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Original

Hangekobokuto, a traditional Japanese herbal medicine, ameliorates postoperative ileus through its anti-inflammatory action

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Abstract

Background/Aims: Gastroprokinetic agents are used for patients with postoperative ileus (POI), and the Japanese traditional herbal medicine daikenchuto (DKT) is one such agent used in the clinical setting. POI is caused by inflammation. DKT and rikkunshito have anti-inflammatory abilities in addition to their gastroprokinetic effects. The efficacy of Kampo formulations, including hangekobokuto (HKT), in patients with POI has been reported recently. Several authors have described the efficacy of honokiol, the primary component of *Magnoliae Cortex,* in HKT in mouse models of POI. We therefore analyzed the effect of HKT on POI model mice to determine the similarities in the mechanism of action between HKT and DKT.

Methods: HKT was administered orally to each mouse before and after intestinal manipulation was performed on the distal ileum. The gastrointestinal transit *in vivo*, leukocyte infiltration, and levels of inflammatory mediators, such as cytokines and chemokines, were analyzed.

Results: HKT significantly inhibited the infiltration of neutrophils and macrophages and led to the recovery of delayed intestinal transit. In addition, it significantly decreased inducible nitric oxide synthase (iNOS) as well as honokiol levels, suggesting anti-inflammatory activity. However, it did not inhibit the increase in levels of interleukin (IL)-1beta and IL-6, which are related to iNOS induction. In contrast, HKT increased levels of nerve growth factor (NGF) and suppressed those of nuclear factor-κB (NFκB), which are related to

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iNOS induction, suggesting the possibility of a neuronal anti-inflammatory mechanism.

Conclusions: HKT exerted a POI-relieving effect similar to DKT in a murine POI model, and findings suggest that it may exert its anti-inflammatory activity through NGF.

Key words: anti-inflammatory action, hangekobokuto, postoperative ileus, nerve growth factor (NGF)

Introduction

Post-operative ileus (POI), a frequent complication of abdominal surgery, causes gastrointestinal dysmotility with abdominal pain and vomiting, which leads to increased costs owing to extended hospitalization (1, 2). Gastroprokinetic agents, such as metoclopramide, cisapride, and mosapride, are commonly used as first-line drugs for POI treatment. POI is induced by intestinal inflammation (3–6). Mosapride exerts anti-inflammatory effects in addition to effects on gastrointestinal hyperactivity (7).

In recent years, clinicians have used daikenchuto (DKT), a Kampo medicine, to treat POI in Japan. We previously focused on Kampo medicine and reported the utility of DKT and rikkunshito (RKT) as gastroprokinetic agents with anti-inflammatory effects (8, 9). However, there have been no reports on similar activities for other Kampo formulations.

Hangekobokuto (HKT) effectively improves abnormal pharyngeal sensation, gastrointestinal upset, and functional dyspepsia or abdominal bloating caused by mental anxiety and nervousness (10–12). A Kampo formulation including HKT was found to be effective in patients with POI (13). Furthermore, honokiol, a major component of the constituent crude drug *Magnoliae Cortex*, which is a primary crude drug of HKT, has shown efficacy in POI model mice (14). Therefore, we speculated that HKT may thus show a similar activity to DKT and RKT, which may be an effective mechanism of action for POI.

In the present study, we explored the effects of HKT on POI model mice.

Methods

Animals

We used seven-week-old male C57BL/6J mice (Japan SLC, Hamamatsu, Japan) for the present study. The mice were maintained under the following conditions: room temperature (RT) of 23 ± 2 °C, humidity at 55% ± 10%, and a 12-h light/dark cycle. Animals were given Lab Diet® 5058-PicoLab Mouse Diet 20 (Lab Diet, St. Louis, MO, USA) and water *ad libitum*. All animal experiments were conducted after obtaining approval of this study protocol from the Institutional Animal Care and Use Committee of Kitasato University in accordance with the Regulations for the Care and Use of Laboratory Animals issued by Kitasato University.

