P2X₇ receptor of rat dorsal root ganglia is involved in the effect of moxibustion on visceral hyperalgesia

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Received: 13 August 2014 / Accepted: 4 December 2014 / Published online: 20 December 2014
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Abstract Irritable bowel syndrome (IBS) and inflammatory bowel disease often display visceral hypersensitivity. Visceral nociceptors after inflammatory stimulation generate afferent nerve impulses through dorsal root ganglia (DRG) transmitting to the central nervous system. ATP and its activated-purinergic 2X₇ (P2X₇) receptor play an important role in the transmission of nociceptive signal. Purinergic signaling is involved in the sensory transmission of visceral pain. Moxibustion is a therapy applying ignited mugwort directly or indirectly at acupuncture points or other specific parts of the body to treat diseases. Heat-sensitive acupoints are the corresponding points extremely sensitive to moxa heat in disease

conditions. In this study, we aimed to investigate the relationship between the analgesic effect of moxibustion on a heat-sensitive acupoint “Dachangshu” and the expression levels of P2X₇ receptor in rat DRG after chronic inflammatory stimulation of colorectal distension. Heat-sensitive moxibustion at Dachangshu acupoint inhibited the nociceptive signal transmission by decreasing the upregulated expression levels of P2X₇ mRNA and protein in DRG induced by visceral pain, and reversed the abnormal expression of glial fibrillary acidic protein (GFAP, a marker of satellite glial cells) in DRG. Consequently, abdominal withdrawal reflex (AWR) score in a visceral pain model was reduced, and the pain threshold was elevated. Therefore, heat-sensitive moxibustion at Dachangshu acupoint can produce a therapeutic effect on IBS via inhibiting the nociceptive transmission mediated by upregulated P2X₇ receptor.

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Keywords Moxibustion · Dorsal root ganglia · Purinergic
2X₇ receptor · Satellite glial cell · Visceral hyperalgesia ·
Colorectal distension

Abbreviations

IBS	Irritable bowel syndrome
ATP	Adenosine triphosphate
CRD	Colorectal distention
VHM	Visceral hyperalgesic model
AWR	Abdominal withdrawal reflex
HSM	Heat-sensitive moxibustion
DRG	Dorsal root ganglia
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
GFAP	Glial fibrillary acidic protein
SDS	Sodium dodecylsulfate
HRP	Horseradish peroxidase
SGC	Satellite glial cells

Introduction

Visceral pain is a symptom of splanchnic disease. Visceral hyperalgesia is the most important pathological scenario in visceral pain. Irritable bowel syndrome (IBS) is one of the most common intermittent or durative functional gastrointestinal disorders characterized by abdominal discomfort or pain and altered bowel habits. IBS and inflammatory bowel disease often exhibit visceral hypersensitivity [1–3]. The intestinal wall of functional bowel disease also has a similar inflammatory response. The chronic inflammatory stimulation of bowel triggered visceral nociceptive response, which caused visceral pain [1, 3–5]. Adenosine triphosphate (ATP) is involved in the neurotransmission via acting on purinergic 2X (P2X) receptors [6–8]. Visceral nociceptors after inflammatory stimulation formed the afferent nerve impulses through primary sensory dorsal root ganglia (DRG) transmitting to the central nervous system [4, 5]. P2X₇ receptor plays an important role in the transmission of nociceptive signal [7–13]. Such purinergic signaling is involved in the sensory transmission of visceral pain [14, 15].

Acupuncture and moxibustion are alternative or complementary therapies used to treat IBS [16, 17]. Moxibustion is a therapy which applies the ignited mugwort (*Artemisia vulgaris* from traditional Chinese medicine) directly or indirectly at acupuncture points or other specific parts of the body to cure diseases [17–19]. The mechanisms of moxibustion action possibly involve the combination of heat (burning pain and heat stress), tar (extract), aroma (fume), and psychological stress [20–22]. Moxibustion on heat-sensitive acupoints is a new therapy [23, 24]. Heat-sensitive acupoints are the corresponding points extremely sensitive to moxa heat in disease conditions. Clinical studies have shown that moxibustion on heat-sensitive acupoints may be a better treatment of irritable bowel syndrome, but its mechanism is unclear. In this study, we investigated the relationship between the analgesic effect of heat-sensitive moxibustion at “Dachangshu” acupoint and the expression levels of P2X₇ receptor in rat dorsal root ganglia after the chronic inflammatory stimulation by colorectal distension.

