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Editorial board member of *World Journal of Gastroenterology*, Alexander Link, MD, PhD, Academic Research, Associate Professor, Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University Hospital Magdeburg, Magdeburg 39120, Germany

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Case Control Study

Increased intestinal mucosal leptin levels in patients with diarrhea-predominant irritable bowel syndrome

De-Rong Liu, Xiao-Juan Xu, Shu-Kun Yao

De-Rong Liu, Graduate School, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100730, China

De-Rong Liu, Shu-Kun Yao, Department of Gastroenterology, China-Japan Friendship Hospital, Beijing 100029, China

Xiao-Juan Xu, Department of Gastroenterology, First Hospital of Shanxi Medical University, Taiyuan 030001, Shanxi Province, China

ORCID number: De-Rong Liu (0000-0002-7052-5246); Xiao-Juan Xu (0000-0001-6658-7279); Shu-Kun Yao (0000-0002-8512-2589).

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Correspondence to: Shu-Kun Yao, MD, PhD, Professor, Department of Gastroenterology, China-Japan Friendship Hospital, No. 2, Yinghua East Road, Chaoyang District, Beijing 100029, China. shukun Yao@126.com

Telephone: +86-10-84205288

Fax: +86-10-84205288

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Abstract

AIM

To measure the leptin levels in patients with diarrhea-predominant irritable bowel syndrome (IBS-D) and analyze the relationship of leptin with clinical features, visceral sensitivity, mast cells, and nerve fibers.

METHODS

Forty-two patients with IBS-D fulfilling the Rome III criteria and 20 age- and sex-matched healthy controls underwent clinical and psychological evaluations using validated questionnaires (including IBS Symptom Severity Scale, IBS-specific Quality of Life, Hamilton Anxiety Scale, and Hamilton Depression Scale), along with colonoscopy, colonic mucosal biopsy, and visceral sensitivity testing. Serum leptin levels were assayed using enzyme-linked immunosorbent assay. Mucosal leptin expression and localization were evaluated using immunohistochemistry and immunofluorescence.

Mucosal leptin mRNA levels were quantified using quantitative real-time reverse transcription polymerase chain reaction. Mast cell counts and activation rates were investigated by toluidine blue staining. Correlation analyses between these parameters were performed.

RESULTS

There were no statistically significant differences in age, gender, or body mass index between the IBS-D group and the control group. The median IBS Symptom Severity Scale score in the IBS-D group was 225.0 (range, 100-475). IBS-D patients had significantly increased anxiety [IBS-D: median, 6.5; interquartile range (IQR), 3.3; control: median, 2.0; IQR, 2.0; $P < 0.001$] and depression (IBS-D: median, 7.0; IQR, 3.0; control: median, 3.0; IQR, 2.0; $P < 0.001$) scores. IBS-D patients had significantly lower first sensation threshold (IBS-D: median, 50.6; IQR, 25.9; control: median, 80.5; IQR, 18.6; $P < 0.001$), defecation sensation threshold (IBS-D: median, 91.5; IQR, 29.3; control: median, 155.0; IQR, 21.1; $P < 0.001$) and maximum tolerable threshold (IBS-D: median, 163.2; IQR, 71.2; control: median, 226.2; IQR, 39.3; $P < 0.001$). Mucosal leptin expression, as reflected by integrated optical density (IBS-D: median, 4424.71; IQR, 4533.63; control: median, 933.65; IQR, 888.10; $P < 0.001$), leptin mRNA expression (IBS-D: median, 1.1226; IQR, 1.6351; control: median, 0.8947; IQR, 0.4595; $P = 0.009$), and mast cell activation rate (IBS-D: median, 71.2%; IQR, 12.9%; control group: median, 59.4%; IQR, 18.88%; $P < 0.001$) were significantly increased in IBS-D patients. The colocalization of leptin and leptin receptors was observed on mast cells and PGP9.5-positive nerve fibers in the intestinal mucosa. Also, leptin expression was positively correlated with anxiety, depression, and the mast cell activation rate, but negatively correlated with the defecation sensation threshold and the maximum tolerance threshold during visceral sensitivity testing (adjusted $P < 0.0038$).

CONCLUSION

Increased levels of mucosal leptin may interact with mast cells and the nervous system to contribute to the pathogenesis of IBS-D.

Key words: Leptin; Irritable bowel syndrome; Mast cells; Diarrhea; Visceral hypersensitivity

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Core tip: Leptin is an important cytokine that exerts significant biological effects on gastrointestinal function and immune system modulation. We found that diarrhea-predominant irritable bowel syndrome (IBS-D) patients had significantly increased psychological symptoms, visceral hypersensitivity, mucosal leptin expression, leptin mRNA expression, and mast cell activation rate. Additionally, leptin expression was positively correlated with anxiety, depression, and the mast cell activation rate, but negatively correlated

with the defecation sensation threshold and maximum tolerance threshold during visceral sensitivity testing. Increased levels of mucosal leptin may interact with mast cells and the nervous system to contribute to the pathogenesis of IBS-D.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a commonly diagnosed functional gastrointestinal disease, with a prevalence rate of 5%-10% in most European countries, the United States, and China^[1]. IBS is a typically recurrent disease characterized by abdominal pain or discomfort, stool irregularities, and bloating^[2]. Symptoms may worsen over time and significantly impact patients' quality of life and work productivity^[3]. A major subtype of IBS is IBS with diarrhea (IBS-D), which accounts for 46% of all IBS cases^[4].

The pathogenesis of IBS-D is complex and poorly understood. Recent studies of the pathophysiology of IBS-D have focused on molecular mechanisms and have suggested that the levels of luminal and mucosal cytokines originating from the gastrointestinal tract may be altered in the gut of IBS-D patients. These alterations could result in dysregulation of gastrointestinal secretion and motility and an increase in visceral hypersensitivity. These cytokines can also affect the peripheral and central nervous systems and disrupt communication between the brain and gut^[5].

Leptin is a 16 kDa non-glycosylated peptide hormone belonging to the type I cytokine superfamily^[6]. It is produced predominantly by mature adipocytes, but also by gastric and colonic epithelial cells, immune cells, placental trophoblasts, amniotic cells, chondrocytes, and synoviocytes^[7]. When bound to receptor sites, leptin not only exerts significant biological effects, such as appetite control, by signaling satiety and increasing energy expenditure, but also modulates the immune system^[8] and gastrointestinal function^[9,10].

