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Original Article

Yokukansan Suppresses Gastric Hypersensitivity and Eosinophil-associated Microinflammation in Rats With Functional Dyspepsia

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Background/Aims
Herbal medicine is an important complementary therapy for functional dyspepsia (FD). However, its effect against gastric hypersensitivity in patients with FD has rarely been evaluated. Yokukansan (YKS), a traditional Japanese herbal medicine, is effective against neuropathic and inflammatory pain. This study aims to use a maternal separation (MS) stress-induced FD model to investigate the effects of YKS against gastric hypersensitivity, gastric motility, and duodenal micro-inflammation.

Methods
The MS stress model was established by separating newborn Sprague-Dawley rats from their mothers for 2 hours a day from postnatal days 1 to 10. At the age of 7-8 weeks, the rats were treated with YKS at a dose of 5 mL/kg (1 g/kg) for 7 consecutive days. After YKS treatment, electromyographic activity in the acromiotrapezius muscle by gastric distention and the gastric-emptying rate were assessed. Immunohistochemical analysis of eosinophils in the duodenum and phosphorylated extracellular signal-regulated kinase (p-ERK) 1/2 in the spinal cord was performed.

Results
YKS treatment suppressed MS stress-induced gastric hypersensitivity and decreased the elevated levels of p-ERK1/2 in the spinal cord. In the gastroduodenal tract, YKS inhibited eosinophil-associated micro-inflammation but did not improve gastric dysmotility.

Conclusions
YKS treatment improved gastric hypersensitivity by alleviating eosinophil-associated micro-inflammation in the gastroduodenal tract. This treatment may be considered an effective therapeutic option for epigastric pain and micro-inflammation in patients with FD.
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Key Words
Dyspepsia; Hypersensitivity; Yokukansan

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Introduction

Functional dyspepsia (FD) is a functional gastroduodenal disorder that is characterized by dyspeptic symptoms, such as early satiety, postprandial fullness, epigastric pain, or epigastric burning, in the absence of organic disease.[1] Dyspeptic symptoms commonly affect approximately 5.3-20.4% of the global population[2] and reduce quality of life and work productivity. Clinical treatment is insufficient as the disease is multifactorial, and its underlying mechanism is unclear. The clinical management of FD, such as the administration of acid-suppressive drugs, prokinetic agents, and central neuromodulators, is primarily aimed at improving the predominant symptoms and not treating the etiology of FD.[3]

Herbal medicine, an important complementary therapy, is clinically effective for relieving FD symptoms. A previous study showed that Rikkunshito (RKT), a traditional Japanese herbal medicine, has beneficial effects against postprandial fullness.[4] A multicenter randomized controlled trial showed that gastric emptying (GE) was significantly improved in patients treated with RKT.[5] However, the beneficial effect of RKT is limited to the improvement of gastric motility dysfunction. As gastric hypersensitivity and gastric motility dysfunction are regarded as the main causes of FD,[6] further studies on herbal medicine therapy for gastric hypersensitivity should be conducted. Yokukansan (YKS), a traditional Japanese herbal medicine, contains dried extracts of the following crude drugs: *Atractylodes lancea* rhizome, 3.0 g; Poria Cnidium, 4.0 g; Cnidii rhizome, 3.0 g; Uncariae cum Uncis ramulus, 3.0 g; Angelica radix, 3.0 g; Bupleurum radix, 2.0 g; and Glycyrrhiza radix, 1.5 g.[7] A study showed that YKS alleviated abnormal behavioral and psychological symptoms in patients with dementia.[8] Thus, it has been used clinically to treat anxiety, neurosis, insomnia, and night crying in children.[7] Recent reports have shown that YKS alleviates pain disorders, including chronic neuropathic and inflammatory pain.[7,9,10] Other studies revealed the analgesic effects of YKS in an animal model of formalin-induced acute inflammatory pain.[7] However, the analgesic effect of YKS against gastric hypersensitivity in FD has not been validated.

In recent years, mucosal microinflammation has been observed in the duodenum of patients with FD.[11-13] The mast cell and eosinophil counts in the duodenal mucosa are high in patients with FD, and this phenomenon is associated with postprandial satiety symptoms and gastric hypersensitivity.[13,14] The activation and degranulation of immune cells lead to the release of inflammatory mediators, which may result in tissue damage and barrier dysfunction in the duodenal mucosa and affect enteric nerve function. Changes in immune response and enteric nerve function may contribute to this sensorimotor abnormality and the development of symptoms.[15-17]

In our previous study, we established a rat model of maternal separation (MS) stress-induced FD accompanied by gastroduodenal microinflammation.[18] Using this rat model, the present study aims to investigate the effects of YKS against gastric hypersensitivity and gastroduodenal microinflammation.

