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ORIGINAL ARTICLE—ALIMENTARY TRACT

Daikenchuto, a traditional Japanese herbal medicine, ameliorates postoperative ileus by anti-inflammatory action through nicotinic acetylcholine receptors

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Abstract
Background Daikenchuto (DKT), a gastrointestinal prokinetic Japanese herbal medicine, is prescribed for patients with postoperative ileus (POI) and adhesive bowel obstruction following abdominal surgery. Several mechanisms for the amelioration of POI by DKT have been suggested; however, it has remained unclear whether DKT shows anti-inflammatory effects in POI. In the present study, we investigated the effects of DKT in a mouse POI model and attempted to clarify the detailed mechanisms of action.
Method Intestinal manipulation (IM) was applied to the distal ileum of mice. DKT was administered orally to the animals 4 times before and after IM. Gastrointestinal transit in vivo, leukocyte infiltration, cytokine mRNA expression and gastrointestinal motility were analyzed. We also investigated the effects of the α7nAChR antagonist methyllycaconitine citrate (MLA) on the DKT-mediated ameliorative action against POI, and we studied the effects of DKT on inflammatory activity in α7nAChR knockout mice.
Results DKT treatment led to recovery of the delayed intestinal transit induced by IM. DKT significantly inhibited the infiltration of neutrophils and CD68-positive macrophages, and inhibited mRNA expressions of TNF-α and MCP-1. MLA significantly reduced the anti-inflammatory action of DKT, and the amelioration of macrophage infiltration by DKT was partially suppressed in α7nAChR knockout mice.
Conclusions In conclusion, in addition to the gastrointestinal prokinetic action, DKT serves as a novel therapeutic agent for POI characterized by its anti-inflammatory potency. The DKT-induced anti-inflammatory activity may be partly mediated by activation of α7nAChR.

Keywords Anti-inflammatory action · Daikenchuto · Macrophage · Nicotinic acetylcholine receptor · Postoperative ileus

Abbreviations

POI	Post-operative ileus
DKT	Daikenchuto
IM	Intestinal manipulation
α7nAChR	α7 Nicotinic acetylcholine receptor
TNF-α	Tumor necrosis factor alpha

Introduction

Post operative ileus (POI) is a problem that results in treatment delays and increased cost burden of hospitalization among patients undergoing abdominal surgery [1]. Gastrointestinal prokinetic agents, such as metoclopramide [2], cisapride [3] and mosapride [4, 5], provide treatment options for POI. At present, however, they are rarely used

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in clinical settings. Therefore, further elucidation of the pathogenesis of POI and establishment of new options for its treatment are required [6].

Daikenchuto (DKT) is a traditional herbal (Kampo) medicine in Japan, and comprises four medical herbs; zanthoxylum fruit, processed dried ginger, ginseng, and malt sugar. This formula is known for its prokinetic action or clinical efficacy against intestinal obstruction subsequent to laparotomy or radiation therapy [7–11]. In experimental studies, DKT showed preventive effects against POI [8, 12, 13]. It is conceivable that DKT stimulates intestinal motility and accelerates delayed intestinal transit through the cholinergic pathway and activation of 5-HT_3R and 5-HT_4R. The main mechanism of contractile action and improvement of gastrointestinal motility mediated by DKT is the release of acetylcholine (ACh) from the cholinergic nerves through 5-HT_3R and 5-HT_4R stimulation [12–14]. This ACh improves delayed intestinal transit and recovers delayed gastric emptying in POI [12–14].

Recent studies have revealed that local inflammation is responsible for prolonging post-operative gastrointestinal motility disorder [6, 15]. Macrophages and neutrophils play a pivotal role in the induction of post-operative ileus. These inflammatory cells express inducible nitric oxide synthase, which in turn produces nitric oxide, thus inducing gastrointestinal motility disorder [16, 17]. As noted above, gastroprokinetic agents such as 5-HT_4R agonists provide effective therapeutic treatment for POI. We recently found that mosapride citrate exerts anti-inflammatory effects by activating alpha7 nicotinic acetylcholine receptors (α7nAChR) on macrophages via released ACh from myenteric plexus, which in turn ameliorates POI [5].

On the other hand, it has been reported that DKT decreases serum C reactive protein (CRP) levels induced by laparoscopic colorectal resection [18], and increases adrenomedullin (ADM) and calcitonin gene-related peptide (CGRP) levels [19, 20]. We therefore hypothesized that DKT prevents POI due to its gastroprokinetic activity, as well as it anti-inflammatory action via α7nAChR stimulation and other pathways. In the present study, we investigated the efficacy and mechanisms of action of DKT primarily on its anti-inflammatory potency, using a mouse POI model. The results indicated that DKT has an anti-inflammatory action, in addition to its prokinetic action, and part of this anti-inflammatory action is mediated through α7nAChR activation.

Materials and methods

Animals

Male BALB/c mice (Japan SLC, Hamamatsu, Japan) weighing 21–26 g were used. Mice were housed under conditions of constant temperature (23 ± 2 °C) and humidity (55 ± 10 %) with standard rodent chow and water ad libitum, and a 12-h light/dark cycle. All animal experiments were performed according to the "Regulations for the Care and Use of Laboratory Animals in Kitasato University" published by Kitasato University. The Institutional Animal Care and Use Committee for Kitasato University approved the study protocol.

Female and male α7nAChR knock out (KO) mice of C57BL/6J background and weighing 21–23 g (The Jackson Laboratory, Bar Harbor, ME) were obtained from backcrossing with wild-type (WT; C57BL/6J) strains. Mice were cared for in strict compliance with the "Guide to Animal Use and Care" published by the University of Tokyo. The Institutional Review Board of the Graduate School of Agriculture and Life Science of the University of Tokyo approved the study protocol.

Preparation of Kampo medicines

DKT was blended in the Oriental Medicine Research Center of Kitasato University. Daily human doses (190 mg/kg) of the crude herbs (ninjin, *Ginsen radix* 3.0 g; kankyo, *Zingiberis Siccatum rhizoma* 3.0 g; sansho, *Zanthoxylum fructus* 2.0 g; and koi, *Saccharum Gramorumb* 20.0 g) in DKT were decocted with 600 ml of distilled water until the filtered decoction was reduced by half. The decocted extract solution was centrifuged at 3,000 rpm for 15 min, and the supernatant was lyophilized. The obtained freeze-dried powder (9.5 g) was dissolved in distilled water to the appropriate dose just before administration. In the current series of experiments using mice, the half daily human dose (95 mg/kg) was used. Manufactured DKT (m-DKT) prepared as a dried powder extract of mixing the crude herbs (*Ginsen radix*, 3.0 g; *Zingiberis siccatum*, 5.0 g; *Zanthoxylum fructus*, 2.0 g) was from Tsumura & Co. Ltd. (Tokyo, Japan) with dried powder extract of *Saccharum gramorumb* at a ratio of 1:8.

Three-dimensional HPLC

DKT was dissolved with H_2O, and was then filtered and analyzed by HPLC (ACQUITY UPLC; Nihon Waters K.K, Tokyo, Japan) under the following conditions. Sample (10 μl) was applied to a COSMOSIL C18-MS-II column (3.0 × 50 mm; Nacalai Tesuque Co., Inc., Kyoto, Japan). The mobile phase was water (H_2O)/acetonitrile (CH_3Hn) (9:1) for the first 10 min, changing to a linear gradient of (1:1) over 85 min. The flow rate was 1.0 ml/min and the oven temperature was 30 °C. HPLC patterns were analyzed by absorbance at 200–340 nm.

Intestinal manipulation (IM) for mouse model of POI

All animals were anesthetized with isoflurane (Escain; Mylan Inc., Tokyo, Japan) or pentobarbital sodium (Somnopentyl; Kyoritsu Seiyaku Corp., Tokyo, Japan). Intestinal manipulation (IM) was performed as reported previously [21, 22]. Briefly, the distal ileal part (10 cm from the ileocecal valve) was exteriorized and then gently scraped with compression along its entire length for about 3–5 min at a strength equal to general writing pressure using a sterile cotton applicator moistened with physiological saline. After manipulation, the abdomen was closed with sutures.

