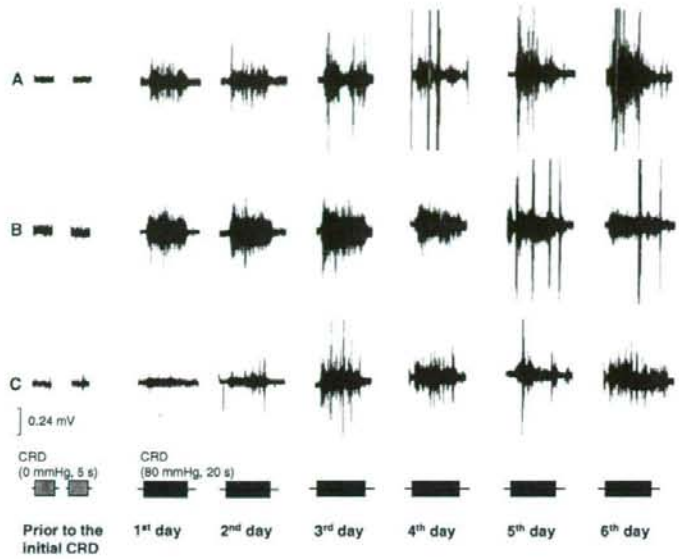

Figure 2 Visceromotor response represented as electromyographic activity prior to the initial distension (0 mmHg, 5 and 15 s before colorectal distention (CRD)) and following repetitive CRD (80 mmHg, 20 s duration). (A) Repetitive CRD alone, (B) repetitive CRD with previous inflammation and (C) repetitive CRD with previous inflammation and CP-154,526 treatment.

(Fig. 2). However, no significant group effect was detected by two-way ANOVA on percentage VMR (Fig. 3). In the rats subjected to repetitive CRD alone, VMR on the fifth and sixth days of CRD was significantly higher than that on the first, second, third and fourth days of CRD ($P < 0.001$) (Fig. 3). In TNBS-treated rats, in contrast, VMR on the third day of CRD was significantly higher than that on the first, second, fourth, fifth and sixth days of CRD ($P < 0.05$). Similarly, VMR on the first, second and third days of CRD was significantly higher than that on the fourth day of CRD ($P < 0.01$) (Fig. 3). On the first day of CRD, VMR in the initial three recordings did not significantly differ between the vehicle-treated rats (group A, Fig. 3) and TNBS-treated rats (group B, Fig. 3). However, repetitive CRD produced significantly robust contractions of the abdominal musculature in both the vehicle-treated rats ($P < 0.01$) and TNBS-treated rats ($P < 0.01$), although TNBS-treated rats showed significantly greater response than the vehicle-treated rats ($P < 0.05$). Although on the second day of CRD VMR in the vehicle-treated rats significantly increased with repeated stimuli ($P < 0.01$), it did not significantly differ from that recorded in TNBS-treated rats. On the third day of CRD, VMR in TNBS-treated rats was significantly greater than that in the vehicle-treated rats ($P < 0.01$). On the fourth day of CRD, however, VMR did not significantly differ between the vehicle-treated rats and TNBS-treated rats. On the fifth day of CRD, VMR in the vehicle-treated rats was, in contrast to the initial 3 days of CRD, significantly greater than that in TNBS-treated rats ($P < 0.05$). Finally on the sixth day of CRD, VMR did not significantly differ between the vehicle-treated rats and TNBS-treated rats.

Effects of specific CRH-R1 antagonist on previous inflammation-induced visceral hypersensitivity

Significant group effect (TNBS-treated rats and TNBS-/CRH-R1 antagonist-treated rats, $F = 12.6$, $P < 0.01$), period effect ($F = 1.5$, $P < 0.01$) and group × period interaction ($F = 2.9$, $P < 0.001$) were detected by two-way ANOVA on percentage VMR (Fig. 3). In the rats treated with TNBS and the CRH-R1 antagonist CP-154,526, VMR on the third, fourth, fifth and sixth days of CRD was significantly higher than that on the first and second days of CRD ($P < 0.05$) (Fig. 3). On the first day of CRD, VMR in the initial three recordings did not significantly differ between TNBS-treated rats (1655.6 ± 146.5 counts 20 s^{-1}) and TNBS-/CRH-R1 antagonist-treated rats (1695.6 ± 98.3 counts 20 s^{-1}). From the first to the third day of CRD, VMR in TNBS-/CRH-R1 antagonist-treated rats was significantly lower than that in TNBS-treated rats ($P < 0.001$). However, from the fourth to the sixth day of CRD, VMR did not significantly differ between TNBS-treated rats and TNBS-/CRH-R1 antagonist-treated rats.

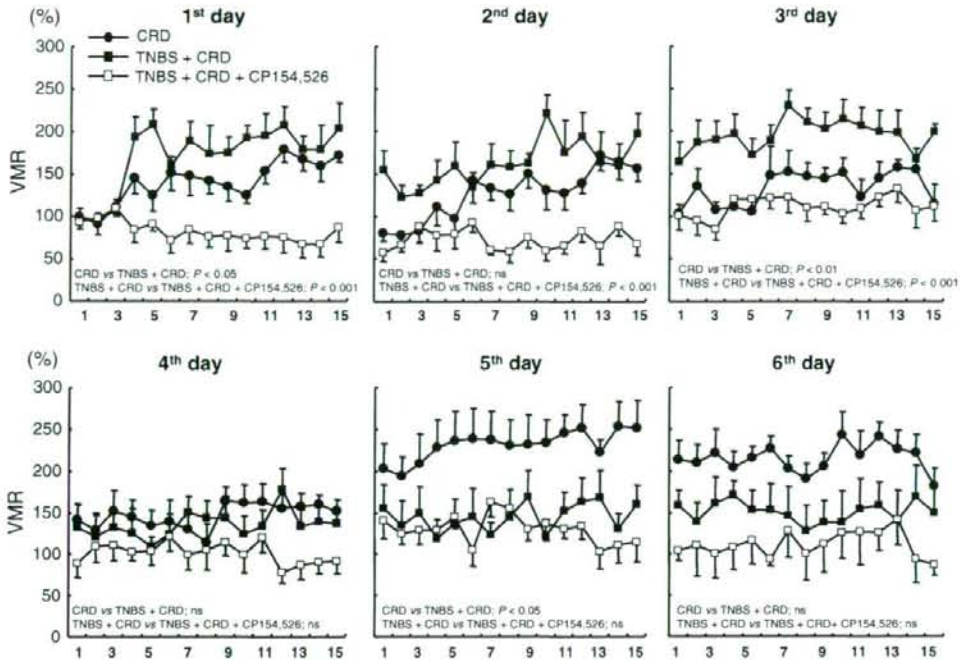

Figure 3 Effects of repetitive colorectal distention (CRD) alone, repetitive CRD with previous inflammation, and repetitive CRD with previous inflammation and CP-154,526 on visceromotor response. Numbers on the abscissa mean ordinal number of stimulation with 80 mmHg for 20 s. Data are expressed as the mean ± SE (n = 5–7 rats per group). P-value for group effect was determined by two-way ANOVA.

Effects of repetitive CRD and repetitive CRD after colitis: histological examination

Light microscopic photomicrographs of H&E-stained colon segments taken on the sixth day of CRD showed colonic inflammation in both the rats that received repetitive CRD rats and those that were treated with TNBS (Fig. 4B,C). However, no overt colonic inflammation was observed in the non-stressed rats and TNBS-/CRH-R1 antagonist-treated rats (Fig. 4A,D). In rats that received repetitive CRD, CRD exposure for 6 days induced a rise in the numbers of neutrophils, eosinophils and intraepithelial lymphocytes compared with the non-stressed rats (Table 1). On the other hand, in TNBS-treated rats, CRD exposure for 6 days induced a rise in the number of intraepithelial lymphocytes compared with the non-stressed rats, and a significant decrease in the number of neutrophils and eosinophils compared with the repetitive CRD rats. Finally, in TNBS-/CRH-R1 antagonist-treated rats, the numbers of neutrophils, eosinophils and intraepithelial lymphocytes did not differ from those in the TNBS-treated rats. There was a significant decrease in the numbers of neutrophils and eosinophils in TNBS-/CRH-R1 antagonist-treated rats when compared with the repetitive CRD rats.

DISCUSSION

This is the first to demonstrate that combination of previous inflammation and repetitive CRD makes the colon hypersensitive. Moreover, the time course of colonic sensitization by repetitive visceral stimulation has clearly been clarified in this study. The most important finding of this study is that treatment with a specific CRH-R1 antagonist dramatically attenuated visceral hypersensitivity induced by a combination of previous inflammation and repetitive CRD.

Previous inflammation makes the colon vulnerable to stress and increases parameters associated with colonic inflammation.[22,23] In addition, it has been reported that inflammation induces visceral

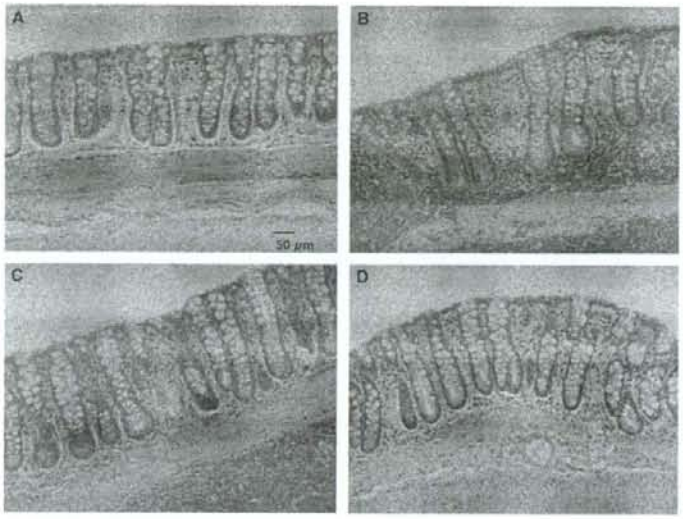

Figure 4 Effects of repetitive colorectal distention (CRD) alone and repetitive CRD with previous inflammation on colonic inflammation. (A) Non-repetitive CRD, (B) repetitive CRD alone, (C) repetitive CRD with previous inflammation and (D) repetitive CRD with previous inflammation and CP-154,526 treatment.