The number of mucosal mast cells has been reported to be increased in IBS-D patients^[11], and mast cell number often correlates with symptoms including abdominal pain^[12] and bloating^[13]. Mast cells are key to the induction and maintenance of low-grade immune activation in IBS-D patients^[14]. Gastrointestinal mast cells can express leptin and leptin receptors, indicating the modulatory effects of leptin on mast cells^[15].

The enteric nervous system (ENS) is important in regulating gut motility, secretion, and nutrient absorption. Leptin has been shown to affect the ENS^[16,17] and

sensory afferent neurons^[18]. Taken together, leptin may be involved in the regulation of both local gastrointestinal functions and the brain-gut axis.

Few studies have specifically addressed the role of leptin in the pathogenesis of IBS^[19–21]. Therefore, the present study measured leptin expression in both the serum and intestinal mucosa of patients with IBS-D, and then analyzed the relationship of leptin with the clinical features, visceral sensitivity, the number and activation rate of mast cells, and nerve fibers in these patients

MATERIALS AND METHODS

Subjects

We recruited 42 IBS-D patients (15 women and 27 men; mean age, 29.4 years; age range, 22–40 years) and 20 healthy controls (8 women and 12 men; mean age, 28.9 years; age range, 20–38 years). All patients were treated at the gastroenterology department of the China-Japan Friendship Hospital from January 2016 to July 2016. IBS-D was diagnosed according to the Rome III criteria. Controls were recruited through public advertisement or from asymptomatic patients undergoing colonoscopy for colorectal cancer screening or polyposis follow-up. Exclusion criteria included the use of anti-spasmodics, analgesics, antibiotics, nonsteroidal anti-inflammatory drugs, corticosteroids, mast cell stabilizers, and anti-depressants or a history of organic diseases (documented by patient history, appropriate consultation, and laboratory testing), celiac disease (documented by celiac serology), allergic disease, major abdominal surgery, and severe psychiatric disorders.

All participants underwent venous blood sampling and colonoscopy. Before colonoscopy, all subjects underwent a standard bowel preparation in the form of 3 L of dissolved polyethylene glycol electrolyte powder (Fortrans, Beaufour Ipsen Industrie, Dreux, France). Four biopsies were obtained in each subject from the rectosigmoid junction in order to standardize the site of sampling. Two biopsy specimens were used for routine hematoxylin and eosin (H and E) staining, mast cell staining, and immunohistochemistry. The other two biopsy specimens were used for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR).

IBS-D patients and control patients both gave informed consent in a written form before entry into the study. The study protocol was approved by the China-Japan Friendship Hospital Ethics Committee (No. 2015-33) and the study was conducted in accordance with the Declaration of Helsinki.

Measures

Questionnaires: Clinical status was evaluated with the use of validated questionnaires. The IBS Symptom Severity Scale^[22,23], a 5-item self-reporting questionnaire designed to measure disease severity, was given to each participant to complete. Questionnaire items

included information regarding abdominal pain, bloating, satisfaction with bowel habits, and overall interference with quality of life. The total score of the questionnaire ranges from 0 to 500.

The IBS-specific Quality of Life questionnaire^[24,25] was used to evaluate changes in patients' quality of life. This scale evaluates 34 broad well-being factors based on variables including feelings of dysphoria, social interactions, body image, and health worries. The Hamilton Anxiety Scale^[26,27] and Hamilton Depression Scale^[27,28] were used to measure anxiety and depression, respectively.

Visceral sensitivity testing: Participants were placed in the left lateral decubitus position. After a digital rectal examination, a lubricated catheter with a latex balloon at the tip (Medical Measurement Systems, Enschede, the Netherlands) was inserted into the rectum such that the balloon was 8 cm proximal to the anal verge. Then, the balloon was inflated at a speed of 10 mL/5 s. During balloon inflation, feelings of initial perception, defecation urge, and discomfort/pain were reported by the participants. The balloon volumes were recorded at the first sensation threshold, defecating sensation threshold, and maximum tolerable threshold. All the tests were performed by the same investigator between 1 and 3 PM in a blinded fashion.

Serum leptin level: All fasting blood samples from both IBS-D patients and controls were collected between 11 AM and 1 PM to avoid diurnal variation. Whole blood was centrifuged immediately at 1151 *g* for 10 min, and stored at -80 °C until assay. Serum leptin levels were measured in duplicate using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Human Leptin Instant ELISA; Bender MedSystems GmbH, Vienna, Austria).

Histology, immunohistochemistry, and immunofluorescence: H and E sections from formalin-fixed and paraffin-embedded tissue samples were blindly assessed by independent observers. Mast cells were stained with toluidine blue. The slides were first soaked in 0.5% toluidine blue, and then differentiated with acetone. Afterwards, five 400 × magnification fields (field area, 0.237 mm²) were chosen randomly and scanned. Mast cells were identified using light microscopy by their metachromatic cytoplasmic granules. Finally, mast cell degranulation was assessed based on unclear or irregular cell membranes and the presence of extruded secretory granules. The mean mast cell number per millimetre square of the mucosal area (/mm²) was calculated. The percentage of degranulated mast cells (mast cell activation rate) was also calculated in each section (degranulated mast cells/the total number of mast cells × 100%).

Immunohistochemistry and immunofluorescence experiments were performed on paraffin-embedded, 4-mm-thick sections. The following primary antibodies

were used: rabbit polyclonal anti-leptin antibody (1:100; Abcam, Cambridge, United Kingdom), rabbit polyclonal anti-leptin receptor antibody (1:50; Abcam, Cambridge, United Kingdom), mouse monoclonal anti-mast cell tryptase antibody (1:50; Abcam, Cambridge, United Kingdom), and mouse monoclonal anti-PGP9.5 antibody (1:50; Abcam, Cambridge, United Kingdom).

