

# Effects of electroacupuncture on expression of c-fos protein in the spinal dorsal horn of rats with chronic visceral hyperalgesia

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**OBJECTIVE:** Acupuncture is widely used in clinics to suppress chronic visceral pain in patients with irritable bowel syndrome (IBS); however, the exact neurobiological mechanisms for its therapeutic effects need further exploration. The aim of this study was to investigate the possible involvement of spinal neurons in the effects of electroacupuncture (EA) in relieving chronic visceral hyperalgesia in a rat model of IBS.

**METHODS:** Colon mechanical irritation was applied to male neonatal Sprague-Dawley rats to establish the IBS model. Behavioral test of the abdominal withdraw reflex (AWR) response to colorectal distention stimuli was conducted to judge the degree of colorectal sensitivity. EA at acupoints Zusanli (ST36) and Shangjuxu (ST37) was applied bilaterally in a total of four times every other day, while sham-EA at similar acupoints was done by inserting needles without electrical stimulation. Immunohistochemical methods were used to display the expression of proto-oncogene protein c-fos in the spinal dorsal horn.

**RESULTS:** It was found that AWR scores were significantly increased in the IBS model rats ( $P < 0.01$ ), accompanied with significant increase in the expression of c-fos protein in the superficial laminae (SDH, laminae I and II) and nucleus proprius (NP, laminae III and IV), the neck of the dorsal horn (NECK, laminae V and VI) at lumbosacral (L6-S2) spinal level, and in NECK at thoracolumbar (T13-L2) spinal level, when compared with normal rats ( $P < 0.05$ ). After EA treatment, AWR scores and the expression of c-fos protein in SDH, NP and NECK at similar spinal levels were significantly decreased in the IBS model rats ( $P < 0.05$ ). No such effects on either AWR scores or the expression of c-fos protein were observed in IBS model rats after sham-EA treatment.

**CONCLUSION:** The abnormally high neuronal excitability in the spinal dorsal horn may be an important reason underlying the visceral hyperalgesia in IBS model rats. EA treatment can relieve the chronic visceral hyperalgesia in IBS rats by suppressing the abnormal neuronal excitability in the spinal dorsal horn.

**KEYWORDS:** hyperalgesia; acupuncture therapy; proto-oncogene proteins c-fos; irritable bowel syndrome; rats

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Among all functional bowel disorders, irritable bowel syndrome (IBS) is the most common and prevalent gastrointestinal disorder, involving 18% to 20% of the patient population<sup>[1]</sup>. Patients classically present chronic abdominal pain associated with an alteration in bowel habits<sup>[2]</sup>. There are different factors that can cause or affect these disorders, such as persistent mental and social stress, a previous episode of infection or inflammation, genetic background, and early-life adverse events<sup>[1,3]</sup>. It is presently accepted that the majority of IBS patients exhibit enhanced sensitivity in the rectum, meaning they may develop hyperalgesia-experiencing excessive pain to painful stimuli, or allodynia-experiencing pain in response to normally nonpainful stimuli<sup>[4,5]</sup>.

Both animal and human studies of IBS clearly point to a spinal mechanism for visceral hypersensitivity involved in primary sensory afferents and spinal neurons to visceral stimuli and their sensitization<sup>[1,6,7]</sup>. However, on the basis of the evidence presented so far, it is not entirely clear to which extent these enhanced responses are the result of a facilitating mechanism confined within the brain, a spinal sensitization maintained by tonic impulse input from the rectum and colon, or a mechanism of descending facilitation from the brain to the spinal cord and gut<sup>[1]</sup>.

Our previous research has reported that there is a suppression effect of electroacupuncture (EA) on chronic visceral hyperalgesia and abnormal intestinal motility in an IBS rat model<sup>[8,9]</sup>, and possible involvement of N-methyl-D-aspartate receptor 1 (NR1) in the central nervous system underlying such an effect of EA<sup>[10,11]</sup>. Visceral neurons in the spinal cord plays a key role in the formation of central mechanisms, while proto-oncogene protein c-fos has been widely used as a marker of increased neural activity in the central nervous system<sup>[12]</sup>. Therefore in this study, c-fos was chosen to measure the specific response of neurons in the spinal dorsal horn underlying the mechanism of acupuncture relieving chronic visceral hyperalgesia.

