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CLINICAL RESEARCH

A preliminary study of neck-stomach syndrome

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Abstract

AIM: To determine the expression of c-Fos, caspase-3 and interleukin-1β (IL-1β) in the cervical cord and stomach of rats with cervical spondylosis, to analyze their relationship, and to offer an explanation of one possible cause for functional dyspepsia (FD) and irritable bowel syndrome (IBS) caused by cervical spondylosis.

METHODS: The cervical spondylosis model in rats was established by destroying the stability of cervical posterior column. The cord segments C4-6 and gastric antrum were collected 3 mo and 5 mo after the operation. Rats with the sham operation were used as controls. The expressions of c-Fos, caspase-3 and IL-1β in the cervical cord and gastric antrum were determined by immunohistochemistry and/or Western blot.

RESULTS: Immunohistochemical staining showed a few c-Fos, caspase-3 and IL-1β-positive cells in the cervical cord and antrum in the control. There was a significant increase in c-Fos, caspase-3 and IL-1β expression in model groups compared to the control groups at 3 mo and 5 mo after operation. More importantly, there was a significant ($P < 0.05$) increase in c-Fos, caspase-3 and IL-1β expression in the model group rats at 3 mo compared to those at 5 mo after the operation (c-Fos: 11.20 ± 2.26 *vs* 27.68 ± 4.36 in the cervical cord, 11.3 ± 2.3 *vs* 29.3 ± 4.6 in the gastric antrum; caspase-3: 33.83 ± 3.71 *vs* 36.32 ± 4.01 in the cervical cord, 13.23 ± 3.21 *vs* 26.32 ± 4.01 in the gastric antrum; IL-1β: 42.06 ± 2.95 *vs* 45.91 ± 3.98 in the cervical cord, 26.56 ± 2.65 *vs* 32.01 ± 2.98 in the gastric antrum). Western blot analysis showed time-dependent changes of caspase-3 and IL-1β protein in the cervical cord and gastric antrum of rats with cervical spondylosis; there was no significant expression of caspase-3 and IL-1β protein in the control group at 3 mo and 5 mo after the sham operation, whereas there was a significant difference in caspase-3 and IL-1β protein levels between the model group rats followed up for 3 mo and those for 5 mo ($P < 0.05$).

CONCLUSION: There is a significant association of c-Fos, caspase-3 and IL-1β expressions in the gastric antrum with that in the spinal cord in rats with cervical spondylosis, suggesting that the gastrointestinal function may be affected by cervical spondylosis.

Key words: Caspase-3; IL-1β; c-Fos; Cervical spondylosis; Gastric antrum

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INTRODUCTION

Many patients with cervical spondylosis complain of gastrointestinal symptoms. Some are caused by NSAIDs, but many patients are not taking any medication. There is a direct or indirect relationship between the neck and the stomach, called the neck-stomach syndrome. Cervical pathology, mediated through sympathetic nerves, has been associated with a number of disorders, which include about 20 kinds of diseases or symptom-groups, such as hypertension, cardiac arrhythmias, dizziness, eyesight malfunction and gastrointestinal dysfunction. Perhaps the clinical symptoms of cervical spondylosis include gastrointestinal disorders mediated through irritated sympathetic nerves. A study reported such a mechanism[1], but offered no supportive evidence. In the present report, expression of c-Fos, caspase-3 and IL-1β in the cervical cord and gastric antrum were examined in rats with cervical spondylosis and the results were analyzed. If the longer duration the cervical vertebrae degeneration is correlated with the increasing levels of c-Fos, caspase-3 and IL-1β in the cord and in the stomach, then the hypothesis might be validated.