Materials and methods

Animals and visceral hyperalgesic models

Neonatal male Sprague-Dawley rats (5 days old) were provided by the Laboratory Animal Center of Medical College of Nanchang University. The use of the animals was approved by the Animal Care and Use Committee of Medical College of Nanchang University and was strictly in accordance with the National Institutions of Health Guide for the Care and Use of Laboratory Animals. In order to investigate mechanisms of

pain in IBS sufferers, we developed a rat model of chronic visceral pain induced by mechanical colorectal irritation. The procedure was as follows. Daily 60 mmHg of colorectal distention (CRD) was performed on neonatal rats beginning 8–21 days after birth. The distention was applied using vascular reconstruction balloon (length 20.0 mm; diameter 2.0 mm) inserted into the descending colon through the rectum of awake rats. The balloon was quickly distended with 60 mmHg for 1 min and then deflated and withdrawn. Neonatal rats received twice daily CRD, at 30-min intervals [17, 25]. After the distention stimulation was terminated, the rats were kept until they reached adulthood (at least 8 weeks old), and then experiments were performed. After the behavioral responses of visceral pain induced by CRD were identified, the rats were used as visceral hyperalgesic model (VHM).

Assessment of visceral hyperalgesic sensitivity

Abdominal withdrawal reflex (AWR) was monitored to determine the visceral hyperalgesic sensitivity [17, 25]. Behavioral responses to CRD in young adult rats were assessed by observing AWR scores. The experimental rats were lightly anesthetized with diethyl ether, and the vessel dilating balloon was inserted from the anus into the rectum and descending colon (6–8 cm in depth) with its balloon catheter fixed at rat tail by a tape. The rats were then housed in a Lucite cubicle (20×10×10 cm) on an elevated Plexiglas platform and allowed to wake up and adapt for 30 min. CRD was produced by gradually inflating the balloon to the desired pressure (20, 40, 60, or 80 mmHg) for a duration of 20 s. When CRD stimulation was performed, pressure was slowly increased to initiate the abdomen lifting in experimental rats for determining the threshold value of pressure pain. Stimuli were applied in an ascending-graded manner (spaced by 5 min). To minimize the bias, the same stimulation paradigm was used in every rat. Afterwards, AWR was measured by visual observation reproduced by three blinded observers. AWR scores were assigned according to the scale of marks [25]: 0, no behavioral response to CRD; 1, immobile during the CRD and appearance of brief head motion after a pause at the onset of the stimulation; 2, a mild contraction of the abdominal muscles, but no lifting the abdomen off the platform; 3, a strong contraction of the abdominal muscles and lifting the abdomen off the platform, but no lifting the pelvic structure off the platform; and 4, body arching and lifting the pelvic structure and scrotum. The mean scores from three measurements were used as the final score.

Acupoint selection and heat-sensitive moxibustion

Acupoint selection Dachangshu (BL 25) is located on the posterior trunk in the lumbar region. The point is located 0.5 cm lateral to the depression immediately inferior to the

spinous process of the fourth lumbar vertebra. Dachangshu was determined according to the national standards, “Nomenclature and Location of Acupoints” [26, 27]. Dachangshu acupoint was chosen because the stimulation of Dachangshu acupoint can produce various effects, such as regulating the large intestine, small intestine, and stomach; benefiting the lower back and knees; removing obstruction from the intestine channel; and relieving fullness and swelling. Dachangshu acupoint is a key acupoint usually used to treat patients with IBS. So Dachangshu acupoint was chosen in this study. Any place 1 cm outside the Dachangshu acupoint was selected as non-acupoint control.

Heat-sensitive moxibustion (HSM) Was used in the experiments [23, 28, 29]. The duration (30–60 min) of heat-sensitive moxibustion is longer than that (15 min) of traditional suspended moxibustion, which enhances its efficiency. Dachangshu acupoint or non-acupoint were heated by suspended moxibustion using a moxibustion cigar produced from mugwort (customer made for animals, length 12 cm, diameter 0.6 cm, in the Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine, Jiangxi, China) at a height of approximately 2 cm over a hairless area of skin four times a day for 8 consecutive days. The time of heat-sensitive moxibustion was about 40 min, and the diameter of acupoint hole was 0.5 cm. Rats were randomly divided into four groups: control group ($n=10$), VHM group ($n=10$), VHM with HSM at Dachangshu acupoint group ($n=10$), and VHM with HSM at non-acupoint group ($n=10$).