Materials and Methods

Animals

Pregnant Sprague-Dawley rats (gestational day 14) were acquired from SLC Inc (Shizuoka, Japan). The rats were maintained in a 12-hour light-dark cycle (light on at 7 AM) and provided with water and food ad libitum. The room temperature was set at 23 ± 1°C, and sterile bedding was provided. The experimental protocols were approved by the Health Sciences Committee on Animal Research (No. 2018-15-2).

Maternal Separation Stress-induced Functional Dyspepsia Model

The MS stress-induced FD model was used according to our previously described protocol.[18] In brief, newborn rats were randomly assigned to the MS or control group. Both male and female rats were included in the study. The newborn rats in the MS group were separated from their mothers and each placed in a clean cup (5 cm in diameter) for 2 hours (9:30-11:30 AM) daily from postnatal days (PND) 1 to 10. PND 0 was considered as the day of birth. After separation, the MS pups were taken back to the mothers' cages. The control pups were kept in the cages with their mothers. The litters were weaned at PND 21. The rats grew up to 7-8 weeks and were used in the experiments described subsequently. Figure 1A shows the diagram of the experimental design.

Drug Treatment

A total of 73 rats were used in the study. The rats were randomly divided into the vehicle-treated control, YKS-treated control, vehicle-treated MS, and YKS-treated MS groups. The rats in the YKS group were treated with YKS (Tsumura & Co, Tokyo, Japan) orally at a dose of 5 mL/kg (1 g/kg) for 7 consecutive days. The vehicle rats received tap water orally under the same procedure. Drugs were administered once a day from 10 AM to 11 AM from PND 49 to 56. The experiments were conducted on PND 57.

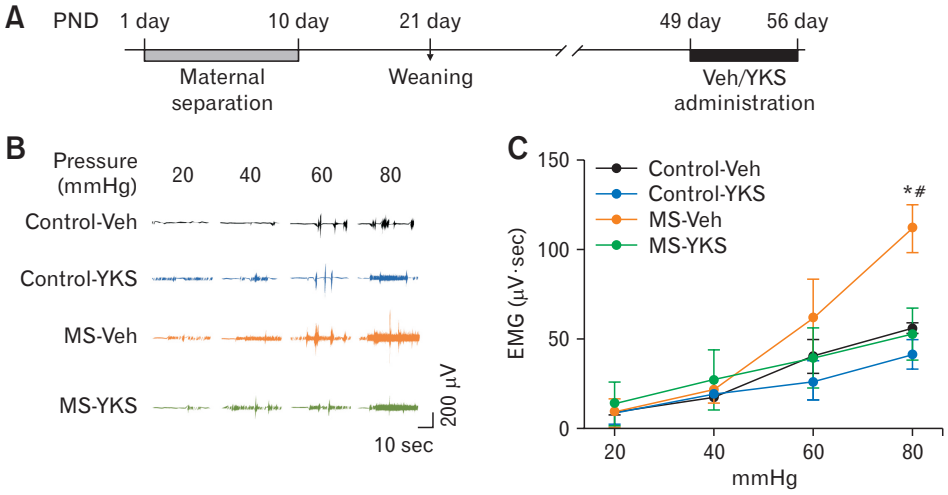

Figure 1. Yokukansan (YKS) inhibited hypersensitivity to gastric distention (GD) induced by neonatal maternal separation (MS) stress. (A) Schedule for MS and drug treatment. MS treatment was performed from postnatal day (PND) 1 to 10, and YKS was administered from PND 49 to 56. (B) Sample trace of electromyography (EMG) response to different GD pressures in each group (black line, control-veh; blue line, control-YKS; orange line, MS-veh; green line, MS-YKS). (C) Doted-line graph shows the statistics of EMG response in each group (control-veh, n = 5; control-YKS, n = 5; MS-veh, n = 5; MS-YKS, n = 7). Data were analyzed by one-way ANOVA. $^{*}P < 0.05$, MS-veh vs MS-YKS at 80 mmHg; $^{\#}P < 0.05$, MS-veh vs control-veh at 80 mmHg. Data are presented as the mean ± SEM.

Surgery and Electrode Implantation

Surgery was performed on PND 52 according to our previous protocol.[18] Briefly, the rats were fasted for 12 hours before surgery. A 2.5-cm long loose elastic latex balloon (Okamoto, Tokyo, Japan) was connected to a 12-cm long catheter (PE240 tube, BD Intramedic; BD, Franklin Lakes, NJ, USA). During surgery, the rats were anesthetized with 2% isoflurane (Wako Pure Chemical Industries, Ltd, Osaka, Japan). A 2-cm incision was made on the left abdominal wall, and the balloon was placed into the stomach via a 0.5-cm incision made above the pylorus. The incision was securely covered, and the catheter was exteriorized at the posterior part of the neck. To record the electromyographic (EMG) response to gastric distention, we prepared three 5-cm long Teflon-insulated flexible wires (AS 633; Cooner Wire, Chatsworth, CA, USA) as electrodes. These electrodes were implanted into the acromiotrapezius muscle. Briefly, 2 flexible wires were implanted into the left acromiotrapezius muscle at a distance of 1 cm, and 1 flexible wire was implanted into the right side. The electrodes were externalized with the catheter. Electrodes and catheter were anchored to the skin in the back with a suture.

