Original Article

Interventional effects of da-cheng-qi decoction on enteric nerve system in a rat model of multiple organ dysfunction syndrome

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Received May 17, 2015; Accepted September 10, 2015; Epub November 15, 2015; Published November 30, 2015

Abstract: In this study, we investigate the morphologic changes of enteric nerve system (ENS) and the expression of neurotransmitters, acetylcholine (ACh), substance P (SP), vasoactive intestinal peptide (VIP) and nitric oxide synthase (NOS), in small bowel of rats undergoing multiple organ dysfunction syndrome (MODS). Undergoing MODS, fluorescence integral optical density (IOD) value of enteric nerve fibers were significantly decreased ($P<0.05$), and the network structure of ENS was destroyed. The expression of ACh, SP, VIP and NOS was inhibited, IOD value of the four neurotransmitters was significantly decreased ($P<0.05$). After intervention of DCQD, the fluorescence IOD value of enteric nerves were significantly increased ($P<0.05$), and the network structure of ENS was repaired. The expression of ACh, SP, VIP and NOS was recovered, fluorescence IOD value of the four neurotransmitters was significantly increased ($P<0.05$). In conclusion, the gastrointestinal motility disorders undergoing MODS may be closely related to the morphology destroy of ENS and down regulation of neurotransmitters (ACh, SP, VIP and NOS) expression. DCQD could promote gastrointestinal motility through protecting the morphology of ENS and up regulation of neurotransmitters (ACh, SP, VIP and NOS) expression.

Keywords: Multiple organ dysfunction syndrome, enteric nerve system, gastrointestinal motility, da-cheng-qi decoction

Introduction

Gastrointestinal motility is reduced during multiple organ dysfunction syndrome (MODS), and also participates in the development of MODS [1]. Studies found that ileus in both the stomach and small bowel caused the proximal gut becoming a reservoir for pathogens and toxins that contributed to sepsis-associated multiple organ failure (MOF) [2]. The gut can be both an instigator and a victim of MODS [2]. Improving the recovery of gastrointestinal motility function could effectively prevent the advance and onset of MODS [3].

Enteric nervous system (ENS) is a peripheral nerve loop with high degree of autonomy, not dependent on the central nervous system (CNS) [4]. ENS is comprised of a large number of glial cells, neurons, nerve fibers and neurotransmitters, which are organized into a network throughout the overall length of the gut wall [5]. ENS provides the intrinsic neural control of the gastrointestinal tract and regulates virtually all gastrointestinal tract functions [6]. Therefore, altered neuronal activity within the ENS is a key contributing factor of various gastrointestinal tract disorders underlies stress [6].

The Chinese medicine recipe Da-Cheng-Qi decoction (DCQD) is a famous herbal formula, having been used in clinical practice for thousands of years, composed of four Chinese herbs: Radix et Rhizoma Rhei, Natrii Sulfas, cortex magnoliae officinalis, and Fructus Aurantii Immaturus. A recent study showed that DCQD could recover gastrointestinal motility by improving gastric dysrhythmia, enhancing gastrointestinal motility, adjusting the synchronized recovery of the alimentary tract, and increasing plasma ghrelin after abdominal surgery [7]. But
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little is known about the pathogenesis of gastrointestinal motility disorder during MODS and the intervention mechanism of DCQD. In this study, we elucidate the morphological changes of ENS and the expression of the neurotransmitters, which determine neuronal excitability within the ENS, such as Ach, SP, NO and VIP, to reveal novel mechanism of DCQD promoting gastrointestinal motility undergoing MODS.

Materials and methods

Animals

Fifty healthy adult Wistar rats, half male and half female with weighting 200-250 g, were supplied by Animal Experimental Center of Dalian Medical University. After conventional breeding for 3 days, rats with no abnormalities were taken into the experiment. Fifty rats were divided into control group (n=10), MODS model group (n=20) and DCQD treated group (n=20) by means of random number table.

Drugs

DCQD was supplied by the pharmaceutical preparation section of Nankai Hospital (Tianjin, China). The formula was composed of Chinese rhubarb, Glauber’s salt, Magnolia, citrus aurantium, in the ratio of 4:2:3:3 (Radix et Rhizoma Rhei 12 g, Natrii Sulfas 6 g, cortex magnoliae officinalis 9 g, Fructus Aurantii Immaturus 9 g). Granules were dissolved into sterile distilled water, to prepare medicament containing crude drug 100%, equivalent to crude drug 1 g/mL.

