

# Therapeutic effect and mechanism of Daikenchuto in a model of methotrexate-induced acute small intestinal mucositis

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## Article

**Keywords:** intestinal mucositis, methotrexate, Daikenchuto, barrier function

**Posted Date:** November 29th, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-2182269/v1>

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# Abstract

Daikenchuto (DKT) has positive therapeutic effects on improving various gastrointestinal disorders. The present study investigated whether or not DKT has a potential therapeutic effect on chemotherapy-induced acute small intestinal mucositis (CIM) in a rat model. Intraperitoneal injection of 10 mg/kg methotrexate (MTX) every 3 days for a total of 3 doses was used for induction of CIM in a rat model. The MTX and DKT-MTX groups were injected with MTX as above from the first day, and the DKT-MTX and DKT groups were administered 2.7% DKT via the diet at the same time. The Control and DKT groups were given the same volume of placebo. The body weights, food intake and plasma diamine oxidase (DAO) levels were measured every three days. The rats were euthanized on day 15, and small intestinal samples were collected for histological, histochemical, and mRNA analyses. The DKT-MTX group showed an improvement in the body weight and conditions of gastrointestinal disorders as well as increased levels of DAO in plasma and in the small intestinal villi. The pathology results showed that small intestinal mucosal injury in the DKT-MTX group was less severe than that in the MTX group. Immunohistochemistry for myeloperoxidase and malondialdehyde and quantitative real-time polymerase chain reaction (RT-qPCR) for TGF- $\beta$ 1 and HIF-1 $\alpha$  showed that DKT attenuated peroxidative damage and inflammatory responses. The crypts in the DKT-MTX group contained more Ki-67-positive cells than MTX group. The ZO-1 immunofluorescence results showed that DKT promoted repair of the small intestinal mucosal barrier. RT-qPCR for the amino acid transporters EAAT3 and BO+AT also confirmed that DKT promoted mucosal repair and thus promoted nutrient absorption in the small intestine. DKT protected against MTX-induced CIM in a rat model by reducing inflammation, stimulating cell proliferation, and stabilizing the mucosal barrier.

## Introduction

Intestinal mucositis (IM) is a common and debilitating side effect of chemotherapy that manifests due to the inability of chemotherapy drugs to differentiate between normal and tumor cells, with the intestinal epithelial cells rapidly proliferating cells and often becoming the target of attack during chemotherapy treatments<sup>1,2</sup>. Chemotherapy-induced IM (CIM) occurs in as many as 25%-75% of cancer patients receiving different chemotherapy, leading to diarrhea, a decreased quality of life, treatment intolerance resulting in discontinuation, and even death<sup>3,4</sup>. Due to the complex and diverse clinical symptoms of CIM and the importance of the reducing this toxic complication of chemotherapy, developing new ways to alleviate or prevent CIM is important.

Methotrexate (MTX), a structural analogue of folic acid, is one of the most widely used therapeutic agents for the treatment of many solid tumors, malignant hematological disorders, and autoimmune diseases<sup>5,6</sup>. MTX causes inhibition of growth and repair activities of epithelium and mucosa where the dividing and proliferating activities are increased, inducing IM, which is the main reason for limiting further use of this drug or prompting its use<sup>7</sup>.

Daikenchuto (DKT) is a traditional Japanese medicine (Kampo) originally described in a Chinese classic article and independently developed in Japan. It is a mixture of extract powders from dried Japanese pepper, processed ginger, ginseng radix, and malt sugar powder and is reported to have the effects of improving gastrointestinal motility, activating anti-inflammatory, increasing intestinal blood flow, and altering the intestinal microbiome<sup>8-11</sup>. The main mechanism underlying the DKT-mediated contraction and improvement of gastrointestinal motility is modulation of intestinal contraction and relaxation via the release of acetylcholine, nitric oxide, releasing of acetylcholine from cholinergic nerves stimulated by 5-HT<sub>3</sub>R and 5-HT<sub>4</sub>R and other excitatory neurotransmitters<sup>9,12-14</sup>. The anti-inflammatory effect of DKT are attributed to the fact that DKT manages the downregulation of cyclooxygenase 2 (COX-2), the upregulation of endogenous adrenomedullin (ADM), and the suppression of eosinophil infiltration<sup>15</sup>. The regulation of intestinal blood flow by DKT is achieved by stimulating epithelial transient receptor potential ankyrin 1 (TRPA1) to induce endogenous ADM release<sup>16,17</sup>. Recent studies have suggested that DKT can alter the gut microbiota, thereby improving long-lasting dysbiosis and gastrointestinal dysfunction after bowel or liver surgery<sup>18,19</sup>.