Table 1 Effects of repetitive CRD and repetitive CRD after colitis on cells-count in H&E-stained colon segments

	Neutrophils (cells mm^{-2})	Eosinophils (cells mm^{-2})	IEL (cells per 100 cells)
Non-stressed	25 (2)	30 (6)	4 (1)
Repetitive CRD	40 (3)[a]	46 (4)[a]	8 (1)[a]
TNBS-treated	29 (2)[b]	23 (3)[b]	8 (1)[a]
TNBS-/CRH-R1 antagonist-treated	22 (3)[b]	24 (3)[b]	8 (1)[a]

CRD, colorectal distention; IEL, intraepithelial lymphocytes; TNBS, 2,4,6-trinitrobenzene sulphonic acid; CRH-R1, corticotropin-releasing hormone receptor 1. The rats were killed on the last day of CRD experiments, and haematoxylin and eosin (H&E)-staining of colon segments was performed. Tissues from five to seven rats per group were scrutinized, one tissue section per rat, and, for each tissue, 10 contiguous non-overlapping areas above the muscularis mucosae were examined. Values are shown as mean (SEM). [a]$P < 0.05$ compared with non-stressed rats. [b]$P < 0.01$ compared with rats that received repetitive CRD rats.

sensitization and hyperalgaesia, and that TNBS-, mustard oil- or acetic acid-induced colitis increases VMR to phasic or tonic CRD.[24–27] Increased pain sensitivity after acute colitis has been suggested to be due to sensitization of peripheral and/or central nociceptors.[37–39] At the peripheral level, gut tissue injury has been shown to induce the release of prostaglandins and bradykinin, which increase sensitivity of afferent nerve terminals.[38,39] Prostaglandins and bradykinin are also known to stimulate the release of histamine, serotonin, nerve growth factor and prostanoids from afferent nerves. These events sensitize the endings of afferent nerve terminals and increase response to painful stimuli.[38,39] At the central level, inflammation increases pain signal transmission in the dorsal horn of the spinal cord and/or in the brain.[38,39] In this study, sensitization in TNBS-treated rats continued for the first 3 days, but was not observed after the fourth day of CRD. The sensitization for the first 3 days seems to be similar to that observed in patients with PI-IBS. We reported that acute CRD induces anxiety-like behaviours in rats.[35] More anxious patients suffered from IBS after acute gastroenteritis.[12] Because rats pretreated with CRH-R1 antagonist did not exhibit colonic sensitization, the present rat model at least in part provides a possible mediator of colonic hypersensitivity in PI-IBS. Acute inflammation has been shown to generate hyperalgesia, whereas repetitive inflammation, which involves lymphocytic infiltration into the mucosal and submucosal tissue accompanied by μ-opioid receptor and β-endorphin upregulation, provides an antinociceptive input that restores normal visceral perception in mice.[40] Patients with active colitis have been reported to exhibit reduced tolerance to balloon distension of the rectum, whereas patients with chronic or quiescent colitis exhibit normal or increased tolerance to distension.[41] These differences have been attributed to changes in descending spinal modulation of sensory input from the periphery during chronic inflammation.[41] This could partly explain the switch in visceral sensitivity that occurred on day 4 in repetitively distended TNBS-treated rats. On the other

hand, detection of visceral hypersensitivity after intestinal inflammation may depend on the programme of viscera stimulation. Accordingly, no difference in VMR was observed between TNBS-treated and TNBS-untreated rats at the initial three stimuli on the first day of CRD, but VMR increased from the fifth distention in the TNBS-treated rats. In addition, no robust differences between the two groups were observed on the initial days of testing. This may be due to the fact that after induction of colitis, rats were allowed to recuperate for 6 weeks, a period reported to be sufficient for recovery from TNBS-induced colonic inflammation.[22] It has also been reported that 6 weeks after administration of TNBS, mild restraint stress for three consecutive days causes a significant increase in myeloperoxidase activity in TNBS-treated rats, but not in the stressed controls.[22] This is the first study that measures VMR under almost the same experimental protocol. There is a possibility that inflammation was induced by stimulation on the first day and differences in VMR appeared after the second day. However, this is only a hypothesis as no histological assessment was conducted on the first and second days of stimulation. Further studies are necessary to clarify the mechanisms behind these phenomena.

Repetitive distention of the sigmoid colon has been reported to induce visceral hypersensitivity[42] and increased contractions of the descending colon[5] in patients with IBS. However, the precise mechanism of visceral hypersensitivity after repetitive visceral stimulation is still unknown. In this study, from the first to the third day of CRD, rats treated with TNBS were the most sensitized, while on the fifth day of CRD, rats that received CRD alone were the most responsive. Repetitive noxious CRD induces colonic plasma extravasation, suggesting minor colonic inflammation.[43–45] In addition, experimental stress can be an initiating factor in intestinal inflammation by impairing mucosal defense against luminal bacteria via mast cell degranulation.[19,46] Moreover, repetitive CRD as an interoceptive stressor can on one hand reactivate colonic inflammation[23] and lead to decrease in sensitivity after a few days, and on the other hand activate other pathways, such as mast cell degranulation, and increase colonic permeability, leading to hyperalgesia. Inflammation can also be responsible for remodelling of the primary afferent neurons innervating the colon, which could explain hypersensitivity to repetitive CRD.[47] Another possible explanation of this phenomenon is that repetitive CRD induces changes in molecular components of the brain–gut axis. Indeed, repetitive noxious CRD has been shown to increase Fos immunoreactivity in the spinal cord[45,48] via *N*-methyl-D-aspartate receptors.[45] Previous studies have also shown that many molecules, including T-type Ca^{2+}channels, γ-amino butyric acid (GABA), $GABA_B$ receptors, opioids and CRH are involved in the control of visceral perception.[35,38,39,49,50] However, the hypothesis that dynamic changes in nociceptive and antinociceptive molecules may account for visceral hypersensitivity after repetitive visceral stimulation remains to be verified.

In this study, treatment with a specific CRH-R1 antagonist attenuated visceral hypersensitivity induced by a combination of previous inflammation and repetitive CRD. We have previously elucidated the role of CRH in visceral hypersensitivity[51] and shown that administration of non-specific CRH receptor antagonist reduces abdominal pain evoked by repetitive electrical stimulation of the rectum in patients with IBS.[34] We have also proved that pretreatment with CRH-R1 antagonist attenuates CRD-induced hippocampal noradrenaline release and visceral perception in rats.[35] In agreement with these results, others have shown that a CRH-R1 receptor antagonist abolishes the activation of locus coeruleus neurons induced by colorectal distension in rats.[52] Other evidence on the other hand contrasts the role of CRH-R1 and CRH-R2 in visceral nociception, i.e. CRH-R1 is involved in pronociception of visceral pain, whereas CRH-R2 is related to antinociception.[53,54] Stress-induced colonic hypersensitivity has been reported to be mediated by CRH and CRH-R1.[55,56] Besides, it has been shown that peripheral CRH may also play an important role in intestinal hypersecretion and inflammation induced by *Clostridium difficile* toxin A and that CRH-R1 antagonists inhibit this response.[57] Activation of CRH-R1 causes pro-inflammatory responses, whereas stimulation of CRH-R2 provokes anti-inflammatory effects.[51,58] In our study, histological findings in animals treated with a CRH-R1 antagonist support this notion. Further studies on CRH and this peptide family, and on CRH receptor subtypes during sensitization (e.g. from first to third day of CRD in TNBS-treated rats) or recovery (e.g. from fourth to sixth day of CRD in TNBS-treated rats) processes in the brain–gut axis are warranted. The results of this study suggest that among the key molecules relating to visceral perception, at least CRH and CRH-R1 play a key role in visceral hypersensitivity caused by a combination of previous inflammation and repetitive CRD.

As indicated by our histological examination of H&E-stained colonic segments, repetitive CRD induced colonic inflammation. The number of intraepithelial lymphocytes was increased in all groups except the non-stressed group. However, the numbers of neutrophils and eosinophils were increased only in the

vehicle-treated groups. It is known that TNBS induces acute colitis, notably transmural inflammatory cellular infiltration associated with a Th1-dominated cytokine profile.[59] On the other hand, under Th2-associated inflammatory conditions, a marked increase in eosinophils occurs not only in the lamina propria but also in Peyer's patches.[60] Therefore, rats with repetitive CRD appear to show a different quality of inflammation from rats with repetitive CRD after TNBS. Further studies are needed to clarify the precise mechanisms underlying these phenomena. Moreover, data from Collins group clarified that transient colitis induced by TNBS full recovers and there is no inflammation in the mucosa.[22] However, TNBS is known to induce transmural inflammation in the colon.[59] Therefore, still there is possibility that sensitization of primary afferent neurons may be due to the sustained inflammatory changes in the muscular layer. This possibility and relating molecule should be clarified in the future study.