Intestinal transit determination

Twenty-three hours after IM with fasting, the non-absorbable marker, 80 μl of 0.25 % (w/v) phenol red (PR) in phosphate buffered saline (PBS), was orally administered to mice via a gastric tube. After 1 h, the gastrointestinal part was isolated and stomach and intestine were separated as a single stomach segment (Sto), ten small intestine segments (SI1–SI10), a single cecum segment (Cec) and three colon segments (Co1–Co3). The contents of each segment were mixed with 0.1 N sodium hydroxide. Proteins in the sample were precipitated by addition of 20 % (w v^{-1}) trichloroacetate. The optical density volume of each supernatant after centrifugation at 1600×*g* for 20 min with 0.6 N sodium hydroxide was then determined at 570 nm. The volume of PR for 14 segments was calculated using a standard curve. Each geometric center (GC) of distribution for PR in 14 segments of the gastrointestinal tract was calculated using the following formula [17, 22]:

$$\mathrm{GC} = \Sigma\{(\%\ \text{of each fluorescence signal}) \times (\text{segment number})\} \div 100$$

^{13}C-acetate breath test

Twenty-two hours after IM with fasting, mice were given a [1-^{13}C] sodium acetate (Cambridge Isotope Laboratories, Woburn, MA, USA) labeled solid test meal and placed in test chambers. To collect air from the chambers, we used the noninvasive breath test system [23], comprising four animal chambers, a pump and breath sampling bags. Expired air was collected and measured at 5-min intervals until 30 min, with additional measurements at 10-min intervals until 60 min. $^{13}CO_2$ levels in the trapped air were measured by POC one (Otsuka Electronics Co., Ltd., Tokyo, Japan) and are given as $\Delta^{13}CO_2$ (‰), as reported previously [24].

Whole mount preparations

Histochemical examination was performed on whole-mount muscularis preparations of the ileum. Whole-mount muscularis (5 cm from ileocecal valve of the distal ileal region) samples were prepared as reported previously [23, 25, 26]. The isolated mucosa-free muscularis tissue sheets were pinned to the silicon base of dishes. The tissue sheets were stretched to 110 % of their resting length on the silicon sheet and then fixed in 4 % paraformaldehyde in PBS for 30 min at 4 °C to make a whole mount preparation (5 mm × 5 mm sheet). After fixation, whole mount were washed in PBS and were then cut and used for staining procedures.

Whole mount immunohistochemistry

Muscularis whole mounts were used for immunohistochemical analysis of CD68 and PGP9.5. Each whole mount was incubated with 0.2 % triton X-100 in PBS at room temperature (RT) for 2 h. After blocking with 2 % BSA in PBS at RT for 1 h, whole mounts were incubated overnight in primary antibody (rat anti-mouse CD68 Ab, dilution 1:1000; Serotec, Düsseldorf, Germany; and rabbit anti-human PGP9.5 poly Ab, dilution 1:1000; Cosmo Bio Co., Ltd, Tokyo, Japan) at 4 °C, washed three times in PBS, incubated in 5 % normal donkey and goat IgG in blocking buffer for 15 min, followed by the appropriate secondary antibody (donkey anti-rat Alexa 488, dilution 1:500; Molecular Probes Inc., Eugene, OR; and goat anti-rabbit Alexa 568, dilution 1:500; Invitrogen, Carlsbad, CA) at RT for 90 min. After washing three times, whole mounts were cover-slipped and inspected by confocal microscopy (ECLIPSE Ti; Nikon, Tokyo, Japan). CD68-positive cells were counted in three randomly selected fields in each specimen at a magnification of 40×. The same experiment was performed four times in order to calculate mean ± SEM.

Myeloperoxidase staining

In order to detect myeloperoxidase (MPO)-positive neutrophils, freshly prepared whole mounts were stained with PBS containing 0.1 %(w/v) Hanker-Yates reagent (Polysciences, Warrington, PA) and 0.03 %(v/v) hydrogen peroxidase (Wako Pure Chemical Industries Ltd., Osaka, Japan), and were then rinsed in PBS [27]. Cells that were obviously MPO-positive in the muscularis were counted under a microscope (BX41; Olympus Corporation, Tokyo, Japan) in three randomly selected fields for each specimen at a magnification of 40×.

Measurement of myeloperoxidase activity

MPO activity in the ileum tissue was measured as described previously, with some modifications [28]. To measure the MPO activity spectrophotometrically, ileal segment tissue homogenates prepared 24 h after IM were combined with TMB substrate reagent. The absorbance at 460 nm was measured on a spectrophotometer (Model 680; Bio Rad, Hercules, CA, USA). The units of MPO activity were divided by the colonic wet weight of the tissue.

Semi-quantitative RT-PCR

Quantitative RT-PCR was performed as described previously [26]. Oligonucleotide primers for GAPDH (NM 008084), MCP-1 (NM 011333), TNF-α (NM 013693), IL-6 (NM 031168), IL-1β (NM 008361) and iNOS (BC 062378) were designed based on the cDNA database. The forward and reverse primers and product sizes were listed in Table 1. Amplification proceeded in a PCR Thermal Cycler (Takara PCR Thermal Cycler MP; Takara Bio, Otsu-shi, Japan) using 32 cycles consisting of 94 °C for 40 s, 58 °C for 60 s, and 72 °C for 90 s. The products of each cycle were resolved on 2 % agarose gels containing 0.1 % ethidium bromide. Detectable fluorescent bands were visualized with an ultraviolet transilluminator (High Performance UV transilluminator; UVP, Upland, CA), and the density of detectable fluorescent bands was measured using NIH Image software (Image J, Ver. 1.44p).

Experimental design

Animals were randomly divided into the following experimental groups: (1) Normal, no treatment, no IM; (2) Control (IM + Vehicle), ultrapure water was orally administered at 3 days, 2 days and 1 day before, and at 6 h after IM; and (3) IM + DKT, 0.2 ml of DKT with doses at one tenth (19 mg/kg) or half (95 mg/kg) the human daily dose were similarly orally administered four times to the mice via gastric tube. The group information was not blinded to the operator. To assess the effects of DKT alone, mice were randomly assigned into 2 groups: 1) Normal; and 2) Normal + DKT. After sacrifice, the muscle layer of the ileum was used for histochemical staining for MPO and immunohistochemical staining for macrophages. The α7nACh receptor antagonist methyllycaconitine citrate (MLA; 0.0125 mg/kg, s.c.) was also applied 30 min before each DKT administration as necessary. Mice were euthanized at 24 h after IM. For analysis of expression of inflammatory mediators, vehicle or DKT was given orally at 3 days, 2 days and 1 day before IM. Samples were taken after at 3 h after IM. Isolated and prepared whole mount smooth intestinal muscle layer was used for analysis of cytokine messenger RNA (mRNA) expression. Intestinal tissue samples were used for MPO staining. In vivo intestinal transit from 23 to 24 h after IM was also determined. All animals recovered rapidly from the bowel manipulation procedure (within 3 days; data not shown). We therefore evaluated the efficacy of DKT at 24 h after IM. Surgery is generally performed according to plan in clinical practice, except in extreme emergencies; prophylactic administration of DKT before IM may therefore have important clinical implications.

Statistics

Results are expressed as mean ± SEM. Data were statistically evaluated using unpaired Student's t test for comparisons between two groups and by one-way analysis of variance (ANOVA) followed by Dunnett's test for comparisons among three or more groups. Values of $P < 0.05$ were considered to be statistically significant.