Preparation of Kampo medicines

We stored the formula of HKT and DKT according to a coded prescription from the Oriental Medicine Research Center, Kitasato University, in the pharmacy department until decoction. Daily human doses (16.5 g) of crude herbs (hange, *Pinelliae Tuber*, lot no. 00912001; Tochimoto Tenkaido Co., Ltd., Osaka, Japan, 6.0 g; bukuryo, *Hoelen,* lot no. 009519012; Tochimoto Tenkaido Co., Ltd., 5.0 g; koboku, *Magnoliae Cortex,* lot no. M3N0140; Uchida Wakanyaku Ltd., Tokyo, Japan, 3.0 g; shisoyo, *Perillae Herba*, lot no. 006917002; Tochimoto Tenkaido Co., Ltd., 1.5 g; shisoyo, *Perillae Herba*, lot no. P28281; Tumura & Co., Tokyo, Japan, 0.5 g; and

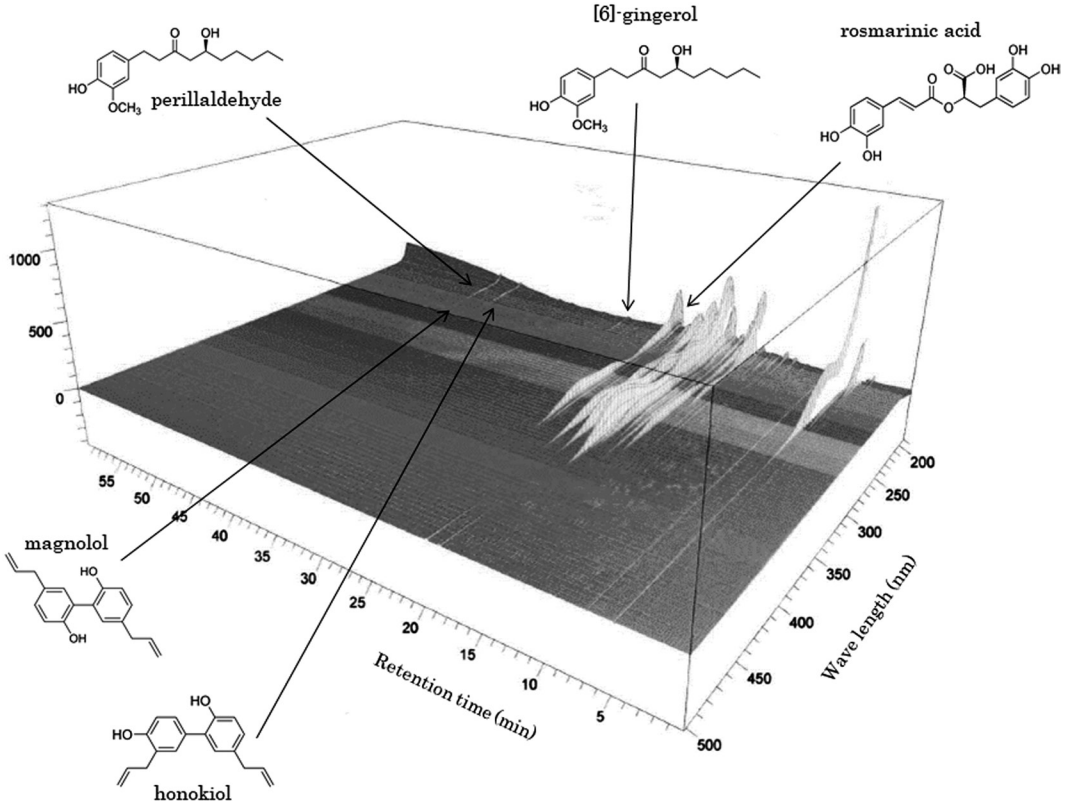

Fig. 1. Three-dimensional-HPLC pattern of a HKT decoction formula. Each peak was identified by matching the retention times and ultraviolet spectra of the major known standard compounds in the crude herbs comprising this formula.

syokyo, *Zingiberis Rhizoma*, lot no. 005820006; Tochimoto Tenkaido Co., Ltd., 0.5 g) in HKT were decocted with 400 ml of distilled water for 40 min (30 min after reaching 100 °C), and 300 ml of the filtered decoction was obtained.