Immunohistochemistry

L₆–S₁ dorsal root ganglia (DRG) isolated from rats were washed by phosphate-buffered saline (PBS). The ganglia were fixed in 4 % paraformaldehyde (PFA) for 24 h and dehydrated with 20 % sucrose for overnight at 4 °C. The ganglia were cut into 10 µm in thickness at a cryostat. The sections were incubated in 3 % H₂O₂ for 10 min to block the endogenous peroxidase. Following incubation with regular goat serum for 1 h, the sections were incubated with rabbit anti-P2X₇ (1:100 diluted in PBS; Abcam) for overnight at 4 °C. After three rinses in PBS, the sections were incubated with biotinylated goat anti-rabbit secondary antibody (Beijing Zhongshan Biotech. Co.) for 1 h at room temperature and then with streptavidin-horseradish peroxidase (Beijing Zhongshan Biotech. Co.) for 30 min. The color was developed in DAB substrate (Beijing Zhongshan Biotech. Co.); sections were then dehydrated and mounted with neutral gum. Image-Pro Plus 6.0 image analysis software (Media Cybernetics Inc.) was used to quantify the expression of P2X₇ receptor.

For immunofluorescence, the preparation of sections was done as described above. After being washed with PBS for three times, the sections were pre-incubated with 10 % normal

goat serum for 40 min at 37 °C, and then incubated with rabbit anti-P2X₇ (1:500) and chicken anti-glia fibrillary acidic protein (GFAP) (1:500, Abcam) diluted in PBS for overnight at 4 °C. After three rinses in PBS, the sections were incubated with fluorescent goat anti-chicken FITC and goat anti-rabbit TRITC secondary antibody (1:200 dilution of both secondary antibodies) at 37 °C for 40 min. After three washes in PBS, sections were mounted with glycerin and examined with fluorescence microscopy. The overlap coefficient was measured by Image-Pro Plus 6.0 image analysis software (Media Cybernetics Inc.) and used to quantify the co-expressions of P2X₇ and GFAP. To specify the immunoreactivity of P2X₇ and GFAP, normal goat serum and PBS were added as a negative control instead of primary antibodies.

RNA preparation and reverse transcriptase (RT)-PCR

L₆–S₁ DRG were isolated immediately and flushed with ice-cold PBS. Total RNA was extracted by using TRIzol Total RNA Reagent (Beijing Tiangen Biotech Co.). Isolated RNA was dissolved in diethylpyrocarbonate (DEPC) water and reversely transcribed to complementary DNA (cDNA) using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. The primers information is as follows: β -actin sense CACCCGCGAGTACAACCTTC, antisense CCCA TACCCACCATCACACC; P2X₇ sense 5'-CTTCGGCG TGCGTTTGTG-3', antisense 5'-AGGACAGGGTGGAT CCAATG-3'. The length of PCR products is 265 bp for P2X₇ and 207 bp for β -actin, respectively. PCR was performed as follows: 1 µl of cDNA, 12 µl DEPC water, 2 µl 10 µmol primers (equal sense and antisense), and 15 µl 2× Easy Taq PCR SuperMix (Beijing TransGen Biotech Co.) in 30 µl. PCR conditions were set as follows: hot start at 94 °C for 3 min, 30 s denaturation (94 °C), 30 s annealing (56 °C), and 30 s extension (72 °C) for 30 amplification rounds and 5 min final extension at 72 °C. PCR products (8 µl) were run in 1 % agarose gels with EB using standard protocols. Gels were scanned with gel imaging system (Beijing Junyi Co., LTD), and the intensity of PCR product bands was quantified using Gel-Pro Analyzer 32. The results are expressed as the ratio of P2X₇ receptors band intensity/ β -actin band intensity.

Western blotting

L₆–S₁ DRG were isolated and flushed with ice-cold PBS. Ganglia were homogenized by mechanical disruption in lysis buffer (50 mM TrisCl, pH 8.0, 150 mM NaCl, 0.1 % dodecyl sodium sulfate, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin) and incubated on ice for 40 min.

