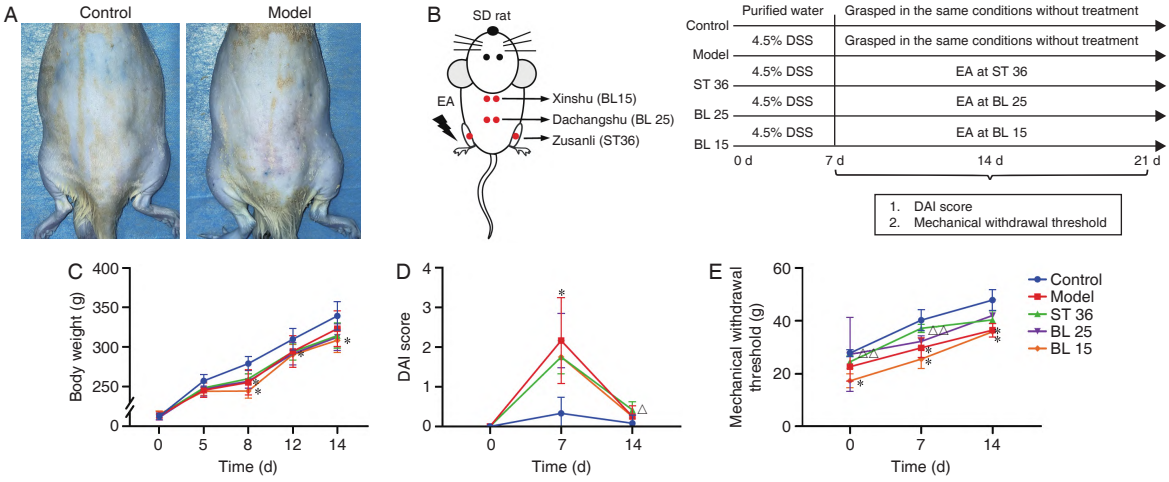

Figure 1. EA Stimulation on Sensitized Acupoint Alleviates Colitis Severity and Hyperalgesia in DSS-Induced Colitis Rats

Notes: A: Determination of sensitized acupoints. B: Experimental designs and animal groups. C: Body weight changes in rats. D and E: Disease Activity Index (DAI) and mechanical withdrawal threshold (MWT) test were measured at 0, 7, and 14 days in DSS-induced colitis rats (n=6, $\bar{x} \pm s$). $^{*}P$<0.01 *vs*. control group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. BL 15 group. EA: electroacupuncture

compared with the model group (P<0.05, Figure 1D).

The results of the PWT test showed that the mechanical withdrawal thresholds of rats in the model group were reduced at 7 and 14 days compared with the control group (P<0.01); while the mechanical withdrawal thresholds in the ST 36 group were increased at 7 days compared with the BL 15 group (P<0.01, Figure 1E).

EA at Sensitized Acupoints Improved Colonic Histopathology in Colitis Rats

As shown in Figure 2, compared with the control group, degenerative necrosis of cells in the mucosal layer and disintegration and fragmentation of nuclei were obviously observed in the colon tissue of the model group (P<0.01). Compared with the model group, the histopathological changes in the colon of ST 36, BL 25, and BL 15 groups were reduced to varying degrees but no significance was found (P>0.05). The degree of histopathological changes in the ST 36 and BL 25 groups was more obvious than that in the BL 15 group.

Effect of EA at Sensitized Acupoints on SSC in Colitis Rats

The SSC in DRG was observed by immunofluorescence staining of TH with CGRP (Figure 3A). The distribution of TH-positive neurons in the DRG increased in the model group and sprouted into the DRG, and TH-positive neurons were observed to wrap around CGRP sensory neurons in the DRG,