Gastric Distention and Electromyography

To keep the rats calm during gastric distention, we placed them into the cylinder box 15 minutes per day for 5 consecutive days for habituation. Gastric distention was performed at day 5 after surgery. We placed the rats into a cylinder box and carefully assessed them for gastric distention. Distention was induced by inflating the balloon with water (37°C) at pressures of 20, 40, 60, and 80 mmHg for 20 seconds each. Pressure was applied at 5-minute intervals. EMG activity in the acromiotrapezius muscle was recorded simultaneously using electrodes with Power Lab (Power Lab/Bio Amplifier; AD Instruments, New South Wales, Australia). The EMG trace was recorded at a sampling rate of 4 Hz. In EMG analysis, the area under the curve (AUC) was calculated for 20 seconds during the baseline and distention periods separately. The visceromotor response was defined as the AUC of the pressure minus the AUC at baseline.

Immunohistochemistry of Phosphorylated Extracellular Signal-regulated Kinase 1/2

The balloon was distended at a pressure of 80 mmHg for 20 seconds, and the rats were perfused with 1% paraformaldehyde and then 4% paraformaldehyde through the heart 2 minutes after distention. The T9-T10 segment of the spinal cord was dissected, post-fixed overnight in 4% paraformaldehyde, and dehydrated in 30% sucrose for 2 days. Then, 20-μm thick sections were prepared. The sections were incubated with the primary antibody phosphorylated extracellular signal-regulated kinase (p-ERK) 1/2 (Cat. No. 4370s; Phospho-p-ERK44/42 Map Kinase; Cell Signaling Technology,

Inc, Danvers, MA, USA) at a ratio of 1:500 overnight. Biotinylated horse anti-rabbit secondary antibody (BA-1000; Vector Laboratories, Burlingame, CA, USA) was incubated at a ratio of 1:200 for 1 hour at room temperature. Then, the sections were incubated with avidin-biotin peroxidase (Strep ABC peroxidase kit; Nacalai Tesque Inc, Kyoto, Japan) for 30 minutes and visualized with diaminobenzidine (DAB). The images used for analysis were obtained at ×20 and ×40 magnification by digital microscopy with NIS-Elements D Imaging Software version 3.2 (Nikon Instruments Inc, Tokyo, Japan). Five sections of each animal were included for p-ERK1/2 immunoreactive cell quantification. Four animals were included in each group. The number of p-ERK1/2 cells in the laminae I-II of dorsal horn was analyzed.

Immunohistochemistry of Eosinophils

Many cationic proteins are present in eosinophils, including mouse anti-major basic protein (MBP), eosinophil peroxidase, and eosinophil cationic proteins. MBP was reported to account for > 50% of the eosinophil cationic proteins and used to represent eosinophil infiltration and degranulation.[19] Therefore, MBP was used as an eosinophil marker in the present study. Fresh-frozen tissues were collected from the stomach and duodenum for immunohistochemical analysis. Ten-micrometer thick sections were prepared using a cryostat for fixation. The sections were placed in acetone for 6 minutes at −20°C and then incubated with MBP primary antibody (Cat. No. MCA5751; AbD Serotec Ltd, Oxford, United Kingdom) at a ratio of 1:500 at 4°C overnight. The following steps were similar to those used for obtaining spinal cord sections. H&E staining was performed after the DAB reaction. For MBP-immunoreactive cell quantification, 3 areas (12, 4, and 8 o'clock positions) were selected from the transection under a light field at ×4 magnification. Images for quantification were obtained under light-field microscopy at ×40 magnification. The number of MBP-positive cells in each micrograph was counted. All micrographs were acquired under the same conditions. The results were presented as immunoreactive cells divided by the area (cells/mm^2). Three sections were analyzed for each group, which comprised of 3 rats each.

Gastric Emptying

The experiments were performed as described in our previous report.[18] Briefly, the rats were fasted for 18 hours to empty their stomachs. On the day of the experiment, the rats received 1.5 mL oral 0.05% phenol red (Wako Pure Chemical Industries, Ltd) via a syringe. After sacrifice, the stomach of the rat was resected out 30 minutes after phenol red administration. The time of sacrifice was the baseline (time 0). To homogenize the stomach, the supernatant was collected and used to measure spectrophotometric absorbance at 560 nm.