For immunohistochemistry studies, the sections were first incubated with the primary antibody overnight. Next, they were incubated at room temperature with a universal secondary antibody (EnVision Detection Systems, Dako, Denmark) for 60 min and then visualized using diaminobenzidine. Finally, the nuclei were labeled by counterstaining the sections with Mayer's hematoxylin. The quantification of immunoreactivity was performed by two operators in a blinded fashion. Three randomly selected fields from each section were scanned under a Nikon Eclipse 80i microscope (Nikon Instruments Co., Ltd., Tokyo, Japan). Photographs were taken with a Nikon DS-Ri1 camera (Nikon Instruments Co., Ltd., Tokyo, Japan). The images were analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, United States). During the analysis, the average integrated optical density (IOD) of positive staining substances of three non-overlapping fields chosen randomly was measured for the final analysis.

For immunofluorescence studies, the sections were prepared sequentially as follows: incubated with primary antibody (double labeling) overnight, rinsed with phosphate-buffered saline, and incubated at room temperature with goat anti-mouse IgG H and L (Alexa Fluor 647) (1/300; Abcam, Cambridge, United Kingdom) or goat anti-rabbit IgG H and L (Alexa Fluor® 488) (1/400; Abcam, Cambridge, United Kingdom) for 2 h. Specimens were examined by two operators in a blinded fashion using a Nikon Eclipse 90i microscope (Nikon Instruments Co., Ltd., Tokyo, Japan). Representative photomicrographs were taken using a Nikon DS-Ri1 camera.

Leptin gene expression analysis using qRT-PCR: The gene expression of leptin and the leptin receptor in the colonic mucosa was analyzed using qRT-PCR. Each supernatant was assayed in duplicate, and altogether three independent experiments were conducted. TRIzol reagent (Invitrogen Life Technologies, Waltham, MA, United States) was used to isolate total RNA. Then, the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, United States) was used to perform reverse transcription. Next, real-time PCR was carried out using the FastStart Universal SYBR Green Master Rox kit (Roche, Shanghai, China) in the StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, United States). Finally, leptin and leptin receptor mRNA expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The following primers were used for PCR amplification:

forward, 5'-ACTTTGGTATCGTGGAAGGACTCAT-3' and reverse, 5'-GTTTTCTAGACGGCAGGTCAGG-3' for GAPDH; forward, 5'-ACACGCAGTCAGTCTCCTCCAA-3' and reverse, 5'-CTGGAAGGCATACTGGTGAGGA-3' for leptin; and forward, 5'-GCCAACAGCCAACTCAACG-3' and reverse, 5'-GGTGGGCTGGACCAAGAAATC-3' for leptin receptor. The results were first normalized to GAPDH expression using the $2^{-\Delta\Delta Ct}$ method^[29]. Then, the leptin and leptin receptor mRNA levels in each sample are expressed as the fold-change relative to the average level of healthy controls, in whom the mean value was used as the reference and considered as 1.

Statistical analysis

All statistical analyses were performed using SPSS for Windows software, version 24.0 (SPSS Inc, Chicago, IL). All data are reported as values (mean \pm SD) or medians [interquartile range (IQR)]. Independent sample *t*-tests or nonparametric Mann-Whitney *U*-tests were used to analyze quantitative data. The χ^2 test or Fisher's exact test were used to analyze qualitative data. Correlations between two parameters were performed using Spearman's correlation coefficient, followed by Bonferroni correction to adjust multiple comparisons, with a corrected significance level of 0.0038 (0.05/13). Two-tailed *P*-values < 0.05 were considered significant.

RESULTS

Demographic and clinical characteristics

There were no statistically significant differences in age, gender, or body mass index between the IBS-D group and the control group. The median IBS Symptom Severity Scale score in the IBS-D group was 225.0 (range, 100-475). There was no significant difference between male and female IBS-D patients in terms of IBS Symptom Severity Scale score (*P* = 0.053). The median duration of disease was 3.5 years (range, 1-13 years) in the IBS-D patients. The IBS-specific Quality of Life score was significantly lower in IBS-D patients (*P* < 0.001), and the differences in the scores of both the Hamilton Anxiety Scale and the Hamilton Depression Scale were also statistically significant between the IBS-D group and the control group (*P* < 0.001) (Table 1).

Visceral sensitivity test

The first sensation threshold, defecation sensation threshold, and maximum tolerable threshold were significantly lower in the IBS-D patients compared with those in the control group (*P* < 0.001) (Table 1).

Serum leptin

Serum leptin levels in the IBS-D patients (median, 1.28 ng/mL; IQR, 1.63 ng/mL) and healthy controls (median, 1.27 ng/mL; IQR, 0.83 ng/mL) were not

Table 1 Demographics, clinical characteristics, and visceral sensitivity of irritable bowel syndrome patients and healthy controls

	IBS-D	Controls	P value
Demographic and clinical feature			
N	42	20	NA
Age (yr)	29.4 ± 4.3	28.9 ± 5.1	0.673
Gender (male: female)	9:5	3:2	0.744
Body mass index (kg/m ²)	22.5 ± 2.5	22.1 ± 2.2	0.553
Duration of disease (yr)	3.5 (3.3)	NA	NA
Questionnaire			
IBS Symptom Severity Scale	225.0 (50.0)	NA	NA
IBS-specific Quality of Life	76.5 (17.3)	100.0 (1.1)	< 0.001
Hamilton Anxiety Scale	6.5 (3.3)	2.0 (2.0)	< 0.001
Hamilton Depression Scale	7.0 (3.0)	3.0 (2.0)	< 0.001
Visceral sensitivity			
First sensation threshold (mL)	50.6 (25.9)	80.5 (18.6)	< 0.001
Defecating sensation threshold (mL)	91.5 (29.3)	155.0 (21.1)	< 0.001
Maximum sensation threshold (mL)	163.2 (71.2)	226.2 (39.3)	< 0.001

The data are expressed as mean ± SD, or median (interquartile range).
IBS-D: Irritable bowel syndrome; NA: Not applicable.

significantly different ($P = 0.657$).

Mucosal histology and mast cell staining

On H and E histology, all colonic mucosal biopsies were reported as normal (Figure 1A and B). Toluidine blue staining revealed that mast cells were often scattered in the lamina propria and submucosa (Figures 1C and D). The mast cell count was not significantly different between the two groups (IBS group: median, 9.62/mm²; IQR, 1.61/mm² vs control group: median, 10.75/mm²; IQR, 4.52/mm²; $P = 0.164$) (Figure 1E). However, the percentage of degranulated mast cells was significantly increased in IBS-D patients (IBS group: median, 71.2%; IQR, 12.9% vs control group: median, 59.4%; IQR, 18.8%; $P < 0.001$) (Figure 1F).