Table 1 Seqences of PCR primers and their T_m values and product sizes

Gene (locus)	Forward primers reverse primers	T_m (°C)	PCR cycles	Size (bp)
GAPDH	TGTTCCTACCCCCAATGTGT	58	32	269
(NM 007778.2)	CCCTGTTGCTGTAGCCGTAT			
TNF-α	ACGGCATGGATCTCAAAGAC	58	32	324
(NM 013693)	CGGACTCCGCAAAGTCTAAG			
IL-6	TCTCTGGGAAATCGTGGAAA	58	32	397
(NM 031168)	GATGGTCTTGGTCCTTAGCC			
IL-1β	TGACGTTCCCATTAGACAGC	58	37	497
(NM 008361)	TGGGGAAGGCATTAGAAACA			
MCP-1	CCCACTCACCTGCTGCTACT	58	32	381
(NM 011333)	AAGGCATCACAGTCCGAGTC			
iNOS	AAGAGAGTGCTGTTCCAGGT	58	37	196
(BC 062378.1)	CCACCAGCTTCTTCAACGTG			

Results

Three-dimensional HPLC

HPLC profile of DKT was shown in Fig. 1. HPLC analysis revealed that the prepared DKT contained ginsenoside-Rg1, [6]-gingerol, ginsenoside-Rb1 and [6]-shogaol. The main component detected at 75 min should be hydroxy-α-sanshool plus hydroxy-β-sanshool, as reported previously [10, 29], although this was not confirmed using standard preparations.

Recovery of IM-induced intestinal transit disorder by treatment with DKT

The effects of DKT on delayed intestinal transit in the mouse POI model are summarized in Fig. 2. Approximately 6 % of the orally administered labeled phenol red (PR) remained inside the stomach, while 94 % was transported down the intestine to the distal end of the ileum, peaking at SI-8 in the normal group (Fig. 2a). The average calculated geometric center and gastric emptying rate in the normal group were 7.16 ± 0.22 and 94.42 ± 0.85 % for the 15 segments of the gastrointestinal tract, respectively (Fig. 2b, c). In the IM + Vehicle group, approximately 44 % of the orally administered labeled PR remained inside the stomach, while 56 % was transported to SI-1 and SI-2 (Fig. 2a). The IM group showed significantly delayed rates for the geometric center at 2.04 ± 0.07 and gastric emptying at 55.56 ± 4.13, as compared with the normal group (Fig. 2b, c). The IM + DKT (95 mg/kg) group showed significant recovery of the delayed intestinal transit caused by IM, in which 22 % of the orally administered content remained in the stomach, while 78 % of the transported content moved between SI-1 and SI-3, peaking in SI-3 (Fig. 2a). Both the geometric center and gastric emptying rate in IM + DKT (95 mg/kg) were significantly higher, reaching 4.11 ± 0.37 and 84.13 ± 2.60, respectively (Fig. 2b, c). In normal mice, DKT slightly but significantly increased intestinal transit and gastric emptying rate (Geometric center: normal, 7.15 ± 0.15, +DKT, 9.53 ± 0.78, $P < 0.05$; Gastric emptying rate: normal 94.96 ± 0.63 %, +DKT, 99.70 ± 0.22 %, $P < 0.05$, $n = 3$–5), thus suggesting the prokinetic

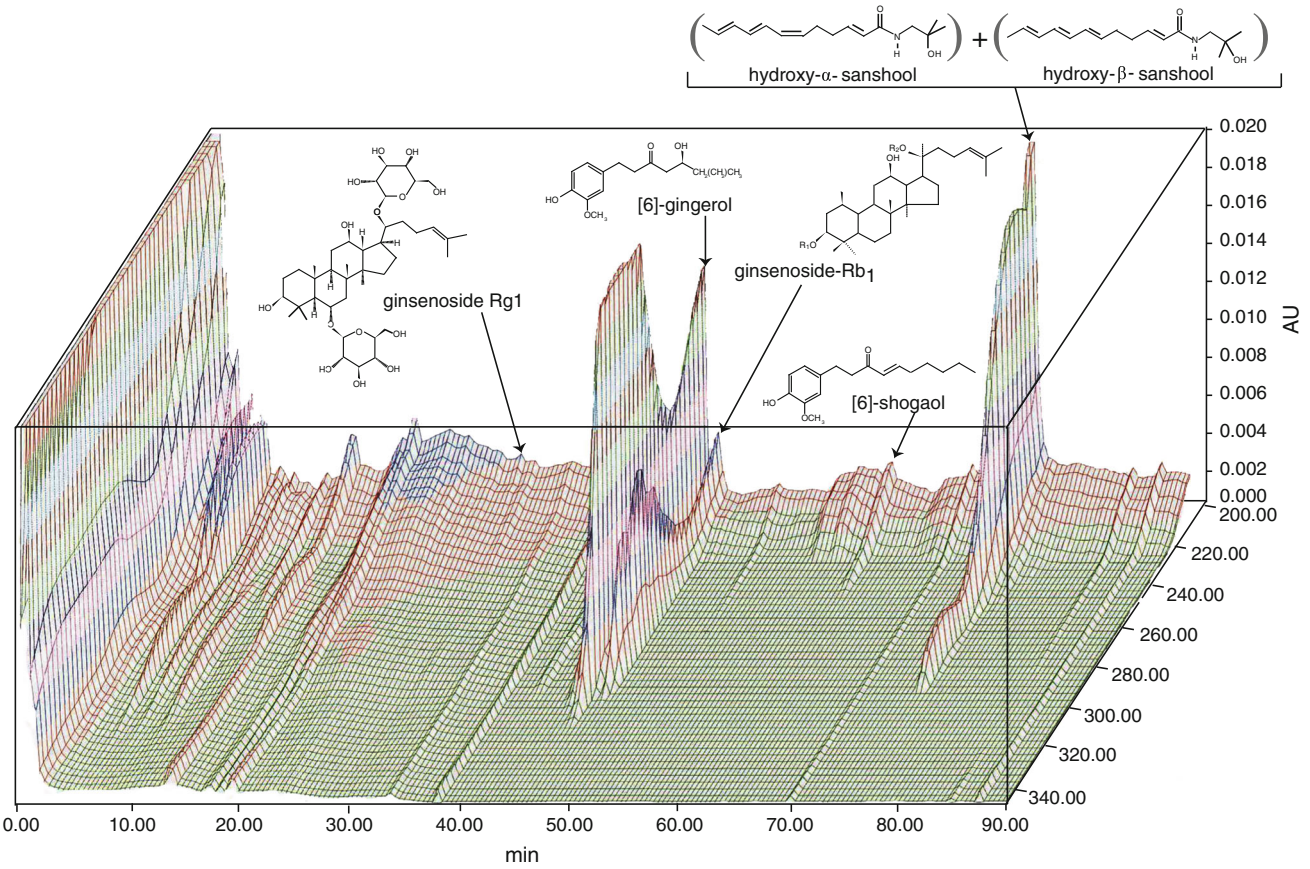

Fig. 1 HPLC profile of DKT. DKT analyzed by HPLC (ACQUITY UPLC; Nihon Waters K.K, Tokyo, Japan) under following conditions: column, COSMOSIL C18-MS-II (3.0 × 50 mm); mobile phase, H_2O:CH_3Hn (9: 1 → 1:1, linear gradient, for 95 min); flow rate; 1.0 ml/min; oven temperature, 30 °C; injection volume, 10 µl

