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RESEARCH ARTICLE

Extracellular signal-regulated kinase, substance P and neurokinin-1 are involved in the analgesic mechanism of herb-partitioned moxibustion

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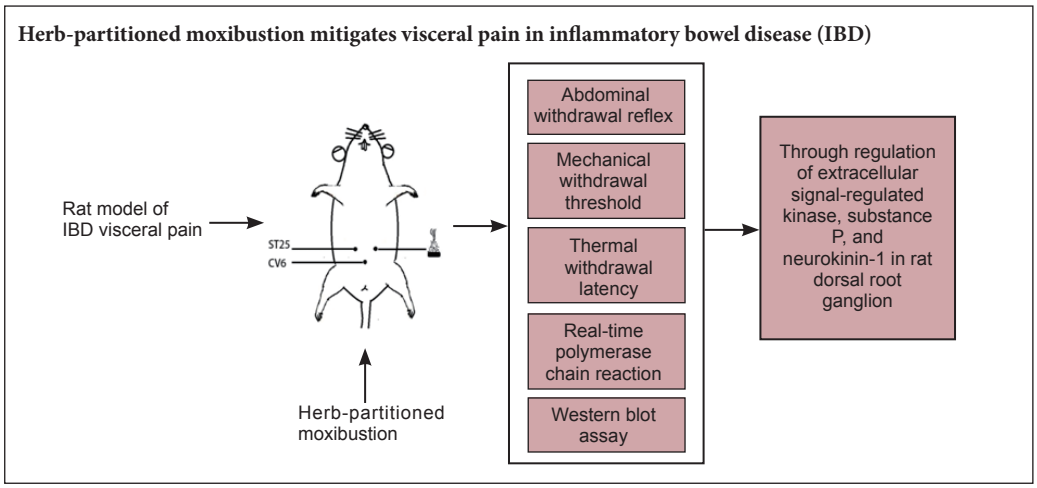
Graphical Abstract



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Abstract

Herb-partitioned moxibustion can effectively mitigate visceral pain, a major symptom in inflammatory bowel disease, but the analgesic mechanism is still unclear. Moreover, extracellular signal-regulated kinase, substance P, and neurokinin-1 are involved in formation of central hyperalgesia. Thus, we postulated that the analgesic effect of herb-partitioned moxibustion may be associated with these factors. Accordingly, in this study, we established an inflammatory bowel disease visceral pain model in rat by enema with a mixed solution of 5% trinitrobenzenesulfonic acid and 50% ethanol. Bilateral *Tianshu* (ST25) and *Qihai* (CV6) points were selected for herb-partitioned moxibustion. Our results showed that herb-partitioned moxibustion improved visceral pain and down-regulated extracellular signal-regulated kinase, substance P, and neurokinin-1 protein and mRNA expression in dorsal root ganglia. These results indicate that down-regulation of extracellular signal-regulated kinase, substance P, and neurokinin-1 protein and mRNA may be a central mechanism for the analgesic effect of herb-partitioned moxibustion.

Key Words: *nerve regeneration; inflammatory bowel disease; visceral pain; herb-partitioned moxibustion; analgesic effect; Tianshu (ST25); Qihai (CV6); dorsal root ganglion; extracellular signal-regulated kinase; substance P; neurokinin-1; neural regeneration*

Introduction

Inflammatory bowel disease (IBD) is an autoimmune disease characterized by nonspecific intestinal mucosal inflammation, majorly comprised of ulcerative colitis and Crohn's disease. It is commonly seen in European and American countries (Molodecky et al., 2012), although incidence of IBD has also been increasing in China (Wang et al., 2010; Zeng et al., 2013; Zhao et al., 2013). Abdominal pain, diarrhea, and purulent or mucus bloody stool are major symptoms of IBD that present recurrently and severely affect patients' quality of life (Volz et al., 2016).

Visceral pain is a major symptom of IBD, and a significant reason for patients to consult a doctor. Accordingly, the effectiveness of herb-partitioned moxibustion (HPM) in

relieving IBD has been verified clinically, with HPM effectively alleviating the symptoms of abdominal pain in IBD patients. The analgesic effect of HPM is comparable to melaclazide enteric-coated tablets, but HPM can also alleviate a patient's anxiety and depression (Shi et al., 2011; Bao et al., 2014, 2016). Moreover, HPM effectively mitigates visceral pain in IBD rats, with the analgesic effect possibly related to down-regulation of serum substance P (SP), tumor necrosis factor-α, and prostaglandin E2 (Huang et al., 2015; Li et al., 2016). Here, we ask what is the central mechanism of HPM in exerting its analgesic effect?