Leptin and leptin receptor expression in the colonic mucosa

In the colonic mucosa, leptin and leptin receptor staining was mostly scattered in the epithelium and lamina propria (Figure 2A-D). The leptin protein level in mucosal biopsies was significantly increased in the IBS-D patients (IOD median, 4424.71; IQR, 4533.63) vs the control group (IOD median, 933.65; IQR, 888.10; $P < 0.001$) (Figure 2E). The leptin receptor expression was not significantly increased in the colonic mucosa obtained from IBS-D patients (12454.27 ± 6946.06) compared to controls (12508.07 ± 8088.46) ($P = 0.979$) (Figure 2F).

Colocalization of leptin and leptin receptors on mast cells and PGP9.5-positive nerve fibers

Leptin and tryptase double-labeling immunofluorescence experiments recorded colocalization of leptin and tryptase, indicating that mast cells were producers of leptin (Figure 3A and B). Moreover, leptin receptor and tryptase colocalization was found to exist in the

colonic mucosa (Figure 4A and B). Double-staining experiments confirmed leptin receptor expression in PGP9.5-immunoreactive nerve fibers (Figure 4C and D).

Leptin and leptin receptor gene expression in the colonic mucosa

Leptin mRNA expression was significantly increased in IBS-D patients (median, 1.1226; IQR, 1.6351) vs that seen in the control group (median, 0.8947; IQR, 0.4595; $P = 0.009$). Similarly, leptin receptor gene expression was also significantly increased in IBS-D patients (median, 1.5491; IQR, 2.0721) vs that seen in the control group (median, 0.9062; IQR, 0.3850; $P = 0.001$).

Correlation analysis

In patients with IBS-D, the leptin protein expression level showed a significantly positive correlation with anxiety, depression, and mast cell activation rate, and a negative correlation with the defecation sensation threshold and maximum tolerable threshold (adjusted $P < 0.0038$). The leptin mRNA level was significantly positively correlated with IBS symptom severity, anxiety, and depression, and negatively correlated with the visceral sensation threshold (adjusted $P < 0.0038$) (Table 2).

DISCUSSION

The present study investigated the possible role of leptin in the pathogenesis of IBS-D. Biopsies from IBS-D patients revealed significant upregulation of both leptin protein and mRNA levels. The increased leptin protein or mRNA expression closely correlated with IBS symptom severity, anxiety, depression, and visceral sensation thresholds, which may provide a putative basis for the development of IBS symptoms. To our knowledge, this was the first study to preliminarily confirm that leptin may play a certain role in the pathophysiology of IBS-D.

Visceral hypersensitivity has been identified in 20%-90% of IBS patients and is defined as a low threshold for the perception of stimuli arising from the gut^[30,31]. Mechanisms of visceral hypersensitivity are complex, including the sensitization of gut wall sensory endings, enhanced nociceptive information, reduced antinociceptive effects at the level of the spinal cord, and hypervigilance of the central nervous system^[31]. In line with these mechanisms, the present study found that visceral sensation thresholds were significantly lower in IBS-D patients than those of healthy controls. Limitations regarding the method of rectal distention used were discussed in our previous study^[32] and include: (1) the sensory threshold, reported by the subject, may be susceptible to a perceptual response bias^[33]; (2) progressive rectal distention may lead to hypervigilance and alter the response of the participants; and (3) volume measurements, rather than balloon pressures recorded with a barostat, may