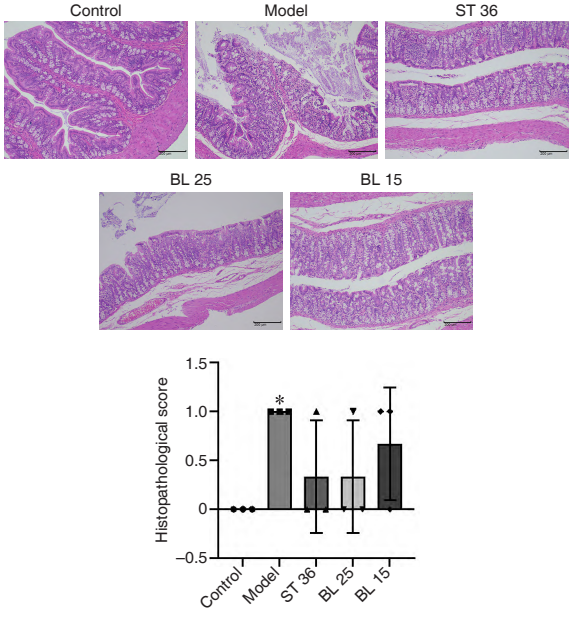

Figure 2. Effect of EA Stimulation of Sensitized Acupoint on Colonic Histopathological Changes in Colitis Rats (H&E staining, 100×)

Notes: Scale bar = 200 μm; n=3 for bar graph. $^{*}P$<0.01 *vs*. control group

forming a SSC-like a "basket structure", which was reduced after EA treatment.

Moreover, the density of CGRP-positive sensory nerve fibers and TH-positive sympathetic nerve fibers was increased in the model group compared with the control group (P<0.01); compared with the model group, the density of CGRP-positive sensory nerve fibers and TH-positive sympathetic nerve fibers in the 3 EA treatment groups were decreased (P<0.01) and