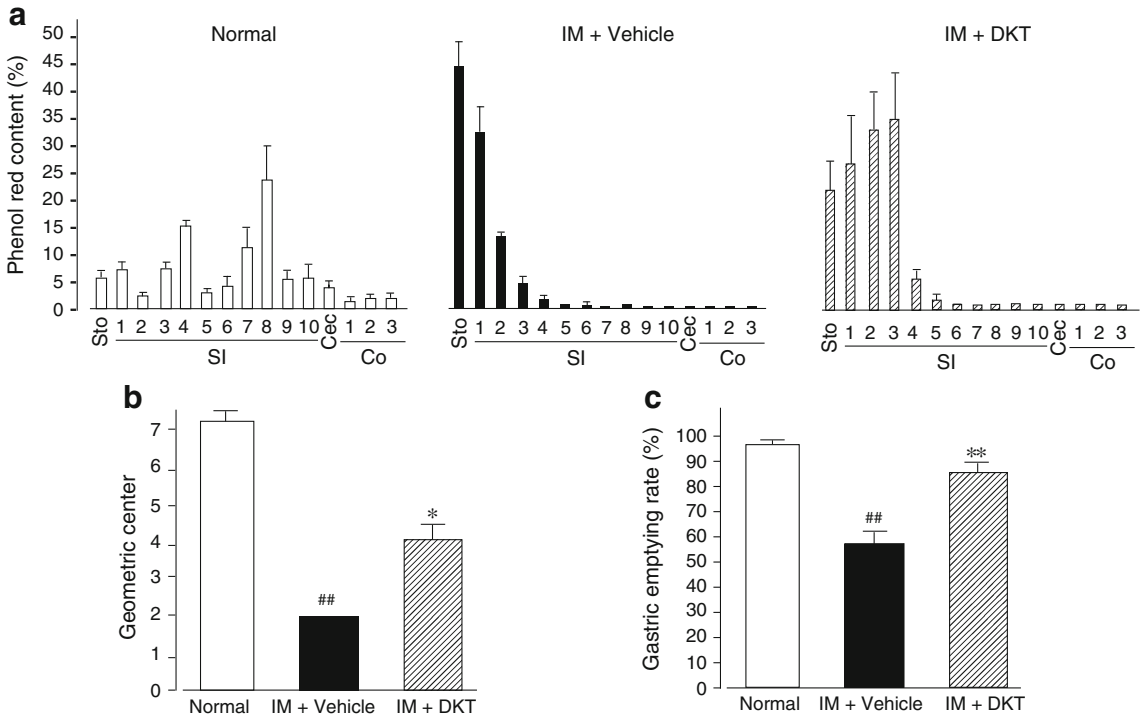

Fig. 2 Ameliorative action of DKT on gastrointestinal transit in mouse POI model. Detailed procedures are described in "Materials and methods. " **a** Shows distribution of PR in the normal group (*left panel*), at 24 h after IM + Vehicle (*middle panel*) and after IM + DKT (95 mg/kg, *right panel*). *Columns* indicate mean ± SEM of $n = 4$/group. **b**, **c** Show geometric center and gastric emptying rate, as calculated from **a**. *Bars* indicate mean ± SEM. ##; significantly different from normal at $P < 0.01$. * and ** are significantly different from IM + Vehicle at $P < 0.05$ and $P < 0.01$, respectively

potential of DKT under the current experimental conditions.

Effect of IM-induced delayed gastric emptying by treatment with DKT

To further examine an effect of DKT on gastric emptying rate by measuring ^{13}C-acetate breath test. Curves obtained for $^{13}CO_2$ excretion in the two different doses of DKT + IM, IM and normal groups are shown in Fig. 3. Excretion (A) and cumulative excretion (B) of $^{13}CO_2$ in the IM group were significantly lower than those in the normal group ($P < 0.01$ by ANOVA). At each time-point from 15 to 40 min, excretion of $^{13}CO_2$ in the IM group was significantly lower ($P < 0.01$ each) than those of the normal group. At each time-point from 15 to 60 min, cumulative excretion of $^{13}CO_2$ in the IM group was significantly lower ($P < 0.01$ each) than that in the normal group. The maximum concentration (C_{max}; Δ ‰) (Normal; 55.24 ± 5.22, IM + vehicle; 21.23 ± 1.41, $P < 0.05$) and the aria under the curve (AUC; Δ ‰/min) (Normal; 1385.33 ± 221.31, IM + vehicle; 180.75 ± 18.71, $P < 0.01$) were significantly decreased and the time to reach the maximum concentration (T_{max}; min) (Normal; 19.00 ± 0.94, IM + vehicle; 28.57 ± 1.71, $P < 0.01$) was significantly increased in IM.

Excretion of $^{13}CO_2$ in the IM + DKT (95 mg/kg) group significantly increased ($P < 0.01$ by ANOVA) the delayed gastric emptying induced by IM but not at each time-point from 0 to 60 min. DKT does not show the efficacy for C_{max} (IM + DKT 19 mg/kg; 28.97 ± 2.24, 95 mg/kg; 32.02 ± 3.29), AUC (19 mg/kg; 317.01 ± 38.59, 95 mg/kg; 441.35 ± 95.53) and T_{max} (19 mg/kg; 25.00 ± 1.15, 95 mg/kg; 32.02 ± 1.70). The cumulative excretion of $^{13}CO_2$ in the IM + DKT (19 mg/kg and 95 mg/kg) group significantly increased ($P < 0.01$ by ANOVA) the delayed gastric emptying induced by IM but not at each time-point from 0 to 60 min.

Amelioration of IM-induced inflammation of intestinal wall by treatment with DKT

After DKT treatment, we immunohistochemically monitored changes in MPO-stained neutrophils, and CD68-positive resident and monocyte-derived macrophages, as shown in Figs. 4 and 5. In Fig. 4, MPO-stained neutrophil infiltration into the ileal muscle layer was increased in the IM + Vehicle group, as compared with that in the normal group. Neutrophil infiltration by IM was significantly ameliorated in the IM + DKT group. In Fig. 5, resident dendritic macrophages [30] stained by CD68 were present

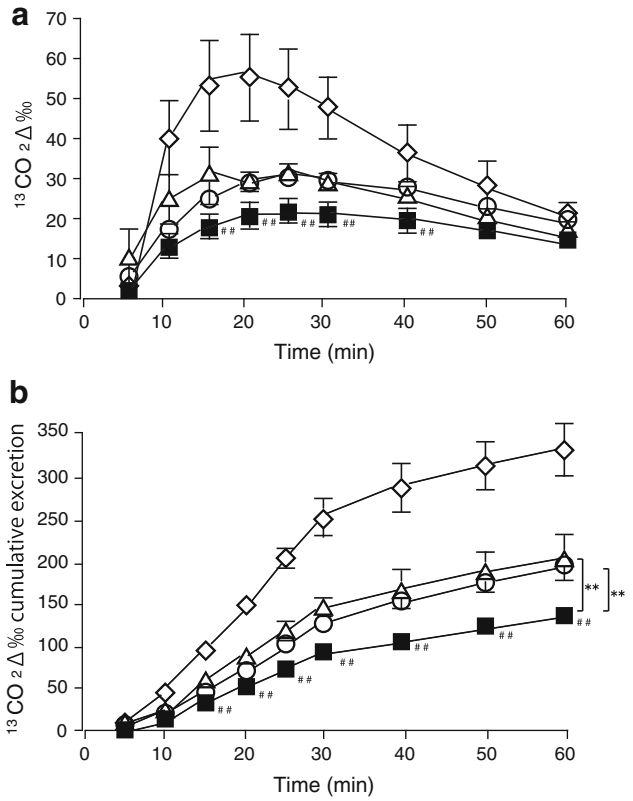

Fig. 3 Effect of DKT on excretion and cumulative excretion of $^{13}CO_2$, as calculated by ^{13}C-acetate breath test in POI model mice. **a**, **b** Showed excretion and cumulative excretion curves for $^{13}CO_2$, respectively. Detailed procedures are described in "Materials and methods". *Open diamonds*, *closed squares*, *open triangles* and *open circles* indicate normal, 24 h after IM, IM with 15 mg/kg DKT and IM with 95 mg/kg DKT groups, respectively. ## are significantly difference from normal ($P < 0.01$). *Symbols* indicate mean ± SEM of 5–7 experiments. ** are significantly different between IM group and IM + DKT group (19 and 95 mg/kg), respectively