## 1 Materials and methods

**1.1 Animals** Thirty-two male Sprague-Dawley neonatal rats (younger than 8 d) were obtained from the Experiment Animal Center, Chinese Academy of Sciences, Shanghai Branch. Rats were housed in plastic cages with one nursing adult female rat per 10 male neonates until they were 25 d old; the adult female rat had access to food and water *ad libitum*. After separation from the adult female rat, every four weaned rats were housed in one cage with access to food and water *ad libitum*. All rats in the study were used strictly in accordance with the *National Institutions of Health Guide for the Care and Use of Laboratory Animals* in order to minimize the number of animals used and their suffering.

**1.2 Production of IBS model** IBS model rats were produced as described in our previous reports<sup>[9,11]</sup>. Daily mechanical colon distention was performed on neonatal rats ( $n = 24$ ) from 9 to 22 d after birth. The distention was applied using silica gel balloons (20.0 mm in length; 2.0 mm in diameter) inserted into the descending colon through the rectum of conscious rats. The balloon was distended with 0.35 mL of air for 1 min and then deflated and withdrawn. The distention was repeated twice a day at 30 min intervals. After cessation of the distention stimulus, the rats were kept in cages until they reached adulthood (at least 6 weeks old), then measurements of visceral pain were conducted using behavioral tests. Eight neonatal rats were raised simultaneously with the IBS model rats; their perianal skin was gently kneaded by balloons as the control procedure.

**1.3 Assessment of abdominal withdrawal reflex** Behavioral responses to colorectal distention (CRD) were assessed in normal control group and IBS model rats over 6 weeks old by observing the abdominal withdrawal reflex (AWR). Semiquantitative scores were used for judgment of the responses to CRD stimulus. A balloon (made from condoms, 20.0 mm



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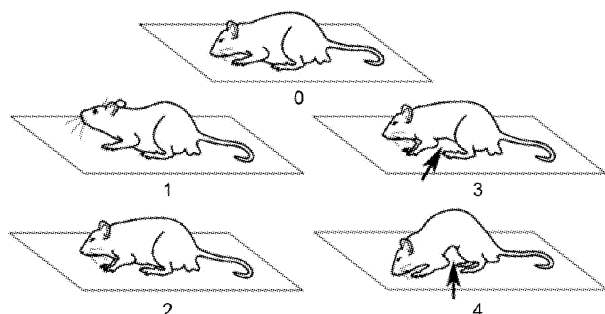
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in length and 33.0 mm in diameter) was inserted with depth of 6 cm from the anus into the descending colon and lightly tied to the root of rat tail to prevent it from dislodging. The rat was then placed in a small cubicle (20 cm×8 cm×8 cm) on a platform and allowed to adapt for 20 min. AWR in response to CRD at strengths of 20, 40, 60, and 80 mmHg was measured. AWR scores were assigned according to the scale of Al-Chaer *et al*<sup>[13]</sup> and our improved method<sup>[9,11]</sup>, whereby 0 indicates no behavioral response to CRD; 1 indicates an immobility response during the CRD at the onset of the stimulus; 2 indicates a mild contraction of the abdominal muscles without lifting the abdomen off the platform; 3 indicates a strong contraction of the abdominal muscles and lifting the abdomen off the platform, but without lifting the pelvic structure off the platform; and 4 indicates body arching and lifting the pelvic structure and scrotum off the platform. See Figure 1.

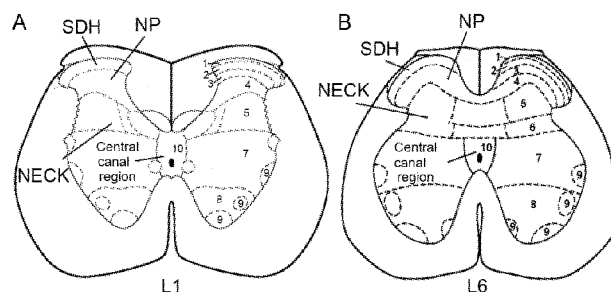


**Figure 1** Schematic diagram for measuring AWR scores in rats  
AWR responses to graded CRD of 20, 40, 60 and 80 mmHg were visually observed and were divided into 5 scores: 0, no behavioral response to CRD; 1, an immobility response during the CRD at the onset of the stimulus; 2, a mild contraction 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, no lifting the pelvic structure off the platform; 4, body arching and lifting the pelvic structure and scrotum. AWR: abdominal withdrawal reflex; CRD: colorectal distension.