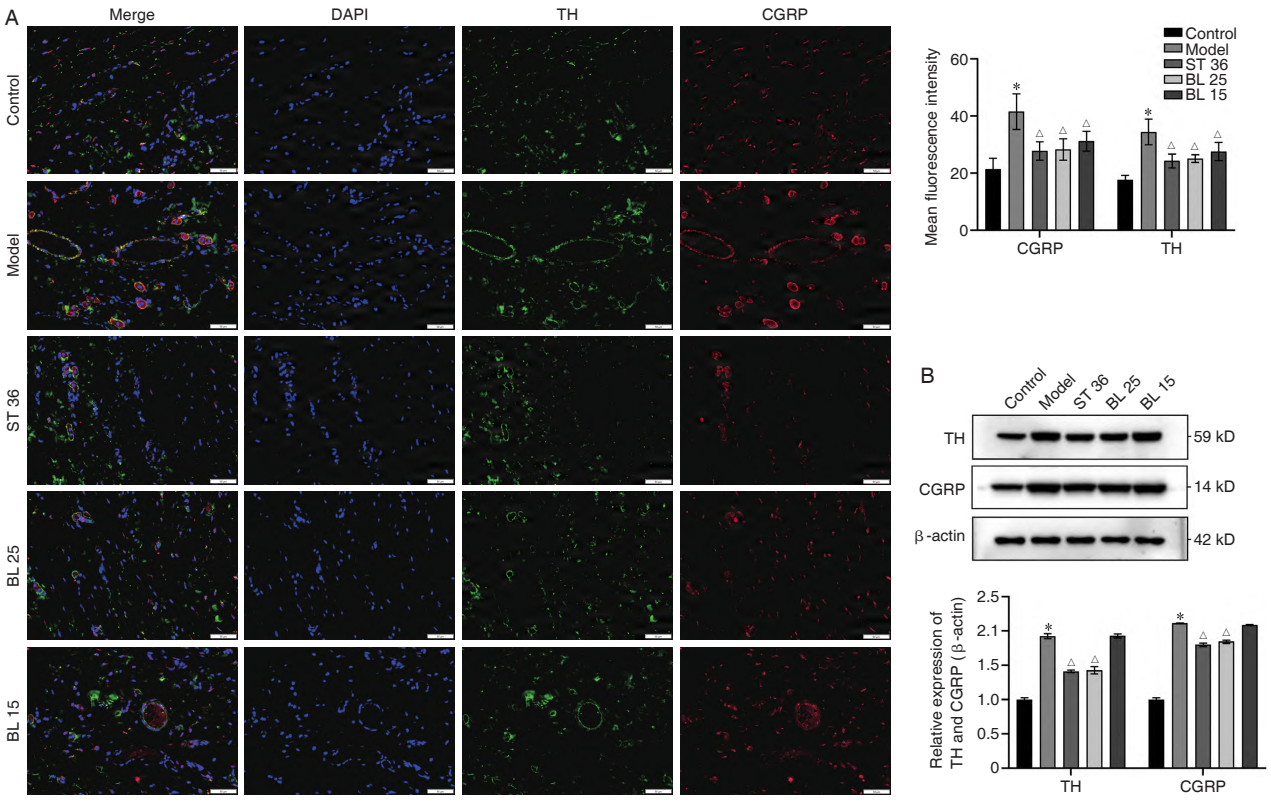

Figure 3. Effect of EA at Sensitized Acupoints on Sympathetic-Sensory Coupling in Colitis Rats ($\bar{x} \pm s$, n=3)

Notes: A: TH-labeled sympathetic neurons and CGRP-labeled sensory neurons in DRG observed by immunofluorescence staining. Scale bar = 50 μm, 20×. CGRP-labeled sensory neurons are red, TH-positive neurons are green, and DAPI is blue. B: The levels of CGRP and TH detected by Western blot. *P<0.01 *vs*. control group ($\bar{x} \pm s$, n=3); △P<0.01 *vs*. model group

no significant difference was found in the BL 15 group compared with the ST 36 and BL 25 groups (P>0.05). In addition, the protein expressions of TH and CGRP in the DRG were detected by Western blot, and the results were consistent with immunofluorescence staining (P<0.01, Figure 3B).

EA at Sensitized Acupoints Improved Colitis in Rats by Inhibiting SSC-Induced Neurogenic Inflammatory Responses and 5-HTergic Neural Pathway

As shown in Figure 4A, compared with the control group, the contents of BK and PGI2 in the skin tissues were increased and HA levels were decreased in the model group (P<0.01). Compared with the model group, the contents of BK and PGI2 were decreased and HA were increased in the ST 36 and BL 25 groups (P<0.05 or P<0.01).

As shown in Figure 4B, the contents of 5-HT, 5-HT3R, and TPH1 in the colonic tissues were elevated, and the contents of 5-HT4R and SERT were lower in the model group compared with the control group (P<0.01). Compared with the model group, decreased contents of 5-HT, 5-HT3R, and TPH1, and increased contents of 5-HT4R and SERT in the colonic tissue were found in ST 36 and BL 25 groups (P<0.05 or P<0.01).

Notably, immunohistochemical results showed that the levels of 5-HT and SP in the skin tissues of rats in the model group were significantly elevated, while the levels of 5-HT and SP were significantly decreased after EA sensitized acupoints intervention (P<0.01, Figure 4C).

EA at Sensitized Acupoints Alleviated SRP Caused by Colitis in Rats through 5-HTergic Neural Pathway

As shown in Figure 5A, compared with the control group, the contents of BK and PGI2 in the model group were increased and HA contents were decreased in the model group (P<0.01). Increased BK and PGI2 contents and decreased HA contents were found in the EA+GR113808 group compared with the EA group (all P<0.05). As shown in Figure 5B, 5-HT and 5-HT3R levels were increased and 5-HT4R levels were decreased in the model group than in the control group (P<0.01); compared with the EA group, administration