As a crucial signaling pathway, extracellular signal-regulated kinase (ERK) is a branch of mitogen-activated protein kinases that can be evoked by multiple stimuli and is involved in transduction of pain signals (Xu et al., 2016). SP and its receptor, neurokinin-1 (NK-1), play an important role in formation and maintenance of hyperalgesia (Zhao and Yin, 2015). In a rat model of inflammatory pain, striatal ERK, SP, and NK-1 were reported to interact and contribute to transduction of pain signaling (Nakamura et al., 2014). In this study, we aimed to gain new insight into the central mechanism of the effect of HPM in relieving visceral pain in IBD rat models, by measuring expression of ERK, SP, and NK-1 protein and mRNA in dorsal root ganglia.

Materials and Methods

Animals

Thirty-six healthy, pathogen-free male Sprague-Dawley rats aged 6 weeks old and weighing 150 ± 20 g were purchased from Shanghai Sippr-BK Laboratory Animal Co., Ltd., Shanghai, China [license No. SCXK (Hu) 2013-0016]. The rats were housed in a well-ventilated room with a 12-hour light/dark cycle (8:00–20:00 light; 20:00–8:00 dark), at 20 ± 1°C and relative humidity of 50%. Before the experiment, the rats were acclimated for 1 week. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), and the protocol was approved by the Committee on Use of Human and Animal Subjects in Teaching and Research, Shanghai University of Traditional Chinese Medicine (license No. 2015000519001).

The 36 rats were randomized into a normal group, IBD group, HPM group (IBD + HPM), and sham HPM group (IBD + sham HPM), with nine rats in each group. IBD visceral pain models were established in all groups except for the normal group.

Preparation of the IBD visceral pain model

The IBD visceral pain model was established by enema with 5% (w/v) trinitrobenzenesulfonic acid (Sigma, Los Angeles, CA, USA) mixed with 50% ethanol (2:1). After fasting for 24 hours, the rats were weighed and then intraperitoneally anesthetized with 2% pentobarbital sodium (30 mg/kg). Trinitrobenzenesulfonic acid enema solution was administered at 3 mL/kg with the needle inserted 6–8 cm into the anus. After the enema, the rats were placed upside down for 1 minute (Morris et al., 1989; Zhou et al., 2008). An enema was performed once every 7 days for a total of 4 times. After modeling, the rats were subjected to abdominal withdrawal reflex (AWR), mechanical withdrawal threshold (MWT), and thermal withdrawal latency (TWL) tests to categorize the pain. One rat from each group was sacrificed to verify successful model establishment by histopathological observation (hematoxylin-eosin staining) of the colon. The intervention began when the model was confirmed successful.

HPM intervention

Rats in the HPM group had HPM intervention at the points, *Tianshu* (ST25, at the same level as the umbilicus and lateral to the anterior midline) (bilateral) and *Qihai* (CV6, located on the midline, 1.5 cun below the umbilicus) (Wang and Ji, 2007). Herb cakes were made of Aconiti preparata powder and yellow wine. Using a specific mold, moxa cones were prepared from approximately 90 mg refined moxa wool (Shanghai Research Institute of Acupuncture and Meridian, Shanghai, China). Two moxa cones were burnt successively for each point (for a total of approximately 10 minutes) per session, once every day for a total of 7 sessions. Rats in the sham HPM group were treated by sham HPM. Here, herb cakes and moxa cones were placed, as in the HPM group, but not ignited, for 10 minutes each time, once a day for 7 times in total (Yu, 2003). Rats in the normal and IBD groups received no treatment but had the same grasping and fixing as the HPM group.

During the intervention, all rats were removed from their cage and fixed onto the table but not anesthetized. No rat died during the process.

Pain measurements

Pain measurements were performed on the second day after model establishment.

AWR

According to Al-Chaer's method (Al-Chaer et al., 2000), AWR was examined under four different levels of colorectal distention stimulus: 20 mmHg, 40 mmHg, 60 mmHg, and 80 mmHg. Each rat was measured three times, while each stimulus lasted 20 seconds, with an interval of 5 minutes. The average value from the three measurements was considered as the final score.