Homogenate was then pelleted at 6000g for 10 min, and supernatant was collected. The quantity of total protein in the supernatant was determined using the Lowry method. After being diluted with sample buffer (100 mM TrisCl, 200 mM dithiothreitol, 4 % sodium dodecylsulfate (SDS), 0.2 % bromophenol blue, 20 % glycerol) and heated to 95 °C for 10 min, samples containing equal amounts of protein (20 µg) were separated by SDS-polyacrylamide gel electrophoresis on Bio-Rad system and 10 % gel. Separated proteins on gel were electrophoretically transferred onto PVDF membrane using the same system. The membrane was blocked with 5 % BSA in 25 mM Tris-buffered saline, pH 7.2, plus 0.1 % Tween 20 (TBST) for 3 h at room temperature, followed by incubation with primary antisera (rabbit anti-P2X₇ 1:1000, Abcam; monoclonal β-actin 1:800) in the same buffer for overnight at 4 °C. The membrane was then washed in TBST and incubated with secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2000) and goat anti-mouse IgG (1:4000) (Beijing Zhongshan Biotech. Co.) in the same buffer for 1 h at room temperature. After washing in TBST, chemiluminescent signals were collected on an autoradiography film using the enhanced chemiluminescence (ECL) kit (Shanghai Pufei Biotech. Co.). The quantification of band intensity was carried out using Image-Pro Plus software. Band densities were normalized to individual β-actin internal control.

Statistical analyses

Statistical analyses of the data were performed on SPSS 11.5. All results were expressed as mean±SD. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Fisher post hoc test for multiple comparisons or by unpaired *t* test for comparisons between two groups. *p*<0.05 was considered significant.

Results

AWR score in visceral hyperalgesic rats

Pain threshold (32.85 ± 11.23 mmHg) in the model of visceral hyperalgesia after 8-week CRD stimuli in neonatal rats was significantly lower in comparison with that (60.65 ± 15.28 mmHg) in the control group (*p*<0.01). Accordingly, AWR scores of 20, 40, 60, and 80 mmHg in the model group were significantly higher than those in the control group (*p*<0.01) (Fig. 1). The results indicated that continuous CRD stimulation in neonatal rats can form visceral hyperalgesic model.

Fig. 1 AWR score in model rats. Control control group (*n*=10), VHM visceral hyperalgesic model group (*n*=10). The AWR scores of VHM group after the CRD stimuli in rats in comparison with those in control group. ***p*<0.01, compared with control group

Effects of HSM at Dachangshu acupoint on visceral hyperalgesia

The pain threshold value (78.06 ± 12.30 mmHg) in HSM at “Dachangshu” acupoint model rats was increased in comparison with that (33.9 ± 11.34 mmHg) in model rats (*p*<0.01). There was no difference between HSM at the non-acupoint group (32.00 ± 13.87 mmHg) and the model group (*p*>0.05). HSM at Dachangshu acupoint also significantly decreased AWR scores at 20, 40, 60, and 80 mmHg in these model rats (*p*<0.01). However, no difference was seen between HSM at the non-acupoint group and model group (*p*>0.05) (Fig. 2).

Effects of HSM at Dachangshu acupoint on P2X₇ mRNA levels

RT-PCR results revealed that P2X₇ messenger RNA (mRNA) levels in DRG of the model group were significantly increased compared to the control group (*p*<0.01). The expression levels of P2X₇ mRNA in HSM at the Dachangshu acupoint group were significantly decreased compared with those in the model group (*p*<0.01). There was no difference between HSM at the non-acupoint moxibustion group and the model group (*p*>0.05) (Fig. 3).

Effects of HSM at Dachangshu acupoint on P2X₇ protein

The results from immunohistochemistry showed that P2X₇ immunoreactivity in the DRG of the model group was significantly higher than that in the control group (*p*<0.01). Compared to the model group, the expression levels of P2X₇ receptor in HSM at the Dachangshu acupoint group were significantly decreased (*p*<0.01). No difference between HSM at the non-acupoint group and model group (*p*>0.05) (Fig. 4).

