Evaluation of a modified rat model for functional dyspepsia

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Abstract

Background/Aim: The purpose of this study was to establish a modified rat model with functional dyspepsia (FD) and analyze the changes in gastrointestinal motility and brain-gut peptide levels in serum and brain-gut axis.

Materials and Methods: Male Wistar rats were divided into control group (Con) and FD model group. FD model was established by stimulating semi-starvation rats via tail damping, provocation, and forced exercise fatigue until gastrointestinal motility disorder appeared, and then levels of motilin, leptin, cholecystokinin (CCK), and vasoactive intestinal peptide (VIP) were detected in serum by enzyme linked immunosorbent assay and in duodenum, antrum, and hypothalamus by immunohistochemistry, reverse transcriptase-polymerase chain reaction, and Western blot.

Results: The results showed rates of intestinal propulsion and gastric emptying slowed down markedly compared to Con ($P < 0.05$), the gastrointestinal electric activity attenuated, and migrating motor complex (MMC) interrupted in the model group. The levels of leptin and VIP markedly increased, but motilin decreased as compared to the Con ($P < 0.05$) in serum and in the above tissues. It is interesting that the level of CCK decreased in the antrum and duodenum but increased in the hypothalamus as compared to Con ($P < 0.05$).

Conclusions: The modified rat model meets the diagnostic criteria of FD and can be used as a method for studying FD in animals.

Keywords: Brain-gut axis, brain-gut peptides, functional dyspepsia, gastrointestinal motility

INTRODUCTION

Functional dyspepsia (FD) is clinically characterized by upper abdominal pain or burning sensation and early satiety without any organic disease. The disorder has a passive effect on the health of patients [1,2] and constitutes a huge economic burden to medical institutions and the society.

In previous studies, gastrointestinal motility disorder [3,4] and abnormal brain-gut interaction, characterized by fullness, upper abdominal discomfort, or nausea [5] have been reported as the main pathogenetic factors in patients with FD.

The brain-gut axis plays an important physiological role in gastrointestinal tract. For example, dysregulation of central nervous system (CNS) or enteric nervous system (ENS) leads to disorder in sensation and motility via brain-gut peptides [6,7,8].

Motilin (MTL), a peptide containing 22 amino acids secreted by upper small intestine during fasting, controls...
gastrointestinal motility by enhancing migrating motor complex (MMC).[9] MTL and MTL receptor have been located in rodent CNS,[10] which indicates that MTL plays an important role as a neurotransmitter in brain. Leptin, a 16-kD product of ob gene synthesized primarily in adipocyte, can inhibit a population of vagal intestinal mechanoreceptors.[11] Leptin in blood regulates energy balance and digestive behavior by penetrating blood-brain barrier via a receptor-mediated transport system[12] to act on leptin receptor in medial hypothalamus.[13] While cholecystokinin (CCK), secreted in the gastrointestinal tract and related to the gastrointestinal motility, cannot cross the blood-brain barrier.[14] CCK signal can reach the hypothalamus by vagal reflex pathway to involve in feedback suppression of gastrointestinal motility.[15] Vasoactive intestinal peptide (VIP), secreted in the CNS and ENS, participates in secretory activity, motility regulation, and vasodilatation, and peristaltic reflex suppression in circular smooth muscle layer.[16] Changes in VIP levels in plasma and mucosa may be concerned with the gastric motility.[17]

Animal model for FD is limited, and the most common is 0.1% iodoacetamide gavage modeling method. Recently, some researchers used tail damping and provocation to establish FD rat model.[18-20] The purpose of the present research was to establish a modified rat model with FD and analyze the changes in gastrointestinal motility and brain-gut peptides levels in serum and brain-gut axis.

MATERIALS AND METHODS

Animal model
All experiments were performed according to protocols approved by the Ethics Committee of Animal Experiments at Lanzhou University and in accordance with the guidelines from the International Association for the Study of Pain.