A limitation of this study is that the short accommodation time before CRD may be responsible for variability of data. It can be difficult to perform CRD without restraining the rats, although the results of the study may be, to a certain degree, influenced by this practice. Both types of studies have been conducted; those where animals are restrained prior to CRD and those where animals are not restrained.[44,61–64] Because the intensity and frequency of CRD differ from study to study, it is difficult to compare directly these studies. However, no report provides direct evidence of the effects of animals' habituation to restrain prior to testing on CRD outcome.

In conclusion, our results show that a combination of previous inflammation and repetitive CRD induces colonic hypersensitivity and that CRH-R1 antagonist attenuates this response in rats. These results can help clarify the mechanisms underlying PI-IBS and would be useful in the development of treatment for IBS patients who suffer from visceral hypersensitivity.

GRANT SUPPORT

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Increased Brain Histamine H1 Receptor Binding in Patients with Anorexia Nervosa

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Background: The central histaminergic neuron system modulates various brain functions, including eating behavior. We hypothesized that women have higher density of histamine H1 receptor (H1R) in the limbic system than men and that the density of central H1R is increased in patients with anorexia nervosa (AN).

Methods: Subjects were 12 female AN patients, 12 healthy female subjects, and 11 healthy male subjects. Positron emission tomography with H1R radioligand [^{11}C]doxepin was performed on all subjects and regions of interest based analysis was conducted to evaluate brain H1R binding potential (BP). Abnormal eating behavior, depression, and anxiety of subjects were evaluated using the Eating Attitude Test-26 (EAT-26), Self-Rating Depression Scale (SDS), and State-Trait Anxiety Inventory (STAI), respectively.

Results: Binding potential of [^{11}C]doxepin in female subjects was significantly higher than that in male subjects at the following brain sites: amygdala, hippocampus, medial prefrontal cortex, orbitofrontal cortex, and temporal cortex. Anorexia nervosa patients showed significantly higher BP of [^{11}C]doxepin in the amygdala and lentiform nucleus than the control female subjects. In AN patients, BP of [^{11}C]doxepin in the amygdala and thalamus negatively correlated with EAT-26 scores. There was a significant negative correlation between BP of [^{11}C]doxepin and SDS or STAI scores in the amygdala, anterior cingulate cortex, and orbitofrontal cortex of AN patients.

Conclusions: These findings support the hypothesis that women have higher H1R density in the limbic system than men and suggest that AN patients may have higher expression of H1R in the limbic brain, particularly in the amygdala.

Key Words: Amygdala, anorexia nervosa, doxepin, histamine, histamine H_1 receptors, positron emission tomography

Anorexia nervosa (AN) is a behavioral disorder characterized by fear of becoming obese, refusal to maintain a minimally normal body weight, disturbance of body image, and denial of the seriousness of the current low body weight (1). Anorexia nervosa occurs mainly in adolescent or young adult females (1). Anorexia nervosa patients start a self-imposed diet with chronic starvation featuring continuous strengthening symptoms and AN signs. The cause and progression of AN may involve biological vulnerability with dysfunction in the central neuron system (CNS) being one of the most important causation factors.

Several candidates including monoamines (serotonin, dopamine, and norepinephrine) (2–4) and neuropeptides (corticotropin-releasing hormone, neuropeptide-Y, peptide-YY, cholecystokinin, beta-endorphin, and leptin) (5–8) have been reported to be involved in the pathogenesis of AN. These neurotransmitters and neuropeptides may collectively play some roles in various features of AN; however, it is unlikely that only one of them is responsible for all features of AN.

Recently, a number of functional neuroimaging studies in AN patients have been conducted (9–15). At rest, single-photon emission computed tomography (SPECT) has shown hypoperfusion of regional cerebral blood flow (rCBF) in the medial prefrontal and anterior cingulate cortices and hyperperfusion of rCBF in the thalamus and amygdala-hippocampus complex (9). In addition, positron emission tomography (PET) has revealed a generally reduced brain glucose metabolism, although higher than the normal glucose metabolism has been observed in the inferior frontal cortex and basal ganglia (10). Moreover, functional magnetic resonance imaging has shown that the prefrontal and anterior cingulate cortices, amygdala, and the parahippocampal gyrus are activated by unpleasant presentation to elicit symptom-related brain processes (11–13). Under recovery conditions, rCBF showed hypoperfusion in the temporal, parietal, occipital, and orbitofrontal cortices in a SPECT study (14), and a recent PET study has shown that serotonin 2A (5-HT2A) receptor binding was reduced in the amygdala, hippocampus, and cingulate cortex (15). These findings suggest that dysfunction of the limbic brain with dysregulation of neurotransmitters/neuromodulators may play a role in the pathophysiological features of AN.

Central histaminergic neurons are located exclusively in the tuberomammillary nuclei of the hypothalamus (16) and their neuronal fibers project extensively into the limbic system and neocortex (17–19). The central histaminergic neuron system modulates various physiological functions such as wakefulness, sleep-awake cycle, fluid balance, body temperature, cardiovascular control, appetite control, stress-related hormone release, learning, memory, aggressive behavior, and emotion (20,21). It has been reported that histamine decreases food intake via H1 receptors (H1Rs) in the ventromedial hypothalamus and the paraventricular nucleus of the hypothalamus (20,21). The H1Rs are mainly postsynaptically located and are present particularly in the cortex and limbic areas, including the hypothalamus, amygdala, and hippocampus (22). Using PET with [^{11}C]doxepin, a potent radioligand for H1R, it has been shown that H1R binding

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is abundant in the prefrontal, temporal, and cingulate cortices of the human brain (23).

Central histaminergic activity is increased by food intake after starvation (24,25). Also, dehydration has been reported to increase the synthesis and release of histamine in the hypothalamus (26). Moreover, H1R concentration has been shown to be inversely correlated with food intake, particularly low protein diets (27,28). In addition, the central histaminergic neuron system is affected by various stressors (29–32) and other central appetite modulators, such as leptin (21,33,34), orexin-A, and neuropeptide-Y (35). These findings suggest the alteration of central histaminergic activity in AN patients. Alterations in the central histaminergic neuron system in some psychiatric disorders such as depressive disorder (36) and schizophrenia (37) have also been reported; however, there has been no report on AN to date.

The central histaminergic neuron system shows sex differences. These differences include H1R densities (male < female) (38), suppressive effect of histidine on food intake (male < female) (39), stress-related hypothalamic histamine release (40) in rat, and arousal-reducing effects of H1R-antagonist in mouse (male < female) (41). A study of human cerebrospinal fluid (CSF) suggests that central histaminergic activity is higher in women than in men (42). These sex differences may partially explain why women are more prone to suffer from AN than men.

We tested the following major hypotheses in this study: 1) women have higher H1R density in the limbic system than men, and 2) the density of central H1R is increased in AN patients. Furthermore, we additionally hypothesized that the central histaminergic activity in AN patients is proportional to abnormal eating behaviors and/or negative emotion.

Methods and Materials

Subjects

Twelve female AN patients, 11 healthy age-matched male volunteers, and 12 healthy age-matched female volunteers were enrolled in this study. Subject screening and diagnosis were conducted by medical doctors (with a diploma from the Japanese Society of Psychosomatic Medicine) in Tohoku University Hospital based on psychiatric interview performed according to the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision* (DSM-IV-TR) criteria (1). All patients were of the restricting type, had no complications, never had any other neuropsychiatric disorders, were drug-free, and received no high-calorie infusion throughout the study. The healthy control subjects, who were recruited through advertisement, never suffered from eating problems, substance abuse, menstrual problems, or physical, neurological, or psychiatric disorders. Psychometric tests including Eating Attitudes Test-26 (EAT-26) (43), Self-Rating Depression Scale (SDS) (44), and State-Trait Anxiety Inventory (STAI) (45) were used to quantify eating attitude, depression, and anxiety, respectively. The Japanese versions of all three tests have already been validated (46–48). The characteristics of each group of subjects are shown in Table 1. Anorexia nervosa patients showed significantly lower body mass index (BMI), higher EAT-26 scores, higher depression scores, and higher anxiety scores than the control subjects. Before the start of the study, all subjects gave a written informed consent. This study was performed in accordance with the policy of the Declaration of Helsinki and was approved by the Ethics Committee of Tohoku University School of Medicine (No. 2000-42).

Table 1. Characteristics of Study Subjects

	Control Male Subjects (n = 11)	Control Female Subjects (n = 12)	AN Patients (n = 12)
Age (years)	21.8 ± 1.3	22.3 ± 2.5	23.4 ± 2.8
BMI	20.4 ± 1.3	20.3 ± 1.1	14.7 ± 1.7[a,b]
Duration of illness (years)(range)	0 ± 0 (0)	0 ± 0 (0)	5.2 ± 2.0 (3–9)
EAT-26 score	3 ± 3.7	1.9 ± 2.7	22.3 ± 13.4[c,d]
SDS score	37 ± 7.2	35 ± 6.2	50 ± 8.1[e,f]
STAI-state score	40.5 ± 9.5	38.8 ± 9.5	49.6 ± 11.5[g,h]
STAI-trait score	46.5 ± 8.9	41.1 ± 11.2	52.1 ± 11.5[i,j]
Estradiol (pg/mL)		67 ± 51.7	13.2 ± 7.3[k]

One-way ANOVA for three group difference and post hoc analysis using the Tukey's test between two groups were performed. Values are expressed as mean ± SD.