**1.4 Administration of EA treatment** All rats were divided into four groups with eight in each, namely, normal control rats without any treatment; IBS model rats without any treatment; IBS model rats with EA treatment; and IBS model rats with sham-EA treatment. The rats were slightly restrained in a box and their hind feet were exposed bilaterally for EA treatment through small holes in the bottom of the box. No anesthetics were applied during the EA treatment. EA was applied by two pairs of stainless steel needles (0.25 mm in diameter) inserted bilaterally at a depth of 5 mm into two acupoints, Zusanli (ST36, 5 mm lateral to the anterior tubercle of the tibia and 10 mm below the knee joint) and Shangjuxu (ST37, 5 mm lateral to the anterior tubercle of the tibia and 15 mm below the knee joint) of each hind limb. Each pair of needles (one in ST36 and the other in ST37) was connected with the output terminals of an EA apparatus (Model SDZ-IV, Suzhou Medical Appliance Factory,

China). Alternating trains of dense-sparse frequencies (5 to 100 Hz, sparse wave time was 5 s, and dense wave time was 10 s alternately) were selected. The intensity of stimulation was adjusted to induce mild shaking in the rats' hind limbs. EA treatments were administered to the IBS model rats for 30 min every other day, with a total of four EA treatments on days 1, 3, 5 and 7. The IBS model rats with sham-EA treatment had needles inserted bilaterally into the same acupoints, then the needles were retained for 30 min without receiving electrical stimulation. As discussed in our previous findings<sup>[9]</sup>, continued EA treatments gradually enhanced to its maximum within 8 to 12 d, then AWR assessment was performed once again in all four groups during 24 h after EA or sham-EA treatment.

**1.5 Tissue preparation** After 5 to 8 h of the CRD stimulation following EA treatment, distended rats were deeply anesthetized with pentobarbital and intracardially perfused with 350 mL of saline followed by 400 mL of 4% paraformaldehyde in 0.1 mol/L of phosphate buffer saline (PBS) at 4 °C. Rat spinal cords were sliced at thoracolumbar (T13-L2) and lumbosacral (L6-S2) segments<sup>[14]</sup>, and the postfixed slices were placed in 4% paraformaldehyde overnight, and subsequently allowed to equilibrate in 30% sucrose with PBS for 48 h. Transverse sections of 30 μm were sliced on a cryostat. See Figure 2.



**Figure 2** The spinal dorsal horn sliced at the level of T13-L2 and L6-S2 segments

A: The sample of transversal photographs at the L1 level; B: The sample of transversal photographs at the L6 level. SDH: superficial laminae (laminae I and II); NP: nucleus proprius (laminae III and IV); NECK: neck of the dorsal horn (laminae V and VI); 1-10: spinal laminae I - X.

**1.6 Immunohistochemistry of c-fos** The spinal cord sections were stained to visualize the c-fos by the streptavidin-biotin complex (SABC) method. Free floating sections were treated in methanol containing 3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity for 10 min at approximately 26 °C. Then sections were blocked with 10% normal goat serum in PBS for 30 min at room temperature, followed by incubation with rabbit polyclonal antibody solutions against c-fos (1 : 400 in PBS containing 0.2% Triton X-100, Wuhan Boster Bio-Engineering Limited Company, China) for 48 h at 4 °C. Following primary antibody incubation, the sections were incubated for 30 min with a biotinylated secondary

antibody (biotin-goat anti-rabbit IgG, 1 : 100 in PBS; Wuhan Boster Bio-Engineering Limited Company, China) at room temperature. Following incubation in a solution containing avidin-biotin complex (SABC kit, Wuhan Boster Bio-Engineering Limited Company, China) for 30 min at room temperature and subsequent reaction with diaminobenzidine for 4 min, sections were mounted on gelatin-coated slides, dehydrated in a series of graded alcohol and coverslipped. The sections were rinsed in 0.01 mol/L of PBS for 3×5 min between transitions of these steps. To measure the levels of intensity of c-fos immunoreactivity, five slices for each rat were selected for the count of c-fos positive neurons on images at ×100 magnifications.

**1.7 Statistical analysis** Data were presented as mean±standard error of mean and analyzed by using a statistical software (SPSS, version 13.0). Data were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni's test among multiple groups.  $P<0.05$  implied statistical significance.

2 Results

**2.1 AWR scores in IBS rats** AWR scores in the IBS model group were significantly increased with

the graded CRD stimulation, when compared with those of the normal control rats ( $P<0.01$ ). AWR scores were significantly decreased after four applications of EA treatment at bilateral ST36 and ST37 acupoints in the IBS model group rats ( $P<0.05$ ). There was no significant difference in the AWR scores of the IBS rats after sham-EA treatment. See Table 1.