We centrifuged the decocted extract solution at 4 °C, 3,000 rpm for 15 min, and the supernatant was lyophilized to obtain 1.5 g of the freeze-dried powder. This powder was dissolved in distilled water immediately before the treatment. Similarly, we dissolved the DKT powder obtained by modifying our previous report (8) in a half-human daily dose (95 mg/kg). Figure 1 depicts three-dimensional high-performance liquid chromatography (HPLC) images of the HKT extracts obtained under identical analytical conditions, as described previously (8).

Intestinal manipulation (IM)

We performed IM as previously reported (5, 15, 16). In brief, the abdomen was opened under anesthesia with isoflurane (Escain; Mylan Inc., Tokyo, Japan), and the ileum was gently rubbed with a sterile cotton applicator soaked in physiological saline. Following IM, we sealed the abdomen.

Experimental schedule

The control and HKT-treated mice underwent IM. The normal mice were not treated with IM. The HKT-treated mice were orally administered a water solution (200 µl/mouse/day) of HKT at a daily human dose (25 mg/kg) once a day for 4 or 7 days before IM. Water was orally administered to the normal and control mice for a similar period.

Intestinal transit analyses

We orally administered 80 µl of phenol red (0.25%) by gavage 23 h following IM. The gastrointestinal tract (stomach to colon), was divided into 15 segments at 1 h after the administration of phenol red (PR). The protein in each of the contents collected in sodium hydroxide (0.1 N) was precipitated by the addition of 20% trichloroacetic acid. Subsequently, the optical density at 560 nm of the each supernatant following centrifugation at 1,600 × *g* for 20 min with sodium hydroxide (0.5 N) was determined. We calculated the PR volume for each of the 15 segments using a standard curve. The following formula was used to calculate the percentage intensity and geometric center (6, 15):

Geometric Center = Σ [(% of each fluorescence signal) × (segment number)] / 100

^{13}C-acetate breath test to determine the gastric emptying rate

At 23 h following IM, a solid test meal labeled with ^{13}C sodium acetate (Cambridge Isotope Laboratories, Woburn, MA, USA) was orally administered by gavage and placed in the test chambers. We used a noninvasive breathe test system to collect the expired air from the chambers (17). We measured the $^{13}CO_2$ levels in the trapped air by POCone (Ohtuska Electronics Co., Ltd., Tokyo, Japan), denoted as $\Delta^{13}CO_2$ (‰) (18).

Whole-mount specimens

We prepared whole-mount muscularis ileum tissue samples using muscularis tissue that had been opened, pinned, and flattened (8, 19, 20). The flatted tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at 4 °C. Following fixation, these preparations were washed 3 times in PBS for 30 min and used for staining.

Histochemical and immunohistochemical analyses

We used muscular whole-mount specimens to detect myeloperoxidase (MPO)-positive neutrophil infiltration. The whole-mount specimens were stained with PBS containing 0.1% (w/v) Hanker-Yates reagent (Polysciences, Warrington, PA, USA) and 0.03% (v/v) hydrogen peroxidase (Wako Pure Chemical Industries Ltd., Osaka, Japan) immediately following whole-mount preparation. Subsequently, they were rinsed in PBS (21), and then the MPO-positive cells in the tissues were counted at ×40 magnification in 3 randomly selected fields for each specimen using a microscope (BX41; Olympus Corporation, Tokyo, Japan).