The GE was calculated according to the following formula:

$$GE(\%) = \left[1 - \frac{Absorbance\ of\ the\ test\ sample}{Absorbance\ at\ baseline\ (GE\ at\ time\ 0)}\right] \times 100\%$$

Statistical Methods

The unpaired *t* test was used to analyze p-ERK expression, eosinophil infiltration, and gastric-emptying analysis, and one-way analysis of variance was performed to analyze the EMG response. All data were presented as the mean ± standard error of the mean. Statistical tests were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). A *P*-value of < 0.05 was considered to be indicative of statistical significance ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results

Yokukansan Inhibited Hypersensitivity to Gastric Distention Induced by Neonatal Maternal Separation Stress

Gastric hypersensitivity is the main pathophysiology of FD. Thus, we investigated the EMG response to gastric distention to represent gastric hypersensitivity. Pressures of 20, 40, 60, and 80 mmHg were applied to the stomach. Distention caused an intensity-dependent EMG response in all groups. The vehicle-treated MS group exhibited gastric hypersensitivity at adulthood. Both male and female litters were included in the study, and no difference was observed in gastric hypersensitivity between the sexes (Supplementary Figure). There was no significant difference in terms of gastric hypersensitivity among the rats with distention induced at low pressure (20, 40, and 60 mmHg). However, the vehicle-treated MS group had a higher EMG response to distention at a pressure of 80 mmHg (111.87 ± 13.54 μV·sec, n = 5, $P = 0.011$) than in the vehicle-treated control group (56.52 ± 2.12 μV·sec, n = 5). Interestingly, we found that YKS treatment inhibited gastric hypersensitivity induced by MS stress. The EMG response to gastric distention at a pressure of 80 mmHg was lower in the YKS-treated MS group (52.98 ± 14.41 μV·sec, n = 7, $P = 0.017$) than in the vehicle-treated MS group. However, there was no significant difference in the EMG response between the YKS-treated con-

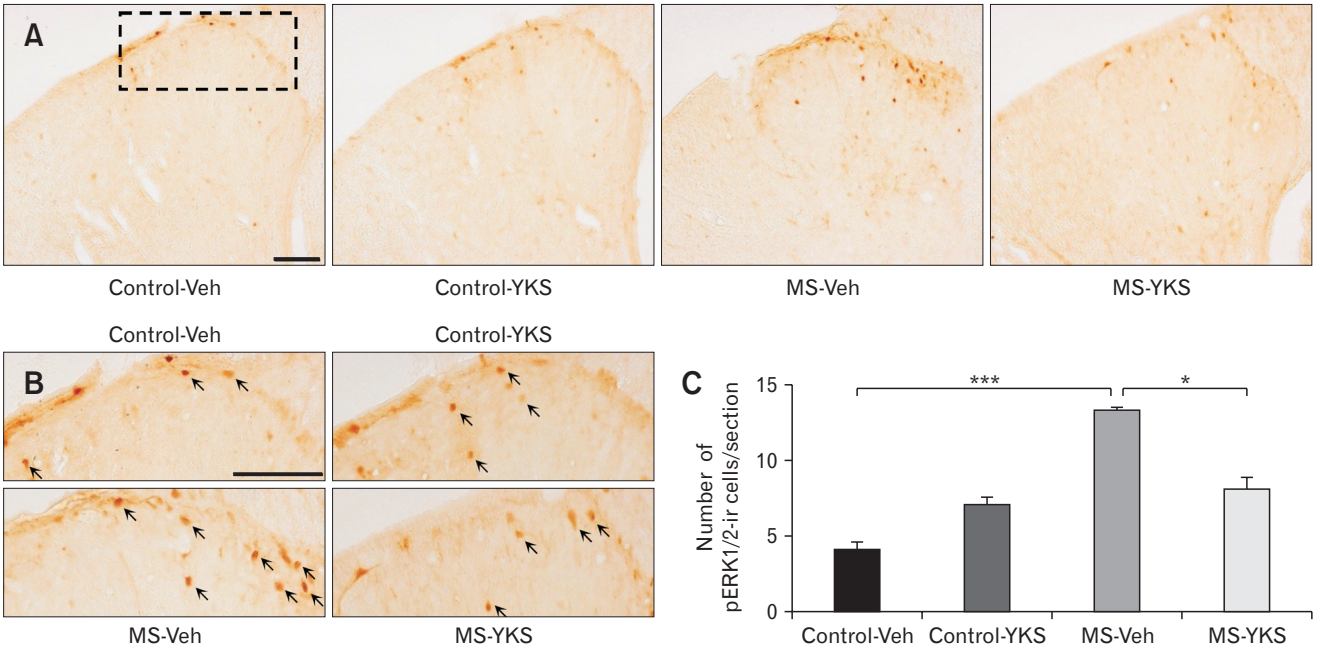

Figure 2. A lower number of phosphorylated extracellular signal-regulated kinase (p-ERK)–immunoreactive (ir) neurons were observed in spinal cord (T9-T10) after treatment with Yokukansan (YKS) in maternal separation (MS) rats. (A) Representative pictures of p-ERK–ir neurons in the dorsal horn 2 minutes after gastric distention at 80 mmHg in each group. We magnified the lamina I and II layer of the dorsal horn (dotted square), (B) the black arrows indicate p-ERK1/2–ir cells. (C) The bar graph shows the statistics of positive cells in each group at a pressure of 80 mmHg (control-veh, n = 3, control-YKS, n = 4, MS-veh, n = 4, MS-YKS, n = 4. Data were analyzed by unpaired *t* test. **P* < 0.05, ****P* < 0.001; data are presented as the mean ± SEM; scale bar: 50 μm).

trol group (41.39 ± 8.00 μV·sec, n = 5, $P = 0.304$) and vehicle group. Hence, YKS alleviated hypersensitivity to gastric distention caused by early life stress (Fig. 1B and 1C).

