

Regulatory Mechanism of Electroacupuncture in Irritable Bowel Syndrome: Preventing MC Activation and Decreasing SP VIP Secretion

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Abstract The efficacy of electroacupuncture (EA) for treating patients with diarrhea-predominant IBS has been confirmed in the authors' former research, but the regulatory mechanism of EA in IBS is still unknown. The aim of this study was to explore the relationship between the effect of EA on treating IBS rats and the activation and proliferation of mast cell (MC), the secretion of substance P (SP), and vasoactive intestinal polypeptide (VIP). The IBS rat model was set up with stress of binding limbs and colorectal distention. All rats were randomly assigned to four groups (Normal, Model, Tegaserod and EA). Hematoxylin and eosin staining has been used to observe the pathological change in the rats' colonic mucosa and an AWR scoring system has been applied to evaluate improvement of visceral hypersensitivity in various methods of the different groups. Toluidine blue improved method (TBI) and immunohistochemistry have also been involved in observations of mucous mast cells in the colon, change of c-fos positive cells, and secretion of SP, SPR, VIP, VIPR in the local colon. Firstly, the threshold of visceral sensitivity in the rats model with IBS was remarkably reduced ($P < 0.01$). The MC count in colonic mucosa and c-fos positive cells count increased significantly ($P < 0.01$) with positive correlation within each. Secondly, EA on ST-25 and Tegaserod pouring into the stomach can inhibit the proliferation and activation of MC in the colon and regulate secretion of SP, SPR, VIP, VIPR

($P < 0.01$, $P < 0.05$), while the effect of EA is obviously superior to Tegaserod. We concluded, firstly, that the abnormal proliferation and activation of mucous mast cells in the colon, and oversecretion of neuropeptides such as SP, VIP and their receptors could be one of key mechanisms of etiology of IBS. Secondly, the inhibition of activation and proliferation and the secretion of SP, VIP could be major effects of EA when treating rats with IBS.

Keywords Irritable bowel disease · Mucosal mast cell · Electroacupuncture therapy · Regulatory mechanism · Substance P Vasoactive intestinal polypeptide

Abbreviations

MC	Mast Cell
IBS	Irritable bowel syndrome
TBI	Toluidine blue improved method
CRD	Colorectal distention
SP	Substance P
VIP	Vasoactive intestinal polypeptide
EA	Electroacupuncture
MMC	Mucous mast cell

Introduction

Irritable bowel syndrome (IBS) is a common chronic gastrointestinal dysfunctional disease, which manifests as intermittent or constant abnormal pain or discomfort, and altered bowel habit and form, which makes treatment difficult. The authors' formal research shows that the general therapeutic effective rates of EA on ST-25 to treat IBS is 84.90% (Project of State Administration of TCM of PRC

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‘Clinical research on diarrhea predominant IBS by EA on ST-25’ No. 03XDLZ26, finished). However, the regulatory mechanism of EA on IBS is still unknown.

IBS results from visceral hypersensitivity with anomalies of the digestive motility. These anomalies are secondary effects of dysfunction of the brain–gut axis modulated by environmental and the psychosocial factors [1]. Moreover, the heightened response to the stressors and the greater epithelial damage in irritable bowel disease patients suggests that stress-induced activation of the brain–gut axis and of mucosal mast cells (MC) is important in the initiation and/or flare up of irritable bowel disease [2]. And the MC of the ileocecal junction maybe a mediator between the gut and the nervous system in IBS [3]. Perhaps activation of MC is the bridge between the neuro-immunological net and brain–gut axis, abducting infection, food allergy, stress, etc. c-Fos is an immediate reactive protein in the process of genetic transcription of MC, and is a mark of MC activation [4, 5]. P-substance (SP) and vaso-inhibitory peptide (VIP) are gastrointestinal peptide hormones. Both reside in the gastrointestinal tract and central nervous system. Also, SP [6] and VIP [7] are interactive signaling molecules between the nervous system and the immune system. SP and VIP could regulate the function of intestinal mucosal mast cells by regulating neurosecretion and paracrine secretion. So the MC count, c-fos positive cells count, and the content of SP, SPR, VIP, VIPR, would be observed in this research.