We also used muscular whole-mount specimens incubated with 0.2% Triton X-100 in PBS for 2 h at room temperature (RT) to detect Cluster of Differentiation 68 (CD68)-positive macrophages and Protein Gene Product 9.5-positive nerve regions, respectively. The specimens were incubated overnight with primary antibodies (rat anti-mouse CD68 Ab, Serotec, Düsseldorf, Germany), dilution 1:1,000, and rabbit anti-human poly Ab (Cosmo Bio Co., Ltd., Tokyo, Japan), dilution 1:1,000, at 4 °C following blocking with 2% bovine serum albumin in PBS at RT for 1 h. The specimens were washed thrice in PBS, incubated with 5% normal donkey and goat IgG in blocking buffer for 15 min, and then incubated further with appropriate secondary antibodies (donkey anti-rat Alexa 488; Molecular Probes Inc., Eugene, OR, USA; and goat anti-rabbit Alexa 568; Invitrogen, Carlsbad, CA, USA; both dilution 1:500) at RT for 90 min. They were then washed thrice, cover-slipped, and inspected under a fluorescence microscope (BX41; Olympus Corporation). The CD68-positive cells in the tissues were counted at ×40 magnification in 3 randomly selected fields for each specimen using a fluorescence microscope (BX41; Olympus Corporation).

Quantitative polymerase chain reaction (PCR)

We extracted total RNA from the ileum tissue and muscle layer using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH, USA) and determined the quantity (260 nm) and purity (280 nm) of RNA using a Nano Drop ND-1000 spectrometer (SCRUM Inc., Tokyo, Japan). Single-stranded complementary DNA (cDNA) was generated from 2 μg of total cellular RNA using a High-Capacity cDNA Reverse Transcription Kit (Rever Tra Ace®; Toyobo, Osaka, Japan). The obtained cDNAs were analyzed by real-time PCR using the Thunderbird®SYBR qPCR Mix (Toyobo) for quantitative PCR with specific primers. The real-time PCR amplifications were monitored using an analyzer (Rotor Gene Q, Qiagen, Tokyo, Japan) for 1 cycle at 95 °C for 1 min followed by 45 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s. We performed the relative quantification of the target gene expression according to a standard curve. For a comparison, the target gene expression in the individual samples was normalized to their glyceraldehyde-3-phosphate dehydrogenase (Gapdh) values. Table 1 summarizes the primers used in this study.

Statistical analyses

The results are expressed as the mean ± standard error (SE). Data were statistically evaluated using an unpaired Student's *t*-test for comparisons between two groups and a one-way analysis of variance followed by the Dunnett's test for comparisons among three or more groups. Statistical significance was set at $P<0.05$.

Results

Ameliorative action of HKT on IM-induced delayed gastric emptying and gastrointestinal transit

Figure 2 shows the effects of HKT on delayed gastric emptying and intestinal transit in the mouse POI model. Figure 2a shows the distribution of the dye in each of the 15 segments, from the stomach to the anus, following 23 to 24 h of IM. In the normal group, approximately 5% of the orally administered labeled PR remained in the stomach, with 95% transported to the distal end of the ileum, peaking at SI-9. In contrast, in the control (IM + vehicle) group, approximately 6% of the labeled PR remained in the stomach, with 94% transported to SI-9, peaking at SI-5. In the HKT-treated (IM + HKT) group, the delayed intestinal transit caused by IM was significantly recovered, with 0.6% of the orally administered content remaining in the stomach while 99.4% of the transported content had moved between SI-5 and C-1, peaking at the cecum. The average geometric center in the control group was significantly lower than that in the normal group, but the IM + HKT group showed recovery of this decrease (Fig. 2b). In normal mice, HKT did not alter the intestinal transit or gastric emptying rate (geometric center: normal, 4.65± 0.275, +HKT, 4.29 ± 0.76 GC; gastric emptying rate: normal, 64.89% ± 3.74%, +HKT, 56.86% ± 11.70%, n=3). The gastric emptying rate was also examined by the ^{13}C-acetate breath test (Fig. 2c). The obtained $^{13}CO_2$ excretion curve in the IM group was lower than that in the

Table 1. Primer list for real-time polymerase chain reaction

Gene	Forward	Reverse
iNOS	TGCAGGTCTTTGACGCTCGGAA	TCATGTTTGGCCGTCACTCCGCT
Il-6	CAACGATGATGCACTTGCAGA	CTCCAGGTAGCTATGGTACTCCAGA
Il-1β	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG
Ngf	AAGGTTTTGCCAAGGACGCA	TGTACGCCGATCAAAAACGC
NfκB	GCTGCCAAAGAAGGACACGACA	GGCAGGCTATTGCTCATCACAG
Gapdh	TGTCCGTCGTGGATCTGAC	CCTGCTTCACCTTCTTG