Reagents and instrument

Mouse IgG antibody (β-tubulin III), rabbit IgG antibody (Ach, SP, VIP, NOS), donkey anti mouse IgG secondary antibody (PE) and donkey anti rabbit IgG secondary antibody (FITC) were supplied by Santa Cruz, USA and mounting medium were supplied by Vector, USA. Tris-Hcl and Triton X-100 were supplied by Amresco, USA. Albumin bovine was supplied by Roche, Germany. Microscopic anatomical tweezers was provided by REGINE EPOXY, Switzerland. Dissection microscope (SMZ800) was provided by Nikon, Japan. Nikon AZ-C1 laser scanning confocal microscope was provided by Nikon, Japan.

MODS rats modeling and grouping

Sixty Wistar rats were randomly divided into control group (n=20), MODS group (n=20) and DCQT treatment group (DCQT group) (n=20). Based on method of Qi QH, et al [8], the rats of control group were injected 1 ml normal saline into abdominal cavity under sterile condition. To establish MODS model caused by bacteria, rats in MODS and DCQD group were injected 1 ml E.coloi ×10⁸ cfu/ml suspension with 10% BaSO₄ adjuvant. Rats in DCQD group were gaveved with DCQD 2 days before establishing MODS model, twice a day, 2 ml for each rat.

Table 1. Fluorescence IOD Value of Nerve Fibers (x±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>IOD value of nerve fibers (x±s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>612.0±145.7</td>
</tr>
<tr>
<td>MODS</td>
<td>10</td>
<td>289.7±80.1a</td>
</tr>
<tr>
<td>DCQD</td>
<td>10</td>
<td>388.6±95.3b</td>
</tr>
</tbody>
</table>

Notes: *P<0.05, compared with control group; *P<0.05, compare with MODS model group.
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All protocols were conducted in accordance with the Guidance Suggestions for Care and Use of Laboratory Animals, formulation by Ministry of Science and Technology of People’s Republic of China [9].

Whole-mount preparation

Twenty four hours after modeled, upper section of the small intestine with 2 cm length from pyloric was taken from survived rats in control group, MODS group, and DCQD group, and rinsed by 4°C PBS solution. After small intestine was cut along the mesentery, contents were removed by PBS solution. The tissue was cut into 1×1 cm blocks, placed into 4% paraformaldehyde solution, storing in 4°C refrigerator overnight. The blocks were rinsed by PBS, and the mucosa and submucosa were dissected by anatomy microscope (Nikon SMZ800, Japan).

Immunofluorescence

Reference to Axel Brehmer’s method [10], briefly described as follows. The preparations were incubated in 0.05 mol/l Tris-HCl containing 0.5% TritonX-100 at 37 for 4 h, 1% BSA was then added at room temperature and kept for 1 h. Mouse IgG antibody (β-tubulin III) was added (1:200), and the preparations were kept at 4°C for 48 h. After rinsed by 0.1 mol/l PBS (3×5 min), donkey anti mouse IgG secondary antibody (PE) (1:100) was added and kept for 2 h. Rabbit IgG antibody (VACHT, VIP, SP, NOS1) was added (1:200), and the preparations were kept at 4°C for 48 h. After rinsed by 0.1 mol/l PBS (3×5 min), donkey anti rabbit IgG secondary antibody (FITC) (1:100) was added and kept for 2 h. The slides were observed by laser scanning confocal microscope.

Data and statistical analysis

Data were analyzed using ANOVA test by SPSS13.0 software. All data were expressed as mean ± standard deviation. P<0.05 was considered as statistically significant.