The IBS rat model with stress of binding limbs and colorectal distention (CRD) had been set up in this research. This model is regarded as an IBS model with visceral hypersensitivity. Antagonism of 5-HT₄ receptors mediates antinociception in enteric viscera [8]. the pain threshold would obviously be heightened after taking 5-HT₄ receptor antagonist [9]. So the effect of EA and Tegaserod (5-HT₄ receptor antagonist) would be compared in this IBS model.

Methods

Forty-eight male Sprague–Dawley rats, SPF class, weighing from 175 g to 225 g, supplied by the experimental animal center in the Shanghai University of TCM, were randomized into four groups (Normal, Model, Tegaserod and EA on ST-25) according to their weights.

Establishment of IBS rats model

On the second day after being fasted, the experiment began. In the normal group, grabbing around the anus was treated, while the others were tied to get stimulations of CRD. Limbs

were tied with medical plaster so that their movements were limited, but the tied rats could crawl without using their rear limbs. Polyethylene tubes had four holes bilaterally 0.5 cm apart, while the finger of disposable rubber glove was tied tightly onto the end of tube with medical silk thread to be a 4-cm-long balloon. The other end of the tube was connected with a rubber tube, 10 cm in length. The rubber tube was also connected to a tri-channel valve, which was connected to a syringe and sphygmomanometer, respectively. The visceral stimulus employed was distension of the descending colon by inflation of a 5-cm-long latex balloon inserted anally and kept in place by taping the polyethylene tube holding the balloon to the base of the tail, such that the tip of the balloon remained 10 cm from the anal verge. The tied period lasted 2 h per day. The CRD was stimulated once every other day, 8 days in total. The whole modeling period lasted 15 days, and three rats died after that.

Group and treatment

EA group (n = 12)

EA on ST-25 bilaterally (ST-25 was located according to Lin WZh, *Experimental theory of acupuncture*, Technical press of Shanghai, 1994), pricked 0.3 cm deep, sparse and intense waveform with frequency of 2/50 Hz, 20 min, once per day, 7 days in a row.

Tegaserod group (n = 12)

Tegaserod solution was used with a drencher. The dosage was according to the scale of 1:0.018, which is the ratio between 70 kg adult human and 200 g rat (Experimental method of pharmacology, YaoLiXue ShiYan FangFa), once per day, 2 ml per rat, 7 days in a row.

Normal group (n = 12)

No treatment, but with the same fixation as the two groups above.

Model group (n = 12)

No treatment, but with the same fixation as the first two groups.

Contraction reaction of rat abnormal scoring test

The model group was fasted in the afternoon of the previous day. Vaseline was smeared on the surface of the

balloon and the balloon inserted into the rats' anus slowly according to the physical curve of the colonrectum until 5 cm deep, and maintained for 5 min. The test began when the rats adapted.

Abdominal withdrawal reflex (AWR) [10] scoring criteria

1. 0 score: no behavioral response to CRD
2. 1 score: immobile during the CRD and occasionally clicked the head at the onset of the stimulus;
3. 2 score: a mild contraction of the abdominal muscles, but no lifting the abdomen off the platform;
4. 3 score: a strong contraction of the abdominal muscles and lifting the abdomen off the platform, no lifting the pelvic structure off the platform;
5. 3 score: body arching and lifting the pelvic structure and scrotum.

With the addition of air into the balloon with the syringe, the stimulation of the rats' rectum in different degrees of contraction reaction were observed. The pressure value (mmHg) during the behavior response of 1, 2, 3, 4 score of rats was recorded, and named as the threshold of sensitivity. It took 20 s to observe constantly, each score was tested three times, and each rat was tested by two different people who were not involved in this research. Means were taken (six values in total). There were 3 min intervals between each two tests, in order to allow enough time for the rats to adapt.

Staining process of HE and toluidine blue improved method (TBI), observation

Making the sample of tissue fixation, dehydration, embedding, section, and staining

Samples were deparaffinized in xylene, hematoxylin and eosin staining, dehydrated in 95%, 90%, 70% ethanol, cleared in xylene, mounted in Permount or Histoclad, checked under 100× and 400× microscope (Olympus-BH2) to observe morphological change of rats' colonic membrane.