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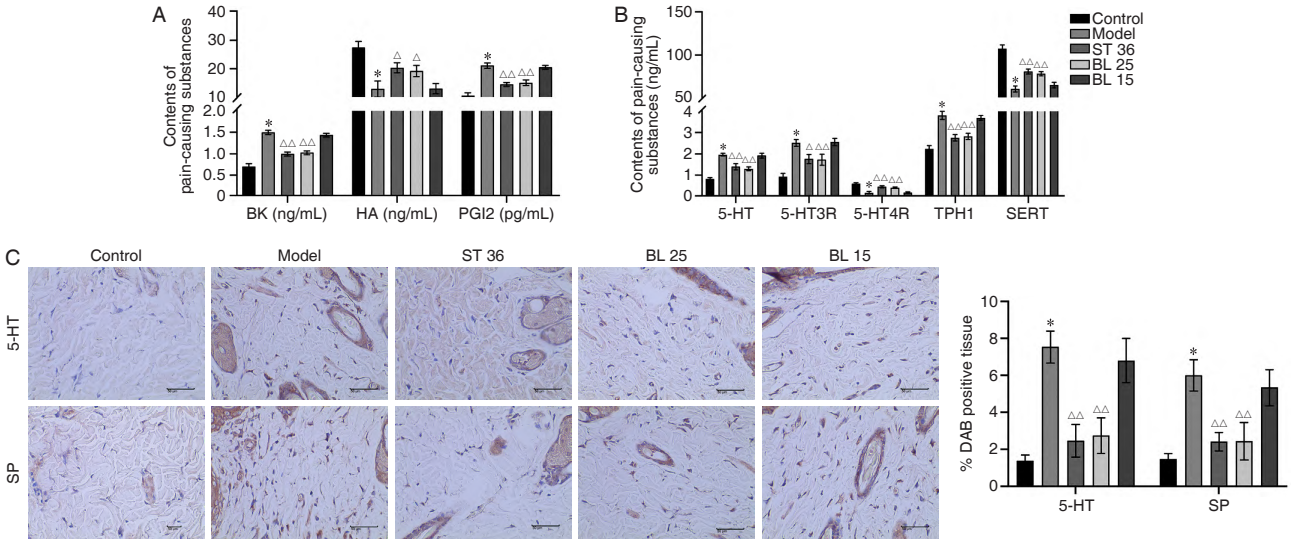

Figure 4. EA Stimulation of Sensitized Acupoints Inhibited Neurogenic Inflammatory Responses and 5-HTergic Neural Pathways ($\bar{x} \pm s$, n=6)

Notes: A: Levels of pain-causing substances BK, HA, and PGI2 in rat L6 DRG detected by ELISA. B: Levels of 5-HTergic neural pathways proteins 5-HT, TPH1, SERT, 5-HT3R, and 5-HT4R in rat colon tissue measured by ELISA. C: Levels of 5-HT and SP in rat L6 DRG determined by immunohistochemistry staining. The hematoxylin stained nucleus is blue, and DAB shows a positive expression of brownish yellow. Scale bar = 50 μm. $^{*}P$<0.01 *vs*. control group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. model group