Results

Morphology of enteric nerve system

In control group, the morphology of small intestine neurons was clear, distributing uniformly and continually. Neurons connected with adjacent neurons through weeny synapses. Some neurons assembled in some region. In MODS group, the morphological structure of intestinal nerve was indistinct, distributing sparsity. Compared with the control group, the number of nerve fibers was significantly reduced (P<0.05). The nerve synapses was short, or even completely disappeared, and the gap between adjacent nerves was increased. The morphological structure and continuity of intestinal nerve was seriously destructed. In DCQD group, compared with MODS group, the number of nerves increased significantly (P<0.05),

Table 2. Fluorescence IOD Value of ACh and SP neurotransmitters (X ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>IOD Value of ACh (X ± s)</th>
<th>IOD Value of SP (X ± s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>540.1±82.4</td>
<td>517.0±98.0</td>
</tr>
<tr>
<td>MODS</td>
<td>10</td>
<td>263.9±85.4</td>
<td>244.7±65.4</td>
</tr>
<tr>
<td>DCQD</td>
<td>10</td>
<td>342.6±75.9</td>
<td>400.6±70.0</td>
</tr>
</tbody>
</table>

Notes: *P<0.05, compared with control group; **P<0.05, compare with MODS model group.

Figure 2. The expression of ACh and the structure of nerve fibers by fluorescence immunostaining. Notes: nerve fibers were marked by red fluorescence; ACh was marked by green fluorescence. A. Normal Group; B. MODS Group; C. DCQD Group (scale = 50 μm).

Table 2.

<table>
<thead>
<tr>
<th>Group</th>
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<th>IOD Value of ACh (X ± s)</th>
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<tr>
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Notes: *P<0.05, compared with control group; **P<0.05, compare with MODS model group.
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Excitatory neurotransmitter pathway

ACh neurotransmitter: In control group, nerves distributed uniformly and continually in intestinal muscle layer. The expression of ACh was densely. In MODS group, nerves distributed sparsely, some discontinued. ACh scattered, and the value of fluorescent IOD was significantly lower than the control group ($P<0.05$). In DCQD group, the morphological structure of nerve was clear, and concentrated. The value of ACh fluorescent IOD was significantly increased than MODS group ($P<0.05$) (Figure 2; Table 2).

SP neurotransmitter: In control group, Nerves ranged closely in muscle layer, and the expression of SP was clear. In MODS group, the morphological structure of nerve was not clear, scattered and discontinuously. The value of SP fluorescent IOD was significantly decreased than control group ($P<0.05$). In DCQD group, compared with MODS group, the morphological structure of nerves was clear, and ranged continuously, and the value of SP fluorescent IOD

**Table 3.** Fluorescence IOD Value of NOS and VIP neurotransmitters ($\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>IOD Value of NOS ($\bar{x} \pm s$)</th>
<th>IOD Value of VIP ($\bar{x} \pm s$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>$632.8 \pm 114.9$</td>
<td>$627.6 \pm 83.9$</td>
</tr>
<tr>
<td>MODS</td>
<td>10</td>
<td>$346.8 \pm 68.2^a$</td>
<td>$301.3 \pm 72.7^a$</td>
</tr>
<tr>
<td>DCQD</td>
<td>10</td>
<td>$457.1 \pm 92.8^b$</td>
<td>$409.8 \pm 73.7^b$</td>
</tr>
</tbody>
</table>

Notes: $^aP<0.05$, compared with control group; $^bP<0.05$, compare with MODS model group.

**Figure 3.** The expression of SP and the structure of nerve fibers by fluorescence immunostaining. Notes: nerve fibers were marked by red fluorescence; SP was marked by green fluorescence. A. Normal Group; B. MODS Group; C. DCQD Group (scale = 50 μm).

**Figure 4.** The expression of NOS and the structure of nerve fibers by fluorescence immunostaining. Notes: nerve fibers were marked by red fluorescence, NOS was marked by green fluorescence. A. Normal Group; B. MODS Group; C. DCQD Group (scale = 50 μm).
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was significantly increased (P<0.05) (Figure 3; Table 2).

Inhibition neurotransmitter pathway

NOS neurotransmitter: In control group, the morphological structure of nerves was clear, and NOS neurotransmitter expressed near by the nerves in intestinal muscle layer neural. In MODS group, the nerves scattered and discontinued. The value of NOS fluorescent IOD was significantly decreased than control group (P<0.05). In DCQD group, compared with MODS group, the morphological structure of nerves was clearly ranged continuously. The value of NOS fluorescent IOD was significantly increased (P<0.05) (Figure 4; Table 3).