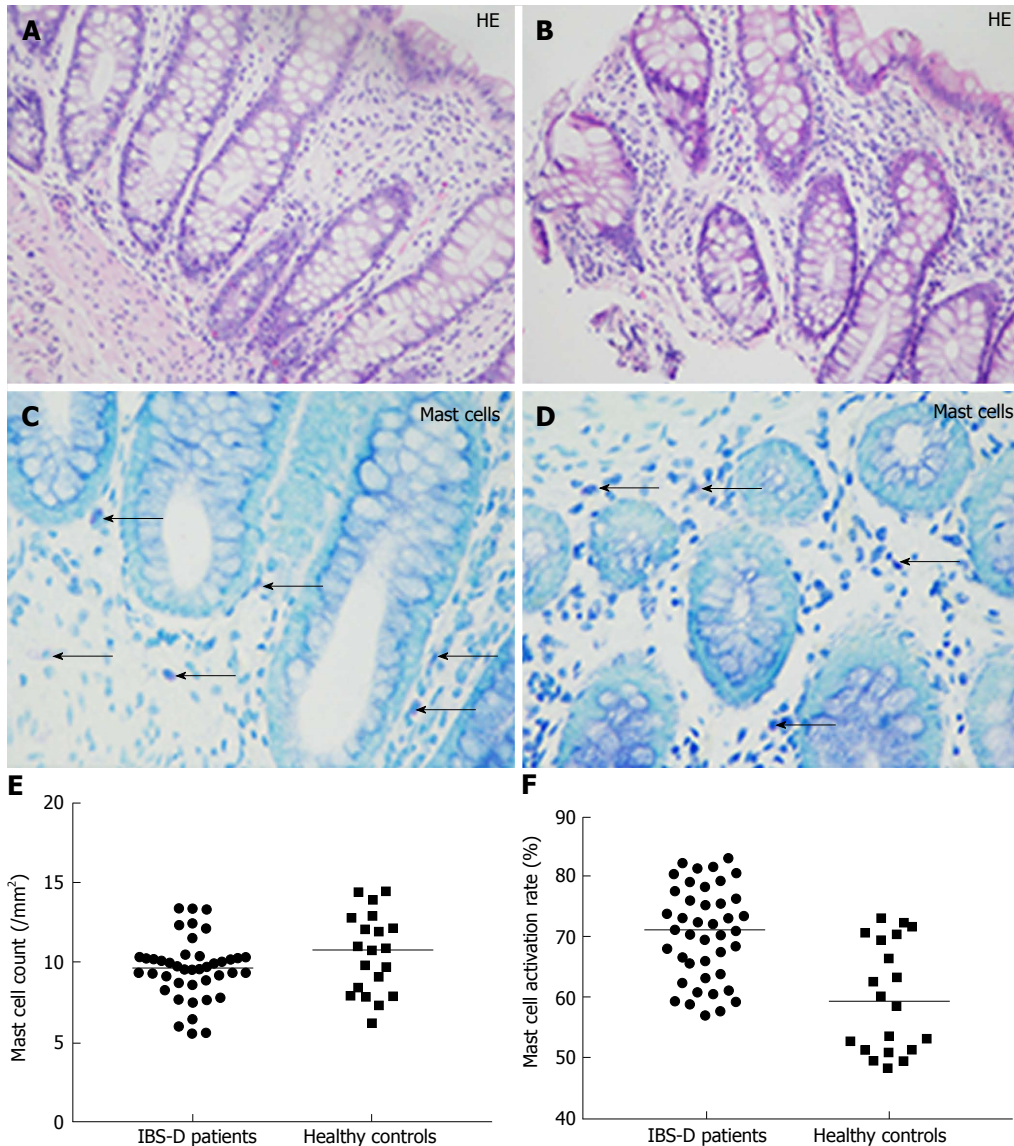


Figure 1 Intestinal mucosal histology (hematoxylin and eosin staining), mast cell toluidine blue staining, mast cell count ($/mm^2$), and mast cell activation rate (%) in irritable bowel syndrome patients and controls. A: Colonic mucosal histology of IBS-D patients ($\times 200$); B: Colonic mucosal histology of healthy controls ($\times 200$); C: Mast cell toluidine blue staining of IBS-D tissue ($\times 400$, arrows represent mast cells); D: Mast cell toluidine blue staining of normal control tissue ($\times 400$, arrows represent mast cells); E: Mast cell count was not significantly different between the two groups ($P = 0.164$); F: Mast cell activation rate significantly increased in IBS-D patients ($P < 0.001$). Line in the scatter plots means the median. IBS-D: Irritable bowel syndrome with diarrhea.

not accurately reflect the biological properties of the rectum, leading to measurement errors^[34]. In spite of these limitations, this method is convenient to perform and well-tolerated by patients. Furthermore, some studies have shown that volume thresholds are a valid measure of rectal sensory function^[35-38].

Mast cell proliferation has been recognized as a common feature of IBS patients^[39-41]. Some studies have suggested that the number of mast cells is increased in IBS patients^[42,43], while other studies have shown that the density of mast cells is not significantly different in these patients^[44-46]. Additional studies have shown that the degranulation or activation rate of mast cells, rather than the number of mast cells, best reflects the severity of IBS in patients^[47]. In IBS patients, activated mast cells release mediators such as histamine, serotonin,

and bradykinin^[41]. These mediators contribute to the development of major IBS-D symptoms through the modulation of visceral sensation, gastrointestinal motility, intestinal immune function, and epithelial permeability and secretion^[41]. In the present study, the mast cell activation rate was found to be significantly higher in IBS-D patients, while the mast cell count was not significantly different from that seen in the control group, indicating that activated mast cells are important in the pathophysiology of IBS-D.

Few studies have specifically analyzed serum leptin levels in IBS patients^[19-21], and the results of these studies have been inconsistent. In the present study, serum leptin levels in patients with IBS-D were not significantly different from those seen in healthy controls. However, both leptin and leptin receptor