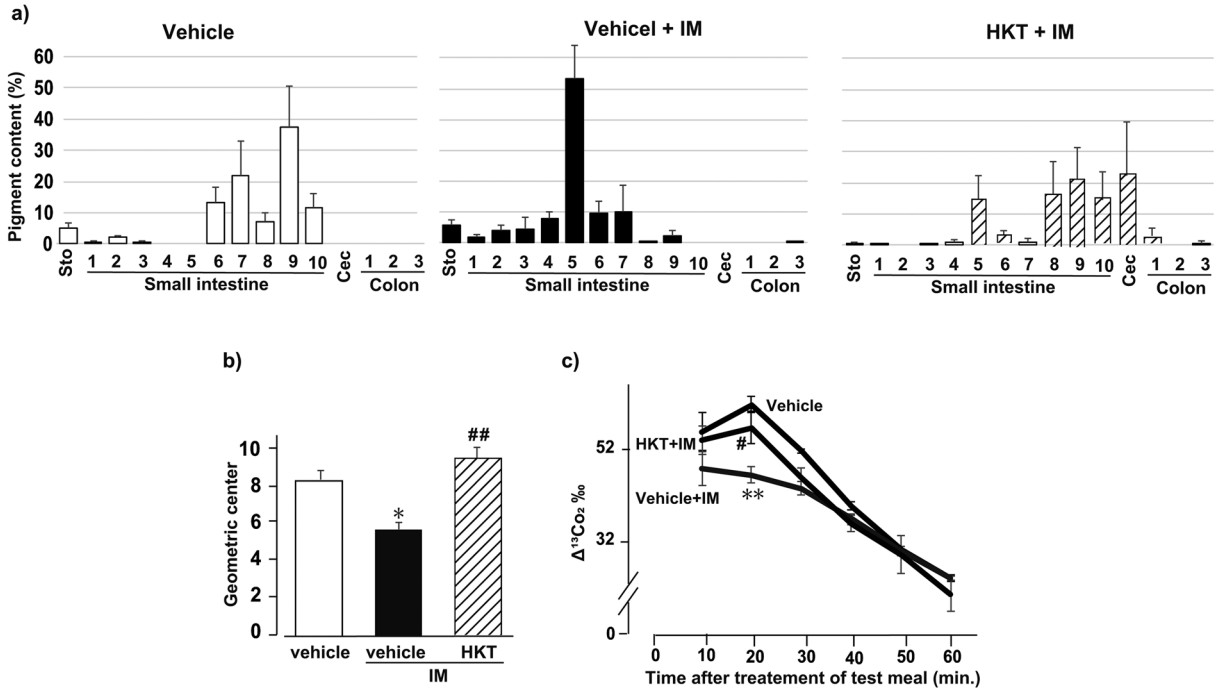

Fig. 2. The amelioration of HKT on delayed gastric emptying and gastrointestinal transit in the POI mouse model. (a) The pigment distribution in the normal group, IM + vehicle and IM + HKT (administered for 4 days) treatment groups following 23 to 24 h of IM is shown on the left, middle, and right panel, respectively. The 15 segments included 1, 10, 1, and 3 segments of the stomach (Sto), small intestine (SI1-SI10), cecum (Cec), and colon (Co1-Co3), respectively. (b) Geometric center calculated from (a). (c) The excretion curves for $^{13}CO_2$. Bars indicate the mean ± S.E.M. (n=4/group). * and ** indicate significant difference from the normal at $P<0.05$ and $P<0.01$, respectively. $^{\#}$ and $^{\#\#}$ indicate significant difference from the IM + vehicle at $P<0.05$ and $P<0.01$, respectively.

normal group, showing a statistically significant difference at the 20-min point following treatment with the test meal. HKT treatment recovered the delayed gastric emptying in the control group.