In addition to these effects, Wada et al. also demonstrated that DKT enhanced anastomotic healing via an anti-inflammatory effect and increased blood flow after intestinal surgery in rats<sup>20</sup>. DKT has been widely used clinically in patients with gastrointestinal symptoms, such as postoperative intestinal obstruction, inflammatory bowel disease, abdominal pain, and pain accompanied by abdominal flatulence<sup>19,21-23</sup>. A randomized, open, controlled trial in patients undergoing hepatectomy with postoperative abdominal pain or bloating is ongoing in Japan<sup>21</sup>. DKT improves gastrointestinal motility disorders and reduces serum C-reactive protein (CRP) levels in patients with grade B liver injury after hepatectomy and is an effective treatment after hepatectomy for hepatocellular carcinoma<sup>24</sup>. A previous report showed that DKT can suppress the adverse effects associated with irinotecan hydrochloride, an anticancer agent with debilitating side effect of severe diarrhea, and improve the function of tight junction proteins, including zonula occluden-1 (ZO-1), occludin and claudin-4<sup>25</sup>.

Based on existing experiments and recent studies on DKT, we hypothesized that DKT could improve CIM through the above mechanisms. Therefore, the present study investigated whether or not DKT could improve CIM and promote recovery from CIM.

## Results

*DKT can enhance the body weight recovery and ameliorate the symptoms of IM induced by MTX.*

MTX was used for induction of CIM, and the optimum dose and frequency (10 mg/kg every 3 days, 3 times total) of MTX to induce CIM was determined. The DKT treatment experiment was performed according to the diagram (Fig. 1a). The body weight of the rats in the MTX and DKT-MTX groups decreased over time compared with control group, but after day 12, the body weight of the rats in the DKT-MTX group increased, although there was no statistically significant difference compared with the DKT

group (Fig. 1b). The changes in food intake were similar to those of the body weight, with rats in both the DKT-MTX and MTX groups showing reductions after the administration of MTX compared to the control group, although values gradually increased in DKT-MTX group after day 12 (Fig. 1c). There was also no significant difference in the survival rate between the MTX group and the DKT-MTX group on day 15 (Fig. 1d).

The MTX group had severe diarrhea by day 15, while the rats in the DKT-MTX group had only slightly soft stool. When the small intestine was observed after euthanasia and opening, the small intestine in the MTX group was observed to be relatively pale in appearance (Fig. 1e). Pathologic changes in the small intestine would be described in detail below.

#### *DKT attenuated MTX-induced inflammation and mucosal damage in the small intestinal mucosa.*

Pathological and inflammation-related factors were used to evaluate the inflammation of the small intestinal mucosa and the therapeutic effect of DKT on acute CIM. HE staining showed that the MTX group had degeneration and vacuolization of the surface and crypt epithelium and villus structure in the jejunum, the digestion and dissolution of epithelial structures, and bleeding and edema in the lamina propria in the ileum. The DKT-MTX group showed the degeneration and vacuolization of the surface and crypt epithelium and villus structure in the jejunum and ileum. There were no marked changes in the control or DKT group (Fig. 2a).

Immunohistochemical (IHC) staining for neutrophil myeloperoxidase (MPO) and malondialdehyde (MDA) showed that the DKT-MTX group had a lower percentage contribution of positivity than the MTX group in the jejunum and ileum, although there was no significant difference in the MDA in the jejunum (Fig. 2b-d). RT-qPCR showed that the expression of inflammation-related mRNA of TGF- $\beta$ 1 was up-regulated in the small intestine of the MTX group compared with the DKT-MTX and control groups; however, there were no significant changes in the HIF-1 $\alpha$  levels in any groups after DKT treatment (Fig. 2e).