**2.2 Immunohistochemistry analysis of c-fos protein in NECK of thoracolumbar segments of IBS rats** The sample images of immunoreactive neurons of c-fos protein in SDH, NP and NECK of thoracolumbar (T13-L2) segments in each group are seen in Figures 3A to 3D. The number of c-fos immunoreactive neurons of IBS model rats was significantly higher than that of the control rats in the NECK of T13-L2 segments ( $P<0.05$ ), and it decreased significantly after four EA treatments ( $P<0.05$ ). There were no significant differences observed in the SDH, NP and central canal region of thoracolumbar segments in IBS rats after EA and Sham-EA treatments (Table 2).

**2.3 Immunohistochemistry analysis of c-fos protein in lumbosacral segments of IBS rats** Figures 4A to 4D show the sample images of c-fos immunoreactive neurons in SDH, NP and NECK of lumbosacral

Table 1 AWR scores in four experimental group rats

(Mean±standard error of mean)					
Group	<i>n</i>	20 mmHg	40 mmHg	60 mmHg	80 mmHg
Normal control	8	0.35±0.10	1.38±0.09	2.04±0.09	2.67±0.07
IBS model	8	1.00±0.09**	2.19±0.07**	2.90±0.08**	3.67±0.12**
EA treatment	8	0.29±0.06△	1.39±0.05△	2.32±0.07△	3.01±0.07△
Sham-EA treatment	8	0.77±0.05	1.91±0.07	2.79±0.07	3.60±0.08

\*\*  $P<0.01$ , vs normal control group; △  $P<0.05$ , vs IBS model group. IBS: irritable bowel syndrome; EA: electroacupuncture; AWR: abdominal withdraw reflex.

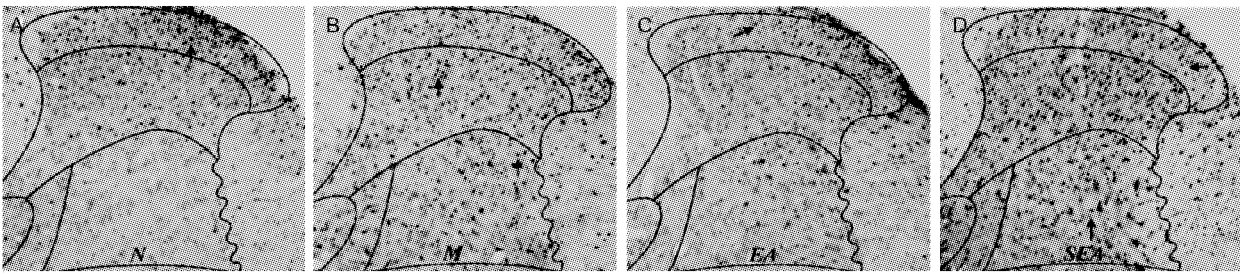


Figure 3 Representative microphotographs of c-fos protein immunoreactive neurons in thoracolumbar segments (Light microscopy, ×100)

N: Normal control group; M: IBS model control group; EA: IBS model rats after EA treatment; SEA: IBS model rats after sham-EA treatment; IBS: irritable bowel syndrome; EA: electroacupuncture.

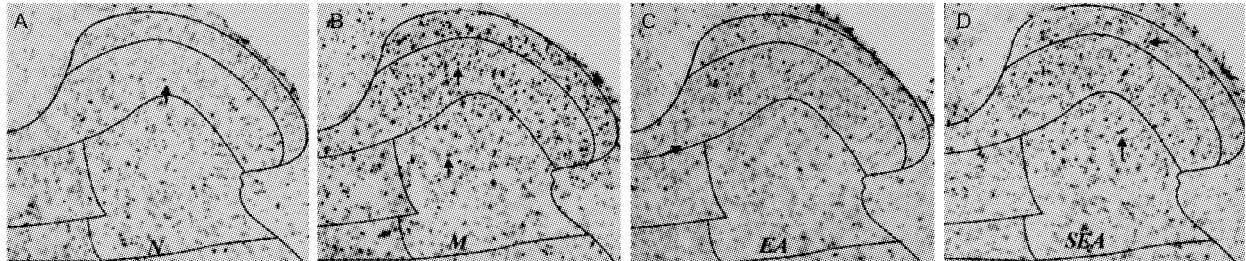
Table 2 Number of positive immunoreactive neurons of c-fos protein in the thoracolumbar (T13-L2) segments