AN, anorexia nervosa; ANOVA, analysis of variance; BMI, body mass index; EAT-26, Eating Attitudes Test-26; SDS, Self-Rating Depression Scale; STAI, State-Trait Anxiety Inventory.

[a] $p < .001$ versus control male subjects.
[b] $p < .001$ versus control female subjects.
[c] $p < .001$ versus control male subjects.
[d] $p < .001$ versus control female subjects.
[e] $p < .001$ versus control male subjects.
[f] $p < .001$ versus control female subjects.
[g] ns versus control male subjects.
[h] $p < .05$ versus control female subjects.
[i] ns versus control male subjects.
[j] $p < .05$ versus control female subjects.
[k] $p < .01$ versus control female subjects.

PET Tracer

Positron emission tomography experiments were conducted at the Cyclotron and Radioisotope Center, Tohoku University, Sendai, Japan. Doxepin, a tricyclic antidepressant, was C-11 labeled and used as a PET tracer. To verify the specific binding of [^{11}C]doxepin to H1R, the characteristics of the binding of doxepin to brain tissues were examined using H1R gene knockout mice (49,50). Doxepin has two saturable binding sites with high and low affinities in wild-type mice. However, only negligible labeling of doxepin was observed in H1R null mice. These data demonstrate clearly that the high affinity component of doxepin binding is associated with H1R. Moreover, a study, which compared the binding ability of a serotonin transporter among several antidepressants, has shown that doxepin possesses the weakest binding ability for a serotonin transporter (51). [^{11}C]doxepin was prepared by [^{11}C]methylation of desmethyl doxepin with [^{11}C]methyl triflate as described previously (52,53). The radiochemical and chemical purities of the ligand were more than 99% and 97%, respectively, and its specific radioactivity at the time of injection was 93.52 ± 50.12 GBq/μmol (2528 ± 1355 mCi/μmol). The injected dose and cold mass of [^{11}C]doxepin were 117.0 ± 23.0 MBq (3.16 ± .62 mCi) and 1.86 ± 1.64 nmol, respectively. [^{11}C]doxepin radiological dose was calculated based on a previous paper on radiological exposure (54).

PET Measurement

All subjects underwent magnetic resonance imaging (MRI) of the brain before PET scanning. No major abnormalities on brain MRI were observed in any subject. All subjects were right-handed, never had long-term treatment with antihistamines, and were free of any medication for at least 2 weeks prior to PET scanning. Before PET scanning, alcohol and nicotine were forbidden for 1 week and caffeinated beverages were not

allowed for at least 1 day. All subjects finished their meals 4 hours before PET scanning. Taking the menstrual cycle into consideration, PET scanning in control female subjects was performed during the follicular phase (within a week after the last menstruation).

Positron emission tomography scanning was performed for 90 min (11:00–12:30 or 15:00–16:30). Subjects were resting awake with closed eyes in the supine position. To maintain arousal throughout the scanning period, the subjects were confirmed to be awake every 15 min. Subjects were positioned in a SET2400W (Shimadzu, Kyoto, Japan) scanner so that the transaxial slices were parallel to the orbitomeatal line, and a 7-min long $^{68}Ge/^{68}Ga$ transmission scan was started for tissue attenuation correction. The subjects were then scanned to detect high-energy photon emission (511 keV) from [^{11}C]doxepin injected intravenously via the median cubital vein. The scanner collected 63 simultaneous transverse slices with a spatial resolution of 4 mm (transaxial) and 4.5 mm (axial) full-width at half-maximum in the center of the field of view. The sensitivity for a 20-cm cylindrical phantom was 48.6 kcps $kBq^{-1}mL^{-1}$ (1.8 Mcps $\mu Ci^{-1}mL^{-1}$) in the three-dimensional mode (55). Dynamic PET images were obtained for 90 min (22 sequential scans; 6 scans × 90 sec, 7 scans × 180 sec, 6 scans × 300 sec, and 3 scans × 600 sec) after [^{11}C]doxepin injection.

Image Analysis

Positron emission tomography dynamic images, after being corrected for tissue attenuation, were reconstructed using a filtered backprojection algorithm. The reconstructed PET images were co-registered to the identical stereotaxic brain coordinate system using its own MRI-T1 image as a reference. The MRI images were obtained using a 1.5 Tesla magnetic resonance scanner (HiSpeed, Ver9.1, General Electric Inc., Waukesha, Wisconsin) at Sendai Seiryo Clinic, Sendai, Japan. T1-weighted images (Vascular time of flight [TOF] spoiled gradient recalled [SPGR]: repetition time [TR]/echo time [TE]: 50/2.4 msec, fractional anisotropy [FA]: 45°, number of excitations: 1, matrix size: 256 × 256, and spatial resolution: x,y,z = .86, .86, 20.0 mm) were collected from all subjects. Regions of interest (ROIs) were first placed on the cerebellum. Information from the ROIs was automatically copied onto the co-registered PET images to obtain time activity curves (TACs). Cerebellar TAC was used as input function to calculate parametric brain images of the binding potential (BP) of [^{11}C]doxepin based on the graphical analysis method introduced by Logan *et al.* (56). The applicability of this method to a human study with [^{11}C]doxepin has been confirmed (57). Finally, brain BP images were created.

Region of interest based analysis was conducted to evaluate brain H1R-BP, minimizing the effects of possible brain atrophy using original BP brain images instead of using transformed BP brain images into standardized brain space. Regions of interest were placed on the following brain regions: the medial prefrontal cortex (MPC; Brodmann's area [BA] 9,10), lateral prefrontal cortex (LPC; BA 44,45,46,47), orbitofrontal cortex (Orb; BA 11), temporal cortex (TC; BA 21,22/41,42), parietal cortex (PC; BA 1,2,3/39,40), occipital cortex (OC; BA 17,18,19), anterior cingulate cortex (ACC; BA 24/32), posterior cingulate cortex (PCC; BA 23/31), insula (Ins), thalamus (Th), caudate nucleus (CN), lentiform nucleus (LN), amygdala (Am), and hippocampus (Hi). All subjects were assigned a number, and imaging data were analyzed in a blinded manner.

Data Analysis and Statistics

Data are expressed as mean ± standard deviation (SD). Region of interest based comparisons of BP of [^{11}C]doxepin were compared between the three groups using one-way analysis of variance (ANOVA) followed by Bonferroni correction (58). To minimize the effect on repeated comparisons, we adjusted the significance levels (total of p value < .05). Thereafter, post hoc analyses between the two groups were performed using the Tukey test only in the brain areas with a significant difference in BP of [^{11}C]doxepin as determined by one-way ANOVA. In AN patients, correlations between BP of [^{11}C]doxepin and eating

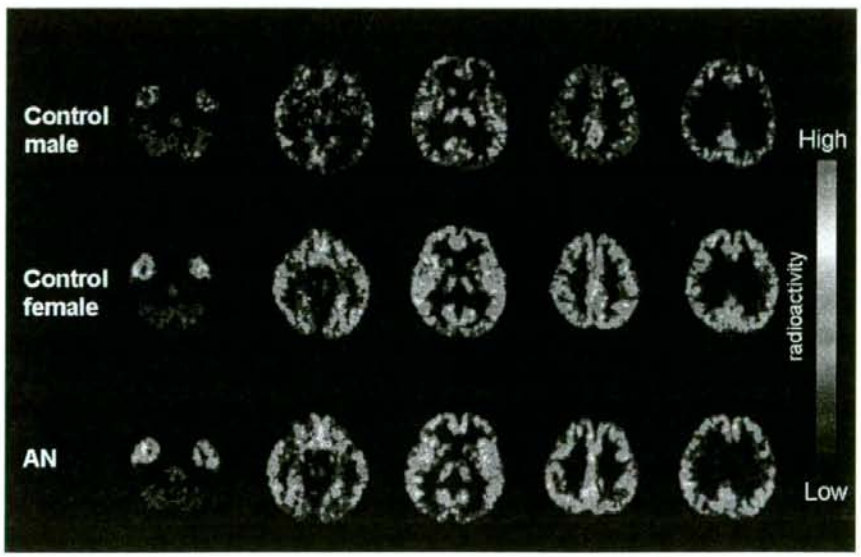

Figure 1. Brain distribution of [^{11}C]doxepin radioactivity in control subjects and AN patients. AN, anorexia nervosa.

Table 2. ROI-Based Comparisons of BP of [^{11}C]Doxepin by One-Way ANOVA

Anatomical Area	Brodmann's Area	*p* Value
L-Medial Prefrontal Cortex	9,10	.028
R-Orbitofrontal Cortex	11	.001
L-Orbitofrontal Cortex	11	.001
R-Temporal Cortex	21,22,41,42	.004
L-Caudate Nucleus		.009
L-Lentiform Nucleus		.001
R-Amygdala		<.001
L-Amygdala		.005
R-Hippocampus		<.001
L-Hippocampus		<.001

Analyses of the three groups were performed by one-way ANOVA.

ANOVA, analysis of variance; BP, binding potential; L, left; R, right; ROI, region of interest.

attitude or psychological status were analyzed using Spearman's rank correlation test; $p < .05$ was considered significant. The SPSS statistical software package for Windows 11.5 (Japanese version, SPSS Japan, Inc., Tokyo, Japan) was used for all statistical analyses.