c-fos is a proto-oncogene or a cellular oncogene,

which exists extensively in genomes of eukaryotes. It participates in normal cell growth and proliferation and regulates message transfer in cells. Most previous studies have focused on c-Fos expression that is induced by the controlled and natural irritations[2-4] Recently, it has been proven that the normal motion of the digestive tube relies on reflex activity controlled by extrinsic nerves and enteric nervous system (ENS). c-Fos as the third messenger, which regulates the target gene, provides a reliable and direct method to study the mechanism of functional gastrointestinal diseases[5]. Our previous study[6] reported that the c-Fos expression in the gastric myenteric plexus was dramatically associated with c-Fos expression of the spinal cord in the rats with cervical spondylosis. It is the sympathetic nerve that results in the c-Fos expression both in the spinal cord and the gastric myenteric plexus in cervical spondylosis and this suggests that the gastrointestinal function may be affected by cervical spondylosis. To provide further evidence, c-Fos, caspase-3 and IL-1β were detected in the cord and stomach. The rationale being that caspase-3 is a potential mediator of apoptosis after central nerve system (CNS) injury[7,8] and its activation may be used as a marker of apoptotic cell death. Several studies have provided evidence that cell death from moderately severe spinal cord injury (SCI) is regulated, in part, by apoptosis that involves the caspase family of cysteine proteases[8,9]. In the hippocampus of aged rats, the concentration of IL-1β is increased and this increase is accompanied by enhanced caspase-3 activity indicative of cell death[10]. These findings suggest that neuronal apoptosis in the CNS is induced by increased IL-1β through the activity of the caspase-3 apoptotic pathway. IL-1β induced apoptosis in neurons *in vitro*[11] and in cultured human astrocytes[12] and oligodendrocytes *in vivo*[13].

In the present study, after establishing the cervical spondylosis model of rats according to a previously described method[6], the cord and stomach were collected at 3 mo and 5 mo to determine the expression of c-Fos, caspase-3 and IL-1β in the cervical cord and gastric antrum by immunohistochemistry and/or Western blot.

MATERIALS AND METHODS

Animal models

Ninety-six four-month-old Sprague Dawley rats (provided by the Experimental Animal Center of Shantou University Medical College, Shantou, China), weighting 250 g (range, 220-280 g), were used in this study. The rats were randomly divided into model and control groups and fed a normal diet, with eight in one big cage, and kept for 3 mo and 5 mo, respectively, after the experimental or sham operations as described below. Each group consisted of 12 male and 12 female rats at each time point.

The rats in the model group were anesthetized by intraperitoneal (ip) injection of 40 mg/kg sodium pentobarbital . The dorsal neck was shaved and a longitudinal incision about 2.5 cm was made. The dorsal muscles were reserved, the spinal processes were removed, as well as the inter-spinal ligaments, the capsule of articular processes and part of the superior and inferior articular processes between C3-7 levels were removed till the movement between the neighboring superior and inferior laminae was obviously increased after the operation, and the incision was closed. Three and five months after the operation, the models were confirmed by evaluating X-ray films and the motion function with oblique board test according to the previous studies[14,15]. X-ray films showed disappeared or stiff nature cervical curve, stenosis of the vertebral space and osteosis spur in the model groups compared with the control groups. To test motion function, the rats were put on a tilted board, and the angle between the board and horizontal plane was recorded, which showed a significant difference in control groups and model groups. The rats in the control group (sham operation group) had only a longitudinal incision on the dorsal neck which was closed without further intervention.

Immunohistochemistry

The rats were euthanized with a lethal dose of pentobarbital sodium (100 mg/kg) and perfused *via* cardiac puncture with 0.1 mol/L phosphate-buffered saline (PBS) (pH 7.4; 150 mL) and subsequently with 40% paraformaldehyde in 0.1 mol/L PBS (250 mL). The cord and gastric antrum tissues were dissected out and post-fixed by immersion in 40 mL/L paraformaldehyde for 3 h and then cryoprotected by immersion in 200 g/L sucrose (in PBS) overnight. Tissue segments were embedded in Tissue-Tek O.C.T. (Lab-Tek Division, Miles Lab, Inc.) and frozen as previously described[16]. Free-floating transverse sections (10 μm) were cut with a cryostat.

Immunohistochemical staining was carried out using the avidin–biotin complex method as previously described[6,17]. The concentrations for rabbit anti-IL-1β, rabbit anti-caspase-3 and rabbit polyclonal anti-c-fos primary antibodies were 1:200, 1:200 (Sigma, Genetimes Technology Inc.) and 1:100 000 (diluted in NGS-T-PBS). Positive immunoreactive labeling was observed qualitatively.