Results by Western blot showed that P2X₇ protein levels in the DRG of the model group were significantly

Fig. 2 Effect of heat-sensitive moxibustion at “Dachangshu” acupoint on visceral hyperalgesia. *Control* control group, *VHM* visceral hyperalgesic model group, *VHM+HSM (“Dachangshu” acupoint)* visceral hyperalgesic model group being treated with heat-sensitive moxibustion at “Dachangshu” acupoint group, *VHM+HSM (Non-acupoint)* visceral hyperalgesic model group treated with heat-sensitive moxibustion at non-acupoint group. Compared with data in control group, AWR scores at 20, 40, 60, and 80 mmHg in VHM group rats were increased ($p<0.01$). The scores in HSM at “Dachangshu” acupoint group were significantly decreased in comparison with those in model group ($p<0.01$). No difference between HSM at non-acupoint group and VHM group ($p>0.05$). ** $p<0.01$, compared with control group; ## $p<0.01$, compared with VHM group

increased in comparison with those in the control group ($p<0.01$). HSM at Dachangshu acupoint could significantly decrease the expression levels of P2X₇ protein in these model rats ($p<0.01$). No difference was found between HSM at the non-acupoint group and the model group ($p>0.05$) (Fig. 5).

Effects of HSM at Dachangshu acupoint on the co-expression of P2X₇ and GFAP

To examine whether the upregulated expression of P2X₇ receptor in DRG is related to the activation of satellite glial cells (SGC), the co-expression of GFAP (a marker of SGCs) and P2X₇ receptor was measured by double immunofluorescence. The overlap coefficient tested by Image-Pro Plus 6.0 image analysis showed the co-expression levels of P2X₇ receptor and GFAP in DRG. Results showed that the overlap coefficient of 82 % in the model group was higher than 73 % in the control group ($p<0.05$). The overlap coefficient of 74 % in HSM at Dachangshu acupoint was lower than 82 % in the model group ($p<0.05$). No difference between 82 % in the model group and 86 % in HSM at the non-acupoint group ($p>0.05$) (Fig. 6). The upregulated expression of GFAP was a typical character of the active state of SGCs.

Fig. 3 Effect of heat-sensitive moxibustion at “Dachangshu” acupoint on the expression of P2X₇ at mRNA level in DRG assessed by RT-PCR. *Control* control group, *VHM* visceral hyperalgesic model group, *VHM+HSM (“Dachangshu” acupoint)* visceral hyperalgesic model group being treated with heat-sensitive moxibustion at “Dachangshu” acupoint group, *VHM+HSM (Non-acupoint)* visceral hyperalgesic model group being

treated with heat-sensitive moxibustion at non-acupoint group. The expression levels of P2X₇ mRNA in the DRG of model group were significantly increased compared with those in control group ($p<0.01$); the expression of P2X₇ mRNA in VHM+HSM (“Dachangshu” acupoint) group was reduced in comparison with that in VHM group. ** $p<0.01$, compared with control group; ## $p<0.01$, compared with VHM group

Fig. 4 Effect of heat-sensitive moxibustion at “Dachangshu” on the expression of P2X₇ receptor in DRG tested by immunohistochemistry. *Control* control group, *VHM* visceral hyperalgesic model group, *VHM+HSM* (“Dachangshu” acupoint) visceral hyperalgesic model group treated with heat-sensitive moxibustion at “Dachangshu” acupoint group, *VHM+HSM (Non-acupoint)* visceral hyperalgesic model group

treated with heat-sensitive moxibustion at non-acupoint group. P2X₇ immunoreactivity in VHM group was significantly higher than that in control group ($p<0.01$). P2X₇ receptor expression levels in HSM at “Dachangshu” acupoint group were significantly decreased compared with those in VHM group ($p<0.01$). ** $p<0.01$, compared with control group; ## $p<0.01$, compared with VHM group. Scale bars 20 μ m

Fig. 5 Effect of heat-sensitive moxibustion at “Dachangshu” on the expression levels of P2X₇ protein detected by Western blotting. *Control* control group, *VHM* visceral hyperalgesic model group, *VHM+HSM* (“Dachangshu” acupoint) visceral hyperalgesic model group treated with heat-sensitive moxibustion at “Dachangshu” acupoint group, *VHM+HSM (Non-acupoint)* visceral hyperalgesic model group treated with heat-sensitive moxibustion at non-acupoint group. The expression levels of P2X₇ protein in VHM group was significantly increased compared to those in control group ($p<0.01$). The expression levels of P2X₇ receptor in HSM at “Dachangshu” acupoint group were significantly decreased compared with those in VHM group ($p<0.01$). ** $p<0.01$, compared with control group, ## $p<0.01$, compared with VHM group