#### *DKT enhanced small intestinal crypt cell proliferation after administration of MTX.*

MTX induced small intestinal mucosal epithelial injury by inhibiting the proliferation of small intestinal mucosal crypt cells, which resulted in the suspension of small intestinal mucosal renewal. IHC staining for Ki-67 of the small intestinal tissue was used to evaluate the cell proliferation in the crypts and epithelia of the small intestinal mucosa. After treatment with DKT in the CIM models, the small intestinal mucosal crypts in the DKT-MTX group contained more Ki-67-positive cells than the MTX group (Fig. 3a). Positive area counting using the image J software program (NIH, Wisconsin, USA) showed that the DKT-MTX group had a higher percentage contribution of Ki-67 positivity than the MTX group in crypts (Fig. 3b).

#### *DKT promoted the repair of the mucosal barrier and nutrient absorption functions.*

DAO can reflect the degree the mucositis induced by MTX. In DKT treatment experiments, the DAO level was decreased in both the MTX and DKT-MTX groups compared with the control group but gradually

recovered after 12 days in the DKT-MTX group (Fig. 4a). IHC staining also showed the positions and relative level of DAO in the jejunum and ileum, with statistically significant differences being noted in the jejunum and ileum between the DKT-MTX and MTX groups (Fig. 4b,c).

To investigate the effect of DKT on the repair of the mucosal barrier function in MTX-induced IM, we examined the ZO-1 protein level in the intestinal tissue by immunofluorescence staining. ZO-1 was predominantly localized along the apical membrane of the intestinal villi in the control, DKT-MTX, and DKT groups. In contrast, a significant reduction in ZO-1 immunostaining was observed along the apical membrane of the intestinal villi, especially along the ileum, in the MTX group (Fig. 4d).

To investigate the effect of DKT on the repair of nutrient absorption function in MTX-induced IM, we examined the expression of genes related to the amino acid transport-related proteins excitatory amino acid transporter 3 (EAAT3) and broad neutral amino acid transporter (BO + AT) in the small intestinal mucosa. The relative gene expression of EAAT3 and BO + AT in the DKT-MTX group was significantly higher than that in the MTX group, especially in the jejunum (Fig. 4e).

## Discussion

IM is a common side effect of chemotherapy, and with the increasing number of cancer diagnoses and the prevalence of chemotherapy drug use, chemotherapy-associated mucositis has become increasingly common. MTX, as an anti-cancer drug widely used for leukemia and other malignancies, is a structural analogue of folic acid that can inhibit the metabolism of folic acid by competitively inhibiting dihydrofolate reductase, thereby inhibiting the *de novo* synthesis of purines and pyrimidines<sup>26,27</sup>. Over the past few decades, MTX has been successfully used alone or in combination with other drugs to treat various cancers and autoimmune diseases<sup>28</sup>. Unfortunately, however, due to its multi-organ toxicity, especially in the gastrointestinal system, the therapeutic potential of MTX can be reduced, with the drug typically having to be ceased in response to bone marrow toxicity, cardiotoxicity, nephrotoxicity, and liver toxicity<sup>29,30</sup>.

Damage to the gastrointestinal mucosa following MTX treatment in cancer patients includes villus shortening and fusion, epithelial atrophy, crypt loss, inflammatory infiltration of the lamina propria, goblet cell depletion, and barrier dysfunction due to loss of mucosal integrity and reduced nutrient absorption<sup>31</sup>. Although various measures to prevent these effects have been adopted, such as reducing the therapeutic dose and duration of treatment to reduce the intestinal damage caused by MTX, there remains no satisfactory therapeutic intervention to prevent MTX-induced small intestinal injury<sup>32,33</sup>.

MTX-induced IM not only causes direct damage by DNA copy inhibition but also induces inflammation and the generation of reactive oxygen species (ROS)<sup>34-36</sup>. Studies have demonstrated that MPO and MDA levels are increased after treatment with MTX, suggesting the possible participation of neutrophil infiltration and ROS in MTX-induced IM<sup>34,37</sup>. In the present study, we found that levels of MPO, a marker of neutrophil accumulation and infiltration during intestinal mucosa damage, were increased in the

epithelial mucosa of rats with IM induced by MTX. Kolli et al. also reported that oxidants such as MDA, which is a product of lipid peroxidation, were increased in MTX-induced small IM<sup>38</sup>. MPO is also an enzyme with peroxidative damage secreted by neutrophils after neutrophil, and it also causes peroxidative damage to tissues. According to our results, oral administration of DKT can inhibit the inflammatory process and ROS damage, as evidenced by the fact that DKT can reduce the production of MPO and MDA.