A 1-cm sample was selected in the descending colon (5 cm above anus) and cecum (junctional ileac and cecum), and cleaned with normal saline, fixed with 10% in formalin, dehydrated, paraffin embedded, continuously slided, toluidine blue improved method stained, deparaffinized and rehydrated, dipped in toluidine blue for 30 min, 2–3 drops of glacial acetic acid, until the nucleus and granulation were pretty clear, dried with cold air, cleared in xylene,

mounted in Permount or Histoclad, checked under 100× and 400× microscope (Olympus-BH2), randomly selected three high-power fields (400×) and counted the number of cells, expressing MC counts with means.

c-fos positive cells in colon, SP/SPR, VIP/VIPR immunohistochemistry test

Paraffin slides were deparaffinized and rehydrated, deparaffinized in xylene I, II, III for 10 min, dehydrated in 95%, 90%, 70% ethanol for 2 min, then, so that the primary antibody bound to the specific antigen (rabbit anti-rat, with concentration 1:150), 4°C for 18 h. The envision immunohistochemistry method was used to stain the samples, controlled by a known positive sample as positive control, while PBS alternated primary antibody as a negative control. Positive results showed brown and dark brown, shaped like granulation, with a background of purple blue. The positive expressing areas of SP and VIP were averaged by picking three fields.

Results

Contraction reaction of rat abnormal scoring test

The threshold pressure level in the model group is remarkably lower than that in the normal group (Table 1), and the threshold pressure level in the EA group is obviously higher than that in the model group ($P < 0.01$, $P < 0.05$). Between the Tegaserod and model groups, there is no significant difference in this indication, while at more than 3 score level, there is a significant difference between the EA and Tegaserod groups ($P < 0.01$), and the level of the Tegaserod group is lower than the EA group (see Fig. 1).

Comparison between MC counts and c-fos positive cell numbers in colonic membrane of rats

MC counts and c-fos positive cell numbers in the model group are higher than those in the normal group ($P < 0.01$) (Table 2). MC counts and c-fos positive cell numbers in the EA group are far lower than those in the model group ($P < 0.01$, $P < 0.05$), while they are obviously higher than in the normal group. In the Tegaserod group, MC counts are obviously lower than in the model group ($P < 0.01$). MC counts and c-fos positive cell numbers in the EA group are remarkably lower than those in the Tegaserod group ($P < 0.01$, $P < 0.05$) (see Fig. 2).

Table 1 Threshold pressure level of rat contraction reaction in each group

Group	Score			
	1 (mmHg)	2 (mmHg)	3 (mmHg)	4 (mmHg)
Normal group (10)	23.0 ± 3.0	38.6 ± 7.5	62.8 ± 8.7	83.3 ± 8.4
Model group (10)	16.2 ± 2.9 [#]	27.6 ± 6.1 [#]	37.9 ± 6.5 [#]	58.7 ± 6.9 [#]
Tegaserod group (10)	17.1 ± 2.6	28.9 ± 6.9	41.6 ± 6.2	62.5 ± 8.0
EA group (10)	19.4 ± 1.8 [*]	32.4 ± 5.0	52.0 ± 6.7 ^{*†}	67.0 ± 9.3 ^{**}