(Mean±standard error of mean)					
Group	<i>n</i>	SDH	NP	NECK	Central canal region
Normal control	8	29.67±1.30	18.94±1.39	10.16±0.95	18.73±1.25
IBS model	8	28.36±1.14	22.10±1.02	14.30±0.80*	18.60±0.87
EA treatment	8	27.09±1.42	19.23±0.94	9.74±0.74△	16.04±0.88
Sham-EA treatment	8	27.84±1.03	22.66±1.14	10.83±0.73	15.37±0.88

\*  $P<0.05$ , vs normal group; △  $P<0.05$ , vs IBS model control. EA: electroacupuncture; IBS: irritable bowel syndrome; SDH: superficial laminae (laminae I and II); NP: nucleus proprius (laminae III and IV); NECK: the neck of the dorsal horn (laminae V and VI); central canal region: lamina X.

(L6-S2) segments in each group and Table 3 summarizes the data obtained from those images in each group, respectively. The number of c-fos immunoreactive neurons of IBS model rats significantly increased as compared to those of the normal rats in the SDH, NP and NECK of L6-S2 segments ( $P <$

0.05), and it significantly decreased after four EA treatments ( $P < 0.05$ ). Similarly to the result of behavioral test, there was no inhibitory effect on the hyperexpression of c-fos protein after sham-EA treatment in each region of lumbosacral segments.



**Figure 4** Representative microphotographs of c-fos protein immunoreactive neurons in lumbosacral segments (Light microscopy,  $\times 100$ )

N: Normal control group; M: IBS model control group; EA: IBS model rats after EA treatment; SEA: IBS model rats after sham-EA treatment; IBS: irritable bowel syndrome; EA: electroacupuncture.

**Table 3** Number of positive immunoreactive neurons of c-fos protein in the lumbosacral (L6-S2) segments

(Mean  $\pm$  standard error of mean)

Group	<i>n</i>	SDH	NP	NECK	Central canal region
Normal control	8	19.37 $\pm$ 0.71	19.39 $\pm$ 0.68	18.25 $\pm$ 0.72	9.09 $\pm$ 0.45
IBS model	8	30.77 $\pm$ 1.29 *	35.38 $\pm$ 1.39 *	24.87 $\pm$ 1.03 *	11.36 $\pm$ 1.0 *
EA treatment	8	22.33 $\pm$ 0.85 $\Delta$	21.48 $\pm$ 0.82 $\Delta$	15.94 $\pm$ 0.88 $\Delta$	7.32 $\pm$ 0.45 $\Delta$
Sham-EA treatment	8	27.89 $\pm$ 1.04	28.85 $\pm$ 1.16	23.05 $\pm$ 1.14	10.58 $\pm$ 0.67

\*  $P < 0.05$ , *vs* normal control;  $\Delta$   $P < 0.05$ , *vs* IBS model control. EA: electroacupuncture; IBS: irritable bowel syndrome; SDH: superficial laminae (laminae I and II); NP: nucleus proprius (laminae III and IV); NECK: the neck of the dorsal horn (laminae V and VI); central canal region: lamina X.

### 3 Discussion

The descending colon of the rat is innervated by sensory afferent fibers in the pelvic nerve projecting to the lumbosacral (L6-S2) spinal cord, and hypogastric and lumbar colonic nerves projecting to the thoracolumbar (T13-L2) spinal cord<sup>[15-17]</sup>. The majority of colonic afferents in the rat pelvic nerve *in vivo* are lower threshold fibers as compared to splanchnic nerve afferents<sup>[18-21]</sup>. Reports show that, in rats, lesioning of the hypogastric and lumbar colonic nerves do not affect the avoidance behavior to noxious CRD<sup>[22]</sup>. Conversely, dorsal rhizotomy at the spinal level of L5-S3 eliminates the visceromotor response<sup>[23]</sup>. These data suggest that acute colorectal pain, such as pain elicited by CRD stimuli, is mainly processed in the lumbosacral spinal cord.

