Original Article

Morphological changes in the enteric nervous system of rats with functional dyspepsia and the therapeutic effects of electroacupuncture at zusanli (ST36)

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Abstract: Introduction: Functional dyspepsia (FD) is characterized by nausea, vomiting, post-prandial fullness and bloating. It commonly induces the dysfunction of gastrointestinal motility, but the mechanism underlying this effect is poorly understood. Previous studies have shown that electroacupuncture (EA) at Zusani (ST36) can correct this dysfunctional gastrointestinal motility and effectively treat FD. This study aimed to create a model of FD in rats, to observe the morphological changes of the enteric nervous system (ENS), and to investigate the therapeutic effects of EA at ST36. Methods: Thirty healthy Wistar rats (including both males and females), that weighed 180 to 220 g, were randomly divided into three groups: A control group (n=10), an FD group (n=10) and an FD+EA-at-ST36 group (n=10). After the establishment of FD, whole-mount preparations of the upper jejunal segments of the rats were labeled by immunofluorescence using antibodies specific for vesicular acetylcholine transporter (VAChT), substance P (SP), vasoactive intestinal polypeptide (VIP) and neuronal nitric oxide synthase (nNOS). Immunofluorescence was detected by confocal laser scanning microscopy. Results: The number and integral optical density (IOD) values of VAChT-expressing, SP-expressing, VIP-expressing and nNOS-expressing neurons were significantly decreased in the FD group compared with the control group (P<0.05), and they were significantly increased (P<0.05) in the EA group compared with the FD group. There was no statistically significant difference between the control group and the EA group. Conclusions: The enteric nervous system was significantly disrupted in rats with FD. EA at ST36 could repair the enteric nervous system damage caused by FD.

Keywords: Functional dyspepsia, electroacupuncture, enteric nervous system

Introduction

Functional dyspepsia (FD) is a clinically common disease with a high incidence [1]. Patients with FD present with symptoms of anorexia, nausea, vomiting, post-prandial fullness, bloating, and early satiety [2]. Although the causes of FD still remain to be determined, FD commonly induces disorders of gastrointestinal motility [3]. Previous clinical and animal studies have shown that patients with FD have serious dysfunctions in gastrointestinal motility and absorption, and also endocrine secretion disorders [4]. In addition, research has shown that gastric electrical activity is abnormal, gastric emptying is delayed, and gastric motor function is diminished in patients with FD, which indicates that gastrointestinal function is impaired [4]. Therefore, the recovery of gastrointestinal motility is critical for restoring normal gastrointestinal function.

The enteric nervous system (ENS) is a relatively independent and integrated nervous system in the gastrointestinal tract, and it is composed of a large number of neurons and glial cells. The enteric nerves interact with each other and form a network that is distributed across the entire gastrointestinal tract. The ENS can regulate the physiological functions of the gastrointestinal tract independently of the central nervous system (CNS), mainly through a complex network of neurotransmitters. Changes in the levels of certain neurotransmitters in the ENS can cause changes in gastrointestinal motility [5].

Acupuncture is an essential part of Traditional Chinese Medicine (TCM) that has been used to treat gastrointestinal problems for more than two thousand years in China. EA is a technique that combines acupuncture with an electric current and is a cost-effective and minimally inva-
sive procedure that has a very low incidence of adverse effects [6]. There is increasing evidence in the literature to show a beneficial effect of acupuncture on FD [7]. EA is able to alter gastrointestinal motility and effectively treat gastrointestinal motility disorders [8]. EA is frequently used in clinical and research settings because of its reproducibility and consistency.

This study aimed to establish a model of FD in rats, to observe morphological changes in the ENS of rats with FD, and to investigate the therapeutic effects of EA at ST36. The mechanism by which FD impairs gastrointestinal motility and the therapeutic effects of EA at ST36 were explored.

Materials and methods

Experimental animals and groups

Thirty healthy Wistar rats (including both males and females) that weighed 180 to 220 g were randomly divided into three groups: A control group (n=10), an FD group (n=10) and an FD+EA-at-ST36 group (n=10). The rats (Animal license number: SCXK (Liao) 2013-0006) were supplied by the specific pathogen-free animal center of Dalian Medical University. In this study, all protocols were approved by the ethics committee of Dalian Medical University.

Establishment of the FD model

The model of FD was established according to Guo’s method [9]. The rats’ tails were wrapped in a bandage and clamped with hemostatic forceps for 30 min every 3 h. This procedure was performed 4 times a day for 7 days. One week after this 7-day period, the rats appeared to be stressed and anxious, and they exhibited poor appetite and low food intake, signs that were consistent with Guo’s report [9]. Thus, the FD model was successfully established.