Yokukansan Inhibited Higher Gastric Distention-evoked Phosphorylated Extracellular Signal-regulated Kinase 1/2 Expression in the Dorsal Horn of Maternal Separation Rats

It has been found that p-ERK1/2 can function as a neural activation marker. When rats receive noxious stimuli, the neurons in the dorsal horns (laminae I-II) are activated and express p-ERK1/2. Thus, we determined p-ERK expression in the dorsal horn of the T9-10 segments after gastric distention at a pressure of 80 mmHg. Our previous study showed that there were few p-ERK1/2 immunoreactive (p-ERK1/2–ir –ir) cells in laminae I-II of the dorsal horn without distention. However, p-ERK1/2–ir cells were expressed after gastric distention at a pressure of 80 mmHg.[15] Therefore, we detected p-ERK1/2-ir cells after gastric distention at a pressure of 80 mmHg. The vehicle-treated MS group expressed more p-ERK1/2-ir cells (13.35 ± 0.21 cells/section, n = 4) than the vehicle-treated control group (4.13 ± 0.53 cells/section, n = 3, $P < 0.001$). Therefore, MS rats exhibited pain hypersensitivity to gastric distension compared to control rats. Interestingly, the YKS-treated MS group expressed lower p-ERK1/2-ir cells (8.16 ± 0.78 cells/section, n = 4, $P = 0.011$) compared with the vehicle-treated MS group. However, the expression did not significantly change after YKS treatment among the control rats (7.10 ± 0.52 cells/section, n = 4, $P > 0.05$). These data showed that YKS suppressed higher gastric distention-evoked p-ERK1/2 expression in MS rats. Therefore, YKS could be used in the treatment of gastric hypersensitivity in patients with FD (Fig. 2A-C).

Yokukansan Suppressed Eosinophil Infiltration in the Gastroduodenal Tract

Although FD is a functional disorder, micro-inflammation was observed in the duodenum of patients with FD according to several recent clinical studies. Based on our previous reports, the MS stress-induced FD model exhibited not only gastric hypersensitivity but also eosinophil-associated micro-inflammation.[15] Thus, the latter may contribute to gastric hypersensitivity in FD.