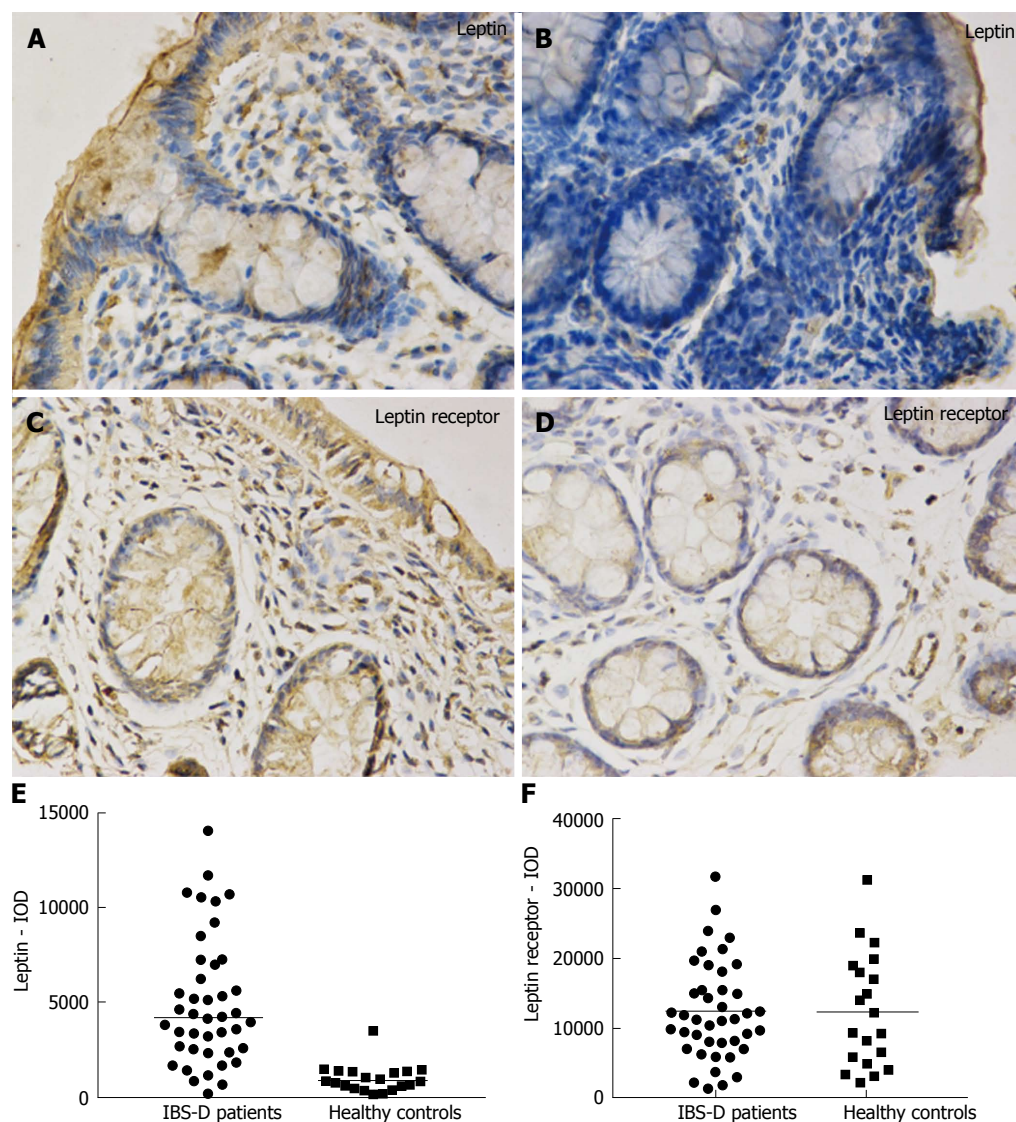


Figure 2 Immunohistochemical staining for leptin and leptin receptor in irritable bowel syndrome patients and healthy controls. A: Colonic mucosal leptin expression in IBS-D patients ($\times 400$); B: Colonic mucosal leptin expression in healthy controls ($\times 400$); C: Colonic mucosal leptin receptor expression in IBS-D patients ($\times 400$); D: Colonic mucosal leptin receptor expression in healthy controls ($\times 400$); E: Colonic mucosal leptin immunoreactivity significantly increased in IBS-D patients than in the healthy controls; F: Colonic mucosal leptin receptor immunoreactivity did not increase significantly in IBS-D patients than in healthy controls. Line in the scatter plots means the median. IBS-D: Irritable bowel syndrome with diarrhea; IOD: Integral optical density.

mRNA levels were significantly increased in IBS-D patients compared to the control group. Complex factors influencing serum leptin levels and the relatively small sample size may have contributed to the lack of a difference in serum leptin levels.

The potential role of immune activation in the pathogenesis and symptom formation in IBS patients has been reported in previous studies^[14,48,49]. Mast cells are a key component in the induction and maintenance of low-grade immune activation in IBS patients^[48]. A recent report has demonstrated that leptin, a regulatory hormone that has multiple modulatory effects on immune cells, including T cells, macrophages, mast cells, dendritic cells, neutrophils, eosinophils, basophils, and NK cells, was able to promote the generation of an inflammatory phenotype of mast cells through the induction of interferon- γ and the suppression of

interleukin-4^[50]. In animal experiments, leptin has been proven to be effective in activating the colonic submucosal and myenteric neurons of guinea pigs^[16] and in modulating the activity of enteric inhibitory and excitatory neurons in the proximal colon of rats^[17]. It has also been reported that leptin can stimulate the activity of sensory afferent neurons^[18]. In this study, the expression and location of leptin, leptin receptors, tryptase, and PGP9.5 were analyzed by immunohistochemistry and immunofluorescence. We found that the expression of leptin, rather than the leptin receptor, increased significantly. In addition, the colocalization of leptin and leptin receptors was observed on mast cells and PGP9.5-positive nerve fibers in the intestinal mucosa. These results indicate that leptin may be involved in the pathogenesis of IBS-D *via* mechanisms similar to those noted in the

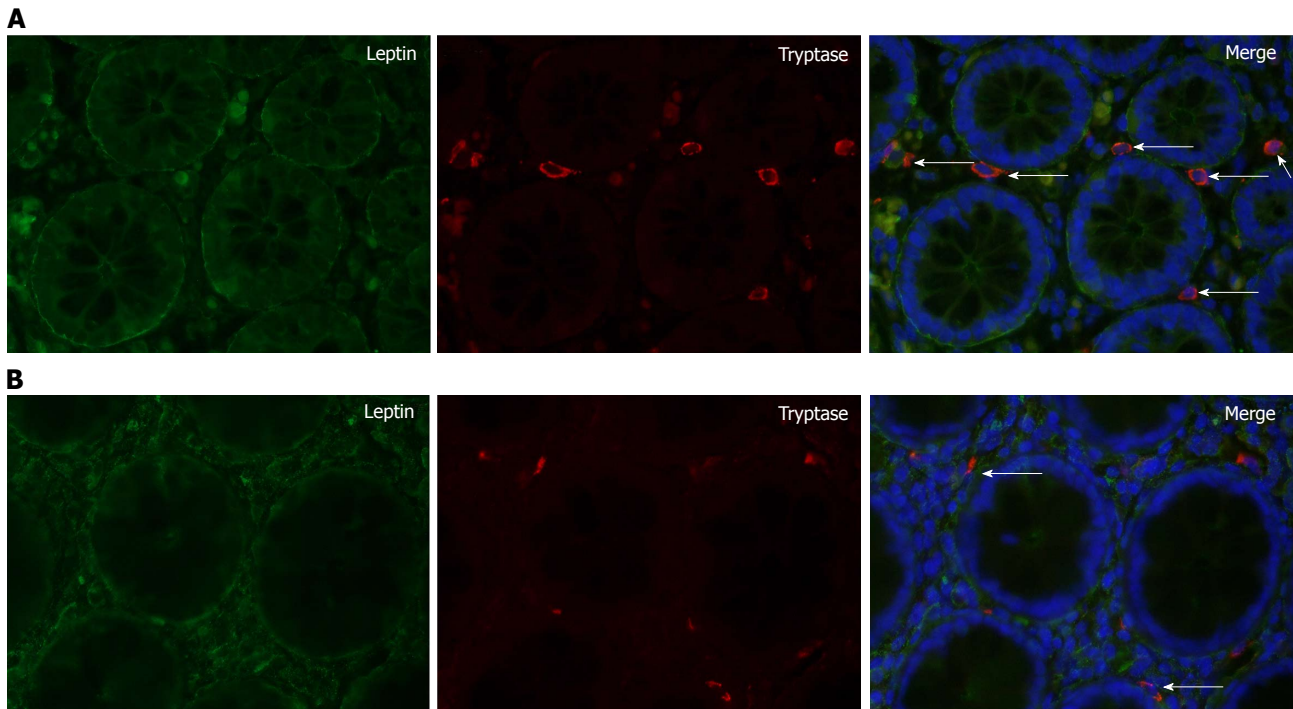


Figure 3 Double-labeling immunofluorescence of leptin and tryptase in irritable bowel syndrome patients and healthy controls. A: Colonic mucosal double-labeling immunofluorescence of leptin and tryptase in IBS-D patients ($\times 400$, arrows represent colocalization of leptin and tryptase); B: Colonic mucosal double-labeling immunofluorescence of leptin and tryptase in healthy controls ($\times 400$, arrows represent colocalization of leptin and tryptase). IBS-D: Irritable bowel syndrome with diarrhea.