In addition to peroxidation-related factors, growth factors secreted from mucosa also played a role in this process. TGF- $\beta$ 1 and HIF-1 $\alpha$  were reported to protect intestinal integrity<sup>39,40</sup>. There have been articles showing that TGF- $\beta$ 1 is dihedral in the homeostasis of the small intestine, having the effect of promoting both inflammation as well as damage repair<sup>41,42</sup>. The mRNA level of TGF- $\beta$ 1 in the DKT-MTX group was higher than that in the control group on day 15, but it was lower in the jejunum and ileum than in the MTX group. This method of mucosal repair was also verified in another experiment of DKT in the treatment of intestinal injury repair in rats<sup>20</sup>.

The changes in the level and localization of ZO-1 induced by MTX may lead to disorder of the barrier function, which leads to increased intestinal permeability, resulting in intestinal mucosal barrier dysfunction<sup>43</sup>. ZO-1, as a tight junction scaffold protein, has been shown to render structure firmness and impermeability to the junction and functions as a link between occludin and the actin, which are the major elements in the structure of the barrier of the small intestine<sup>44</sup>. However, Kuo et al. reported that the tight junction protein ZO-1 was dispensable for the barrier function but critical for intestinal mucosal repair<sup>45</sup>. An intravital confocal microscopy study suggested that the redistribution of ZO-1 contributed to the intestinal barrier function and was the earliest cellular event identified as a herald of physiological cell shedding. Furthermore, redistribution of this tight junction function along the lateral plasma membrane sustained the epithelial barrier during cell shedding<sup>46</sup>. This evidence suggested that ZO-1 was associated with tight junctions in the small intestinal mucosal epithelium as well as with the physiological renewal and essential function of pathological repair of the small intestinal mucosa. In the present study, an immunohistochemical analysis revealed that ZO-1 was predominantly localized along the apical membrane of the intestinal villi in the control and DKT groups. In contrast, a significant increase in ZO-1 immunostaining was observed along the apical membrane of the intestinal villi in the DKT-MTX group compared with the MTX group. DKT has the potential to promote the expression of ZO-1 in the small intestinal mucosa epithelium to facilitate the repair of tight junctions and improve the mucosal nutrient absorption.

The expression of genes related to the amino acid transport-related proteins EAAT3 and B0 + AT was up-regulated after the administration of DKT in the DKT-MTX group. A prospective study of the effects of DKT on the blood flow in the superior mesenteric artery (SMA) and portal vein (PV) reported that DKT may modulate the SMA and PV blood flows by acting on intestinal microvessels<sup>47</sup>. The absorption of nutrients around the ileocecal region was related to the blood flow of the intestine and PV. A prospective

open-labeled randomized exploratory study also demonstrated that DKT can improve the perioperative nutritional status of patients with colorectal cancer <sup>48</sup>.

DAO is a highly active intracellular enzyme in the upper villi of the human and mammalian small intestinal mucosa that plays a role in the metabolism of histamine and various polyamines, and its activity is closely related to nucleic acid and protein synthesis in mucosal cells, reflecting the integrity and degree of damage to the mechanical barrier of the intestine <sup>49</sup>. DAO regulates cell proliferation by degrading polyamines, which are essential substances in the processes of mitosis and meiosis <sup>50,51</sup>. Plasma DAO activity was reported to be associated with the degree of small intestinal injury and had potential utility for measuring mucositis during chemotherapy <sup>49,50</sup>. A previous study showed that an increase of DAO activity in the intestine of mature rats leads to an increase in the degree of DAO activity in plasma <sup>52</sup>. In contrast, intestinal mucosal damage caused by hypertonic sodium sulfate solution or atrophy caused by a low-fiber diet reduces plasma DAO activity <sup>53</sup>.

In the present study, with the occurrence of MTX-induced IM, the DAO content in rat plasma decreased, and with the onset of gastrointestinal tract symptoms aggravated and reduced to a lower level. In the DKT-MTX group, as DKT promoted the recovery of rat mucosa, the serum content of DAO also increased. The IHC results also confirmed that the rat small intestinal mucosal epithelial villi of the DKT-MTX group contained higher DAO levels in the mature apical membrane than that in MTX group.