Discussion

Visceral hyperalgesia and inflammatory bowel disease induce irritable bowel syndrome (IBS) [1, 3]. Visceral nociceptive signal activates the ion channel receptor in the visceral sensory afferent nerve endings which then transmit the excitatory nervous impulse to the central nervous system [4]. However, visceral noxious stimulation lacks a specific signaling pathway, and the pathological mechanism of neuropathy for the functional bowel disorders is still unclear. Peripheral inflammation is often implicated in peripheral sensitization after nerve injury [30]. Consequently, the phenotype of visceral afferent neuron may be changed, and the sensitization or the expression of ligand or voltage-gated ion receptors may be increased after inflammatory injury. To address these issues, an appropriate disease model is required. In this study, we have demonstrated that continuous CRD stimulation in neonatal rats can establish the visceral hyperalgesic model. The pain threshold in this visceral hyperalgesic model developed by an 8-week CRD stimulation was significantly reduced in comparison with that in the control group. In addition, AWR scores at 20, 40, 60, and 80 mmHg in the model group were significantly higher than those in the control group.

All P2X receptors are ATP-gated ion channels, and seven subtypes (P2X_{1–7}) have been identified [6–8, 14]. P2X₇ has been observed to play an important role in inflammatory and nociceptive responses, rendering this receptor as the therapeutic targets of anti-inflammation and anti-nociception [9–12,

Fig. 6 Effect of heat-sensitive moxibustion at “Dachangshu” acupoint on the co-expression of P2X₇ and GFAP in DRG tested by double-label immunofluorescence staining. *Control* control group, *VHM* visceral hyperalgesic model group, *VHM+HSM* (“Dachangshu” acupoint) visceral hyperalgesic model group treated with heat-sensitive moxibustion at “Dachangshu” acupoint group, *VHM+HSM* (Non-

acupoint) visceral hyperalgesic model group treated with heat-sensitive moxibustion at non-acupoint group. The co-expression levels of P2X₇ receptor and GFAP in VHM group were significantly increased compared with those in control group. The co-expression levels of P2X₇ receptor and GFAP in HSM at “Dachangshu” acupoint group was significantly lower than those in VHM group. Scale bar 100 μ m

31–33]. It was reported that the colonic distention irritation increased the expression of P2X₇ receptor in sacral spinal cord of rats, whereas pretreatment with P2X₇ receptor antagonists inhibited the nociceptive transmission of visceral pain induced by the intraperitoneal injection of acetic acid and the visceral pain response of the model rats, suggesting that P2X₇ receptor has a facilitating effect in visceral painful transmission [34–36]. Our experimental results showed that the expression of P2X₇ at both mRNA and protein levels in the DRG of the model rats was significantly increased in comparison with that in the control group, indicating that P2X₇ receptor in DRG was involved in the transmission of nociceptive signal induced by the continuous CRD stimulation.

Each DRG neuron has its own satellite glial cells (SGCs). DRG neurons and their surrounding SGCs form a morphologically distinct functional unit [37, 38]. SGCs are closely surrounding DRG neurons [38, 39]. It is possible that the cross talk between neurons and SGCs within sensory ganglia serves as a mechanism for the modification of neuronal signals in the sensory pathway [37, 38]. There is histological evidence that SGCs are altered in inflammatory and painful animal models [38–40]. There is strong evidence that the activation of SGCs within the DRG plays an important role in the initiation and maintenance of inflammatory and neuropathic pain [37, 40]. GFAP is the marker of satellite glial cells in DRG. Our

observations from double immunofluorescence staining analysis showed that the co-expression levels of P2X₇ receptor and GFAP in DRG of the model rats was significantly increased compared with those in the control rats. P2X₇ co-expressed with GFAP suggested that P2X₇ receptor was expressed in satellite glial cells. It indicated that P2X₇ receptor expressed in satellite glial cells was involved in the nociceptive transmission of visceral pain. The expression levels of GFAP in the model group were higher than those in the control group, indicative of the activation of SGCs in DRG. The activation of SGCs following nerve injury and inflammation may trigger the enhancement of neuronal excitability, which is associated with hyperalgesia [41–43]. ATP that is released from neurons and also from SGCs may act on P2X receptors in SGCs [7, 14, 41, 42]. It is possible that following the continuous CRD stimulation, SGCs in DRG are activated by ATP. Thus, the activation of SGCs and the upregulated expression of P2X₇ receptor within the DRG could affect the transmission of nociceptive signal.