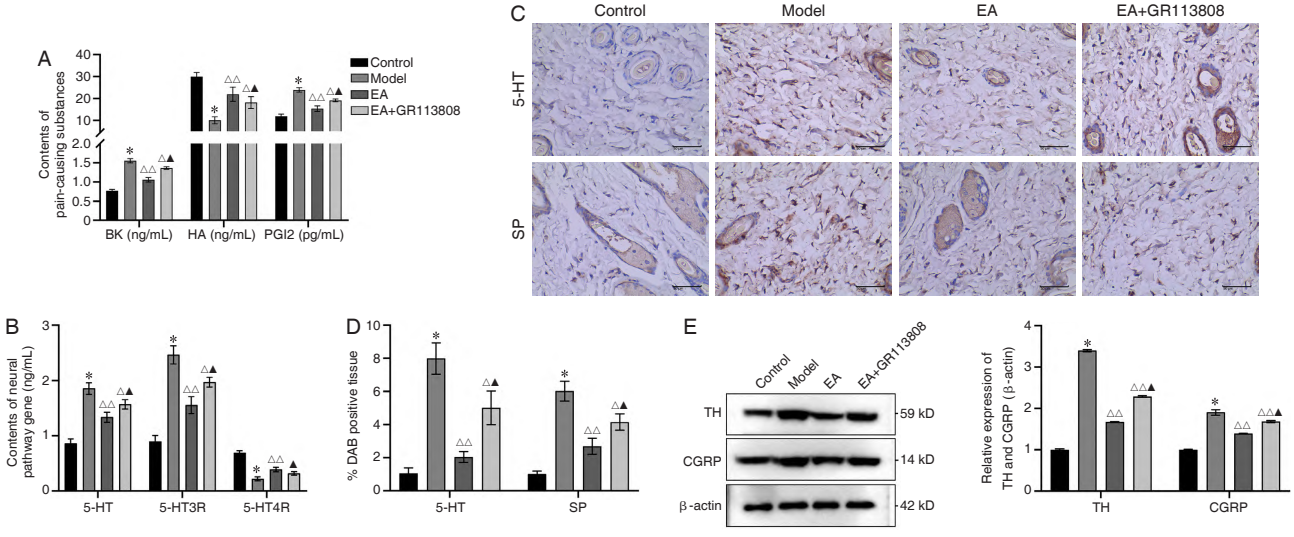

Figure 5. EA Stimulation of Sensitized Acupoints Alleviated Colitis in Rats through Intervention of 5-HTergic Neural Pathways ($\bar{x} \pm s$, n=6)

Notes: A: GR11380 treatment increased BK and PGI2 contents and decreased HA contents compared with the EA group. B: Administration of 5-HT inhibitors increased levels of 5-HT and 5-HT3R, and decreased 5-HT4R levels compared with the EA group. C and D: Levels of 5-HT and SP determined by immunohistochemistry staining. E: The expressions of TH and CGRP in the skin tissues determined by Western blot. $^{*}P$<0.01 *vs*. control group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. model group; $^{\blacktriangle}P$<0.05 *vs*. EA group

of 5-HT inhibitors resulted in increased levels of 5-HT and 5-HT3R, and decreased 5-HT4R level (all P<0.05).

Immunohistochemical staining results showed that the levels of 5-HT and SP in the model group were significantly higher than those in the control group (P<0.01); the administration of 5-HT inhibitors led to an increase in 5-HT and SP levels compared with the EA group (P<0.05, Figures 5C and 5D).

Compared with the control group, the expression of TH and CGRP significantly increased in the model group, whereas the expression of TH and CGRP in the EA group decreased compared with the model group (P<0.01). Compared with the EA group, administration of 5-HT inhibitors increased the expressions of TH and

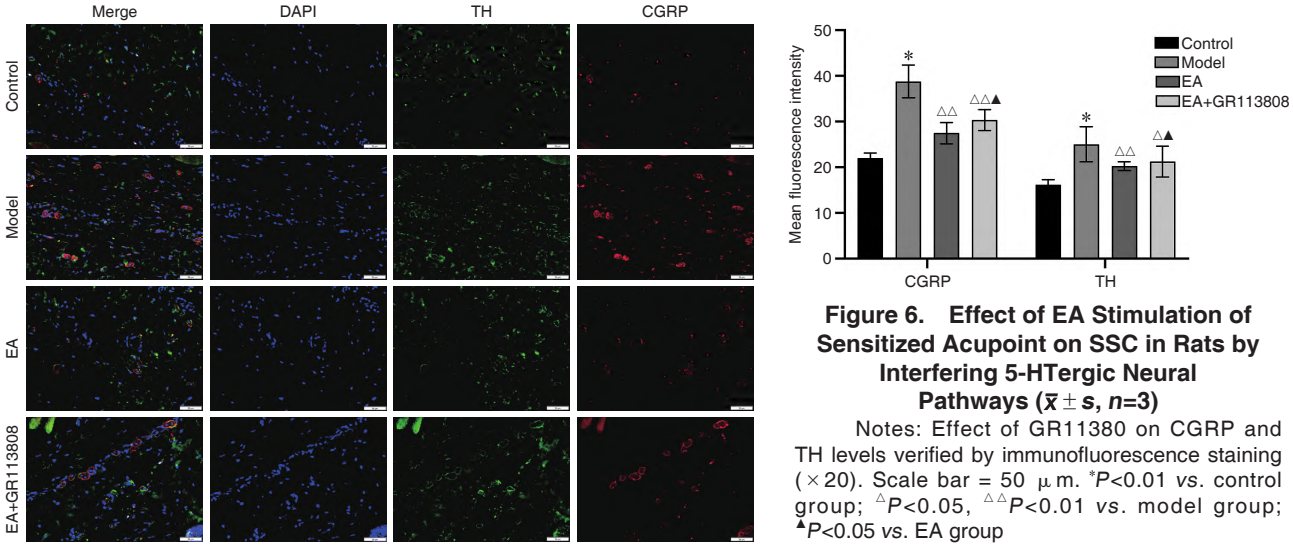

Figure 6. Effect of EA Stimulation of Sensitized Acupoint on SSC in Rats by Interfering 5-HTergic Neural Pathways ($\bar{x} \pm s$, n=3)