MWT

In MWT, a Von Frey filament (grades of 2.0 g, 4.0 g, 6.0 g, 8.0 g, and 15.0 g; Stoelting Co., Kiel City, WI, USA) was used to stimulate the center of the rat's hindpaw, lasting ≤ 4 seconds. When the rat lifted or licked its paw, it was considered positive, otherwise it was negative. The test started with 2 g stimulation, which was followed by a higher stimulus level with a negative result, until the first positive reaction occurred. Each stimulation grade was tested five times, with 30 seconds between each stimulation. The maximum MWT value

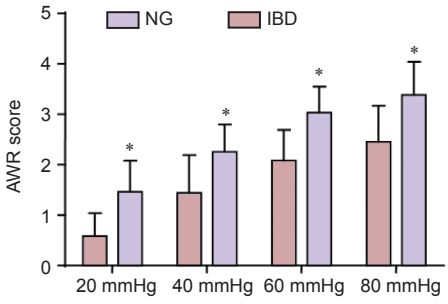


Figure 1 Comparison of abdominal withdrawal reflex (AWR) between the normal group (NG) and inflammatory bowel disease group (IBD).
Data are presented as the mean ± SD (n = 8 in NG, n = 24 in IBD; independent samples t-test). *P < 0.05, *vs.* NG.

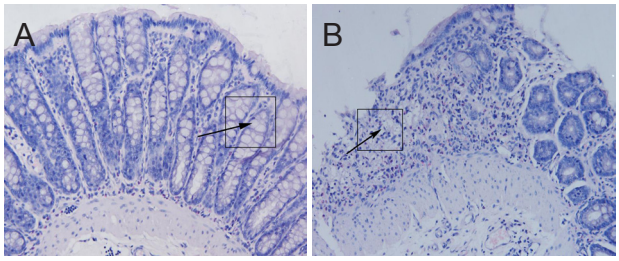


Figure 3 Histopathological observation of the colon using hematoxylin-eosin staining (original magnification, 200×).
(A) Normal group. Arrow represents normal tissue cells. (B) Inflammatory bowel disease group. Arrow represents inflammatory cell infiltration.

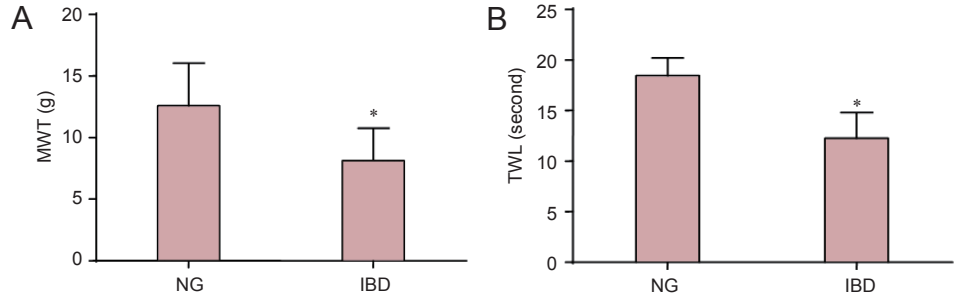


Figure 2 Comparison of mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) between the normal group (NG) and inflammatory bowel disease group (IBD).
(A) MWT test was performed using the Von Frey filament. (B) TWL test followed Hargreaves method. Data are presented as the mean ± SD (n = 8 in NG, n = 24 in IBD; independent samples t-test). *P < 0.05, *vs.* NG.