Male Wistar rats weighing between 180 g and 220 g were delivered from the Experimental Animal Center of Lanzhou University. Two pairs of bipolar stainless-steel electrodes, 3 mm apart, were implanted onto the serosal surface of the gastrointestinal tract in the rat. One pair was on antrum at 1 cm from pylorus and the other was on small intestine at 1–2 cm distal to pylorus after acclimatization for 1 week. The free ends of the electrodes were brought subcutaneously to the back of the neck of each rat. Rats were divided into a control group and an FD model group (10 animals in each group). The FD model was built by stimulating semi-starvation in the rats via tail damping, provocation, and forced exercise fatigue four times a day for 10 days. The detailed method was to use a long sponge holding forceps and clamping the distal one-third of the tail with no damage to the skin. The stimulation lasted 30 min each time, then the rats were forced exercise to fatigue by running on an experimental treadmill at an appropriate speed for 10 min; these procedures were performed at 9:00, 12:00, 15:00 and 18:00 hours for 10 days. After 10 days, the gastrointestinal electric recordings was performed by a BL-420S experimental system biological function analyzer (TaiMeng Technology, Chengdu, China) with a microcomputer including frequency and amplitude of slow wave and spike activity in duodenum and antrum for 1 h.

Measurement of intestinal propulsion and gastric emptying
After abrosia for 24 hours, five rats were killed. Then, the blood samples were collected from femoral arteries, and serums were separated and conserved at −80°C for enzyme linked immunosorbent assay (ELISA). Other animals were given 5% graphite powder with milk and glucose in water (weighed and recorded as A1) by oral gavage and absorbed for 30 min and then sacrificed. After exposed by laparotomy, the stomach and small intestine were carefully removed to observe the leading edge of the graphite powder in the intestine after ligation of esophagogastric, gastroduodenal, and ileocecal junctions. Length of the small intestine from pylorus to ileocecal and the length of graphite powder promoting were measured. The formula of calculating intestinal propulsion was as follows: intestinal propulsion rate = length of graphite powder/whole length of small intestine. The stomach was cut, weighed (recorded as A2), and immersed into 0.9% saline solution to clean the rest of the graphite powder. After blotting dry with absorbent paper to remove any surface moisture, the stomach was weighed again (recorded as A3). The formula for calculating gastric emptying rate was as follows: gastric emptying rate = (A2 − A3)/A3.

Tissue preparation
Duodenum, gastric antrum, and hypothalamus were removed and frozen rapidly in liquid nitrogen and then stored at −80°C until reverse transcription-polymerase chain reaction (RT-PCR) analysis or Western blot analysis was performed.

Enzyme linked immunosorbent assay
The levels of MTL, leptin, and VIP protein in serum were quantitated by ELISA (R and D Systems, Minneapolis, United States) according to the manufacturer's protocol. Optical densities were determined within 30 min on a microplate reader set to 450 nm.
Immunohistochemistry
The fresh duodenum, antrum, and hypothalamus were fixed in 4% paraformaldehyde, embedded in paraffin blocks, and cut into 5-µm sections on slides. The slides were incubated in 0.01 M citrate buffer in a microwave for heat-induced epitope retrieval for 5 min. Endogenous peroxidase activity was blocked by immersing the sections in a solution of 3% H2O2, and nonspecific binding was blocked by incubation with 5% milk in phosphate-buffered saline for 5 min. The samples were then incubated with anti-MTL antibody, anti-leptin antibody, and anti-CCK antibody (Bioss, Beijing, China) overnight at 4°C. After washing, the slides were incubated with secondary biotinylated antibody for 20 min at room temperature. Staining was visualized with a DAB detection kit (Solarbio, Beijing, China).

Reverse transcriptase-polymerase chain reaction
Total RNA was extracted from the samples with Trizol reagent (Invitrogen, Carlsbad, United States) and reverse transcription reaction was performed with a reverse transcription kit (Promega, Madison, United States) according to the manufacturer’s protocol.

Thermal cycling consisted of initial denaturation at 95°C for 2 min followed by 32-36 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Subsequently, a final 10 min elongation step at 72°C was performed. PCR products were separated on 2% agarose gel (Invitrogen), and imaged.