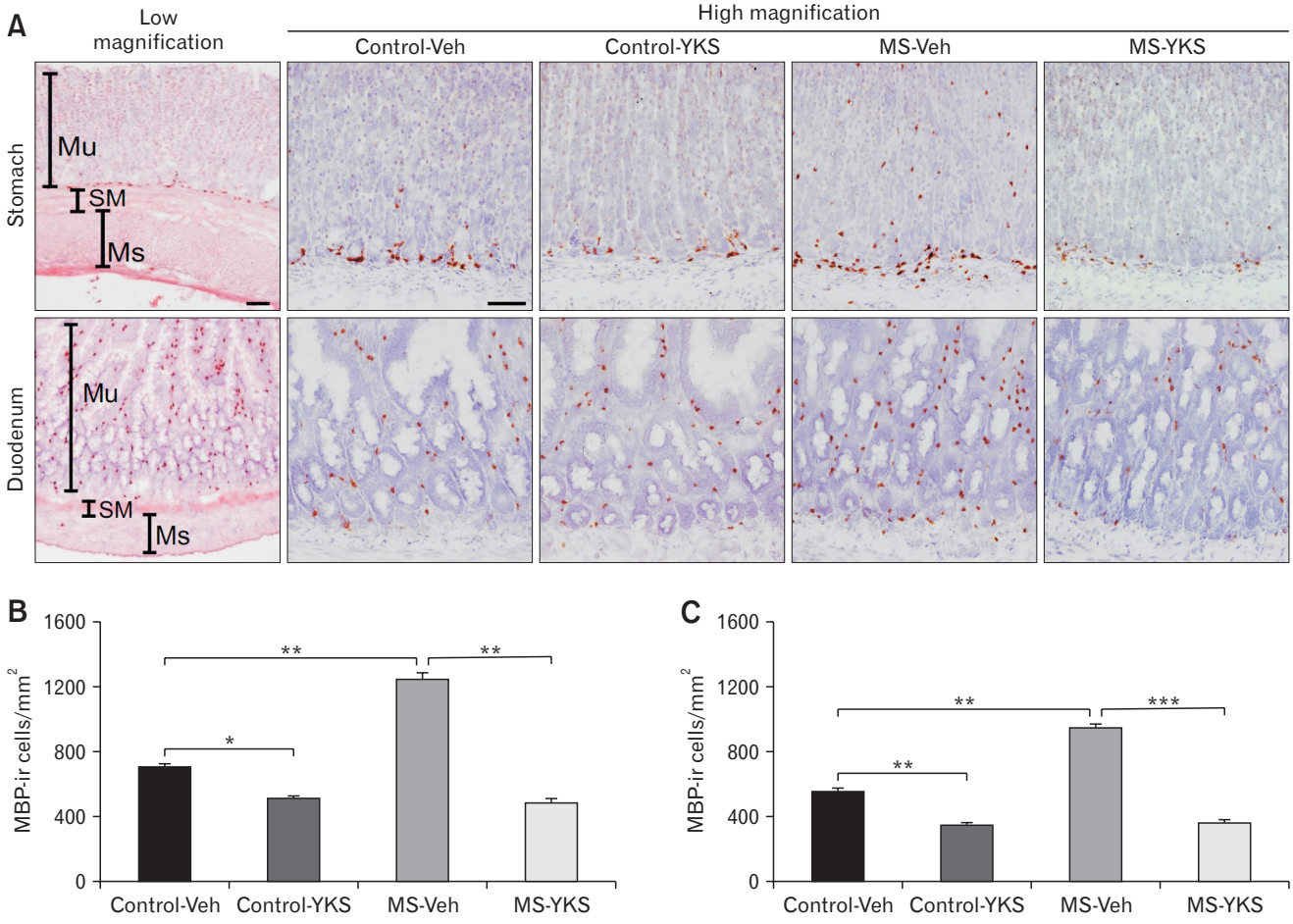

Figure 3. Yokukansan (YKS) decreased the infiltration of eosinophils into the gastroduodenal tract of maternal stress (MS) rats. (A) Low-magnification pictures show that the major basic protein (MBP)–immunoreactive (ir) cells are mainly located in the mucosa (Mu) of the stomach and duodenum, and there are very few cells in the submucosa (SM) and muscle (Ms) layer. High-magnification pictures show the MBP-ir cells in the stomach of vehicle- and YKS-treated rats in each group. (B) The bar graph shows the statistics of MBP–ir cells in the stomachs of each group. (C) The bar graph shows the statistics of MBP–ir cells in the duodenums of each group (n = 3 in each group, data analyzed by unpaired *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Data are presented as the mean ± SEM; scale bar: 50 μm).

Therefore, in the present study, it was determined whether YKS affected eosinophil infiltration in MS rats. As indicated by H&E and DAB immunohistochemistry staining, the eosinophils were mainly located in the mucosa in both the stomach and duodenum (Fig. 3A), and few cells were observed in the submucosa and muscle layers. We then confirmed the presence of eosinophil infiltration in the stomach and duodenum in the vehicle-treated MS group (stomach, 1243.8 ± 42.3 cells/mm^2, n = 3, P = 0.003; duodenum, 942.1 ± 27.6 cells/mm^2, n = 3, P = 0.002) compared with that in the vehicle-treated control group (stomach, 697.6 ± 25.4 cells/mm^2, n = 3; duodenum, 549.4 ± 17.2 cells/mm^2, n = 3) (Fig. 3A-C). The infiltrated eosinophils were also located in the mucosal layer in both the stomach and duodenum. After YKS treatment, the degree of eosinophil infiltration decreased in the gastroduodenal tract of both the control and MS rats (stomach: control-YKS, 507.3 ± 11.2 cells/mm^2, n = 3, P = 0.020; MS-YKS, 475.9 ± 31.7 cells/mm^2, n = 3, P = 0.001; duodenum: control-YKS, 338.5 ± 16.4 cells/mm^2, n = 3, P = 0.004; MS-YKS, 356.9 ± 17.0 cells/mm^2, n = 3, P < 0.001) (Fig. 3A-C). Therefore, YKS could inhibit eosinophil-associated microinflammation in the gastroduodenal tract.

Yokukansan Had No Effect on Dysmotility in Maternal Separation Rats

Gastric dysmotility is another important characteristic of FD. Patients with FD present with delayed GE and impaired gastric accommodation. Previous reports showed that RKS can improve