Compared with normal group: [#] $P < 0.01$

Compared with model group: ^{*} $P < 0.01$, ^{**} $P < 0.05$

Compared with Tegaserod group: [†] $P < 0.01$

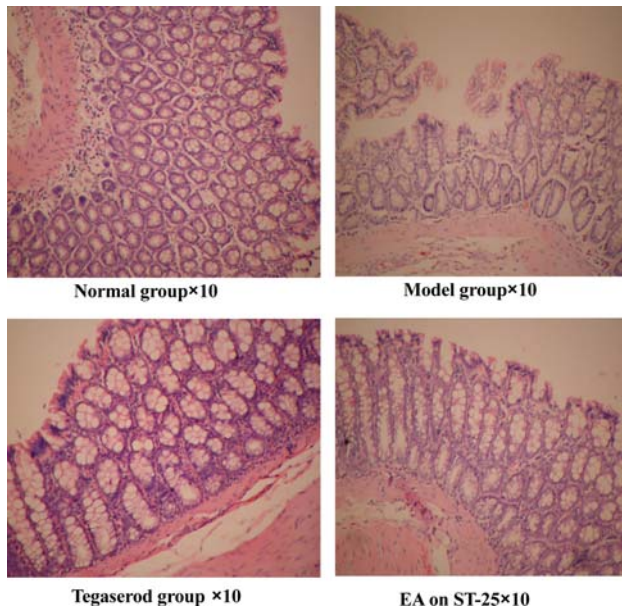


Fig. 1 Results of HE stain in rat colonic tissue. (a) Normal group ($\times 10$): colonic membrane structure is complete without epithelial coming off, trim glands, a few RBC appearing in mucous layer. (b) Model group ($\times 10$): mucous epithelium has been destroyed slightly, trim glands, with a few mucous membrane and glands coming off, and a few RBC and inflammatory cells appearing in mucous layer. (c) Tegaserod group ($\times 10$): complete mucous epithelium, trim glands, a few RBC and inflammatory cells. (d) EA on ST-25 ($\times 10$): complete mucous epithelium, trim glands, a few RBC in mucous and submucous layers

SP expression in mucous layer and SPR expression in submucous layer in rats' colon

The expressing areas of SP and SPR in colonic tissue in the model group are higher than in the normal group ($P < 0.01$) (Table 3). The expressing areas of SP and SPR in the Tegaserod and EA groups are remarkably lower than in the normal group ($P < 0.01$), while the expressing area of SP in the Tegaserod and EA groups is obviously higher than in the normal group. There is no significant difference between the two treatment groups and the normal group, while the expressing areas of SP and SPR are obviously lower than in the normal group ($P < 0.01$) (see Fig. 3).

Results of VIP and VIPR expression in rats colonic tissue

The expressing areas of VIP and VIPR in the model group are obviously higher than in the normal group ($P < 0.01$) (Table 4). The expressing areas of VIP and VIPR in the Tegaserod group and EA on ST-25 are remarkably lower than those in the model group ($P < 0.01$, $P < 0.05$), but expressing area of VIP in the two groups is much higher than that in the normal group ($P < 0.01$), while the expressing area of VIPR is not significantly different between the EA on ST-25 group and the normal group. The expressing areas of VIP and VIPR in the EA on ST-25 group are obviously lower than those in the Tegaserod group ($P < 0.01$) (see Fig. 4).

Discussion

IBS is one of the most common gastrointestinal disorders seen in primary care and specialist practices. The primary symptoms of IBS are chronic recurrent abdominal pain and alterations in bowel function. Such alterations can present as diarrhoea or constipation, or can alternate between the two (referred to as “alternating IBS” in this paper). IBS has a negative impact on patients' daily activities and quality of life, and incurs substantial health-care costs. Since the cause and mechanism are still unclear, the treatment focuses on the expected one, even though the therapeutic effect is not satisfied. Therefore, for further research on causes, mechanisms, and better drug treatment, an objective, justified, repeatable animal model of IBS should be set up. In this research, the most common double model of IBS has been adopted, which is binding limbs [11] and colorectal distention (CRD) [10]. With local distention stimulation of colorectal as unconditional stimulation, and with binding limbs to set up psychological stimulation as conditional stimulation, physical and psychological stimulation are set up together to replicate the pathology of gastrointestinal functional disorders and visceral hypersensitivity.