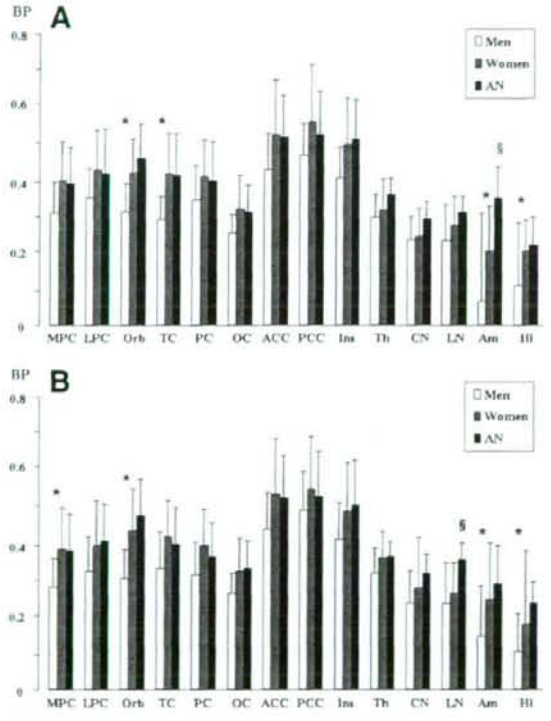

Figure 2. ROI-based comparisons of BP of [^{11}C]doxepin in the right **(A)** and left **(B)** cerebral hemisphere. One-way ANOVA and post hoc analysis using the Tukey's test were performed. *Men versus women ($p < .05$). §Women versus AN ($p < .05$). ACC, anterior cingulate cortex; Am, amygdala; AN, anorexia nervosa; ANOVA, analysis of variance; BP, binding potential; CN, caudate nucleus; Hi, hippocampus; Ins, insula; LN, lentiform nucleus; LPC, lateral prefrontal cortex; MPC, medial prefrontal cortex; OC, occipital cortex; Orb, orbitofrontal cortex; PC, parietal cortex; PCC, posterior cingulate cortex; ROI, region of interest; TC, temporal cortex; Th, thalamus.

Results

ROI-Based Comparisons of BP of [^{11}C]Doxepin Between the Three Groups

Binding potential of [^{11}C]doxepin showed a significant difference in some brain areas between the three groups as determined by one-way ANOVA (Table 2, Figure 1, Figure 2).

Post Hoc Comparisons of BP of [^{11}C]Doxepin

Male Versus Female Subjects. Binding potential of [^{11}C]doxepin in control female subjects was significantly higher than that in control male subjects in the left (L)-MPC, right (R)-Orb, L-Orb, R-TC, R-Am, L-Am, R-Hi, and L-Hi. On the other hand, there was no area where BP of [^{11}C]doxepin was significantly lower in control female subjects than in male subjects (Table 3, Figure 2).

Female Subjects Versus AN Patients. Binding potential of [^{11}C]doxepin in AN patients was significantly higher than that in control female subjects in L-LN and R-Am. On the other hand, there was no area where BP of [^{11}C]doxepin was significantly lower in AN patients than in control female subjects (Table 3, Figure 2).

Male Subjects Versus AN Patients. Binding potential of [^{11}C]doxepin in AN patients was significantly higher than that in control male subjects in R-Orb, L-Orb, R-TC, L-CN, L-LN, R-Am, L-Am, R-Hi, and L-Hi. On the other hand, there was no area where BP of [^{11}C]doxepin was significantly lower in AN patients than in male subjects (Table 3).

Correlation Between BP of [^{11}C]Doxepin and Eating Behavior or Psychological Status in AN Patients. There was no area where BP of [^{11}C]doxepin significantly correlated with BMI or length of illness.

EAT-26. There was a significant negative correlation between BP of [^{11}C]doxepin and EAT-26 scores in R-Th ($r = -.596$, $p < .05$), L-Th ($r = -.667$, $p < .05$), and L-Am ($r = -.646$, $p < .05$). On the other hand, there was no area with significant positive correlation between BP of [^{11}C]doxepin and EAT-26 scores.

Table 3. ROI-Based Comparisons of BP of [^{11}C]doxepin by Post Hoc Analysis Using the Tukey's Test

	Anatomical Area	Brodmann's Area	*p* Value
	L-Medial prefrontal cortex	9,10	.042
	R-Orbitofrontal cortex	11	.019
	L-Orbitofrontal cortex	11	.011
Men < Women	R-Temporal cortex	21,22,41,42	.006
	R-Amygdala		.021
	L-Amygdala		.047
	R-Hippocampus		.001
	L-Hippocampus		.002
Women < AN	L-Lentiform nucleus		.036
	R-Amygdala		.004
	R-Orbitofrontal cortex	11	.001
	L-Orbitofrontal cortex	11	.001
	R-Temporal cortex	21,22,41,42	.013
	L-Caudate nucleus		.007
Men < AN	L-Lentiform nucleus		.001
	R-Amygdala		<.001
	L-Amygdala		.004
	R-Hippocampus		<.001
	L-Hippocampus		<.001

Post hoc comparisons were made using the Tukey's test.

AN, anorexia nervosa; BP, binding potential; L, left; R, right; ROI, region of interest.

SDS. There was a significant negative correlation between BP of [11C]doxepin and SDS scores in R-Orb ($r = -.581$, $p < .05$), R-ACC ($r = -.704$, $p < .05$), and R-Am ($r = -.809$, $p < .01$). On the other hand, there was no area with significant positive correlation between BP of [11C]doxepin and SDS scores.

State Anxiety of STAI. There was a significant negative correlation between BP of [11C]doxepin and state anxiety scores of STAI in L-MPC ($r = -.608$, $p < .05$), R-Orb ($r = -.615$, $p < .05$), R-ACC ($r = -.678$, $p < .05$), and R-Hi ($r = -.671$, $p < .05$). On the other hand, there was no area with significant positive correlation between BP of [11C]doxepin and STAI-state scores.

Trait Anxiety of STAI. There was a significant negative correlation between BP of [11C]doxepin and trait anxiety scores of STAI in R-Orb ($r = -.698$, $p < .05$), L-PC ($r = -.681$, $p < .05$), R-ACC ($r = -.754$, $p < .01$), and R-Am ($r = -.733$, $p < .01$). On the other hand, there was no area with significant positive correlation between BP of [11C]doxepin and STAI-trait scores.

Discussion

The first point that can be drawn from our results is that the histaminergic neuron system in the human brain is different between male and female subjects. In fact, female subjects had significantly higher BP of [11C]doxepin than male subjects in several brain areas, and there was no area where BP of [11C]doxepin was significantly lower in female subjects than in male subjects. In support of this finding, a human CSF study has shown that women have higher levels of histamine metabolites than men (42). In addition, animal studies have also shown sex differences in the central histaminergic neuron system. In particular, it has been reported that H1R density is greater in female rats than in male rats (38), that the suppressive effect of histidine on food intake is greater in female rats than in male rats (39), that hypothalamic histamine release in normal and stressed rats is affected by sex (40), and that female mice are more sensitive to the arousal-reducing effect of pyrilamine, an H1R antagonist, than male mice (41). These findings together with the present results suggest that central histaminergic activity is higher in female subjects than in male subjects in both animals and humans. One possible reason for this difference is the presence of ovarian steroids in female subjects. As mentioned above, H1R density has been reported to be higher in female rats than in male rats (38). However, ovariectomy decreases H1R density and estradiol replacement reverses this effect in female rats (59). In female animals, food restriction induces higher histaminergic activity and reduces food intake (24,25,27,28). Furthermore, estradiol has been reported to facilitate histamine-induced excitation of ventromedial hypothalamus neurons (60). Thus, female animals may adapt better to starvation through the central histaminergic neuron system than male animals. Until now, CNS disturbances seen in AN were mainly considered to be secondary changes due to chronic starvation. However, in the present study, higher BP of [11C]doxepin in several brain areas was observed in normal female subjects. The risk of developing AN may be increased by not only the social background that women want to be thin because people tend to admire a thin figure but also biological vulnerability associated with central histaminergic activity.

The second point is that AN patients showed significantly higher BP of [11C]doxepin in the amygdala and lentiform nucleus than healthy female subjects, and there was no area where BP of [11C]doxepin was significantly lower in AN patients than in control female subjects. These findings are unique to AN patients. However, it is difficult to assume that increased BP of [11C]doxepin in the amygdala and lentiform nucleus in AN patients is the cause or result of AN. Actually, previous functional neuroimaging studies have shown altered rCBF, glucose metabolism, and brain area activation in AN patients (9–13). These alterations cannot be discriminated as the cause or result of AN. In our previous study, chronic food deprivation-induced stress in rats, which can be an AN model, reduced central H1R density (32). In addition, a human PET study in patients with depressive disorder, which is one of the representative stress-related disorders, has shown lower BP of [11C]doxepin in several brain areas in these patients than in healthy control subjects, and there were no brain areas where BP of [11C]doxepin was higher in these patients than in healthy control subjects (36). The decreased H1R density and BP of [11C]doxepin in these studies have been explained by the sustained release of endogenous histamine and the downregulation of H1R as a consequence of endogenous ligands. Low plasma estradiol reduces brain H1R (59), and AN patients in this study showed lower plasma estradiol (Table 1). Therefore, it was predicted that BP of [11C]doxepin in AN patients would be decreased. However, the present results show the opposite. If the increased BP of [11C]doxepin in the amygdala is the cause of AN, female subjects with higher H1R in the amygdala may be more susceptible to AN. The amygdala certainly plays an important role in emotional responses (61–63), and histamine facilitates anxiety via H1R in the rat amygdala (64). The increased BP of [11C]doxepin in the amygdala of AN patients may also be a result of AN. Central histaminergic activity is increased by food intake after starvation (24,25), and H1R concentration is increased by feeding low-protein diets (27,28). Starvation and feeding in AN patients may facilitate the increase in H1R concentration in the amygdala. Whatever the final mechanism or explanation is, the higher BP of [11C]doxepin in the amygdala of AN patients is a novel finding. Further studies, particularly in patients who have recovered from AN, are needed to confirm whether higher BP of [11C]doxepin is the cause or result of AN.