Electroacupuncture treatment

The location of ST36 is approximately 5 mm lateral to the head of the fibula under the knee joint [10]. The EA needles (0.32 mm in diameter and 15 mm in length) were inserted into ST36 at a depth of 3-5 mm and connected to the EA device (HANS-200E, Nanjing Jisheng Medical Technology Co. Ltd, Nanjing, China). Disperse-dense waves at a frequency of 2/15 Hz and an output current density of 0.1-0.4 mA were used to stimulate the muscles with slight vibration, and the current was adjusted to the rats’ endurance. EA treatment was applied for 20 min daily for 15 consecutive days.

Intestinal tissue whole-mount preparation

After FD was successfully established and the EA group had received the EA treatment programme, the rats were killed with anesthesia. The proximal 10 cm segment of the jejunum, beginning 2 cm distal to the pylorus, was surgically removed from all the rats in each group and was incubated in phosphatebuffer solution (PBS) at 4°C. The intestinal canal was clipped along the mesostenium and rinsed clean. The intestinal tissue was cut into 1 cm × 1 cm segments and incubated in 4% paraform for 24 h. Mucosal and submucosal tissues were removed using a stereomicroscope (Nikon, Japan). An intestinal tissue whole mount was prepared.

Immunofluorescence labeling

In accordance with the method of Brehmer et al. [11], immunofluorescence staining was performed. The procedure was as follows: (1) Whole mounts were pre-incubated in 0.05 M Tris-HCl (pH 7.6, 37°C) containing 0.5% Triton X-100 for 4 h; (2) After 3 10-min rinses in PBS, the whole mounts were incubated in 1% BSA (20°C) for 1 h; (3) The BSA was removed, and after rinsing in PBS, the whole mounts were incubated respectively with primary antibodies that were specific for the following molecules for 48 h at 20°C (all purchased from Santa Cruz, USA): VAChT (sc-7717; Goat polyclonal IgG; 1:200); SP (sc-9758; Goat polyclonal IgG; 1:200); VIP (sc-21041; Goat polyclonal IgG; 1:200); And nNOS (sc-648; Goat polyclonal IgG; 1:200); (4) The whole mounts were rinsed in PBS 3 times for 10 min each; (5) The whole mounts were incubated in a solution containing secondary antibodies (sc-2014; Donkey anti-goat IgG-FITC; 1:500) at 20°C and protected from light for 2 h; (6) The whole mounts were rinsed in PBS 3 times for 10 min each; (7) The whole mounts were placed on a slide covered with 0.1% polylysine and fluorescent mounting medium; and (8) negative-control wholemounts were incubated in solutions lacking the primary antibodies listed in step (3) with equivalent dilutions used; Immunofluorescence staining of the negative-control whole mounts then followed steps (4)-(7).
Relevant nerve fibers were stained using the primary antibodies specific for vesicular acetylcholine transporter (VACHT), substance P (SP), vasoactive intestinal polypeptide (VIP) and neuronal nitric oxide synthase (nNOS), as described above. Wholemounts were observed with a confocal laser scanning microscope (TCS-SP5II; LEICA, Germany) that was equipped with a confocal system (Nikon Digital Eclipse C1 with 3 laser lines). A 488-nm argon laser was applied. Objective lenses (40 × dry; Numerical aperture 1.3) were used. Green fluorescence indicated the expression of VACHT, SP, VIP and nNOS. Three wholemounts were observed in each group and twofields of vision from each wholemount were observed randomly.

**Statistical analysis**

Image-Pro Plus 6.0 software (Media Cybernetics, USA) was used for semiquantitative analysis. All data were analyzed using the SPSS 19.0 software package and are presented as the mean ± standard deviation (SD). Differences between the groups were analyzed using the independent samples t-test. P<0.05 was considered statistically significant.

**Results**

**VACHT-expressing nerves**

The number (Figure 1) and IOD values (Figure 2) of VACHT-expressing nerves were significantly decreased (P<0.05) in the upper jejunal wholemounts from the FD group compared with those from the control group. The number and IOD values of VACHT-expressing nerves were significantly increased (P<0.05) in the upper jejunal wholemounts from the EA group compared with those from the FD group. There was no statistically significant difference between the control group and the EA group (Figure 3).