VIP neurotransmitter: In Control group, nerves distributed density in intestinal muscle layer, and the expression of VIP is clear. In MODS group, compared with the control group, nerves distributed sparsely and discontinuously. The value of VIP fluorescent IOD decreased significantly (P<0.05). In DCQD group, compared with the MODS group, the morphological structure of nerves was clearly and distributed densely. The value of VIP fluorescent IOD was significantly increased (P<0.05) (Figure 5; Table 3).

Discussion

Enteric nervous system (ENS) is formed by a large number of neurons and ganglion cells, as well as the nerve fibers and neurotransmitters in the gut wall [11]. ENS is a complete peripheral nerve loop, with high degree of autonomy. Anatomic study found that ENS is mainly composed by the myenteric plexus and submucous plexus [11, 12]. The myenteric plexus distribute between the circular and longitudinal muscle larger, containing a large number of neurons, and can regulate secretion of glands and movement of smooth muscle within gastrointestinal [5]. Submucosal plexus contain less neurons with smaller volume, and connections among ganglions are also very subtle. Submucosal plexus contains sensory neurons, which can receive afferent signals from myenteric plexus, and also can stimulate secretion of the gastric intestinal crypt cells [11]. ENS is different from other peripheral nervous systems, not depending on the brain to regulate the glandular secretion, blood supply and smooth muscle contraction and other physiological functions of alimentary system. The current view is that, ENS has a relative independence, a large number of different neuronal subtypes cells compose a local loop. That is similar to the information processing in the brain and spinal cord, thereby ENS can autonomously regulating gastrointestinal physiological activity [13].

ENS can regulate movement of gastrointestinal smooth muscle mainly through the release of excitatory and inhibitory neurotransmitter. The major excitatory neurotransmitters including acetylcholine (ACh), substance P (SP), and the inhibitory neurotransmitter is mainly the nonadrenergic noncholinergic (NANC) neurotransmitters, including nitric oxide (NO), vasoactive intestinal peptide (VIP) [14-16].

This study showed that the IOD value of nerve fibers in the small intestine of MODS rats decreased significantly, the continuity of nerve
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fibers interrupted and distributed sparsely, and the connection among nerves was loose. Gastrointestinal motility disorder was closely related with the damaged structure of ENS, which caused conduction disturbance of gastrointestinal nerve signals. DCQD could protect the intestinal nerves obviously of MODS rats; the number of nerve fibers was close to control rats. Compared with MODS rats, nerves distributed more intensively and continuously, and the connections among nerves were increased. This study suggested that DCQD could recover gastrointestinal motility by protecting the morphology structure of small intestinal ENS in MODS rats.

Our further study showed that, excitatory neurotransmitters (ACh and SP) were decreased significantly in small intestine muscle layer of MODS rats, causing relaxation of gastrointestinal smooth muscle which resulted in motility disorder. The inhibitory neurotransmitters (NO and VIP) was also significantly reduced under MODS state. Gastrointestinal motility did not recover due to the reduction of the inhibitory neurotransmitters, but further weakened. The reason might be due to excessive destruction of inhibitory neurotransmitters, which causing part of the intestine excessive contraction, even contracture [17]. These factors weaken effective gastrointestinal peristalsis. In addition, VIP can bind to high affinity receptors on intestinal epithelial cells, leading to activation of cellular adenylate cyclase and cAMP production, and secretion of fluid and electrolytes into the intestinal lumen [18].

There is a very complicated neural signal transduction mechanism in ENS. Recent studies proved that purinergic neurotransmission, in particular, has emerged as a key contributor in the efficient control mechanisms in the nervous system [19]. The physiological functions of ENS are multi-target and multi-path, also interacted with the immunological cells. Mast cells can intervention ENS neurotransmitter by the secretion of histamine (H1-4 receptors), proteases (PAR1 receptors), several cytokines and chemokines and probably also serotonin (5-HT (3) receptors) [20]. Although our study revealed that the famous Chinese herbal formula DCQD has the ability to promote the gastrointestinal motility function by protecting the morphological structure of ENS and the expression of neurotransmitters, the precise mechanisms involved are still to be elucidated. DCQD as a potential therapeutic drug for gastrointestinal motility disorders underlying MODS needs to be investigated in the future.

Acknowledgements

Supported by National Natural Science Foundation of China, No. 81273920.

Disclosure of conflict of interest

None.

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