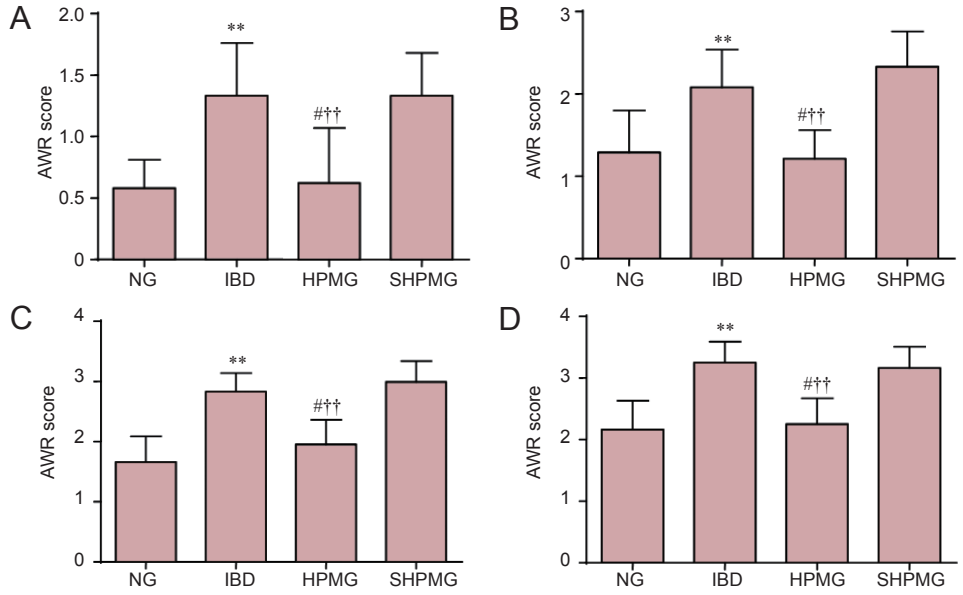


Figure 4 Effect of herb-partitioned moxibustion (HPM) on abdominal withdrawal reflex (AWR) score of inflammatory bowel disease (IBD) rats.
(A) AWR score under 20 mmHg colorectal distention stimulation; (B) AWR score under 40 mmHg colorectal distention stimulation; (C) AWR score under 60 mmHg colorectal distention stimulation; and (D) AWR score under 80 mmHg colorectal distention stimulation. Data are presented as the mean ± SD (n = 8 per group; one-way analysis of variance followed by the least significant difference test). **P < 0.01, *vs.* normal group (NG); #P < 0.01, *vs.* IBD group; ††P < 0.01, *vs.* sham HPM group (SHPMG).

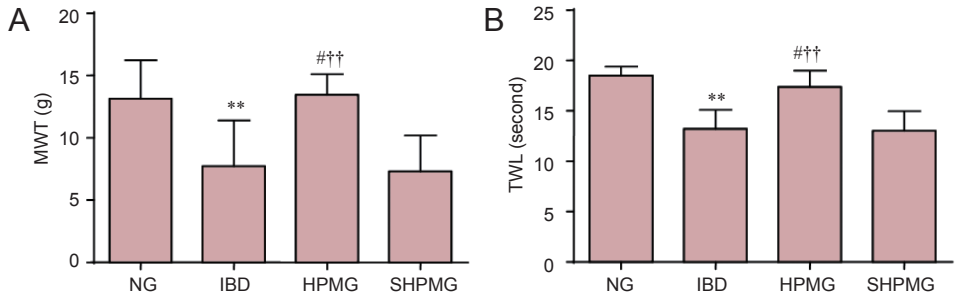
is 15 g (Chaplan et al., 1994).

TWL

Rat TWL was measured according to a previous method (Hargreaves et al., 1988) to investigate hind paw withdrawal under radiation from a thermal stimulator (Institute of Medical Biology, Chinese Academy of Medical Sciences, Beijing, China). Thermal stimulation was controlled at the same intensity and automatically cut off at 20.1 seconds in case of tissue damage. Each rat was examined five times, with 3-minute intervals. The mean value was taken as the TWL.

Sampling

At the end of intervention, the rats were all subjected to visceral and body pain detection. Afterwards, the rats were sacrificed and distal colons (6–8 cm) harvested under anesthesia. The colons were cut longitudinally, rinsed with 4°C normal saline, and then fixed in 10% neutral-buffered formalin for morphological observation. Spinal cords were exposed to collect L_6–S_2 dorsal root ganglia, which were stored at −80°C.



(A) MWT test was performed using the Von Frey filament. (B) TWL test followed Hargreaves method. Data are presented as the mean ± SD (n = 8 per group; one-way analysis of variance followed by the least significant difference test). **P < 0.01, *vs.* normal group (NG); #P < 0.01, *vs.* IBD group; ††P < 0.01, *vs.* sham HPM group (SHPMG).

Figure 5 Effect of herb-partitioned moxibustion (HPM) on mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) of inflammatory bowel disease (IBD) rats.