The method for gastric neuronal counterstaining was adapted from previous studies[6,18,19]. c-Fos cells were counted under microscopy in 25 ganglia from each antral preparation and expressed as a mean percentage count per myenteric ganglion. Myenteric ganglia were recognized as clearly delineated groups of neurons separated by well-defined internodal fiber tracts. The mean from all animals in each group was used to calculate the group mean. Data were expressed as mean ± SD of the number of cells or neurons per ganglion. Buffy spots or particles in cells were regarded as positive expression of caspase-3 and IL-1β. The random sections were observed under the optical microscope at 200 × magnification, the positive cells were counted in ten random visual fields, and then the mean in each group was calculated.

Western blot analysis

At 3 mo and 5 mo after the destabilizing operation, an 8 mm spinal cord segment was dissected 4 mm rostral and 4 mm caudal from the center of the C3-7 cord from each group. Five millimeters of the gastric

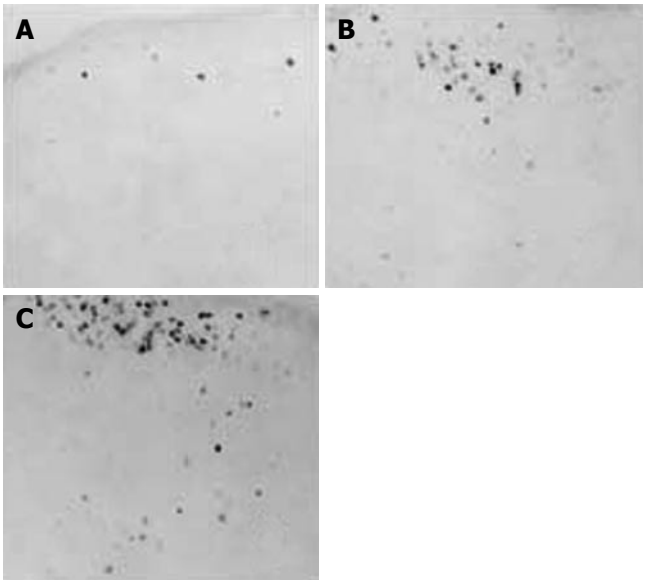

Figure 1 Expression of c-Fos in the cervical cord. **A**: Control group; **B**: Model group at 3 mo; **C**: Model group at 5 mo. There were only a few c-Fos expression in the cervical cord of the control group, but an increased c-Fos expression in the model groups at 3 mo and 5 mo after the operation.

Table 1 c-Fos-positive neurons in the cervical dorsal horn in the two different groups (%, mean ± SD)

Groups	Number of c-Fos-positive neurons	
	3 mo	5 mo
Control group	1.25 ± 0.25	1.98 ± 0.60
Model group	11.20 ± 2.26	27.68 ± 4.36

antrum including the anterior and posterior wall was then extracted longitudinally and opened at the greater curvature, thoroughly rinsed by 0.15 mol/L PBS. The cord and gastric antrum tissues were resuspended in a lysis buffer (Cell Signaling Technology) and homogenized in a homogenizer on ice. Tissue homogenate samples were centrifuged at 1000 r/min for 10 min at 4℃, and the supernatants were stored at -30℃. Protein concentrations in the cell lysates were determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA) as following manufacturer's instructions. For Western blot analysis, 20 μL of each suspension sample was separated on 120 g/L sodium dodecyl sulphate-polyacrylamide gel electrophoresis and the proteins were transferred onto nitrocellulose membranes. Blots were blocked with 50 g/L non-fat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature and then the membranes were incubated with 1:200 diluted monoclonal rabbit anti-rat antibodies against IL-1β (Sigma-Aldrich) or caspase-3 (Sigma-Aldrich) overnight at 4℃. The membranes were then processed with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:500; Sigma-Aldrich). Immunoreactive bands were detected by ECL chemiluminescence kit (Amersham, USA).

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA. The significant difference between pairs of groups was tested by post-hoc analysis. $P < 0.05$ was considered statistically significant.