Binding potential of [11C]doxepin in the orbitofrontal cortex, amygdala, and hippocampus was higher in female subjects and AN patients than in male subjects. These brain areas may play important roles in the central modulation of eating behavior because they are parts of the limbic system controlling emotion, cognition, and decision making (61,62,63,65). Previous functional neuroimaging studies in AN patients have also demonstrated disturbances in the limbic brain (9–13). Moreover, in patients after recovery from AN, 5-HT2A receptor binding was reduced in the limbic brain (15), and serotonin 1A (5-HT1A) receptor binding in the limbic brain positively correlated with a measure of anxiety (66). These findings, together with the present findings, suggest that dysfunction of the limbic brain with dysregulation of neurotransmitters/neuromodulators may play a role in the pathophysiological features of AN. These are very interesting results associated with the characteristics observed in AN patients, such as distorted cognition and emotional changes to food and body image. Therefore, the central histaminergic neuron system may play a role in AN, not only through stimulation of the satiety center, but also through activation of advanced psychological systems.

However, our additional hypothesis that BP of [11C]doxepin in AN patients is proportional to abnormal eating behavior and/or negative emotion is unlikely. Rather, BP of [11C]doxepin in AN patients is negatively correlated with abnormal eating behavior and/or negative emotion. These results were unexpected. In fact,

there were cases in which clinical evaluation of eating behavior and psychological status did not correspond to the results of the questionnaires, particularly in the EAT-26 results where the subjects did not show their eating attitude and psychological status. As there are traits such as lack of consciousness of disease and distorted cognition in AN patients, evaluation of the patient's characteristics using self-rating questionnaires has limitations. However, the relationship between H1R and severity of negative emotion in AN may be similar to that in depressive disorder (36). Indeed, our previous PET study in patients with depressive disorder demonstrated that BP of [^{11}C]doxepin decreases in proportion to SDS scores in the frontal and cingulate cortices (36). The actual relationship between BP of [^{11}C]doxepin in the brain and emotional/behavioral symptoms of AN needs further investigation.

There are several limitations of this study. First, the small number of subjects limits statistical power. The present number of AN patients (n = 12) cannot disprove the firm conclusion regarding the relationship between BP of [^{11}C]doxepin and emotional/behavioral symptoms. Second, comparisons among many ROIs also have statistical limitations. These may increase the risk of having type I errors. Third, we cannot completely rule out the possibility of the effect of brain atrophy on the results in AN patients. This is because the relationship between brain atrophy and brain H1R has not been sufficiently clarified to date. Finally, the results are not all encompassing to enable clear explanation of the specific increase in BP of [^{11}C]doxepin in the amygdala and lentiform nucleus in AN patients. Binding potential may reflect changes in receptor density (B_{max}) and/or receptor affinity (K_D). A previous animal study showed that the B_{max} of H1R was higher in female rats than in male rats, while K_D remained unchanged (38). Higher BP of [^{11}C]doxepin in human female subjects may induce increased B_{max} of H1R in the brain. However, we could not specifically conclude which abnormalities in B_{max} and/or in K_D values were more attributable to the increased BP observed in AN patients. Further studies, such as those employing larger samples, focusing on specific brain regions (e.g., amygdala), using patients who have recovered from AN, or carrying out postmortem binding assays in the brain of AN patients, are needed to arrive at a definitive conclusion.

In conclusion, the present study demonstrates that female subjects have higher BP of [^{11}C]doxepin in the limbic system than male subjects and that AN patients have higher BP of [^{11}C]doxepin in the amygdala and lentiform nucleus than normal female subjects. These findings suggest that the central histaminergic neuron system may play an important role in the pathophysiology of AN.

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Changes in salivary physiological stress markers induced by muscle stretching in patients with irritable bowel syndrome

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Abstract

Background: Psychophysiological processing has been reported to play a crucial role in irritable bowel syndrome (IBS) but there has been no report on modulation of the stress marker chromogranin A (CgA) resulting from muscle stretching. We hypothesized that abdominal muscle stretching as a passive operation would have a beneficial effect on a biochemical index of the activity of the sympathetic/adrenomedullary system (salivary CgA) and anxiety.

Methods: Fifteen control and eighteen untreated IBS subjects underwent experimental abdominal muscle stretching for 4 min. Subjects relaxed in a supine position with their knees fully flexed while their pelvic and trunk rotation was passively and slowly moved from 0 degrees of abdominal rotation to about 90 degrees or the point where the subject reported feeling discomfort.

Changes in the Gastrointestinal Symptoms Rating Scale (GSRS), State Trait Anxiety Inventory (STAI), Self-rating Depression Scale (SDS), ordinate scale and salivary CgA levels were compared between controls and IBS subjects before and after stretching. A three-factor analysis of variance (ANOVA) with period (before vs. after) as the within-subject factor and group (IBS vs. Control), and sex (men vs. female) as the between-subject factors was carried out on salivary CgA.

Results: CgA showed significant interactions between period and groups ($F[1, 31] = 4.89$, $p = 0.03$), and between groups and sex ($F[1, 31] = 4.73$, $p = 0.03$). Interactions between period and sex of CgA secretion were not shown ($F[1, 3] = 2.60$, $p = 0.12$). At the baseline, salivary CgA in IBS subjects (36.7 ± 5.9 pmol/mg) was significantly higher than in controls (19.9 ± 5.5 pmol/mg, $p < 0.05$). After the stretching, salivary CgA significantly decreased in the IBS group (25.5 ± 4.5 pmol/mg), and this value did not differ from that in controls (18.6 ± 3.9 pmol/mg).

Conclusion: Our results suggest the possibility of improving IBS pathophysiology by passive abdominal muscle stretching as indicated by CgA, a biochemical index of the activity of the sympathetic/adrenomedullary system.

Background

Paying attention to the gut may magnify perceptions of abdominal symptoms and symptom related emotion [1,2]. Irritable bowel syndrome (IBS) is associated with an increased incidence of psychological disorder in patient populations [3], and while the cause and nature of this association are a matter of discussion, several possible mechanisms, both psychological and physiological, have been proposed to account for the finding [4-6]. Although there are many treatment strategies [7], traditional IBS therapy is mainly symptom oriented and often unsatisfactory. Increasing knowledge of brain-gut physiology [8], mechanisms, and neurotransmitters and receptors [9] involved in gastrointestinal motor and sensory function have led to the development of several new therapeutic approaches [10,11].

No single medication has proven to be universally effective, and the multiple therapeutic approach of gastrointestinal neurophysiology has led to promising advances in medical and non-medical approaches to IBS. Most studies have examined the association between mood state and IBS symptom severity using between-subjects design. The mechanisms involved suggest an association between mood state and IBS symptom severity within the individual. For example, although self-report measures of symptom severity cannot distinguish between the effect of mood state on physiology and on symptom perception, both mechanisms would lead to a situation in which a worsening of mood would occur before a worsening of IBS symptoms when both are measured longitudinally.

Autonomic imbalance has been proposed as a pathophysiological factor of IBS. Adrenergic neural activity and rectal sensitivity are more pronounced in IBS patients than in normal controls [12]. The stress response system includes the sympathetic/adrenomedullary (S/A) system and the hypothalamic-pituitary-adrenal (HPA) axis. The activities of the HPA axis and the S/A system can be biochemically evaluated by measuring catecholamines and cortisol, and we can measure these hormones as objective markers of stress. Recently, as a result of investigating the derivatives of catecholamines that are detectable in saliva, chromogranin A (CgA) was determined to be a useful index of psychological stress. CgA is a member of a family of highly acidic proteins, chromogranins, which are co-stored in the adrenergic neurons and paraneurons and co-released with adrenaline and noradrenaline in response to stimulation [13,14]. The changes in salivary CgA secretion resulting from exposure to a cognitive task may indicate psychological stress in humans [15].

Colonic stimulation results in brain activation of the somatosensory, insular, anterior cingulate and prefrontal cortices [2]. The somatosensory cortex receives direct anatomical projections from the ventral posterior thalamic nucleus, it is generally assumed that the somatosensory cortex is involved in parallel processing of tactile sensory information derived from this thalamic source of input [16]. In contrast, psychological stress influences pain thresholds via activation of the prefrontal cortices. Corticotropin releasing hormone is released from the hypothalamus, binding to visceral muscles and causing abnormal movement of the colon [9,17]. A stress marker of the S/A system, CgA, is released in saliva due to negative feelings such as aversive stimuli and psychological stress [15,18]. Mental activity may modulate gut perception [18,19] and override the effect of somatic stimulation on gut perception. For example, afferent signals from muscle stretching might modulate visceral perception and emotion via the spinothalamic pathway.

Skeletal muscle stretching is a unique method for relaxation [20-22]. The effect of hypnotherapy on IBS has been well documented [23], but specific psychotherapy usually needs long-range training for therapists at much cost. On the other hand, skeletal muscle stretching is simple and applicable in daily practice. Skeletal muscle stretching improved subjective pain scores of the patients with low back pain, and salivary cortisol concentrations were also significantly decreased during exercise [24]. However, the effects of skeletal muscle stretching on IBS are still unknown.

We hypothesized that IBS subjects would show abnormal salivary CgA and that skeletal muscle stretching would have beneficial effects on the pathophysiology of IBS.