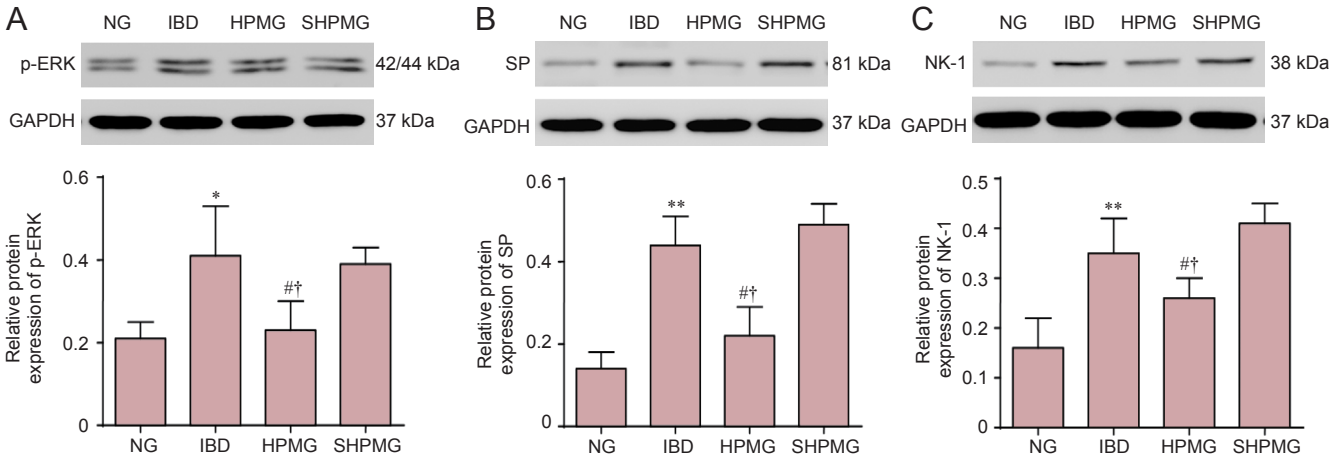


Figure 6 Effect of herb-partitioned moxibustion (HPM) on phosphorylated extracellular signal-regulated kinase (p-ERK) (A), substance P (SP) (B), and neurokinin-1 (NK-1) (C) protein expression in the dorsal root ganglion of inflammatory bowel disease (IBD) rats (western blot assay).
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Protein expression is expressed as target protein/GAPDH gray values. Data are presented as the mean ± SD (n = 8 per group; one-way analysis of variance followed by the least significant difference test). *P < 0.05, **P < 0.01, *vs.* normal group (NG); #P < 0.01, *vs.* IBD group; †P < 0.05, *vs.* sham HPM group (SHPMG).

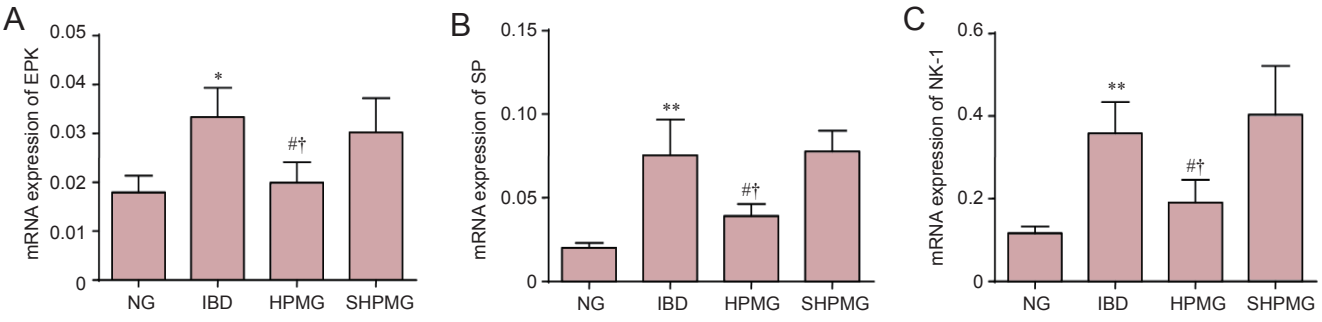


Figure 7 Effect of herb-partitioned moxibustion (HPM) on extracellular signal-regulated kinase (ERK) (A), substance P (SP) (B), and neurokinin-1 (NK-1) (C) mRNA expression in the dorsal root ganglion of inflammatory bowel disease (IBD) rats (real-time polymerase chain reaction).
Data are presented as the mean ± SD (n = 8 per group; one-way analysis of variance followed by the least significant difference test). *P < 0.05, **P < 0.01, *vs.* normal group (NG); #P < 0.01, *vs.* IBD group; †P < 0.05, *vs.* sham HPM group (SHPMG).