Table 2 MC counts and c-fos positive cell numbers in colonic membrane of rats in each group ($\bar{X} \pm S$)

Group	<i>n</i>	MC counts in visual field (per field)	c-fos positive cell numbers (per field)
Normal group	8	2.21 ± 0.31	1.75 ± 0.35
Model group	8	10.00 ± 1.21 [#]	5.46 ± 1.36 [#]
Tegaserod group	8	7.17 ± 1.13 ^{#,*}	4.75 ± 0.61 [#]
EA on ST-25	8	5.67 ± 0.62 ^{#,*†}	3.46 ± 0.83 ^{#,**‡}

Compared with normal group: [#] $P < 0.01$

Compared with model group: * $P < 0.01$, ** $P < 0.05$

Compared with Tegaserod group: † $P < 0.01$, ‡ $P < 0.05$

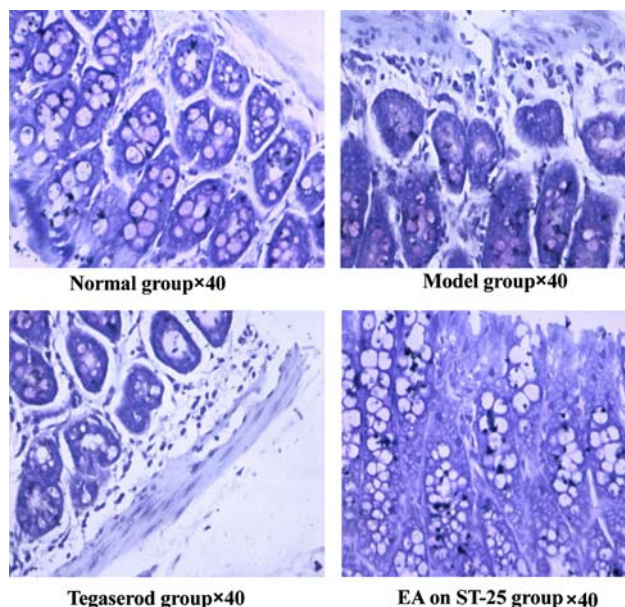


Fig. 2 Results of MC toluidine blue improved method in rat colonic membrane. MC plasma stains into purple, and nucleus shows dark blue, scattering in mucous and submucous layers, or gathering into group, or lining up; cell shape appears round, oval, shuttle-like, erose; small cell with few plasma, clear shape, big cell with more plasma, unclear shape. (a) Normal group ($\times 40$), (b) model group ($\times 40$), (c) Tegaserod group ($\times 40$), (d) EA on ST-25 group ($\times 40$)

In the valuation of the model, anaesthesia is not chosen, and instead colorectal distention stimulator has been adopted, and the distention stress processes as the pace from lower to higher. To avoid the effect to rats' visceral hypersensitivity to anaesthesia, or individually different results of the CRD test in rats, the AWR scoring system has

been applied to objectively evaluate improvement of visceral hypersensitivity. At the same time, during the evaluation process, there are two people, who are not the researchers, in the experiment to duplicate the observations, which is the best way to reduce and difference in results due to subjective causes.

The threshold of visceral sensitivity in the rat model with IBS has been remarkably reduced, compared with the normal group ($P < 0.01$). It shows that after binding limbs and colorectal stimulation, the visceral sensitivity obviously increased with abdominal pain and hypersensitivity. The threshold of visceral sensitivity in the EA group improved remarkably, compared with the model group ($P < 0.05$), while the Tegaserod and model groups showed no significant differences in various score levels of sensitivity thresholds. There was a significant difference between the EA and Tegaserod groups at the 3 score level ($P < 0.01$), while the threshold in the Tegaserod group was lower than in the EA group. EA on ST-25 could regulate visceral hypersensitivity of IBS model rats, while the therapeutic effect of Tegaserod was not as remarkable as EA.

The regulation of EA on mast cells in IBS model rats

The IBS patients may show intolerance to various things, e.g., the symptoms can be evoked or aggravated by items in their diet, and this phenomena hints that the immunosystem may be associated with IBS. Formal research shows mucous mast cells (MMC) have a considerable role in pathogenesis of IBS. Researchers, including Weston, Yang, Li, et al., have identified that MC increases in the ileum

Table 3 SP, SPR expression levels in rats' colonic membrane in each group ($\bar{X} \pm S$)

Group	<i>n</i>	Expressing area of SP (μm^2)	Expressing area SPR (μm^2)
Normal group	10	1,739.19 ± 161.46	359.87 ± 35.51
Normal group	10	3,201.44 ± 279.25 [#]	533.51 ± 92.29 [#]
Tegaserod group	10	2,788.11 ± 186.31 ^{#,*}	411.89 ± 85.42 [*]
EA group	10	2,224.43 ± 186.15 ^{#,*†}	365.21 ± 52.12 ^{*†}