Noxious stimulation of hollow viscera induces a specific pattern of c-fos expression in the rat spinal cord that reflects the intensity of the stimulation<sup>[24]</sup>. In the rat, repetitive noxious CRD increases fos-labeled cells in the lumbosacral segments, but few in the thoracolumbar segments, further suggesting that transient colonic nociceptive input is transduced primarily in the lumbosacral spinal cord<sup>[23]</sup>. Inflammation of the colon prior to CRD induces c-fos expression in neurons in the thoracolumbar spinal cord segments and the lumbosacral spinal cord segments<sup>[25]</sup>. The results in this study has

demonstrated that the expression of c-fos protein in the SDH, NP and NECK of L6-S2 spinal cord segments is significantly increased after CRD stimulation. There is a slightly increase of the expression of c-fos protein in the NECK of T13-L2 spinal cord segments in IBS rats. These findings may imply that acute colorectal nociceptive information in IBS model rats is mainly processed in the spinal cord at L6-S2 level, and abnormally high neuronal excitability in the lumbosacral segments may be an important reason underlying visceral hyperalgesia.

It is generally accepted that multiple supraspinal sites of the descending pain modulatory system exert powerful effects on the inhibitory response of acupuncture to the visceral nociceptive messages at the spinal level<sup>[26-28]</sup>. Acupuncture can significantly inhibit the noxious visceral response at the spinal level in intact rats, but not in spinalized rats, suggesting that supraspinal mechanisms are important in mediating the nociceptive inhibition produced by acupuncture<sup>[27]</sup>. Recent studies have suggested that EA-activated spinal neurons convey acupuncture signals to the brain and activate a descending inhibitory system, which in turn inhibits the c-fos, p38, 5-hydroxytryptamine and NR1 expression in the spinal cord, thus inhibiting hyperalgesia<sup>[11,26,29,30]</sup>. In this study, behavioral tests and immunohistochemical examinations showed that EA treatment decreased rectal hyperalgesia in the rat, as well as the c-fos

expression in L6-S2 spinal segments and NECK in T13-L2 spinal segments.

In conclusion, abnormally high neuronal excitability in the lumbosacral segments may be an important reason underlying visceral hyperalgesia in IBS model rats. EA treatment can relieve chronic visceral hyperalgesia, and such an effect may be correlated with its suppression of abnormal neuronal excitability in the spinal dorsal horn of the IBS rats.

#### 4 Competing interests

The authors declare that they have no competing interests.

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## 电针对慢性内脏痛敏大鼠脊髓背角 c-fos 蛋白表达的影响

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**目的:** 针刺治疗对肠易激综合征(irritable bowel syndrome, IBS)患者的慢性腹痛具有良好疗效,然而,其神经生物学机制仍不清楚。本研究在电针缓解 IBS 模型大鼠慢性内脏痛敏基础上,观察和分析 IBS 大鼠脊髓背角中内脏反应神经元的兴奋性在针刺治疗前后的改变,探讨针刺缓解 IBS 模型大鼠慢性内脏痛敏的机制。

**方法:** 采用新生 9 d 的 Sprague-Dawley 雄性幼鼠,通过结直肠机械扩张刺激制作 IBS 慢性内脏痛敏模型。饲养至 6~8 周后,观察大鼠由结直肠扩张诱发的腹部撤回反射(abdominal withdraw reflex, AWR)评分变化。电针组穴位取双侧“足三里”和“上巨虚”,连续隔日治疗 4 次,假电针组不通电,其余与电针组同。治疗结束后,取材并用免疫组化方法观察各组大鼠脊髓背角中 c-fos 蛋白表达的变化。

**结果:** 与正常大鼠比较,IBS 模型大鼠 AWR 评分明显升高( $P < 0.01$ ),电针治疗后 AWR 评分明显降低( $P < 0.05$ );与正常大鼠相比,IBS 模型大鼠脊髓 L6-S2 节段浅层(superficial laminae, SDH, laminae I and II)、固有层(nucleus proprius, NP, laminae III and IV)、背角颈段(neck of dorsal horn, NECK, laminae V and VI),以及 T13-L2 节段的 NECK 中,c-fos 阳性神经元明显高于正常对照组( $P < 0.05$ ),而电针治疗后 IBS 大鼠脊髓 L6-S2 节段 SDH、NP、NECK 以及 T13-L2 节段的 NECK 亚区中,c-fos 阳性神经元明显下降( $P < 0.05$ );假电针对 AWR 评分、c-fos 蛋白表达没有影响。

**结论:** 电针可以明显抑制 IBS 模型大鼠脊髓背角中内脏反应神经元异常增高的兴奋性,这可能是针刺缓解慢性内脏痛敏的机制之一。

**关键词:** 痛敏; 针刺疗法; c-fos 蛋白; 肠易激综合征; 大鼠