Histopathological score of the colon

Colons were observed under an optical microscope (Olympus, Tokyo, Japan) after sectioning and staining with hematoxylin and eosin.

Western blot assay

Dorsal root ganglion tissue was removed using the –80°C freezer, weighed, and mixed with lysate solution at a proportion of 100 mg:1 mL. Samples were centrifuged at 4°C at a speed of 12,000 r/min for 15 minutes, with the supernatants obtained representing total protein. The bicinchoninic acid method (Qian et al., 2012) was used for determination of total protein content. Electrophoresis was performed, with each well containing 80 μg total protein. Afterwards, protein was transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a wet transfer method (Zhao et al., 2012). Primary rabbit anti-rat phosphorylated ERK monoclonal antibody (1:1,000; CST, Danvers, MA, USA), primary rabbit anti-SP polyclonal

Table 1 PCR primers for ERK, SP, and NK-1

Gene	Sequence (5′–3′)
ERK	Forward: ATC AAC ACC ACC TGC GAC CTT Reverse: TGG CCA CAT ACT CGG TCA GAA
SP	Forward: GGA GCC CTT TGA GCA TCT TCT Reverse: GAT CTG ACC ATG CCC AGC AT
NK-1	Forward: CCA TCT TGT GTT GCT TAT TCA TGG A Reverse: TTC CTT AAA CCA TTG TGA CCC TTC T
GAPDH	Forward: GGA GAA ACC TGC CAA GTA TG Reverse: GAC AAC CTG GTC CTC AGT GT

All primer and probe sequences are expressed as 5′ to 3′. ERK: Extracellular signal-regulated kinase; SP: substance P; NK-1: neurokinin-1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

antibody (1:1,000; Abcam, Cambridge, UK), and primary rabbit anti-NK-1 polyclonal antibody (1:400; Abcam) were incubated overnight at 4°C followed by secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG; 1:1,000; Beyotime Biotechnology, Haimen, China) for 2 hours at room temperature. After electrochemiluminescence incubation for 2 minutes, bands were imaged using a Bio-Rad imaging system (Bio-Rad Laboratories, Inc.). Blots were then subsequently probed using rabbit anti-rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (1:1,500; CST) as an internal control for normalization of protein loading. Gray values of target protein and internal reference protein bands were quantified. The ratio between the target protein and internal reference protein bands was used as the relative expression level.

Real-time fluorescence quantitative PCR (FQ-PCR)

Expression of ERK, SP, and NK-1 mRNA in rat dorsal root ganglia was examined by FQ-PCR. RNA extraction in peritoneal cells was performed by successively adding Trizol, chloroform, isopropanol, 75% ethanol, absolute ethanol, and DEPC water. Internal amplification was performed using an ABI PCR instrument (Biosystems, Foster City, CA, USA) at 95°C for 10 minutes with 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. ABI Prism 7300 SDS software was used to analyze mRNA expression levels of the target genes. Relative mRNA expression of a target gene = $2^{-\Delta CT} \times 100\%$, with ΔCT = CT value of target gene − CT value of internal reference (GAPDH). The primers and probes (**Table 1**) were purchased from Shanghai Generay Biotech Co., Ltd., China. PCR and RT-PCR kits were purchased from Thermo, Waltham, MA, USA.

Statistical analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for data analysis. Data are expressed as the mean ± SD. One-way analysis of variance followed by the least significant difference test was used. A value of $P < 0.05$ was considered statistically significant.

Results

Verification of the IBD model

Detection of AWR, MWT, and TWL

AWR scores under 20, 40, 60, and 80 mmHg colorectal distention stimulation were significantly higher in the IBD group compared with the normal group ($P < 0.05$; **Figure 1**). Meanwhile, MWT and TWL results were significantly lower in the IBD group compared with the normal group ($P < 0.05$; **Figure 2**). Thus, these pain behavior tests all show successful IBD model establishment.

Histopathological observation of the colon

In the normal group, the colonic epithelium had a clear structure with complete mucosa and well-arrayed glands without congestion, swelling, or ulcer. Only mild inflammatory cell infiltration was occasionally detected in mucosal lamina propria. However, in the IBD group, the colonic structure was unclear with defective mucosa, ulcer-like alterations, and inflammatory cell infiltration in mucosa and submucosa coupled with congestion, swelling, and gland damage (**Figure 3**). Thus, these hematoxylin-eosin staining results confirm successful IBD model establishment.