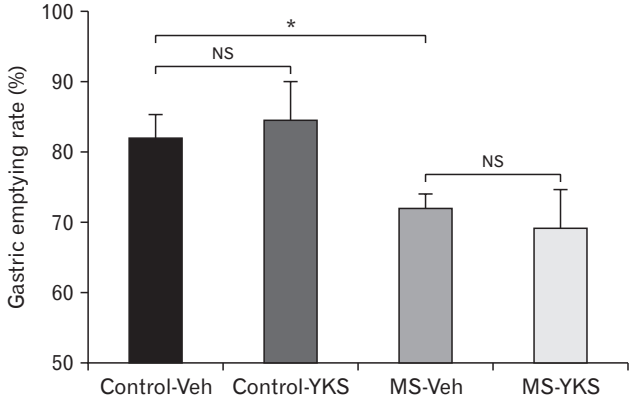

Figure 4. Yokukansan (YKS) treatment did not affect gastric motility in the control and maternal stress (MS) rats. The bar graph shows the gastric-emptying rate of each group. The MS-veh group showed delayed gastric emptying compared with the control-veh; YKS treatment did not affect motility in the MS and control groups (control-veh, n = 6; control-YKS, n = 6; MS-veh, n = 6; MS-YKS, n = 6. Data are analyzed by unpaired test, $*P < 0.05$. Data are presented as the mean ± SEM). NS, no significant difference.

dyspepsia in patients with FD. We investigated the effect of YKS on gastric dysmotility. The vehicle-treated MS group exhibited delayed GE (71.75 ± 2.10%, n = 6) compared with the vehicle-treated control group (81.53 ± 3.42%, n = 6, $P = 0.040$) (Fig. 4). However, YKS treatment did not affect motility in the control (84.21% ± 5.42%, n = 6) and MS rats (68.96% ± 5.47%, n = 6, $P > 0.05$). Hence, YKS relieved gastric hypersensitivity but not delayed GE in MS rats.

Discussion

Herbal medicine is an important complementary therapy for FD that has attracted significant attention. To the best of our knowledge, this study is the first to show that the herbal medicine YKS inhibited gastric hypersensitivity and eosinophil-associated microinflammation in an MS stress-induced FD rat model. Thus, YKS could be an effective therapeutic for the treatment of FD symptoms in clinical practice.

YKS is a traditional Japanese herbal medicine that is useful in treating behavioral and psychological symptoms, such as dementia, anxiety, depression, and in Alzheimer's disease.[20] Although FD is a functional gastrointestinal disease, a previous study reported that 34% of patients with FD had a psychiatric diagnosis, such as anxiety or depression.[21] The present study findings indicate that YKS is a promising therapeutic approach for FD symptoms, and it is already being used clinically. However, the mechanism of YKS in the treatment of FD remains unclear.

Because patients with FD present duodenal microinflammation induced by eosinophil infiltration and degranulation, which may be correlated with postprandial satiety and gastric hypersensitivity,[13] this mechanism has begun to be considered as the mechanism involved in the main pathophysiology.[11-13] A study showed that the number of eosinophils in the stomach and duodenum increased in children with functional GI disorders.[12] Friesen et al[22] found that eosinophil activation increased in the antral biopsies of patients with pediatric FD. Thus, eosinophil infiltration in the stomach and duodenum may be involved in the pathogenesis of FD. Furthermore, our previous study demonstrated that dexamethasone (DEX) inhibited eosinophil-associated microinflammation of the gastroduodenal mucosa and relieved gastric hypersensitivity.[18] Although YKS has a milder inhibitory effect on eosinophil infiltration into the gastroduodenal mucosa than DEX, YKS is useful in clinical practice because it does not lead to the side effects associated with DEX. Therefore, YKS may be an effective treatment option for epigastric pain and micro-inflammation in patients with FD.

Some previous reports described the mechanism underlying the effect of YKS against somatic pain. Repeated YKS treatment promotes glutamate reuptake via activation of glutamate transporters in the spinal cord, thereby inhibiting hyperalgesia or allodynia induced by the enhanced glutamatergic system.[23] A study showed that YKS treatment decreases the expression of 5-hydroxytryptamine 2A receptors in the prefrontal cortex in rats, which is a possible mechanism underlying the inhibitory effect of YKS against visceral pain.[24] Another study showed that YKS can relieve inflammatory pain by inhibiting substance P, which is induced by interleukin (IL)-6 and IL-8, and by reducing cyclooxygenase-2 expression.[25] Most studies focused on the anti-allodynia effect of YKS, but our study is the first to demonstrate an anti-hyperalgesia effect. This additional anti-hyperalgesia mechanism of YKS should be further investigated. Regarding the components of YKS, the combination of *A. lanceae* rhizoma and Glycyrrhizae radix extracts has shown significant antiallodynic effects.[23] Beta-Eudesmol, a component of *A. lanceae* rhizome, inhibits IL-6 expression by suppressing p38 mitogen-activated protein kinase, thereby alleviating allodynia.[26] Atractylodin is a bioactive component of *A. lanceae* that mediates an antinociceptive effect through desensitization of the TRPA1 channel.[27] Hence, *A. lanceae* rhizome and Glycyrrhizae radix may be the main extracts that relieve visceral pain. However, the components of YKS that contribute to gastric hypersensitivity in FD have not been identified, which warrants further studies. Because YKS inhibits the neurotransmitter in the spinal cord, it is possible that YKS inhibits

the neural system and inflammation simultaneously.