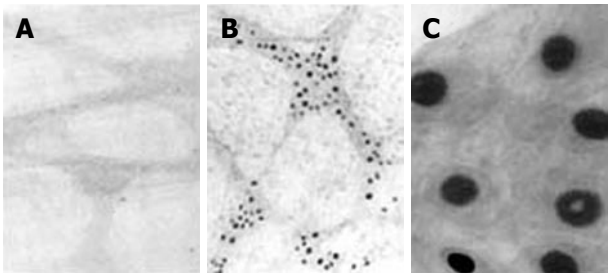

Figure 2 Expression of c-Fos in the gastric antrum. **A**: Control group; **B**: Model group; **C**: Enlarged figure of the model group. Neurons were defined as c-Fos-positive when the nucleus was stained with intensity clearly above the faint background stain, whereas neurons were defined as c-Fos-negative when the nucleus was either not stained at all or stained with intensity close to background stain.

Table 2 c-Fos-positive neurons in the antral ganglia between the two different groups (%, mean ± SD)

Groups	Number of c-Fos-positive neurons	
	3 mo	5 mo
Control group	2.4 ± 0.6	3.2 ± 0.8
Model group	11.3 ± 2.3	29.3 ± 4.6

RESULTS

c-Fos expression in the cervical cord

There were only a few c-Fos-expressing neurons in the cervical cord of the control group. However, an increased c-Fos expression was observed in the model groups at 3 mo and 5 mo after the operation (Figure 1, Table 1). There was no significant spontaneous c-Fos expression in the spinal cord in the control group at 3 mo and 5 mo after sham operation, whereas there was a significant increase in c-Fos expression in the model group rats. More importantly, there was a significant difference in c-Fos expression between the model group rats at 3 mo and those at 5 mo after the operation ($P < 0.05$).

Expression of c-Fos in the gastric antrum

The number of c-Fos-positive neurons in the gastric antrum was expressed as a percentage of the total number of neurons per ganglion as assessed by cuprolinic blue counterstaining. There was no significant spontaneous c-Fos expression in the antrum in the control group at 3 and 5 mo after the sham operation; whereas there was a significant increase in c-Fos expression in the model groups. More importantly, a significant difference in c-Fos expression was observed between the model group rats at 3 mo and those at 5 mo after the operation ($P < 0.05$) (Table 2). c-Fos expression was seen in Figure 2.