Notes: Effect of GR11380 on CGRP and TH levels verified by immunofluorescence staining (×20). Scale bar = 50 μm. $^{*}P<0.01$ *vs*. control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs*. model group; $^{\blacktriangle}P<0.05$ *vs*. EA group

CGRP in skin tissues (both $P<0.05$, Figure 5E).

The effect of intervention with 5-HT on CGRP and TH levels was verified by immunofluorescence staining (Figure 6). The results showed that the densities of CGRP-positive sensory nerve fibers and TH-positive sympathetic nerve fibers were increased in the model group compared with the control group ($P<0.01$); whereas the densities were increased after administration of 5-HT inhibitors compared with the EA group ($P<0.05$).

DISCUSSION

This study selected acupoints according to meridian system theory. ST 36 is located on the Foot-Yangming Stomach Meridian in Chinese medicine and is a distal acupoint.[20] ST 36 can regulate qi, strengthen Pi (Spleen) and stomach function, which is often used in the treatment of abdominal pain, constipation, diarrhea, and indigestion.[21] BL 25 acupoint belongs to the Urinary Bladder meridian, which is located at the Back-Shu point of the Large Intestine meridian.[22] BL 25 has the effects of harmonizing Wei (Stomach), replenishing qi, and lowering adverse qi, which is often used in the treatment of enteritis, dysentery, constipation, diarrhea, and dyspepsia. In addition, EA at BL 25 acupoint has a good analgesic effect.[23] BL 15, which also belongs to the Urinary Bladder meridian, is the Back-Shu point of Xin (Heart). The studies have shown that acupuncture at the BL 15 acupoint has a certain therapeutic effect on cardiovascular diseases, and can improve cardiac referred pain, myocardial ischemia, and myocardial infarction.[24] However, there are no reports on the use of EA at the BL 15 acupoint for colitis. Therefore, in this study, we selected ST 36 and BL 15 acupoints as the EA sensitized acupoints groups, as well as BL 15 acupoints as the EA non-sensitized acupoints group.

When a lesion occurs in the internal organs, it is manifested by the appearance of pain or hyperesthesia on the body, also called SRP.[25] The sensitized area often overlaps or adjacent acupoints, such as colitis patients often appear nodules in the ST 36 acupoint. The DRG is an important structure in the injurious sensory pathway, transmitting sensory information from the viscera and body surface to the central nervous system.[26] Under pathological conditions, sympathetic nerves distributed in the DRG, nerve injury areas, and tissue inflammation areas may develop sprouts, which then wrap around surrounding sensory neurons to form SSC structures.[27,28] The sprouting sympathetic nerves may produce abnormal electrical activity, leading to neuronal damage. Studies have reported sympathetic sprouting around neurons in the DRG and the formation of "basket structures" outside the neurons.[29,30] Sympathetic sprouting and association with sensory neurons are closely associated with increased sympathetic activity and sensitization of surrounding tissues in pathological states, which may be closely related to the pathological process of colitis. In our previous study, we found that colitis rats exhibited secondary hyperalgesia with sensitization at ST 36 acupoint. EA at ST 36 acupoint significantly reduced colonic lesions and relieved SRP in colitis rats.[11] Based on previous studies, ST 36 and BL 25 were selected as

EA sensitization points in this study.[11,12] The results showed that EA treatment increased the mechanical pain threshold of rats to play an analgesic role, and had a good effect on reducing colonic pathological damage in colitis rats, which was consistent with the previous study.