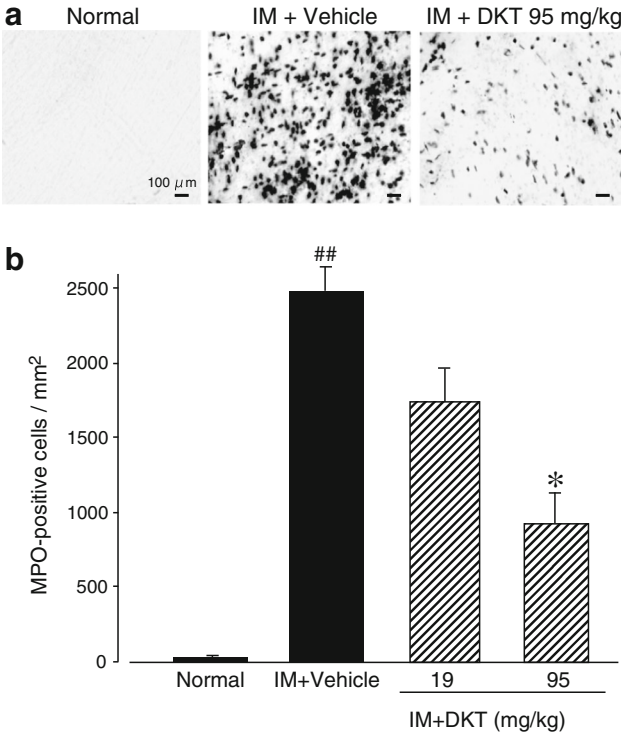

Fig. 4 Ameliorative effects of DKT on MPO-stained neutrophil infiltration in IM. **a** Indicates representative results for whole mount immunohistochemical staining of myenteric plexus region to detect MPO-positive neutrophils. **b** Shows quantification of neutrophil cells in $n = 5$/normal and IM + DKT (95 mg/kg) group, $n = 7$/IM + Vehicle and IM + DKT (19 mg/kg) group. *Columns* indicate mean ± SEM. ## and *: significantly different from normal and IM + Vehicle at $P < 0.01$ and $P < 0.05$, respectively

in the myenteric plexus region in normal intestine [26, 31]. At 24 h after IM, many infiltrating monocyte-derived macrophages and activated round [30] resident macrophages were observed, as reported previously [5]. The CD68-positive macrophage population increased 6-fold in the inflamed ileal muscle layer of the intestine of the IM + Vehicle group, as compared with the normal group. The increased CD68-positive macrophage population was significantly inhibited in the IM + DKT group, as compared with the IM group. However, DKT had no effects on MPO-stained neutrophils or CD68-positive macrophages in the control ileal muscle layer (MPO-positive cells: normal, 3.26 ± 1.46 cells/mm^2, +DKT, 4.88 ± 1.49 cells/mm^2; CD68-positive cells: normal, 646.16 ± 16.59 cells/mm^2, +DKT, 626.63 ± 29.52 cells/mm^2). Neutrophil infiltration and increased CD68-positive macrophage population by IM was significantly ameliorated in manufactured DKT, m-DKT treated group as it was in our hand-made DKT (MPO-positive cells: normal 1.05 ± 0.39 cells/mm^2, IM 1653.66 ± 121.24 cells/mm^2, IM + DKT 1047.47 ± 92.27 cells/mm^2, $P < 0.05$, IM + m-DKT 603.0 ± 90.70 cells/mm^2, $P < 0.01$, $n = 4$; CD68-positive cells: normal 186.71 ± 13.15 cells/mm^2, IM 2095.99 ± 144.04 cells/mm^2, IM + DKT 1484.53 ± 70.11 cells/mm^2, $P < 0.05$, IM + m-DKT 1515.12 ± 71.51 cells/mm^2, $P < 0.05$, $n = 4$).

Inhibition of IM-induced mRNA expression of inflammatory mediators by treatment with DKT

It has been reported that monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor alpha (TNF-α) and IL-6 are the most important chemokines/cytokines for inflammation of POI in mice [17, 32]. We thus investigated the effects of DKT on mRNA expression of MCP-1, TNF-α, IL-6 at 3 h after IM by semi-quantitative RT-PCR. In case of IL-1β, we measured mRNA expression at 6 h after IM, because preliminary data indicated that IL-1β mRNA expression did not increase at 3 h after IM (data not shown). In addition, motility disorder mediated by IM is

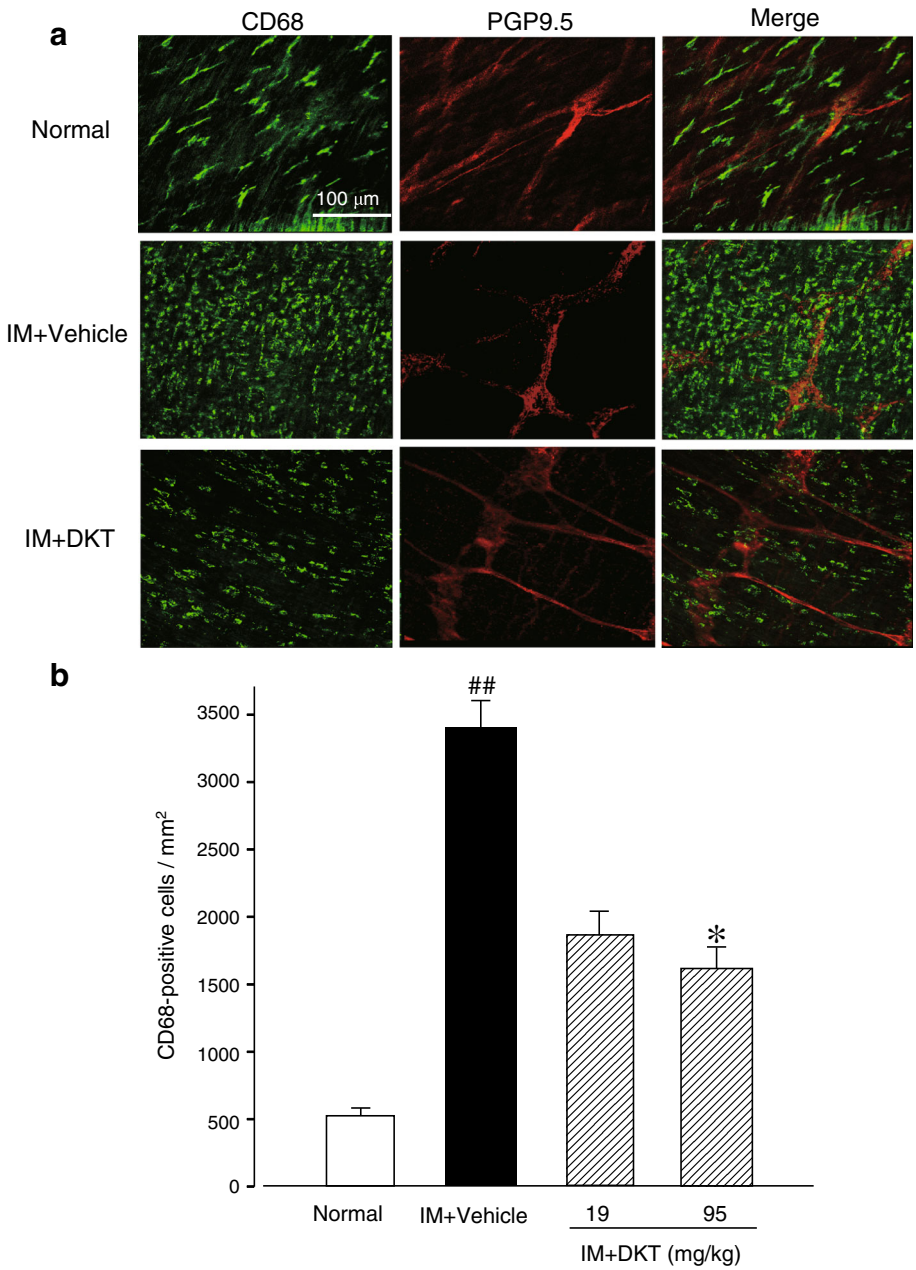

Fig. 5 Ameliorative effects of DKT on CD68-positive macrophage infiltration in IM. **a** Indicates representative results for whole mount immunohistochemical staining of myenteric plexus region to detect CD68-positive macrophages. **b** Shows quantification of macrophage cells in $n = 5$/normal and IM + DKT (95 mg/kg) group, $n = 7$/IM + Vehicle and IM + DKT (19 mg/kg) group, independent experiments. *Columns* indicate mean ± SEM. ## and *: significantly different from normal and IM + Vehicle at $P < 0.01$ and $P < 0.05$, respectively. Position of myenteric plexus region was confirmed by staining with protein gene product 9.5, which detects myenteric nerve plexus. *Red signals* indicate PGP9.5-positive myenteric nerves. *Green signals* indicate CD68-positive macrophages

known to be induced by nitric oxide (NO) through inducible nitric oxide synthase (iNOS) [17, 32] in smooth muscle cell. So we also investigated effect of iNOS mRNA expression at 6 h after IM. It has been reported that IL-1β upregulated in inflammatory lesion of POI model [5, 17]. In this study, however, mRNA expression of IL-1β did not significantly increased in POI model mice.