Compared with normal group [#] $P < 0.01$, compared with model group * $P < 0.01$, compared with Tegaserod group † $P < 0.01$

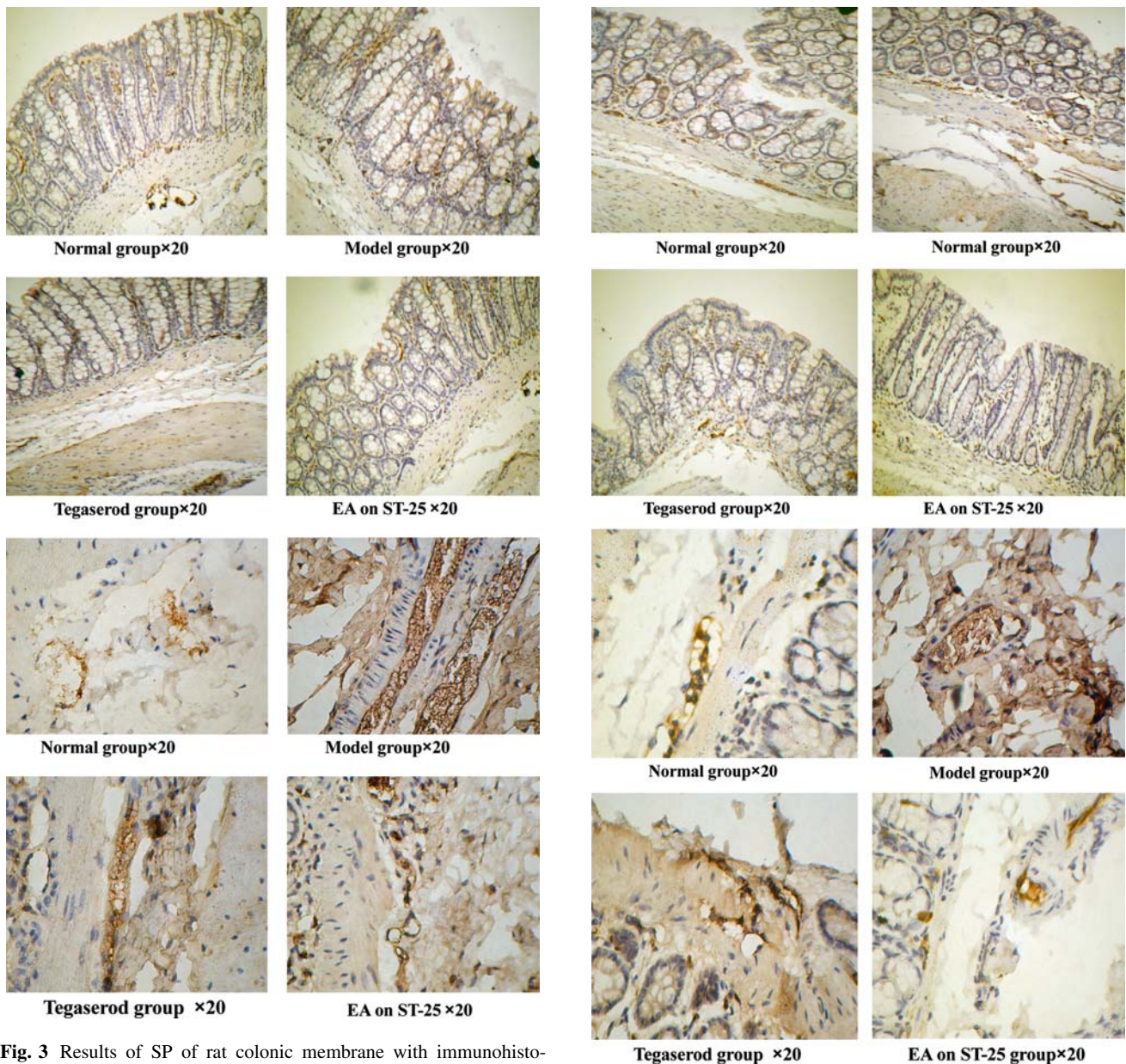


Fig. 3 Results of SP of rat colonic membrane with immunohistochemistry method: (a) normal group (×20), (b) model group (×20), (c) Tegaserod group (×20), (d) EA on ST-25 (×20). Result of SPR in rats' colonic submembrane with immunohistochemistry: (e) normal group (×20), (f) model group (×20), (g) Tegaserod group (×20), (h) EA on ST-25 (×20)