Table 2 Correlation between leptin level and clinical and experimental parameters in irritable bowel syndrome patients

	Leptin expression	Leptin mRNA
Questionnaire		
IBS-SSS	0.417 (0.006) ¹	0.678 (< 0.001)
HAMA	0.540 (< 0.001)	0.675 (< 0.001)
HAMD	0.488 (0.001)	0.589 (< 0.001)
Visceral sensitivity		
First sensation	-0.438 (0.004) ¹	-0.604 (< 0.001)
Defecating sensation	-0.654 (< 0.001)	-0.480 (0.001)
Maximum tolerance	-0.576 (< 0.001)	-0.706 (< 0.001)
Mucosal parameter		
Mast cell activation rate (%)	0.510 (0.001)	0.430 (0.005) ¹
Leptin mRNA	0.666 (< 0.001)	NA
Leptin expression	NA	0.666 (< 0.001)

¹Without significance after Bonferroni correction (adjusted $P < 0.0038$). Spearman's correlation coefficients are expressed with P values in parentheses. IBS-D: Irritable bowel syndrome with diarrhea; IBS-SSS: IBS Symptom Severity Scale; HAMA: Hamilton Anxiety Scale; HAMD: Hamilton Depression Scale; NA: Not applicable.

above studies.

Correlation analyses were performed between intestinal mucosa leptin levels and other parameters. Leptin expression was positively correlated with anxiety, depression, and the mast cell activation rate, and was negatively correlated with the defecation sensation threshold and the maximum tolerable threshold during visceral sensitivity testing. Leptin mRNA levels were positively correlated with disease severity, anxiety, and depression, and negatively correlated with the visceral

sensation threshold. The correlation analyses indicate that leptin might be an important factor involved in the pathogenesis and symptom formation of IBS-D patients. However, correlations cannot be interpreted as causal associations, and further studies are needed to confirm this finding.

Based on the results of our study, we speculate that psychosocial and other stimulations cause disruption in the brain-gut axis^[51]. Corticotrophin-releasing hormone (CRH) and cortisol are released with activation of the hypothalamic-pituitary axis^[51]. CRH and cortisol are also increased with the production of leptin^[51,52]. Also, intestinal mucosal mast cells produce histamine, serotonin, and bradykinin when activated. As a key component of low-grade mucosal inflammation, mast cells also cause increased epithelial permeability, visceral hypersensitivity, and a disruption of gastro-intestinal secretory and motility functions. Leptin-stimulated mast cells modulate low-grade mucosal inflammation, activate nervous system, and increase epithelial tight junction permeability^[53], all of which contribute to the development of IBS-D symptoms.

There were several limitations in this study. First, because of the nature of our research foundation^[32,54], the present study focused on patients with the IBS-D subtype. Therefore, the findings may not be generalizable to constipation-predominant IBS, mixed-type IBS, and untyped IBS patients. Second, leptin has been shown to increase during the process of inflammation^[55]. However, we cannot be sure that all postinfective IBS patients were excluded from the