As shown in Fig. 6, mRNA expressions of MCP-1 and TNF-α were significantly elevated in the IM + Vehicle group. IL-6 and iNOS genes also showed an upward trend in IM (Dunnet's test values: IL-6; $P = 0.06$, iNOS; $P = 0.44$). mRNA expression of TNF-α and MCP-1 were significantly inhibited in the IM + DKT group, as compared with the IM + Vehicle group. The IM-induced increase in IL-6 and iNOS mRNA expressions also showed a downward trend in DKT-treated mice (Dunnet's test values: IL-6; $P = 0.25$, iNOS; $P = 0.29$).

Attenuation of DKT-induced anti-inflammatory activity by MLA

The effects of MLA on anti-inflammatory activity of DKT in the mouse POI model are summarized in Figs. 7 and 8. As shown in Fig. 6b and c, MLA partly attenuated anti-inflammatory activity by DKT, as tissue MPO activity and MPO-positive neutrophil numbers were inhibited when compared with the IM + Vehicle group, respectively.

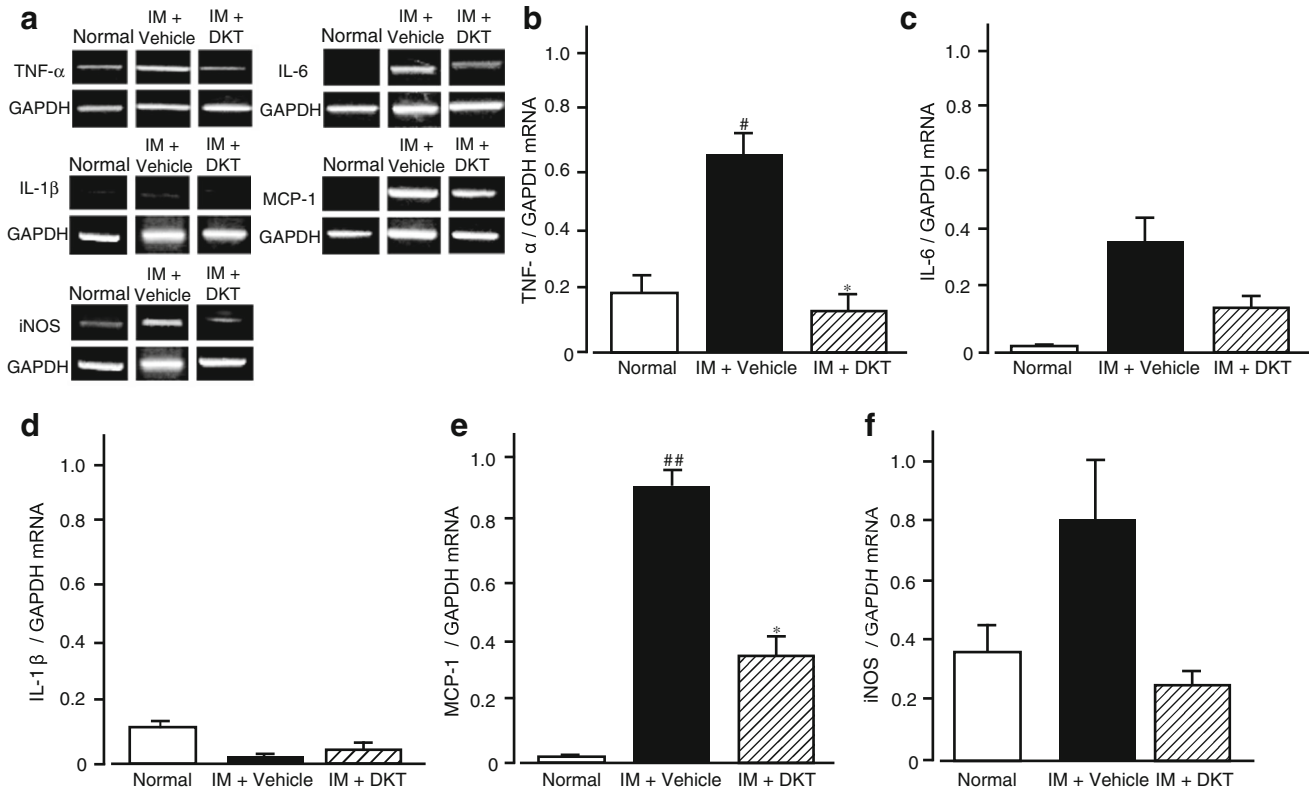

Fig. 6 Effects of DKT on mRNA expression of inflammatory mediators in inflamed muscle layer of small intestine in mouse POI model. **a** Showed typical results of RT-PCR. **b**–**f** Showed quantitative results of mRNA expression of TNF-α, IL-6, IL-1β, MCP-1 and iNOS. *Bars* indicate mean ± SEM from $n = 4$/group. # and ##; significantly different from normal at $P < 0.05$ and $P < 0.01$, respectively. *: significantly different from IM + Vehicle at $P < 0.05$

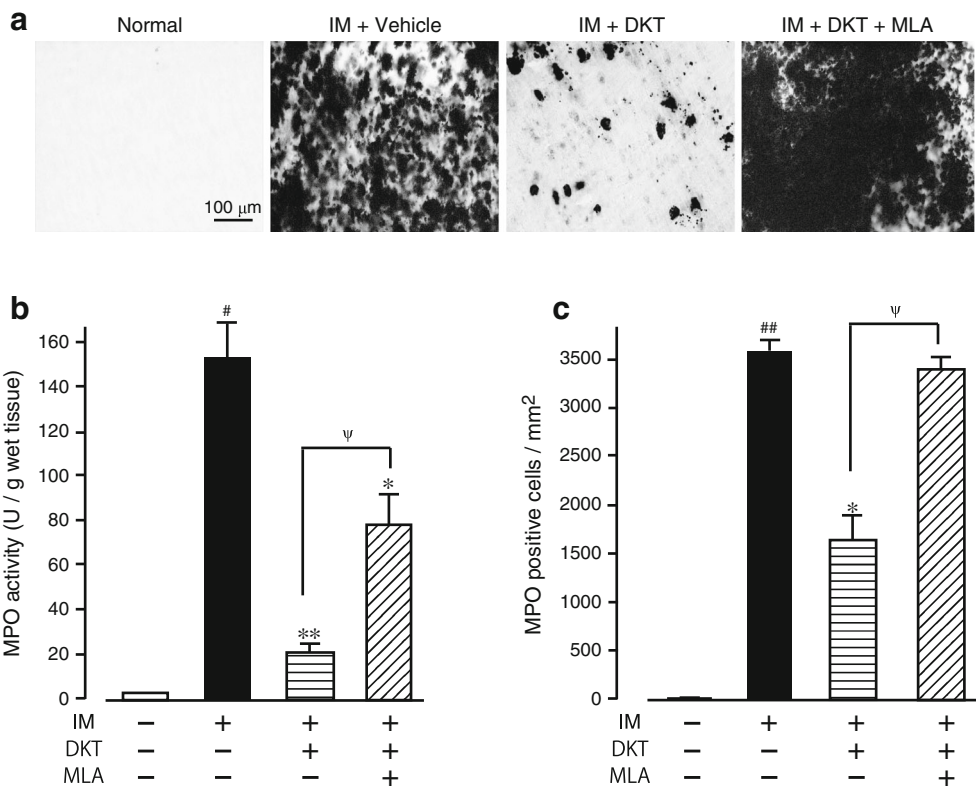

Fig. 7 Ameliorative effects of DKT and negative effects of MLA on MPO activity and MPO-positive neutrophil infiltration in mouse POI model. **a**, **c** Show representative images of MPO-positive neutrophil infiltration into myenteric plexus region and quantification results from $n = 4$/normal and IM + Vehicle group, $n = 5$/ IM + DKT + MLA group. **b** Shows results for MPO activity. *Columns* indicate mean ± SEM from $n = 4$/ group. ##: significantly different from normal at $P < 0.01$. * and ** are significantly different from IM at $P < 0.05$, $P < 0.01$.ψ: significantly different between IM + DKT and IM + DKT + MLA at $P < 0.05$