Amelioration of IM-induced inflammation of ileal muscle layer by HKT treatment

Figure 3 shows the MPO-stained neutrophils (Fig. 3a) and CD68-positive macrophages (Fig. 3b) in the ileal muscle layer 24 h after IM infiltration in the control group compared to the normal group. The increased neutrophil and macrophage populations were significantly inhibited in the IM + HKT group treated with HKT for four or seven days.

Effects of HKT on IM-induced increase in the gene mRNA expression of inflammatory mediators

We determined the effect of HKT on inflammatory mediators, neutrophil homing-related molecules, pro-inflammatory cytokines, and neutrophil-migrating chemokines associated with POI using their mRNA expression as an indicator (4, 15, 22, 23). This helped us investigate the mechanism underlying the anti-inflammatory effects of HKT in the POI model.

IM treatment significantly increased the mRNA expression of the iNOS and IL-1β genes at 6 h following IM and the IL-6 gene at 3 h following IM in the ileum (Fig. 4). The administration of HKT for four days significantly reduced the increase in iNOS mRNA, whereas the increase in the IL-1β mRNA level remained unchanged. Furthermore, HKT administration for seven days did not markedly alter the increase of IL-6 mRNA. Although the expression of IL-6 in the HKT-treated group varied markedly, as shown in Fig. 4b, none of the

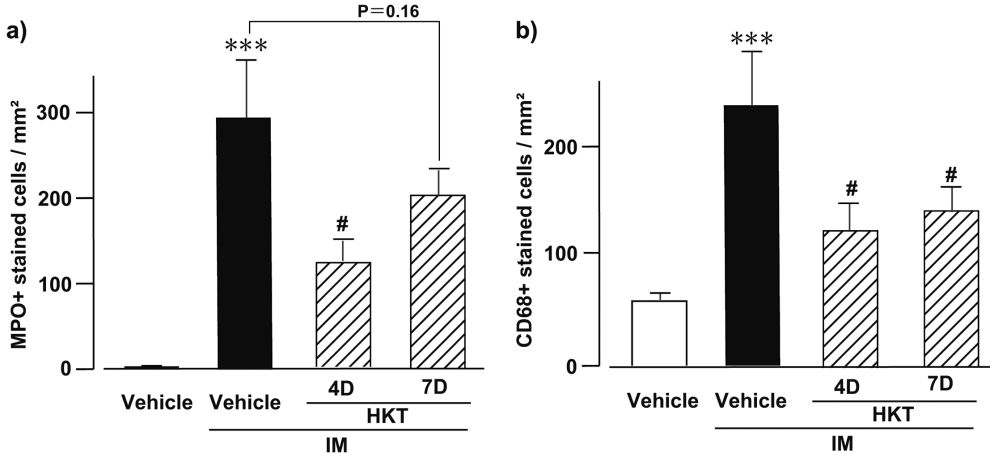

Fig. 3. The amelioration of IM-induced neutrophil infiltration and macrophage infiltration by HKT treatment for four or seven days. (a) The quantification of neutrophil cells at 24 h following IM. (b) The quantification of macrophage cells at 24 h following IM. Columns indicate the mean ± S.E.M. (n=6/group). *** indicates significant difference from the normal at P<0.001. # indicates significant difference from the IM + vehicle at *P*<0.05.