Fig. 4 Results of VIP expression of rat colonic membrane with immunohistochemistry: (a) Normal group (×20), (b) Model group (×20), (c) Tegaserod group (×20), (d) EA on ST-25 (×20). Results of VIPR expression of rat colon submembrane with immunohistochemistry (e) Normal group (×20), (f) Model group (×20), (g) Tegaserod group (×20), (h) EA on ST-25 (×20)

Table 4 VIP, VIPR expression of rats colon in each group ($\bar{X} \pm S$)

Group	n	Expressing area of VIP (μm^2)	Expressing area of VIPR (μm^2)
Normal group	10	488.26 ± 42.87	368.95 ± 69.36
Model group	10	2,086.95 ± 154.96 [#]	546.36 ± 106.72 [#]
Tegaserod group	10	1,588.77 ± 55.39 ^{#,*}	474.72 ± 64.51 ^{#,**}
EA on ST-25	10	1,042.77 ± 171.43 ^{#,*†}	313.20 ± 55.80 ^{*†}

Compared with normal group [#] $P < 0.01$, compared with model group ^{*} $P < 0.01$, ^{**} $P < 0.05$, compared with Tegaserod group [†] $P < 0.01$

and cecum, which is close to the nomyelin nerve fiber which conducts visceral slow pace pain. MC may release bioactivity cytokine to affect the intestine and nerve system to induce a sense of pain in the intestine. Perhaps MMC is the bridge between the nerve-immuno axis and brain-gut axis. MMC is located in lamina propria and submucosa. With TBI, observation under light microscope shows MC plasma stain into purple, and the nucleus shows dark blue, scattering in mucous and submucous layers, or gathering into a group, or lining up; cell shape appears round, oval, shuttle-like, erose; small cell with few plasma, clear shape, big cell with more plasma, unclear shape. Under electron microscope, there are many higher density granulars in the plasma. Active MMC is the key cell of gastrointestinal disfunction and abnormal sensitivity [12–15], producing large portions of chemical cytokine, participating in connections with nerve and immunosystems and in signal conduction within the brain-gut axis, and having an important role in intestinal hypersensitivity and dynamic disorder [16–19]. It could lead to functional abnormal movement-secretion in the intestine, and improve visceral hypersensitivity of pain. Transcription and expression of Protooncogene c-fos seem to be one sign of activity of MMC [20–22].

Formal results in this research shows that the general therapeutic effective rates of EA on ST-25 to treat IBS is 84.90% (Project of State Administration of TCM of PRC “Clinical research on diarrhea predominant IBS by EA on ST-25” No. 03XDLZ26, finished). In this research, the IBS model was set up by binding limbs and colorectal distention, and colon tissue was collected in continuous samples, TBI and c-fos immunohistochemistry stain. Results show that the MC count in mucous membrane of the colon and c-fos positive cells counts have increased significantly ($P < 0.01$), when compared between the normal and model groups. EA on ST-25 and Tegaserod could reduce MMC counts and c-fos positive cells counts significantly, compared with the model group ($P < 0.01$, $P < 0.05$, respectively), and EA is superior to Tegaserod ($P < 0.01$). It shows that MMC activity and proliferation could induce visceral hypersensitivity, and both EA and Tegaserod can inhibit the proliferation and activation of MC in the colon at various levels.

Therefore, it can be concluded that the signal of acupuncture maybe balances the internal disorder of the body, and inhibits MMC activity and proliferation to reduce abnormal secretion of active cytokine, while the effect of EA is obviously superior to Tegaserod to inhibit MMC activity and proliferation.

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