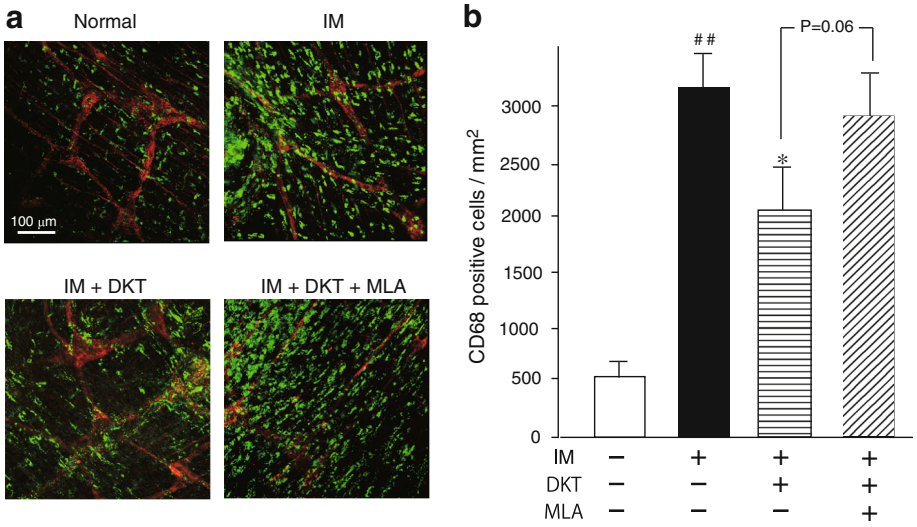

Fig. 8 Ameliorative effects of DKT and negative effects of MLA on CD68-positive macrophage infiltration in mouse POI model. **a**, **b** Show representative images of CD68-positive macrophage infiltration into the myenteric plexus region and quantification results. *Columns* indicate mean ± SEM from $n = 4$/normal, IM + Vehicle and IM + DKT group, $n = 5$/ IM + DKT + MLA group. ## and *: significantly different from normal and IM at $P < 0.01$, $P < 0.05$, respectively

Similar results were obtained for CD-68-positive macrophages. As shown in Fig. 8, MLA came in predisposed to attenuated the DKT-induced inhibition of macrophage infiltration by IM, although IM + MLA group was no significantly different from IM group ($P = 0.06$, $n = 5$). We confirmed that MLA itself had no effect on MPO activity, the immunohistochemical properties of neutrophils or CD68-positive macrophages in normal mice (MPO activity: normal, 10.01 ± 0.97 U/g wet tissue, +MLA, 36.80 ± 4.26 U/g wet tissue; MPO-positive cells: normal, 15.47 ± 1.53 cells/mm^2, +MLA, 15.19 ± 1.49 cells/mm^2; CD68-positive cells: normal, 429.67 ± 45.95 cells/mm^2, +MLA, 261.50 ± 9.76 cells/mm^2). In addition, MLA did not contribute to increasing inflammation by IM, as assessed by MPO activity, MPO-positive neutrophils and CD68-positive macrophages at the concentrations used in this study ($n = 2$, data not shown).

Reduction in DKT-induced inhibition of macrophage populations in α7nAChR KO mice

Figure 9 shows the anti-inflammatory activity of DKT in α7nAChR KO mice, with reference to that in wild-type mice (C57BL/6J). In wild-type mice, the MPO-positive infiltrating neutrophil and CD68-positive macrophage populations increased in the IM + Vehicle group, as was seen in BALB/c mice, and these increases were significantly inhibited in the IM + DKT group, as compared with the IM + Vehicle group.

In α7nAChR KO mice, MPO-positive infiltrating neutrophils and CD68-positive macrophages were also elevated in the IM + Vehicle group. This inflammatory cell infiltration in IM + Vehicle group was not altered in KO mice when compared with wild-type mice. The attenuating effects of DKT on increased MPO-positive infiltrating

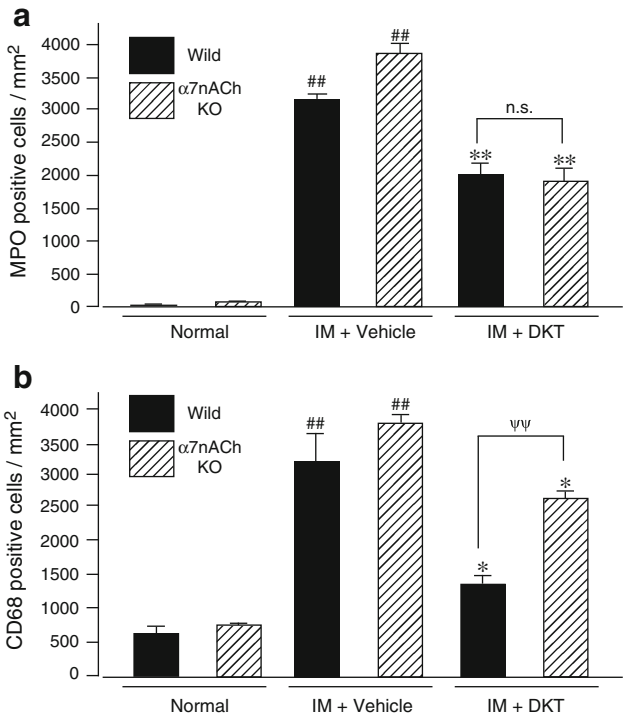

Fig. 9 Ameliorative effects of DKT on MPO- and CD68-positive neutrophils and macrophage infiltration in wild-type and α7nAChR KO mice. **a**, **b** Show the effects of DKT on MPO-positive neutrophil and CD68-positive macrophage infiltration induced by IM, respectively. *Columns* represent mean ± SEM from $n = 7$/wild-type mice, $n = 8$/normal and IM + Vehicle group in α7nAChR KO mice, $n = 9$/IM + DKT group in α7nAChR KO mice. Wild-type mice are C57BL/6J mice and α7nAChR KO mice are of C57BL/6J background. ##: significantly different from normal at $P < 0.01$. * and **: significantly different between IM and IM + DKT at $P < 0.05$ and $P < 0.01$, respectively. ψψ: significantly different between wild-type mice and α7nAChR KO mice at $P < 0.01$

neutrophils were maintained, which was similar to the trend seen in wild-type mice. On the other hand, the attenuating effects of DKT on infiltrating CD68-positive

macrophages by IM were significantly reduced in α7nAChR KO mice when compared with wild-type mice, indicating that the DKT-induced ameliorative effects on CD68 macrophage infiltration by IM is partly dependent on α7nAChR activation. DKT had no effects on cell numbers of either neutrophils or macrophages in the control ileal muscle layer (MPO-positive cells: normal, 7.63 ± 1.23 cells/mm^2, +DKT, 6.63 ± 0.90 cells/mm^2; CD68-positive cells: normal, 692.68 ± 51.93 cells/mm^2, +DKT, 706.75 ± 68.17 cells/mm^2).