Influence of HPM on pain behavior in IBD rats

AWR

After treatment, AWR scores were significantly higher in the IBD group compared with the normal group under different levels of colorectal distention stimulation ($P < 0.01$). Further, AWR scores were significantly lower in the HPM group compared with the IBD and sham HPM groups under four different levels of colorectal distention stimulation ($P < 0.01$). There were no significant differences in AWR scores between the IBD group and sham HPM group ($P > 0.05$; **Figure 4**).

MWT and TWL

After treatment, MWT and TWL scores were significantly lower in the IBD group compared with the normal group ($P < 0.01$). MWT and TWL scores were significantly higher in the HPM group compared with the IBD and sham HPM groups ($P < 0.01$). There were no significant differences in MWT and TWL scores between the sham HPM and IBD groups ($P > 0.05$; **Figure 5**).

Influence of HPM on ERK, SP, and NK-1 protein and mRNA expression in dorsal root ganglia of IBD rats

p-ERK, SP, and NK-1 protein

After treatment, p-ERK, SP, and NK-1 protein was significantly higher in dorsal root ganglia from the IBD group compared with the normal group ($P < 0.05$ or $P < 0.01$). p-ERK, SP, and NK-1 protein was markedly lower in dorsal root ganglia from the HPM group compared with the IBD and sham HPM groups ($P < 0.01$ or $P < 0.05$). There were no significant differences in p-ERK, SP, and NK-1 protein in dorsal root ganglia from IBD and sham HPM groups ($P > 0.05$; **Figure 6**).

ERK, SP, and NK-1 mRNA

After intervention, ERK, SP, and NK-1 mRNA expression was significantly higher in dorsal root ganglia from the IBD group compared with the normal group ($P < 0.05$ or $P < 0.01$). Further, ERK, SP, and NK-1 mRNA expression was significantly lower in rat dorsal root ganglia from the HPM group

compared with the IBD and sham HPM groups ($P < 0.01$ or $P < 0.05$). There were no significant differences in ERK, SP, and NK-1 mRNA expression in dorsal root ganglia from sham HPM and IBD groups ($P > 0.05$; **Figure 7**).

Discussion

Pain is an unpleasant subjective feeling and emotional experience associated with tissue injury or potential injury. Depending on the cause, location, and duration, pain can be classified into nociceptive pain and pathological pain (Han, 2012). As a type of pathological pain, IBD visceral pain is a common symptom in IBD patients and has become a major complaint for patients seeking treatment (Fan et al., 2015). Although the causes of visceral pain are not clear, it has been found that sensitization of peripheral nociceptors and central neurons is the major pathological basis (Anand et al., 2007). In IBD, peripheral nociception exists because of continuous stimulation from intestinal inflammation. As a key structure in the bridge between the central nervous system and peripheral nervous system, and a crucial location in sensitization of central neurons, dorsal root ganglia play a significant role in onset and development of visceral pain. Therefore, in this study, we aimed to determine how HPM influences IBD visceral pain by focusing on changes in ERK, SP, and NK-1 in dorsal root ganglia of a rat model.

ERK is a member of the mitogen-activated protein kinase family, and is crucial in signal transduction from cell membrane receptors to the nucleus. When activated, the ERK signaling pathway contributes to cellular proliferation and differentiation, playing an important role in nociception transduction and central sensitization (Galan et al., 2003). In dorsal root ganglia, activated ERK regulates target proteins *via* transcription, and thereby participates in hyperalgesia development. Research has shown that in the spinal cord and dorsal root ganglia, ERK is strongly associated with neuropathological pain induced by chronic sciatic compression. Appropriately, after treatment, increased p-ERK declines with increased pain threshold, indicating a link between the analgesic effect and p-ERK inhibition (Shi et al., 2016). Another study, based on a rat model of inflammatory pain induced by complete Freund's adjuvant, found increased activated ERK and NK-1 mRNA expression in the superficial dorsal horn of the spinal cord. After intrathecal administration of U0126, a MEK blocker, NK-1 mRNA expression was reduced with a concomitant increased pain threshold. This suggests the ERK pathway has been induced, with hyperalgesia *via* regulation of gene transcription, and suggests NK-1 as a potential target gene (Ji et al., 2002). By intraplantar formalin, Fang et al. (2014) developed a rat model of inflammatory pain to observe the analgesic action of electroacupuncture and its mechanism. Their results revealed that the ERK signaling pathway contributes to development of inflammatory pain. The activated pathway promoted secretion of cyclooxygenase-2 (COX-2) and NK-1, and consequently lead to increased hyperalgesia. After electroacupuncture and U0126 injection, p-ERK decreased, as well as COX-2 and NK-1 expression, indicating that the analgesic effect of electroacupuncture may inhibit activation of the ERK pathway and COX-2 and NK-1 secretion. Induced ERK promotes release of NK-1 receptors and pain development, and vice versa, activated NK-1 enhances ERK activation via G proteins (Jorgensen et al., 2008). The G protein β and γ subunits induce components of the ras pathway, such as shc and src, and ultimately activate raf-1 and ERK1/2 to transmit the signal into the nucleus (Tang, 2013).