**SP-expressing nerves**

The number (Figure 1) and IOD values (Figure 2) of SP-expressing nerves were significantly decreased (P<0.05) in the upper jejunal wholemounts from the FD group compared with those from the control group. The number and IOD values of SP-expressing nerves were significantly increased (P<0.05) in the upper jejunal wholemounts from the EA group compared with those from the FD group. There was no statistically significant difference between the control group and the EA group (Figure 4).
VIP-expressing nerves

The number (Figure 1) and IOD values (Figure 2) of VIP-expressing nerves were significantly decreased ($P<0.05$) in the upper jejunal whole-mounts from the FD group compared with those from the control group. The number and IOD values of VIP-expressing nerves were sig-
nNOS-expressing nerves

The number (Figure 1) and IOD values (Figure 2) of nNOS-expressing nerves were significantly decreased ($P<0.05$) in the upper jejunal wholenumts from the FD group compared with those from the control group. The number and IOD values of nNOS-expressing nerves were significantly increased ($P<0.05$) in the upper jejunal wholenumts from the EA group compared with those from the FD group. There was no statistically significant difference between the control group and the EA group (Figure 5).

Discussion

There are two main types of dyspepsia: Organic dyspepsia and functional dyspepsia (FD), the latter of which is commonly encountered in clinical practice. No specific cause of the symptoms or the organic abnormality has been identified in FD patients. The widely accepted pathophysiological mechanism underlying the symptoms of FD is dysfunctional gastrointestinal motility, especially delayed gastrointestinal emptying or antral dysmotility [12-15].

The enteric nervous system (ENS) is located in the myenteric and submucosal plexuses in the digestive tract and is of great importance in digestive function. The ENS contains a large quantity of neurons and glial cells [16], and the activity of these neurons can modify gastrointestinal motility. Gastrointestinal activity is controlled by the ENS, which consists of both excitatory and inhibitory neurons, and is mediated by neurotransmitters. Excitatory neurons and inhibitory neurons regulate the contraction and relaxation of gastrointestinal smooth muscle to influence gut motility.

Excitatory neurons include those that release acetylcholine (ACh), substance P (SP) and other neurotransmitters, whereas non-adrenergic non-cholinergic (NANC) inhibitory nerves play a critical role by releasing inhibitory neurotransmitters such as nitric oxide (NO), vasoactive intestinal peptide (VIP) and adenosine triphosphate (ATP), for example [17].

Cholinergic neurons represent the majority of excitatory neurons in the ENS [18], and ACh is the most common excitatory neurotransmitter in the ENS. Previous studies have indicated that disordered intestinal cholinergic activity might decrease gut contractility and thus interfere with gastrointestinal motility [19]. Peptidergic neurons contain SP and are mostly found in the bowel wall. SP is an excitatory neurotransmitter and is an effective contractile substance that acts on smooth muscle [20, 21]. As previously reported, a reduction in the number of SP-expressing nerve fibers contributes to gut motility disorders [22]. VIP-expressing neurons are another important type of peptidergic neuron. VIP exerts inhibitory actions in the intestinal tract. The decreased VIP levels observed in idiopathic constipation induce gastrointestinal dysfunction [23]. NANC neurons can release NO, which exhibits neural inhibitory responses that often relax the smooth muscle in the digestive system [24]. Grider et al. [25] have reported that the interaction between and

Figure 6. Morphology of nNOS-expressing Nerves. A: Control group, B: FD group, C: EA group. Figure scale: 100 μm.
interdependence of VIP and NO are important in the gastrointestinal tract. ENS neurons release VIP after the activation of nNOS [26], and VIP can also induce the release of NO [27].

Electroacupuncture is widely used in treating dysfunction of the digestive system. Intestinal motility disorders can be relieved by EA at ST36 [28]. Many investigators have suggested that EA at ST36 can increase gastrointestinal myoelectric activity in Wistar rats and thus modify gut dysfunction [29].

According to TCM, Zusanli (ST36) belongs to the gastric meridian and is believed to be one of the most important acupuncture points in the context of gastrointestinal diseases [30]. ST36 is widely used in acupuncture treatment for gastrointestinal motility disorders. Many investigators have suggested that acupuncture at Zusanli can increase gastrointestinal myoelectric activity in Wistar rats in order to modify gut dysfunction. Some studies have indicated that EA stimulation at ST36 can cure FD [31]. However, the mechanism underlying FD-induced gastrointestinal motility disorders remains unclear and requires further study. Furthermore, the therapeutic effect of ST36 EA observed in this study requires further investigation.

Conclusions

The ENS was disrupted significantly in rats with FD. The number and IOD values of VAChT-expressing, SP-expressing, VIP-expressing and nNOS-expressing nerves were decreased in the FD group; EA at ST36 significantly repaired this ENS damage.

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Disclosure of conflict of interest

None.

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