Discussion

We investigated here whether DKT ameliorates the intestinal transit dysfunction and the delayed gastric emptying seen in the mouse POI model. DKT significantly recovered the intestinal transit. The calculated gastric emptying rate was also recovered by DKT as shown in Fig. 2c. These results in the mouse POI model agree with the DKT data obtained in the rat POI model [12, 13]. We further directly measured gastric emptying ability by using ^{13}C-acetate breath test, and investigated effect of DKT on the delayed gastric emptying induced by IM. Results indicated that DKT appears to have a little ameliorative effect on the delayed gastric emptying by IM as monitored with ^{13}C-acetate breath test. These results support the view that DKT accelerates motility in the lower gastrointestinal tract, but has no effects on gastric emptying in healthy humans [7]. DKT-induced recovery of delayed gastric emptying rate monitored by intestinal transit test (Fig. 2c) might be apparent recovery mediated through amelioration of intestinal transit by DKT. Phenol red was just retained inside stomach by delayed intestinal transit induced by IM. Ameliorative action of DKT for lower intestinal transit might be able to recover the delayed gastric emptying rate by IM. In this study, we further showed for the first time that DKT significantly suppresses neutrophil and macrophage infiltration induced by IM, thus suggesting that DKT exerts an anti-inflammatory effect in POI. These findings indicate that clinical amelioration of POI by DKT is related to improvements in both in gastrointestinal motility and inflammation. Our data may therefore provide new insights into the use of DKT in the treatment of POI.

To date, several mechanisms for the gastroprokinetic action of DKT have been posited. First, DKT accelerates ACh release from cholinergic myenteric neurons mediated by activation of 5-HT$_3$R and 5-HT$_4$R [12–14], and smooth muscles contract due to the released ACh through stimulation of muscarinic receptors (M$_2$R and M$_3$R). Second, it has been reported that DKT raises plasma levels of motilin, a gastrointestinal polypeptide hormone, and this improves morphine-induced constipation in cancer patients and intestinal motility dysfunction in conscious dogs [33, 34]. Third, DKT induces the release of substance P from primary sensory nerves through the vanilloid receptors on intramucosal terminal sensory nerves, and this contracts smooth muscle [35, 36]. In recent years, it was reported that pharmacological modulation of transient receptor potential vanilloid type1 (TRPV1) is a possible therapeutic option in POI [37], and this may be one of the mechanisms responsible for the gastroprokinetic activity of DKT [37]. The efficacy of DKT against POI has largely been explained to date by improvements in gastrointestinal motility and increased blood flow [19].

On the other hand, there have also been several reports on the anti-inflammatory effects of DKT in inflammatory diseases. It has been reported that DKT improves intestinal blood flow by increasing CGRP and substance P levels in plasma, which regulate the growth of bacterial flora, as well as inflammatory cytokine and cyclooxygenase-2 (COX-2) production in the intestine [38]. In an another report, the vasodilatory effects due to up-regulation of the ADM and CGRP system were thought to have therapeutic and preventive effects on intestinal inflammation in Crohn's disease (CD) [39], and DKT was thought to improve CD by increasing ADM and CGRP levels [19, 20]. In addition, it was reported that postoperative DKT administration significantly suppressed CRP and postoperative inflammation following surgery for colorectal cancer [18]. However, it still remains unclear how DKT acts against inflammation on POI.

In the present study, we found that DKT markedly reduces inflammatory cell infiltration into the inflamed muscle region, thus suggesting potent anti-inflammatory effects of DKT on POI. Local inflammation in the intestinal muscle layer is known to be closely correlated with gastrointestinal motility disorder [3, 17, 23, 40], and amelioration of inflammation in the intestinal muscle layer improves motility disorder [5, 17, 30]. Although intestinal transit in healthy mice is improved by DKT in this study, improvement by DKT in intestinal transit in POI mice is more effective than in healthy mice. Taken together, these results have led us to hypothesize that the ameliorative effects of DKT on gastrointestinal motility in POI are mediated by anti-inflammatory action against IM, in addition to the gastrointestinal prokinetic action of DKT.

We next investigated effect of DKT on mRNA expressions of inflammatory mediators induced by IM. IM upregulated the mRNA expression of cytokine/chemokine such as TNF-α, IL-6, IL-1β and MCP-1 [17]. Inducible NOS (iNOS) gene is also upregulated by IM, which in turn induces motility disorder in POI [17]. Our observations suggest the inhibition of TNF-α and MCP-1 by DKT, and this agrees with a report on the anti-inflammatory efficacy of DKT via inhibition of TNF-α in rat and mouse CD

models [29, 41]. The IM-induced increase in IL-6 and iNOS mRNA expressions also showed a downward trend in DKT-treated mice. Taken together, DKT has an inhibitory action of inflammatory mediator genes expression induced by IM.

DKT exerts its gastroprokinetic activity through activation of 5-HT_3R and/or 5-HT_4R of the vagal afferent, which stimulates cholinergic transmission in the myenteric plexus [13]. We previously found that the 5-HT_4R agonist mosapride citrate induced anti-inflammatory effects via activation of α7nAChR on muscularis-activated macrophages through the release of ACh from cholinergic nerves in the myenteric plexus [5]. Subsequently, activation of α7nAChR suppressed inflammatory cytokine production by macrophages, which improved POI [42]. So we further investigated the effects of the α7nAChR antagonist MLA on DKT-induced anti-inflammatory actions in POI. The results suggested that MLA significantly inhibited the DKT-mediated anti-inflammatory activity, as monitored by infiltrating macrophages and neutrophils. These results suggest that DKT-induced anti-inflammatory activity in POI may be mediated through α7nAChR activation. In fact, another report supports our observation that the selective α7nAChR agonist AR-R17779 prevents inflammation in POI [43]. In the rat POI model, we confirmed that infiltrating macrophages and activated resident macrophages, but not infiltrating neutrophils, had an affinity for α-bungarotoxin [5]. We therefore speculated that these α-bungarotoxin-bound macrophages may be effector cells in the anti-inflammatory action of DKT.

We further confirmed this conclusion by using α7nAChR KO mice. Interestingly, DKT-induced anti-inflammatory actions were partly suppressed in α7nAChR KO mice. With regard to macrophage infiltration, the DKT-induced ameliorative effects were partly but significantly reduced in α7nAChR KO mice, suggesting that, at least in part, DKT-induced inhibitory effects on macrophage infiltration could be mediated through α7nAChR. In contrast, in the case of neutrophil infiltration, the DKT-induced inhibitory action was not affected in α7nAChR KO mice, indicating that DKT-induced inhibitory action for neutrophil infiltration may not be mediated through α7nAChR. Taken together, these results suggest that α7nAChR and other subtypes of nicotinic receptors are involved in the DKT-induced anti-inflammatory effects. Recent work also supports the notion that nicotinic inhibition of macrophage activation involves other receptors, in addition to α7nAChR [43]. One possible candidate is α4β2 heteropentameric nAChR [44]. It has been reported that activation of this nAChR subtype also inhibits transactivational activity of the transcription factor NF-κB p65. However, it has been reported that MLA only has binding affinity for the α6 and α7 isoforms of nAChR [45]. Further investigation is thus necessary in order to clarify other types of nAChR activated by DKT.

In conclusion, DKT may serve as a novel therapeutic agent against POI, as characterized by its anti-inflammatory potency, in addition to its gastrointestinal prokinetic action. The anti-inflammatory potency of DKT in POI may be mediated through the activation of nAChRs via ACh release from the myenteric plexus nerve. DKT-induced anti-inflammatory activity may be partly mediated by the activation of α7nAChR. Interestingly, it was reported that herb zanthoxylum fruit and maltose syrup include target component to induce prokinetic ability [12]. So we are preparing to undertake a process to identify the target herb(s) in DKT to induce anti-inflammatory ability.

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Conflict of interest The authors declare that they have no conflict of interest.

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