The acupoints are usually changed into the sensitizing state during disease. Dachangshu acupoint in clinical therapies was selected to treat abdominal pain, fullness or distension, rumbling intestines, borborygmi, enteritis, diarrhea, dysentery, constipation, acute or chronic lumbar pain, low back pain or sprain, pain in the sacro-iliac joint, sciatica, muscular atrophy,

pain, numbness or motor impairment of the lower limbs, and chronic disorders of the large intestine. The acupoints for HSM are the effective therapeutic targets, which are the corresponding points sensitive to moxa heat in disease condition [23, 28, 29]. HSM has been used to treat IBS and chronic visceral hyperalgesia [24, 44]. The treatment outcome of moxibustion not only is the warm effect but also contains a comprehensive result of spectral radiation, thermal biological effects, and biological non thermal effects [23, 28, 29]. The action of HSM treatment includes hanging moxibustion characterized by heat infiltration (the moxa heat transmits from the skin to the deep tissue), heat diffusion (the moxa heat transmits from the center to the surrounding area), heat transmission (the moxa heat transmits along a direction from a point), obvious heat in distant area but no or slight heat in local area, and obvious heat in deep area but no or slight heat in superficial area [23, 24, 28, 29]. Our results showed that AWR scores at 20, 40, 60, and 80 mmHg in HSM at the Dachangshu acupoint group were significantly decreased in comparison with those in the model group. The expression levels of P2X₇ mRNA and protein in HSM at the Dachangshu acupoint group were significantly decreased in comparison with those in the model group. HSM may have anti-inflammatory effects by downregulating the enhanced expression of P2X₇ receptor due to CRD stimulation. The co-expression levels of P2X₇ receptor and GFAP in HSM at the Dachangshu acupoint group were significantly lower than those in the model group. The upregulated expression of GFAP in DRG is the character of SGC activation. The expression levels of GFAP in DRG in the model rats were decreased after HSM at Dachangshu acupoint. Therefore, P2X₇ receptor plays an important role in the inflammatory and nociceptive responses. Moreover, HSM at Dachangshu acupoint could inhibit the up-expression levels of P2X₇ receptor and the activation of SGCs within the DRG, decrease neuronal excitability following visceral hyperalgesia, and increase the pain threshold in visceral hyperalgesic model due to CRD stimulation.

In conclusion, HSM at Dachangshu acupoint inhibited the nociceptive transmission by decreasing the upregulated expression levels of P2X₇ mRNA and protein in DRG induced by visceral pain and by reducing the abnormal expression of GFAP in DRG. Consequently, HSM at Dachangshu acupoint in rats of visceral pain model could reduce AWR scores and elevate their pain threshold. Therefore, HSM at Dachangshu acupoint can produce a therapeutic effect on IBS via inhibiting the nociceptive transmission mediated by upregulated P2X₇ receptor.

Acknowledgments This work was supported by the grants (Nos: 81171184, 31060139, 30860086, 30860333, 30660048, 81460200, and 81200853) from the National Natural Science Foundation of China, the grants (Nos: 2010BSA09500 and 20111BBG70009-1) from the Technology Pedestal and Society Development Project of Jiangxi Province, the grant (Nos.: 20142BAB205028 and 20142BAB215027) from the Natural Science Foundation of Jiangxi Province, the second batch of Jiangxi

province “Gan Po excellence talent 555 project”-leading talent project, the grant (No: 2009CB522902) from National Basic Research Program of China, and the grants (No: GJJ13155 and GJJ14319) from the Educational Department of Jiangxi Province. GL’s research was supported by the NMRC of Singapore (NMRC/CIRG/1334/2012).

Conflict of interest The authors declare that there are no conflict of interest.

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