SSC and neurogenic inflammatory responses occur after acupoint sensitization, accompanied by the release of inflammatory factors and pain-inducing substances, which are important mechanisms for the maintenance and exacerbation of somatic referred pain.[13,31] TH is a specific marker of sympathetic nerves that is widely distributed in the cytoplasm of noradrenergic axons and is the rate-limiting enzyme for catecholamine synthesis.[32] SP and CGRP are mainly found in sensory nerve fibers, as markers for nociceptive afferent neurons, which play an important role in pain transmission processes and neurogenic inflammatory responses.[33,34] In addition, tissue damage or injurious stimuli cause the release of large amounts of the inflammatory and pain-causing substances, such as HA, BK, and PGI2, which participate in the activation and sensitization of the nociceptor.[35-37] In the current study, we found that DSS induced sympathetic sprouting in the DRG of colitis rats, and TH-positive nerve fibers wrapped around DRG sensory neurons, forming SSC structures and inducing neurogenic inflammatory responses on the body surface. Surprisingly, EA at sensitized acupoints significantly reduced the SSC phenomenon to relieve SRP, decreased the expression of TH and DRG in the DRG, and decreased the levels of HA, BK, and PGI2 associated with neurogenic inflammatory responses. Therefore, we concluded that the DSS-induced colitis model caused abnormal sympathetic excitation, and also amplified the SRP caused by colitis by promoting sympathetic sprouting and SSC structure production. EA at sensitized acupoints could reduce the sensitivity to nociception and sympathetic excitation in rats, which contributed to the recovery of colitis rats.

Modern studies have found that 5-HT3 receptor agonists increased long-term secondary allodynia and nociceptive hyperalgesia in rats, while selective 5-HT3 receptor antagonists prevented secondary mechanical allodynia and hyperalgesia.[38,39] In contrast, activation of 5-HT4 receptors improved gastrointestinal dysfunction and reduced visceral nociceptive hypersensitivity.[40] TPH1 is an important rate-limiting enzyme in the 5-HT synthesis pathway. SERT is a transporter protein responsible for transporting serotonin from the synaptic gap to presynaptic neurons, and increasing colonic 5-HT levels and decreasing SERT expression in IBS may lead to diarrhea and visceral hypersensitivity.[41]

In our study, it was found that the expression levels of 5-HT, 5-HT3, and TPH1 in colitis rats were much higher than those in the control group, and 5-HT4 and SERT levels were lower than those of the control group. EA at sensitized acupoints significantly reversed this alteration. Furthermore, it was confirmed that 5-HT pathway inhibitors GR113808 could significantly reduce SSC and neurogenic inflammatory responses in colitis rats. The results revealed that the levels of 5-HT and related receptors in the colon were closely associated with referred pain in colitis rats. The possible mechanism was that traumatic sensory stimulation caused the release of 5-HT from peripheral sensory neurons, mast cells, and platelets, promoting the release of nociceptive substances such as HA, BK, and PGI2 to cause pain; 5-HT could also bind to 5-HT3 and 5-HT4 receptors in sensory nerve terminals, thus causing nociceptive hypersensitivity. EA stimulation at sensitized acupoints might reduce the SSC phenomenon and neurogenic inflammatory response by interfering with 5-HTergic neural pathways, thus alleviating SRP triggered by inflammatory injury in colitis rats.

In conclusion, EA stimulation at sensitized acupoints could reduce the pathological injury of the colon and decrease the sensitivity to nociception in colitis rats. Mechanistically, EA stimulation of sensitized acupoints alleviated DSS-induced SRP in colitis rats by interfering with the 5-HTergic neural pathway, and reducing the SSC structure and neurogenic inflammatory response. This study revealed the therapeutic mechanism of EA stimulation of sensitized acupoints on DSS-induced colitis, which provides an important experimental basis for the clinical application of EA at sensitized acupoints.

Data Availability

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors report no conflicts of interest in this work.

Author Contributions

Yang Y, Qu JY, Guo H, Zhou HY and Ruan X performed the experiments; Wang YL designed the experiments; Peng YC, Shen XF and Xiong J analyzed the data; Yang Y wrote the manuscript. All authors contributed to this article, and gave approval of the final version to be published.

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