The following primers were used for PCR amplification:

**CCK gene:** forward: 5'-GCCTATTGCGAGAATGACA-3'; reverse: 5'-TTTCTCATGATCCTCGCTTC-3'.

**VIP gene:** forward: 5'-TGCCTTAGCGGAGAATGACA-3'; reverse: 5'-CTTACGTCTCCTCTCCA-3'.

**Actin gene:** forward: 5'-TCCTGTGGCATCCATGAAACT-3'; reverse: 5'-GAAGCACTTGCGGTGCACGAT-3'.

Western blot
The gastrointestinal tissues were washed three times with 0.9% saline solution and were lysed in RIPA (Applygen Technologies, Beijing, China). Protein levels were quantified with a BCA Protein Assay Kit (Applygen Technologies). Proteins in an equal amount of each lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Darmstadt, Germany). After blocking nonspecific binding sites for 1 h with 5% dried skim milk (B and D, Ballarat, Australia) and dissolved in TBST, the membranes were individually incubated overnight with anti-leptin (Santa Cruz Biotechnology, Dallas, United States), anti-VIP antibody (Bioss), and anti-actin antibody (Bioss).
Table 1: Inhibition of gastrointestinal electric activity in FD rats

| Groups | Antrum | Duodenum | Hypothalamus |
|--------|--------|----------|--------------|
|        | SA μv  | SF num/min | SPA μv | SSWR % | SA μv | SF num/min | SPA μv | SSWR % |
| Control | 468.836±24.441 | 4.487±0.011 | 141.015±10.928 | 71.133±6.069 | 159.547±36.403 | 37.051±4.031 | 240.024±69.708 | 44.354±3.682 |
| Model  | 314.431±33.487* | 3.615±0.277* | 97.140±5.878 | 140.218±58.972 | 71.340±5.878* | 27.931±4.277 | 153.262±34.521* | 32.564±7.344* |

FD = functional dyspepsia; SA = Slow wave amplitude; SF = Slow wave frequency; SPA = Spike amplitude; SSWR = Spikes/slow wave num ratio. All data are expressed as mean±SD (n=8). Statistical significance was determined compared to control group (*P<0.05).

Figure 3: Abnormal levels of brain-gut peptides in FD rats. Immunohistochemistry for MTL (a), leptin (b) and CCK (c) protein in the antrum, duodenum and hypothalamus of rats (magnified × 100 and × 400). FD = functional dyspepsia; MTL = motilin; CCK = cholecystokinin.

appropriate secondary antibody was used at 10000-fold dilution for 1 h. Target proteins were visualized and representative images were presented.

Statistical analysis
The data were summarized by using the mean ± standard deviation (SD) and were compared using Students t-test. Statistical significance was determined compared to the control group (*P<0.05).

RESULTS

Rates of intestinal propulsion and gastric emptying
As shown in Figure 1a, the rate of intestinal propulsion was decreased (P < 0.05) and gastric emptying slowed down significantly (P < 0.05) in the model group compared with the control group.

Gastrointestinal electric activity
The slow-wave amplitude (SA), slow-wave frequency (SF), spike amplitude (SPA), and spikes/slow-wave ratio (SSWR) were decreased considerably in the model group compared with the control group in the duodenum and antrum, indicating that the gastrointestinal electric activity was decreased in the model rats [Table 1]. Compared with the control group, the MMC cycle in the model group disordered and the phase III disappeared in both antrum and duodenum, as shown in Figure 1b and c.
Levels of MTL, leptin and VIP protein in serum

Figure 2 shows that plasma MTL protein level was significantly decreased in the model group compared with the control group ($P < 0.05$). However, the plasma leptin and VIP protein levels were increased significantly in the model group compared with the control group ($P < 0.05$).

Localization of MTL, leptin, CCK proteins

MTL, leptin, and CCK proteins were found in the submucosa and muscular layer in the duodenum and antrum [Figure 3]. Compared with the control group, MTL protein in the model group was decreased in the duodenum, antrum, and hypothalamus. However, the leptin protein level was higher in the above three kinds of tissues. In the model group, CCK protein level was lower in the duodenum and antrum and higher in the hypothalamus, when compared to the control group.

Levels of CCK and VIP mRNA

As shown in Figure 4, CCK mRNA levels varied in different parts. In the model group, it was decreased significantly in the duodenum and antrum ($P < 0.05$) and increased significantly in the hypothalamus ($P < 0.05$) vs the control group. Compared with the control group, VIP mRNA level in model group was increased significantly in the duodenum, antrum, and hypothalamus ($P < 0.05$).