SP is a neuropeptide distributed widely in nerve fibers. Binding of SP to its receptor, NK-1, contributes to various physiological and pathological processes in the central nervous system including pain, inflammation, depression, and degenerative nervous disease (Munoz and Covenas, 2014). SP is released in both central and peripheral terminals upon nociceptive stimulation, playing an important role in ascending transmission and descending modulation of pain *via* NK-1 (Xiao, 2012). A novel study identified the interaction between SP, NK-1, and ERK during pain onset and release by examining their expression in different pain stages in an inflammatory pain rat model induced by intraplantar formalin (Nakamura et al., 2014). Moreover, under nociceptive stimulation, ERK is phosphorylated, while SP is released and binds to NK-1, which can potently shorten the pain perception period. Conversely, the pain perception period is prolonged with administration of CP96345, a NK-1 antagonist. It was also found that injection of SP plus a blocker of ERK, PD0325901, or injection of PD0325901 alone, also prolonged the pain perception period. Altogether, these results suggest that nociceptive stimulation causes SP to activate NK-1 and phosphorylate ERK, with the SP-NK-1-ERK1/2 system effectively shortening the duration of pain perception (Nakamura et al., 2014).

Based on this interaction between ERK, SP, and NK-1 and their function in pain, we examined their expression in dorsal root ganglia before and after HPM intervention in a rat model of IBD visceral pain. Trinitrobenzenesulfonic acid-induced IBD visceral pain rats had significantly lower pain thresholds, as shown by increased AWR and decreased MWT and TWL. In contrast, after HPM at *Tianshu* (ST 25) and *Qihai* (CV 6), AWR score decreased, while MWT and TWL increased, indicating an increased pain threshold and mitigation of visceral pain. Regarding ERK, SP, and NK-1 detection in dorsal root ganglia, protein and mRNA expression increased significantly in the model but declined after HPM intervention, suggesting that HPM at *Tianshu* and *Qihai* effectively inhibits p-ERK, SP, and NK-1 protein and mRNA expression in rat dorsal root ganglia.

The action of moxibustion features multiple layers and targets. Pain development is also complex, involving the interaction of various pain-inducing and analgesic factors in both the central and peripheral nervous systems. Indeed, there are some drawbacks to our study: we only investigated ERK, SP, and NK-1 in dorsal root ganglia, and further, did not use an ERK blocker. Hence, further studies are needed to unveil the analgesic mechanism underlying HPM intervention in IBD visceral pain.

In conclusion, the analgesic effect exerted by HPM in treating IBD visceral pain in rats may be associated with

p-ERK, SP, and NK-1 inhibition in dorsal root ganglia. Our study provides scientific experimental data for identification of the mechanism of moxibustion analgesia and supports its clinical application and promotion.

Author contributions: *XPM, ZS and HGW designed the study. ZYL, YTY, XFH and LJW implemented the experiments. DZ, JL and YZ analyzed the data. ZYL and JH wrote and revised the paper. XPM supervised all the research and edited the paper. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*
Research ethics: *The study protocol was approved by the Committee on Use of Human and Animal Subjects in Teaching and Research, Shanghai University of Traditional Chinese Medicine of China (approval No. 2015000519001).*
Data sharing statement: *The datasets analyzed during the current study are available from the corresponding author on reasonable request.*
Plagiarism check: *Checked twice by iThenticate.*
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