Methods

Subjects

This study was approved by the Ethics Committee of Niigata University of Health and Welfare. All subjects gave their written informed consent. The subjects were university students, including 15 healthy volunteers as controls (7 males, 8 females: university students at Niigata University of Health and Welfare) and 18 subjects with IBS (not receiving medical treatment for IBS, 7 males, 11 females) aged 20 to 23 years old. The IBS subjects were 20 subjects selected from 245 volunteers selected through a predesigned questionnaire based on the Rome III criteria [25] for functional gastrointestinal diseases. Two IBS subjects were excluded from the results because of incomplete examinations due to cold and headache.

As recommended by the Rome III committees [25], patients with IBS were classified by the predominant stool pattern: IBS with diarrhea (IBS-D) was defined as loose (mushy) or watery stool >25% and hard or lumpy stool <25% of bowel movements; and IBS with constipation (IBS-C) as hard or lumpy stool 25% and loose or watery

stool <25% of bowel movements. Based on questions about the proportion of bowel movements that were either loose or watery, or hard or like a ball (lumpy), IBS subjects were classified by Rome III criteria [25] as IBS-D (n = 4), IBS-C (n = 8), IBS-M (n = 2) and IBS-U (n = 4).

Stretching of the abdominal muscles

Subjects attended a preliminary test session that included the measurement of psychological characteristics and maximal abdominal muscle stretch. Subjects relaxed in a supine position with their knees fully flexed while their pelvis and trunk were passively and slowly moved from 0 degrees of abdominal rotation to about 90 degrees or until the subject reported feeling discomfort.

Participants attended one of several 30-minute experimental stretch sessions that were conducted at the same time of day. Subjects were instructed not to begin a stretching program session and to reschedule their session, if symptoms of their IBS prevented the stretching. During the session, subjects engaged in a 1-minute cyclic stretching protocol, 2 times right and left side rotation of their pelvis and trunk, and a 4-minute static stretching protocol. For the 4-minute static stretching protocol, the subject's knees were moved at a rate of 30°/s from 90 degrees of trunk and pelvic rotation (neutral) to a static hold at 80% of the subject's maximal passive rotation angle for one minute [26]. Immediately following the static stretching, the knees were returned to neutral, then moved to 80% of maximal angle on the other side, and again returned back to neutral [27]. The last stretching sequence was necessary so that measurements of stiffness and abdominal discomfort before the stretching could be compared with measurements of sensation and emotion after the stretching.

Measurement of symptoms and psychological status

Before the experiment, gastrointestinal (GI) symptoms and psychological status were evaluated using the Gastrointestinal Symptoms Rating Scale (GSRS) [28], Zung's Self-rating Depression Scale (SDS) [29], and the State-Trait Anxiety Inventory (STAI) [30]. In addition, the subjects were asked to report the following seven items of visceral sensation or emotion [2]: abdominal discomfort, abdominal distention, abdominal pain, urgency for defecation, perceived stress, sleepiness, and anxiety before and after perceived stretching. Each sensation was evaluated on a scale from 0 (no sensation) to 10 (maximal sensation) as previously described [2,31].

GSRS is a 15-item instrument designed to assess the symptoms associated with common GI disorders. It has five subscales (Reflux, Diarrhea, Constipation, Abdominal Pain, and Indigestion Syndrome). Subscale scores range from 1 to 7 and higher scores represent more discomfort. A total score is derived by summing the individual item scores, and ranges from 15 to 105 [32].

SDS is a 20-item self-report questionnaire. Each item is scored on a Likert scale ranging from 1 to 4. A total score ranges from 20 to 80. Most people with depression score between 50 and 69, while a score of 70 and above indicates severe depression [33].

Mean scores of STAI for normal subjects were substantially lower than those reported in the English STAI Manual (State 24.95 ± 11.36 vs. 36.54 ± 10.22 and the Trait score was 27.88 ± 11.43 vs. 35.55 ± 9.76). The reported scores for depressed patients were 56.22 ± 8.86 and 53.83 ± 10.87, The state score for healthy subjects was 34.30 ± 10.79 and the Trait score was 36.07 ± 10.47 [34].

Salivary CgA sampling

Salivary samples were collected immediately before and after stretching. Saliva samples were extracted from cotton wads that subjects held in their mouths for 2 min by centrifuging at 3,000 rpm for 15 min. The tactile stimulation of the presence of the cotton wad in the oral cavity tends to stimulate a rather uniform salivary flow [35]. During collection, the cotton wad was rolled around like a hard candy in the oral cavity. The samples were stored at -20°C until the assay. Salivary CgA levels were determined using an enzyme-linked immunosorbent assay (EIA) kit (YK070 Human Chromogranin A EIA, Yanaihara Institute, Inc., Shizuoka, Japan), using the method of Yanaihara et al. [36] of Yanaihara Laboratories (Fujinomiya, Shizuoka, Japan). The corrected values of CgA (pmol/mg) were calculated by dividing by the raw results of EIA with the protein concentration of the saliva in the samples (pg/mg).

Levels of salivary CgA were evaluated, according to a previously described method [35,37,38]. Salivary CgA levels are reported as being within the range of 50.0 ± 40.0 pmol/mg (protein corrected) in healthy subjects [39]. Salivary CgA might be a sensitive and promising index for psychosomatic stress. Therefore, an understanding of the circadian rhythm of salivary CgA in normal humans is important. According to a recent report, CgA does not show any obvious circadian rhythm. Salivary CgA levels peak upon awakening, and then quickly decrease to the nadir after 1 hour and maintain a low level throughout the day [40]. The circadian variation of CgA is still not fully established.

Statistic analysis

A three-factor analysis of variance (ANOVA) with period (before vs. after) as the within-subject factor and group (IBS vs. Normal) and sex (men vs. female) as the between-subject factors, was carried out on salivary CgA. Changes in revised salivary CgA levels between before and after

stretching sessions for each group were analyzed statistically by related-measures 3-factor ANOVA, followed by Bonferroni protected least significant difference for multiple comparisons; values of $p < 0.05$ were considered significant.

Values of visceral perception and emotion were compared between groups with the Mann-Whitney U test. Spearman's rank correlation was used for evaluating the intragroup correlation coefficient between CgA and psychological status and perceptional/emotional ratings; values of $p < 0.05$ were accepted as significant.

Results

GI symptoms and psychological status

Table 1 shows the scores for GI symptoms and psychological status in the Normal and IBS groups. The GSRS score of IBS subjects was significantly higher than that of Normal subjects (Mann-Whitney's U test, $p = 0.01$). State anxiety and trait anxiety scores of STAI of IBS subjects were significantly higher than those of normal subjects ($p < 0.01$). SDS scores did not significantly differ between Normal and IBS subjects.

Abdominal discomfort in IBS subjects before and after stretching was significantly higher than in Normal subjects ($p = 0.03$) (Table 2). After stretching, there was no difference in abdominal discomfort between the groups. Anxiety was significantly decreased by stretching in Normal subjects ($p = 0.04$).

Two-way ANOVA of abdominal discomfort showed that there were significant period × group interactions ($F[3, 62] = 6.82$, $p = 0.005$). Before stretching, abdominal discomfort in IBS subjects (1.84 ± 0.43) was significantly higher than in Normal subjects (0.27 ± 0.12, post-hoc, $p = 0.02$). After stretching, abdominal discomfort in IBS subjects (1.74 ± 0.37) was significantly higher than in Normal subjects (0.20 ± 0.11, post-hoc, $p = 0.01$). Abdominal discomfort changes between before and after stretching in Normal controls were not significant (post-hoc, $p = 0.89$). There were no changes in the other scales before and after stretching or between normal controls and IBS subjects.

Table 1: Comparisons of GI symptoms and psychological status between IBS subjects and normal controls

	Controls (n = 15)	IBS (n = 18)	p
GSRS	32.47 ± 5.21	51.16 ± 4.95*	<0.01
STAI-S	23.20 ± 3.23	41.90 ± 1.62*	<0.01
STAI-T	25.53 ± 3.22	42.53 ± 1.80*	<0.01
SDS	42.00 ± 1.60	45.21 ± 1.73	0.23

Mean ± Standard Error. *Comparison between normal group vs. IBS group by the Mann-Whitney's U test. GSRS: Gastrointestinal Symptoms Rating Scale, STAI-S: state anxiety, STAI-T: trait anxiety, SDS: Self-rating Depression Scale.

Changes of salivary CgA

For multiple group comparisons, homogeneity of variance was assessed by the Levene test. Three-way repeated-measures ANOVA of CgA showed significant interactions between period and groups ($F[1, 31] = 4.89$, $p = 0.03$), and between groups and sex ($F[1, 31] = 4.73$, $p = 0.03$). Interactions between period and sex of CgA secretion were not significant ($F[1, 3]$ 2.60, $p = 0.12$). Before stretching, salivary CgA in IBS subjects (36.7 ± 5.9 pmol/mg) was significantly higher than in Normal subjects (19.9 ± 5.5 pmol/mg, post-hoc, $p = 0.006$) (Fig. 1). CgA changes before and after stretching in Normal subjects were not significant (post-hoc, $p = 0.60$). In contrast, CgA was significantly decreased after stretching in IBS subjects (22.5 ± 4.5 pmol/mg, post-hoc, $p = 0.02$). After stretching, there was no significant difference in CgA between Normal and IBS subjects (post-hoc, $p = 0.22$).