Levels of leptin and VIP protein in the gastrointestinal tissues

Figure 5 shows that leptin and VIP protein levels in the model group were increased significantly in the duodenum and antrum ($P < 0.05$) vs the control group.
The dysmotility-like FD rat model was established by stimulating semi-starvation rat via tail damping in our experiment. After stimulation with rat tail damping for 10 days, the rats showed symptoms of malaise, irritability, nervousness, and anxiety. Then, we observed the gastrointestinal electric activity, intestinal propulsion, and gastric emptying and found that hypomotility appeared in the model group, which indicates that the experimental model was successful.

There are four major pathways for interactions between the brain and gut in the brain-gut axis, which include immune, neural, and endocrine messages and nervous system related to brain-gut peptides and signalling molecules. In previous studies, bidirectional brain-gut interactions play an important role in FD patients and the brain-gut peptides such as MTL, leptin, CCK, and VIP originating from the gastrointestinal tract delivered into the circulation. The hypothalamus regulates physical functions by hormonal signals and peripheral neural messages from ENS.

MTL is a gut hormone secreted during the interdigestive phase and can act directly on MTL receptor distributed in smooth muscle to induce muscle contraction by triggering the phase III of MMC. The phase III of MMC may enhance the gastrointestinal motility to clean up food residues in the stomach and intestine. In addition, changes in MTL level are closely related with abnormal gastric emptying. All these indicate that reduced MTL might also induce FD by decreasing motility. In this study, we found that MTL level in the model group was lower than that of the control group in the plasma, antrum, duodenum, and hypothalamus, matching the previous study well. The phase III of MMC disappearance in both duodenum and antrum was related to the reduction of MTL in the model group.

VIP, one important brain-gut peptide released from the ENS to many gastrointestinal tract regions, has been shown to regulate the gastrointestinal motility by relaxing the smooth muscle. VIP promotes the production of NO by stimulating stomach muscle cells, enhances inner cAMP and cGMP and finally induces smooth muscle relaxation. In this study, we found that VIP level in the model group was higher than that of the control group in plasma, duodenum, antrum, and hypothalamus, which is consistent with previous reports.

Serum leptin may play a vital role in FD and was raised in dysmotility-like dyspepsia characterized by early satiety, nausea, and fullness. CCK may participate in the fundus relaxation and the inhibition of antral motility.
Intravenous CCK octapeptide (CCK-8) and CCK-33 infusions result in delayed gastric emptying.\textsuperscript{[23]} Leptin in the gastrointestinal tract stimulates CCK production, and in turn, CCK further increases gastrointestinal and plasma leptin levels in a positive feedback loop.\textsuperscript{[31]} In this study, consistent with the previous reports, leptin concentration in FD rats was higher in serum, duodenum, antrum, and hypothalamus. However, the CCK level conflicts with the previous studies. CCK level in FD rats was weaker than that of the control rats in the duodenum antrum, and greater in the hypothalamus. Although the result conflicts with the studies indicating that CCK inhibits the gastric motility, it conforms with the finding that depressed CCK concentration in FD patients was found in response to duodenal lipid.\textsuperscript{[34]} Lower gastrointestinal CCK level may be due to a higher CCK release or a lower basal CCK concentration compared with normal rats. In addition, leptin signaling appears to be involved in the satiating effect of CCK.\textsuperscript{[23]} All these indicate that endogenous CCK plays a weaker role in the regulation of gastrointestinal motility. Two different receptor subtypes, CCKAR and CCKBR, have been found in the CNS and ENS. CCKAR, widely distributed in the gastrointestinal tract regulates the gastrointestinal motility, while CCKBR that is widely distributed throughout the CNS regulates stress and emotion.\textsuperscript{[32]} Our results showing that higher CCK level was found in hypothalamus may indicate that FD rats were anxious and nervous and the regulation of CCK in the hypothalamus may involve multiple pathways in the CNS and ENS.

**CONCLUSION**

In conclusion, rats in the modified model appear an obvious parasecretion of brain-gut peptides and gastrointestinal motility disorder, which is in accordance with the symptoms of FD.

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**Conflicts of interest**

There are no conflicts of interest.

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