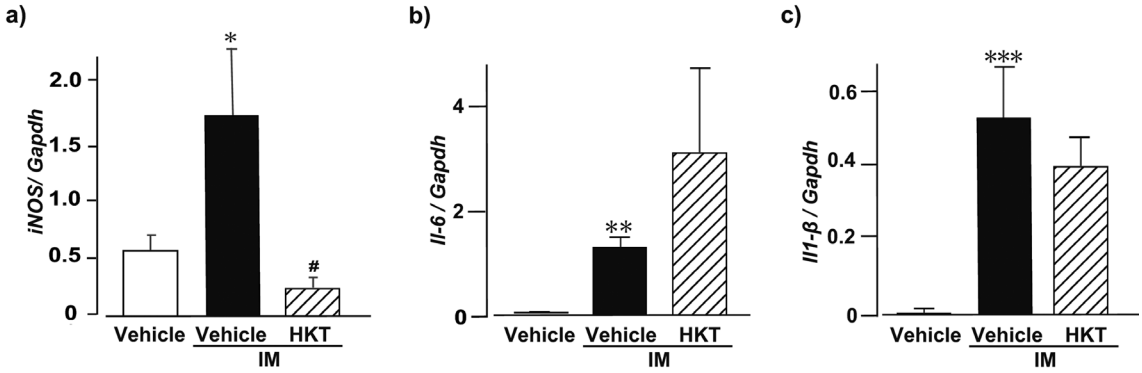

Fig. 4. The effect of HKT on the mRNA of pro-inflammatory cytokines and neutrophil-migrating chemokines. (a)–(c) iNOS and IL-1β gene mRNA expression at 6 h after IM of ileum treated with HKT for 4 days and IL-6 gene mRNA expression at 3 h after IM of ileum treated with HKT for 7 days, respectively. Columns indicate the mean ± S.E.M. (n=6/group). *, **, and *** indicate significant difference from the normal group at *P*<0.05, 0.01, and 0.001, respectively. # indicates a significant difference from the IM + vehicle group at *P*<0.05.

groups treated with different doses of HKT that showed inhibition of neutrophil infiltration had an improvement in the increased expression of IL-6 without individual data differences within each group (data not shown).

Effects of HKT on the mRNA expression of nerve growth factors (NGFs)

NGFs have both pro- and anti-inflammatory effects (24). We therefore focused on NGFs other than homing molecules of macrophages and neutrophils. We examined the effects of HKT on the mRNA expression of the NGF gene (Ngf) in the ileal muscle layer at 3 h following IM (Fig. 5). In the mouse model, we observed a decrease in the ngf expression following IM, and HKT administration resulted in a significant recovery of this gene expression (Fig. 5a). In contrast, the administration of DKT (95 mg/kg), half of the daily human dose, did not increase the Ngf level (Fig. 5b). In normal mice, HKT did not markedly alter the NGF gene expression (normal, 1.032 ± 0.393, +HKT, 1.407 ± 0.819, n=4 and 3).

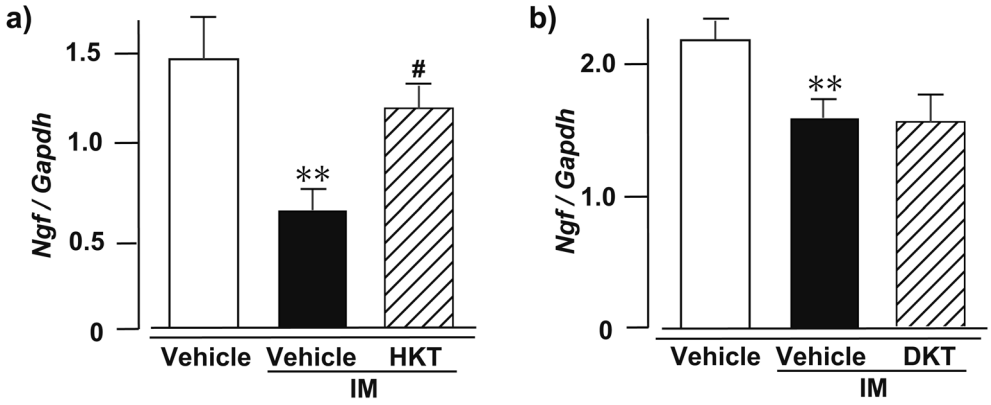

Fig. 5. The effect of HKT and DKT on NGF mRNA. (a) and (b) mRNA expression of the NGF gene at 3 h following IM of the ileum muscle layer treated with HKT or DKT for 4 days, respectively. Columns indicate the mean ± S.E.M. (n=5–6/group). ** and *** indicate significant difference from the normal group at $P<0.01$ and $P<0.001$, respectively. # indicates a significant difference from the IM + vehicle group at $P<0.05$.