Although the mechanism underlying the anti-inflammatory effects of YKS is also unclear, downregulation of the expression of 5-hydroxytryptamine receptors is a potential pathway. YKS was shown to suppress central nervous system inflammation and reduce inflammatory responses by attenuating microglial activation and promoting neurogenesis in the hippocampus of Gunn mice.[28] YKS also inhibited demyelination in the CNS.[29] The anti-inflammatory properties of YKS may be attributed to rhynchophylline and isorhynchophylline in Uncaria hook and liquiritigenin in Glycyrrhiza.[30] However, the active ingredients with anti-inflammatory properties have still not been identified. Thus, studies must be performed to identify such components and related signaling pathways.

Gastric dysmotility is another important pathophysiology in patients with FD.[31] Impaired GE of liquids and solids are related to postprandial fullness and early satiety. The proportion of patients with FD reported to have delayed solids emptying is 23% and to have delayed liquids emptying is 35%. Thus, GE of liquids or solids can represent impaired gastric motility, and liquid emptying is more sensitive. Therefore, we performed liquid GE. We found that YKS did not improve MS stress-induced motility disorder, implying that YKS has no effect on delayed liquid emptying. Gastric motility is mainly regulated by the enteric nervous system (ENS), which is linked to the CNS and autonomic nervous system. Dysmotility in FD is associated with an imbalance in the ENS.[32] YKS is not effective in improving abnormal movements, indicating that its efficacy is limited in the ENS. In contrast, RKT was effective against dysmotility in FD via enhancement of ghrelin secretion.[33] Thus, YKS is effective against gastric hypersensitivity, whereas RKT is useful against dysmotility in FD. Therefore, the co-application of YKS and RKS may be a useful strategy to address all FD symptoms.

Evaluation of the EMG response to gastric distention is a reproducible, reliable, and quantifiable method to assess gastric hyperalgesia. During gastric distention, the EMG response recorded from the acromiotrapezius muscle is increased pressure-dependently compared with that from the external oblique muscle.[34] Regarding the molecular mechanism underlying gastric pain, neurons in the T9-T10 segment receive primary afferent fiber innervating the stomach;[35] thus, noxious gastric distention altered activity of neurons in T9-T10 spinal cord through primary afferent and induced pain. Phosphorylated ERK1/2 and c-fos are neural activation markers after noxious stimulation.[36] c-fos begins to be induced only in the nuclei at 30-60 minutes, whereas p-ERK1/2 can reach its peak 2-5 minutes after receiving noxious stimulation. The expression of p-ERK1/2 is broader than that of c-fos, including nuclei, cytoplasm and axons. Although both can be used as neuronal activation markers, p-ERK1/2 expression is more rapid and dynamic, which is considered to be better for indicating gastric hyperalgesia.[37] Therefore, we investigated the EMG response to gastric distention from the acromiotrapezius muscle and compared p-ERK1/2 expression in the T9-T10 segment at 2 minutes after gastric stimulation. We found that YKS inhibited the increased EMG response to gastric distention and increased p-ERK1/2 in the spinal cord of MS stress-induced FD rats, which indicate an anti-hyperalgesia effect of YKS. This result revealed that the anti-hyperalgesia effect occurred peripherally.

A sex difference has been reported in patients with FD. Symptoms occur more frequently in females than in males and cause lower quality of life.[38] A sex difference in MS rats has also been reported and may induce different behavior profiles because female rats show higher sensitivity to emotions, stress, and pain.[39] However, in the present study, we confirmed that both males and females expressed gastric hypersensitivity in adulthood, and there was no difference between the sexes. Thus, both sexes were included in the research.

In conclusion, YKS was found to suppress gastric hypersensitivity and eosinophil-associated micro-inflammation in an MS stress-induced FD rat model, which is the first evidence from a pathophysiological point of view. Thus, YKS may be a possible clinical treatment option for epigastric pain and micro-inflammation in patients with FD.

Supplementary Material

Note: To access the supplementary figure mentioned in this article, visit the online version of *Journal of Neurogastroenterology and Motility* at http://www.jnmjournal.org/, and at https://doi.org/10.5056/jnm21204.

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Conflicts of interest: None.

Author contributions: Shaoqi Duan and Nobuko Imamura performed experiments, analyzed data, and drafted the manuscript; Takashi Kondo designed the research, analyzed data, and drafted the manuscript; Hiroto Miwa and Yi Dai designed the research, interpreted data, and revised the manuscript; Hirosato Kanda, Yoko Kogure, Takuya Okugawa, Masashi Fukushima, Toshihiko Tomita, Tadayuki Oshima, and Hirokazu Fukui interpreted data and revised the manuscript; and Koichi Noguchi revised the manuscript; and all authors contributed to the interpretation of data, revised it critically, and approved the final version of the manuscript.

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