Caspase-3 and IL-1β expression in the cervical cord and stomach

The caspase-3 and IL-1β expression in the cervical cord

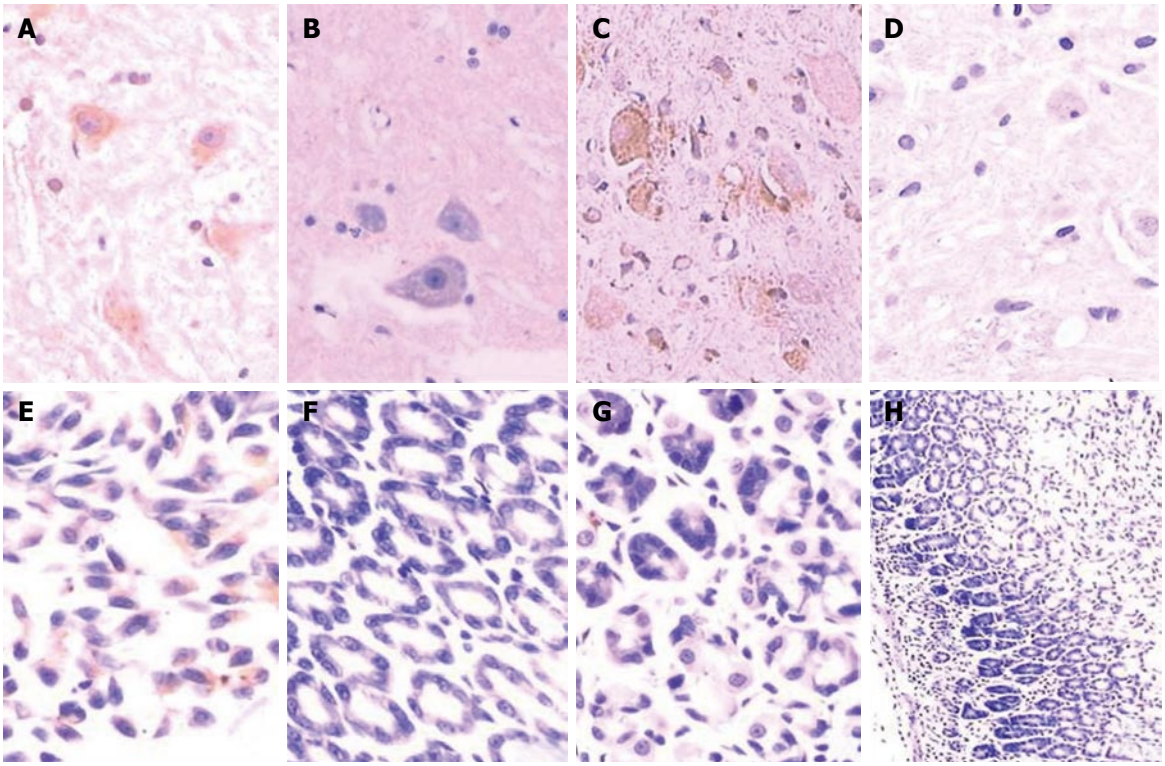

Figure 3 Expression of caspase-3 and IL-1β in the cervical cord and stomach. **A**: An increased immunoreactivity and positive neurons of IL-1β in model group; **B**: Negative expression of IL-1β in control group; **C**: An increased expression of caspase-3 in model group; **D**: Negative immunoreactivity of caspase-3 in control group; **E**: Positive expression of IL-1β in the stomach of model group; **F**: Negative expression of IL-1β in the stomach of control group; **G**: An increased expression of caspase-3 in the stomach of model group; **H**: Negative immunoreactivity of caspase-3 in the stomach of control group.

Table 3 Cells positive for caspase-3 and IL-1β in the stomach of rats with cervical spondylosis mean ± SD

Groups	Caspase-3-positive cells	IL-1β-positive cells
3-mo group	33.83 ± 3.71	42.06 ± 2.95
5-mo group	36.32 ± 4.01	45.91 ± 3.98

There was no significant difference between the controls, but a significant difference between the control groups and the model groups at 3 mo and 5 mo after the operation ($P < 0.01$).

Table 4 Cells positive for caspase-3 and IL-1β in the spinal cord of rats with cervical spondylosis mean ± SD

Group	Caspase-3-positive cells	IL-1β-positive cells
3-mo control group	2.01 ± 1.36	1.98 ± 2.01
3-mo model group	13.23 ± 3.21	26.56 ± 2.65
5-mo control group	3.26 ± 3.02	2.31 ± 2.48
5-mo model group	26.32 ± 4.01	32.01 ± 2.98

There was no significant difference between the controls, but a significant difference between the model groups at 3 and 5 mo after the operation ($P < 0.05$) and also the control groups vs the model groups at 3 mo and 5 mo, respectively ($P < 0.05$).

and stomach was examined by immunohistochemistry (Figure 3, Tables 3 and 4). Buffy spots or particles in cells were regarded as positive expression of caspase-3 and IL-1β. The positive cells were increased both in the cervical cord and stomach of model group rats. There was no significant expression in the control group at 3 mo and 5 mo after the sham operation. However, there was a significant increase both in the cervical cord and stomach of the model group rats. More importantly, there was a significant difference in caspase-3 and IL-1β expression both in the cervical cord and stomach between the model group rats at 3 mo and those at 5 mo after the operation ($P < 0.05$) (Tables 3 and 4).

Western blot analysis of caspase-3 and IL-1β expression

Western blot analysis showed time-dependent changes of caspase-3 and IL-1β protein in the cervical cord of rats with cervical spondylosis (Figure 4). Densitometry readings of gel bands were expressed as arbitrary units. There was no significant expression of caspase-3 and IL-1β protein in the control group at 3 and 5 mo after sham operation, however, there was a significant expression in the model group rats. More importantly, there was a significant difference expression between model group rats at 3 and those at 5 mo after the operation ($P < 0.05$).