The Spearman rank correlation coefficient showed a significantly positive correlation between CgA secretion before stretching and SDS score in IBS ($r = 0.51$, $p = 0.03$) (Table 3). The change in CgA after stretching compared with before stretching was positively correlated to the SDS score in both groups (IBS: $r = 0.52$, $p = 0.03$. Normal: $r = 0.53$, $p = 0.04$). In ratings of perception and emotion, perceived stress to stretching was negatively correlated with CgA secretion in the Normal group ($r = -0.66$, $p = 0.007$).

Discussion

This is the first study to demonstrate that the salivary CgA level of IBS subjects is higher than that of normal subjects. We also demonstrated that, after stretching, the CgA level of IBS subjects became comparable with that of normal subjects. CgA is a major soluble protein in adrenal medullary chromaffin granules and adrenergic neurons and is co-released with catecholamines, which are considered to be a good index of sympathetic activity [41,42]. In particular, salivary CgA is reported to be a sensitive and substantial marker of psychological stress, which does not respond well to physical stress [15]. The results of this study suggest that abdominal muscle stretching may improve sympathetic arousal in IBS subjects.

Patients with IBS show more psychiatric disorders and pathologic behavioral patterns than normal subjects [4,43]. A correlation between CgA secretion and the depression score was observed in this study. This result suggests a potential mechanism connecting events in the nervous system (central or enteric) with IBS symptoms. Noradrenaline in the brain plays a crucial role in anxiety, and colorectal distention induces noradrenaline release in the hippocampus [44]. Not only central but also periph-

Table 2: Ratings of perception and emotion

Perception and emotion	Normal (n = 15)		IBS (n = 18)	
	Before	After	Before	After
Abdominal discomfort	0.27 ± 0.12	0.20 ± 0.11	1.84 ± 0.43*a	1.74 ± 0.37*b
Abdominal distention	0.40 ± 0.16	0.40 ± 0.16	1.32 ± 0.39	1.05 ± 0.35
Abdominal pain	0.20 ± 0.11	0.27 ± 0.15	0.74 ± 0.30	0.47 ± 0.25
Urgency	0.33 ± 0.16	0.47 ± 0.19	0.32 ± 0.23	0.26 ± 0.19
Stress	2.40 ± 0.54	2.27 ± 0.57	2.21 ± 0.41	2.37 ± 0.50
Sleepiness	3.00 ± 0.39	3.53 ± 0.45	4.42 ± 0.72	5.00 ± 0.64
Anxiety	2.20 ± 0.48	0.87 ± 0.35*c	2.42 ± 0.47	1.90 ± 0.48

Mean ± Standard Error. *a: Comparison between Normal group vs. IBS group before stretching (p = 0.03), *b: Normal group vs. IBS group after stretching (p = 0.03), *c: before vs. after stretching of the Normal group (p = 0.04) by Mann-Whitney's U test.

eral adrenergic/noradrenergic functions may contribute to the pathophysiology of IBS. Elsenbruch et al. reported that IBS patients demonstrated significantly greater postprandial increases in plasma noradrenaline and systolic blood pressure [45]. In inflammatory bowel diseases, disturbed adrenergic regulation of interleukin-10 (IL-10) could be part of the mechanism underlying the modulation of disease activity due to psychological stress [46]. Disturbed autonomic or neuroendocrine modulation of cytokine production, may play a role in the pathogenesis of IBS [9]. Increased salivary CgA in IBS subjects suggests that IBS subjects have sympathetic arousal due to increased signaling to the gut afferent neurons.

IBS symptoms are generally worsened by stress and often improve with physical exercise and medications affecting serotonin function [47-49]. Sugano et al. reported that the skeletal muscle stretching program improved subjective pain scores of the patients with low back pain and that salivary cortisol concentrations were also significantly decreased up to 90 min after exercise [24]. Exercise may have beneficial effects on IBS symptoms [50].

CgA secretion before stretching was negatively correlated with the stress score of normal subjects. Additionally, the anxiety score was reduced after stretching in the normal subjects. Psychological factors influencing symptom reporting have been identified in the constructs of visceral perceptional amplification and alexithymia [51]. From a psychological viewpoint, IBS may be conceived as an abnormal cognitive processing of emotional stimuli, via verbal responses, and a tendency to perceive somatic stimuli as evidence of symptoms of disease.

Ghoncheh et al. examined the psychological and physical effect of passive muscle stretching and yoga stretching exercises for relaxation [22]. Muscle relaxation displayed higher levels of relaxation states, physical relaxation, disengagement and higher levels of joy as a post-training effect [52]. Muscle stretching provides sensation contrasts for learning relaxation in addition to fostering relaxation through the stretching of muscles [53]. Muscle stretching for patients with IBS may be of benefit to the patients and could be used as part of a multi-component approach to the treatment of IBS.

Evidence of a physiological component of IBS is based on gender differences in GI symptoms, central nervous system pain processing, and specific effects of estrogen and progesterone on gut function [54,55]. Additional factors may play a role, including gender-related differences in neuroendocrine, S/A system, and stress reactivity, which

Table 3: Correlation between (r) CgA and GI symptoms and psychological status before and after stretching for the Normal and IBS groups.

	GSRS	SDS	STAI-S	STAI-T	Abdominal Discomfort	Abdominal Pain	Stress	Anxiety
IBS (n = 18)								
before	-0.16	0.51*	-0.17	-0.30	0.11	-0.13	-0.20	0.05
after	-0.34	0.19	-0.41	-0.29	0.24	-0.20	-0.37	-0.21
ΔCgA	0.08	0.52*	0.04	0.09	0.10	-0.05	0.18	0.36
Normal (n = 15)								
before	0.07	0.24	0.16	0.44	-0.23	0.13	-0.53*	-0.01
after	-0.20	0.08	0.15	0.07	-0.36	-0.04	0.09	0.18
ΔCgA	0.26	0.53*	-0.01	0.40	0.09	0.25	-0.66*	0.04

Significance, *p < 0.05. ΔCgA: CgA secretion before stretching – CgA after stretching.

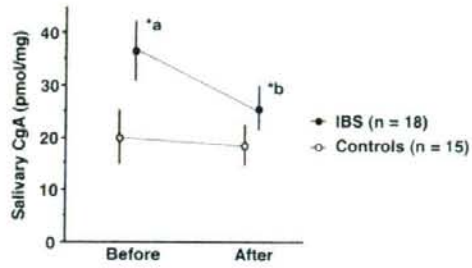

Figure 1
Three-way repeated-measures ANOVA of CgA showed significant interactions between period and groups ($F[1, 31] = 4.89$, $p = 0.03$). *a: Before stretching, salivary CgA in IBS subjects (36.7 ± 5.9 pmol/mg) were significantly higher than in Normal controls (19.9 ± 5.5 pmol/mg, post-hoc, $p = 0.006$). A significant CgA change between before and after stretching in Normal controls was not shown (post-hoc, $p = 0.60$). *b: CgA was significantly decreased after stretching in IBS subjects (22.5 ± 4.5 pmol/mg, post-hoc, $p = 0.02$). After stretching, there was no significant difference in CgA between Normal controls and IBS subjects (post-hoc, $p = 0.22$). Error bars are mean ± standard error.

are related to bowel function and pain. Although gender differences in the therapeutic benefits of serotonergic agents have been observed [56], less is known about potential differences in responsiveness to non-drug therapies for IBS. Multiple comparisons between CgA and gender related information suggest that stretch intervention may have gender dependent effects on IBS.

The following three points can be cited as limitations of this study. The first is that sample size was very small. The levels of CgA found in our subjects were somewhat different from the reported mean value [39], and our findings could not exclude the effect of the sample size. Additionally, we could not examine the effects on subtypes of IBS (i.e. constipation-predominant or diarrhea-predominant), because the sample sizes of the subtypes were too small to analyze them separately. However, a long follow-up study [57] proved the inconsistency of IBS subtypes, suggesting that whole IBS analysis is more important than subtype analysis. The second limitation is that the duration of muscle stretching might be too short. The duration of the effect of contraction-relaxation stretching on range of motion (ROM) in the lower extremities is 15 min and the increase in ROM usually remains for 90 min [58]. Proprioceptive neuromuscular facilitation (PNF) stretching techniques produced greater increases in ROM than static or dynamic stretching exercises. The stretching hold time at the hip is 3–10 sec in one hold-relax PNF stretch [59]. There is no study that clarifies the stretch duration required of the abdominal muscle for relaxation. Thus, it will be necessary to examine how long we should stretch the abdominal muscles for IBS treatment. Lastly, we could not analyze the effect of lifestyle and medical history on CgA in this study. Many stress-related biomarkers are affected by lifestyle or medical history [60]. Such relationships might contribute to increased knowledge about strategies to prevent progression of IBS.

Conclusion

Our results suggested that it is possible to improve IBS pathophysiology by passive abdominal muscle stretching using a biochemical index of the activity of the S/A system (salivary CgA). In this study, we verified only the effects of stretching and presence of IBS on CgA levels. Further study of the S/A system and muscle stretching in IBS is warranted.

Abbreviations

IBS: irritable bowel syndrome; CgA: chromogranin A; GSRS: Gastrointestinal Symptoms Rating Scale; STAI: State Trait AnxietyInventory; SDS: Self-rating Depression Scale; ANOVA: analysis ofvariance; S/A: sympathetic/adrenomedullary.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TH was the main investigator and wrote the first draft of the manuscript. SF supervised the study, analyzed the data and wrote the final draft of the manuscript. MT and TT supervised the study. KS, MO and KS contributed to the study design. KS contributed to the data collection. All authors contributed to the preparation of the article and approved the final manuscript.

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