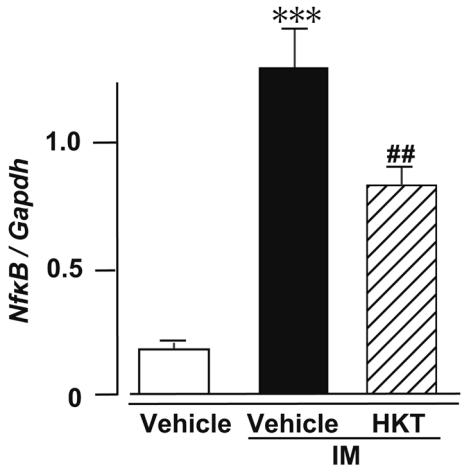

Fig. 6. The effect of HKT on NFκB mRNA. Columns indicate the mean ± S.E.M. (n=5–6/group) of NFκB mRNA expression at 3 h following IM of the ileum muscle layer treated with HKT for 4 days. *** indicates a significant difference from the normal group at $P<0.001$. ## indicates a significant difference from the IM + vehicle group at $P<0.01$.

Effects of HKT on the mRNA expression of nuclear factor-κB (NFκB)

As shown in Fig. 6, the mRNA expression of NFκB in the ileal muscle layer at 3 h following IM was significantly increased, and HKT administration reduced it.

Discussion

In the present study, HKT ameliorated gastrointestinal dysmotility in addition to neutrophil and macrophage infiltration in a POI mouse model. HKT directly restored the delayed gastric emptying ability induced by IM according to the ^{13}C-acetate breathe test, not only improving intestinal transit dysfunction. This result is in agreement with the finding of HKT improving gastric emptying in patients with functional dyspepsia, although the details of how HKT exerts this effect are unclear (25).

Regarding the expression of inflammatory mediators, such as proinflammatory cytokines in gastroin-

testinal tissues, HKT markedly decreased the mRNA expression of iNOS. iNOS plays an essential role in the POI model not only by inhibiting gastrointestinal motility via NO production but also by initiating intestinal inflammation (5). Taken together, these findings suggest that iNOS suppression may be an important pharmacological effect of HKT in this model. Honokiol, a major component of *Magnoliae Cortex* in HKT, exerted its therapeutic effect by suppressing iNOS gene expression in a POI model (14). In contrast, HKT did not suppress the increase in IL-1β or IL-6, which are known to induce iNOS (26, 27) (8, 14). HPLC revealed that 1 mg of freeze-dried HKT contained 0.8 μg of honokiol (data not shown); the total amount of honokiol in the HKT used in the present study was thus only a tiny fraction of the dose of honokiol capable of suppressing IL-1β and IL-6. The presence of another anti-inflammatory pathway different from that of honokiol is thus suspected.

To investigate the possibility of an alternate anti-inflammatory route, we focused on NGF, as enhanced NGF production is associated with anti-inflammatory effects, including the suppression of neutrophils or macrophage infiltration and IL-10 production in intestinal epithelial cells (28–30). In addition, the functional blockade of NGF worsens experimental colitis by decreasing calcitonin gene-related peptide, which enhances iNOS release from proliferated lymphocytes (28, 31, 32). The NGF-induced phosphorylation of protein kinase B inhibits the translocation of NF-κB, an iNOS inducer, into the nucleus (33, 34). In the present study, IM significantly reduced the mRNA expression of ngf, while HKT significantly increased it; however, DKT did not increase the expression in a similar model. HKT also significantly reduced the IM-induced increase in mRNA expression of NF-κB. Accordingly, this finding may be attributed to a unique anti-inflammatory pathway involving HKT that differs from that of DKT. Further analyses will be required to confirm the mechanism of NGF reduction by IM and the anti-inflammatory mechanism of NGF.

Conclusions

In the present study, HKT suppressed POI in a murine model by inhibiting iNOS induction, possibly by increasing the NGF levels. HKT may exert its POI-relieving action through an anti-inflammatory route different from that involved in DKT.

Acknowledgements

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