DISCUSSION

The definition of neck-stomach symptoms is gastrointestinal disorders resulting from cervical spondylosis. The sympathetic fibers are distributed in the periphery of the dorsal root ganglion (DRG). The adventive nerves interact with the neurons of the DRG by a non-synaptic signaling[20,21]. The sympathetic fibers are distribution in the Luschka, articular capsule, cervical facet joints, cervical posterior longitudinal ligaments, posterior annulus fibrosus

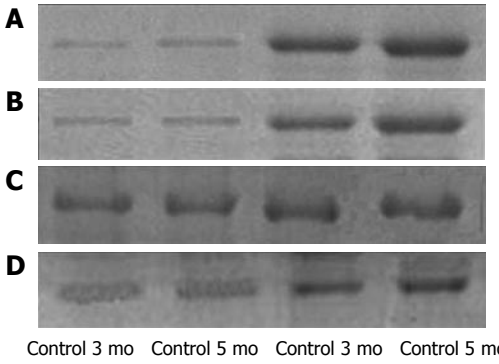

Figure 4 Expression of caspase-3 and IL-1β protein detected by Western blot. **A**: Caspase-3 expression in the cord at 3 mo and 5 mo in different groups; **B**: IL-1β expression in the cord at 3 mo and 5 mo in different groups; **C**: Caspase-3 expression in the gastric antrum at 3 mo and 5 mo in different groups; **D**: IL-1β expression in the gastric antrum at 3 mo and 5 mo in different groups.

and vertebral artery. Parts of cervical nerve roots connect with superior cervical ganglion *via* postganglionic fibres. When the sympathetic fibers are irritated, the clinical syndromes result from the spinal and brain-spinal reflex pathways[22].

The mechanism of neck-stomach syndrome is that when the sympathetic nerve is irritated by nerve roots, degenerated disc and facet joints disorders due to osteophytes, cervical muscle overexertion and/or injury, the irritation reaches to the brain cortex by nerve reflex and produces a higher or lower sympathetic irritability, and then results in multiple dysfunctions of the neck, upper limbs, cardiac and gastrointestinal reflexes, *etc*.

c-Fos as the third messenger provides a reliable and shortcut method to study the mechanism of functional gastrointestinal diseases[5]. Gilby *et al*[23] reported c-Fos, as a third messenger, regulates target gene expression and plays a key role in nerve system signal transmission. A study showed that expression of c-Fos proto-oncogene in fetus cerebral nerve cell cultured in vitro was regulated specially by IL-1β in time course[24].

Many studies have shown that c-Fos expression is related to functional gastrointestinal diseases, such as c-Fos abnormal expression in intestinal myenteric plexus and CNS in inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and Crohn's disease[25,26]. Enteric nervous system (ENS) owns a strong biological compatibility, and c-Fos may be a good index of ENS to short and/or chronic gastrointestinal irritation[25-27].

In our study, Western blot analysis showed time-dependent changes of caspase-3 and IL-1β protein in the cervical cord and gastric antrum of rats with cervical spondylosis. We observed an increased c-Fos, caspase-3 and IL-1β expression in model group rats at 3 and 5 mo after operation by immunohistochemistry staining. Interestingly, we found a significant difference in c-Fos, caspase-3 and IL-1β expression between the model group rats at 3 mo and those at 5 mo after the operation. Expression of c-Fos, caspase-3 and IL-1β in the gastric antrum were dramatically associated with that in the spinal cord of rats with cervical spondylosis, suggesting that the gastrointestinal function may be affected by cervical spondylosis and that the c-Fos expression may be regulated by IL-1β. Further studies need to validate this hypothesis.

Because of the accumulated knowledge of diseases, there is a rapid transition in modern medical sciences from the simple biological pattern based on unity biology to the biology-psychology-society pattern. The traditional "functional gastrointestinal disorders" in the past are now known as multiple physical and patho-physiological diseases involving gastrointestinal motility, gut sensitivity, brain-intestine axis, brain-intestine peptides, society-physiology factors and stress. Especially, the conception of brain-intestine axis and neurogastroenterology has been put forward, which indicates that the alimentary canal is controlled by both motor and sensory nerves. When a nerve is injured, the lesion will impact on both the sense and motion realms. Even though the lesion is limited in only one realm, the change will result in the change of other region's function[28,29]. Theoretically, only when the gastrointestinal tract and CNS are integratively investigated, can the multiple physical and pathophysiological diseases be further understood.

The previous studies had proven mechanisms of FD and IBS were associated with CNS[28,30]. In the present study, we found the same results and believe that there are relationships between the neck and stomach.

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