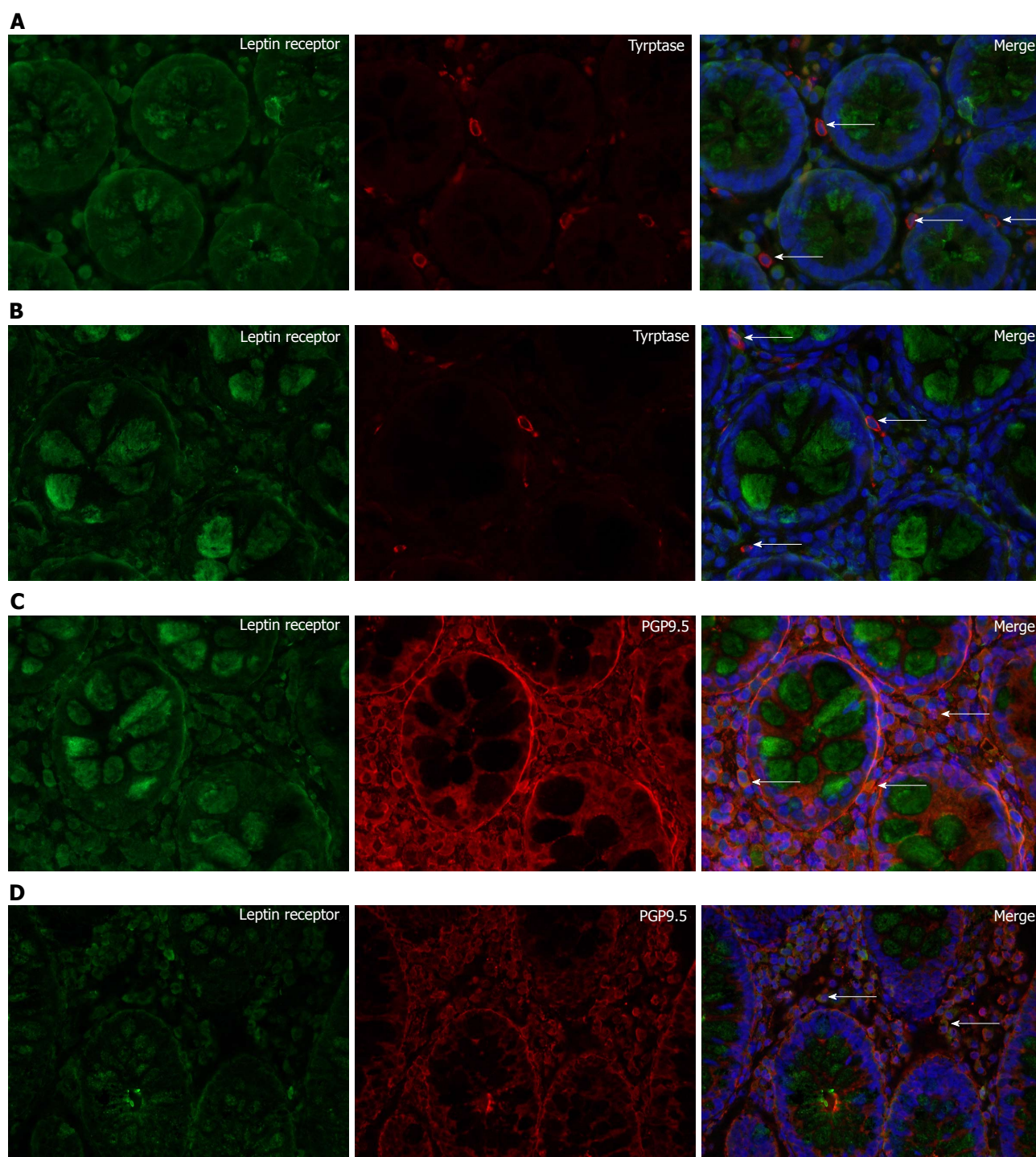


Figure 4 Double-labeling immunofluorescence of leptin receptor with tryptase and PGP9.5 in irritable bowel syndrome patients and healthy controls ($\times 400$ magnification). A: Colonic mucosal double-labeling immunofluorescence of leptin receptor and tryptase in IBS-D patients ($\times 400$, arrows represent colocalization of leptin receptor and tryptase); B: Colonic mucosal double-labeling immunofluorescence of leptin receptor and tryptase in healthy controls ($\times 400$, arrows represent colocalization of leptin receptor and tryptase); C: Colonic mucosal double-labeling immunofluorescence of leptin receptor and PGP9.5 in IBS-D patients ($\times 400$, arrows represent colocalization of leptin receptor and PGP9.5); D: Colonic mucosal double-labeling immunofluorescence of leptin receptor and PGP9.5 in healthy controls ($\times 400$, arrows represent colocalization of leptin receptor and PGP9.5). PGP9.5: Protein gene product 9.5; IBS-D: Irritable bowel syndrome with diarrhea.

study. Because these two subgroups may be different in pathogenesis, future experiments should study these two types separately. Third, due to the lack of previous data related to our study, we did not estimate sample size and power, which may reduce the reliability of our conclusion. Fourth, in qRT-PCR, we used only

one reference gene (GAPDH), which may influence the accuracy of the results. Finally, we cannot make a cause and effect inference on the basis of our findings; additional studies of the effects of leptin on mast cells and the nervous system as well as larger clinical studies are needed.

ARTICLE HIGHLIGHTS

Research background

Irritable bowel syndrome (IBS) is a commonly diagnosed functional gastrointestinal disease. Symptoms may worsen over time and significantly impact patient quality of life and work productivity. A major subtype of IBS is IBS with diarrhea (IBS-D). The pathogenesis of IBS-D is complex and poorly understood. Leptin not only exerts significant biological effects, such as appetite control, by signaling satiety and increasing energy expenditure, but also modulates the immune system and gastrointestinal function. Few studies have specifically addressed the role of leptin in the pathogenesis of IBS. To our knowledge, this was the first study to preliminarily investigate the possible role of leptin in the pathophysiology of IBS-D.

Research motivation

The main topics of the present study include evaluating clinical symptoms, psychological characteristics, and visceral sensitivity of IBS-D patients and healthy controls; examining leptin expression, mast cells, and PGP9.5 nerve fibers in IBS-D and healthy controls; and performing correlation analyses between these parameters. Our study proposed a mechanism by which leptin may contribute to the pathophysiology of IBS.

Research objectives

This study aimed to measure leptin expression in both the serum and intestinal mucosa of patients with IBS-D subtype disease and to analyze the relationship of leptin with the clinical features, visceral sensitivity, and number of mast cells and nerve fibers in these patients.

Research methods

Participants underwent clinical and psychological evaluations using validated questionnaires (including IBS Symptom Severity Scale, IBS-specific Quality of Life, Hamilton Anxiety Scale and Hamilton Depression Scale), along with colonoscopy, colonic mucosal biopsy, and visceral sensitivity testing. Serum leptin levels were assayed using enzyme-linked immunosorbent assay. Mucosal leptin expression and localization were evaluated using immunohistochemistry and immunofluorescence. Mucosal leptin mRNA levels were quantified using quantitative real-time reverse transcription polymerase chain reaction. Mast cell counts and activation rates were investigated by toluidine blue staining. Correlation analyses between these parameters were performed. All statistical analyses were performed using SPSS for Windows software, version 24.0 (SPSS Inc, Chicago, IL).

Research results

The authors found that IBS-D patients had significantly increased psychological symptoms and visceral hypersensitivity, and their mucosal leptin expression, leptin mRNA levels, and mast cell activation rates were significantly increased. Also, leptin expression was positively correlated with anxiety, depression, and the mast cell activation rate, but negatively correlated with the defecation sensation threshold and the maximum tolerance threshold during visceral sensitivity testing. Increased levels of mucosal leptin may interact with mast cells and the nervous system to contribute to the pathogenesis of IBS-D.

Research conclusions

This study presents evidence that leptin levels are increased in the intestinal mucosa of IBS-D patients, proposes a mechanism by which leptin may contribute to the pathophysiology of IBS, and provides some potential avenues for more specific and effective treatments in these patients. The authors believe that this study makes a significant contribution to the literature because it is an original research that provides information that not only contributes to the understanding of the pathogenesis and pathophysiology of IBS, but also suggests area for future research and therapeutic intervention.

Research perspectives

This study preliminarily investigated the possible role of leptin involved in the pathogenesis of IBS-D. Future studies should focus on the following aspects. First, the present study focused on patients with the IBS-D subtype. Therefore, the findings may not be generalizable to constipation-predominant IBS, mixed-

type IBS, and unsubtyped IBS patients. Future research should investigate the possible role of leptin in the pathophysiology of the other three subtypes. Second, leptin has been shown to increase during the process of inflammation. However, we cannot be sure that all postinfective IBS patients were excluded from the study. Because these two subgroups may be different in pathogenesis, future experiments should study these two types separately. Finally, we cannot make a cause and effect inference on the basis of our findings, therefore, additional studies of the effects of leptin on mast cells and the nervous system as well as larger clinical studies are needed.

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