DKT is mainly composed of dried Japanese pepper extract, ginger extract, and ginseng extract. Ginger can reportedly improve ileum damage caused by MTX, shortened villus fusion, inflammatory cell infiltration, and goblet cell depletion <sup>54</sup>. The present findings support the efficacy and safety of ginger, possibly due to the antioxidant properties, as was observed in our experiments. As one of the main components of DKT, ginger extract has been verified to have a variety of intestinal effects, including anti-inflammatory and antioxidative effects in ulcerative colitis <sup>55-57</sup>. The mechanism underlying the restoration of the intestinal barrier function by ginger extract involves the increased expression of ZO-1 and claudin-1 protein <sup>55</sup>. Regarding dried Japanese pepper extract (*Zanthoxylum fructus*), there have been articles reporting that *Zanthoxylum fructus* extract inhibits the reduction in mast cell activation by inhibiting sphingosine kinase 1, mainly reducing the release of inflammatory mediators <sup>58</sup>. In research on DKT, it is reported that DKT depolarizes the pacemaker potential of Cajal interstitial cells in an internal or external Ca<sup>2+</sup>-dependent manner by stimulating the 5-HT<sub>4</sub> and muscarinic M<sub>3</sub> receptors. Its main ingredients are ginseng and ginger root, which help DKT regulate the activity of the intestine and reduce the dysfunction of the small intestine <sup>14</sup>. Some researchers have screened cytoprotective agents against MTX-induced cell genotoxicity from among biologically active phytochemicals and found that agents such as Siberian ginseng and curcumin have cytoprotective effects <sup>59</sup>. Total ginsenosides was reported to promote intestinal epithelial cell proliferation, presumably via the regulation of the cell cycle and of the expression of proliferation-related proteins by polyamines <sup>60</sup>. Specifically, DKT appears to be less effective in reducing rat mortality due to severe mucosal necrosis but shows marked efficacy in another aspect, such as reducing injury and promoting mucosal repair, as well as improving symptoms.



Furthermore, DKT may also have some efficacy against chronic mucositis of the small intestine, so a further investigation regarding chronic mucositis in the clinical setting or as a daily disease will be needed in the future, with particular focus on the therapeutic effect of DKT on chronic mucositis of the small intestine.

In conclusion, DKT may be able to protect against MTX-induced acute small intestinal mucosal injury in a rat model via anti-peroxidation, stimulating cell proliferation, and stabilizing the mucosal barrier. Although DKT comprise numerous chemically diverse compounds with multi-target effects, it has been shown to be able to protect and treat intestinal injury, regardless of administration as a single component or a mixture of multiple components.

## Materials And Methods

### Animals

Male Sprague-Dawley rats (6 weeks, 160–190 g; CLEA Japan Inc., Tokyo, Japan) were used in this study. They were bred and housed at the rat facility in standard rat cages exposing to 12-h light-dark cycles and allowed *ad libitum* access to water and rat chow.

All animal experiments were conducted according to protocols approved by the institutional animal care committee of Nagasaki University and all methods were performed in accordance with the relevant guidelines and regulations of Nagasaki University. The study was reported in accordance with ARRIVE guidelines.

### Experimental protocol

All animals were divided randomly into 4 groups: the control group (Col, n = 5), MTX-induced model group (MTX, n = 5), DKT treatment group (DKT-MTX, n = 5), and DKT-only group (DKT, n = 5). In the MTX and DKT-MTX groups, MTX was administered (10 mg/kg every 3 days, 3 times total) with intraperitoneal injection. In the MTX-DKT and DKT groups, DKT at 2.7% of the total mass (Tsumura & Co., Tokyo, Japan) mixed in the feed (CE-2 feed; CLEA Japan Inc.) was administered orally from the beginning of the administration of MTX, while the other two groups received non-DKT CE-2 feed. In the control group, the rats were intraperitoneally injected with the same volume of normal saline at the same time.

All rats had their body weight measured every three days. All surviving rats were euthanized by cutting the vena cava to induce exsanguination after abdominal collection of all of the small intestinal tissues under deep isoflurane (Wako Pure Chemical, Osaka, Japan) respiratory anesthesia (All-in-one Anesthetizer, Muromachi Kikai CO. LTD, Japan).

### Intestinal histology

The rats were sacrificed under anesthesia at day 15, and the small intestinal tissues were collected immediately. The rat small intestinal tissue was fixed with 4% paraformaldehyde phosphate-buffered

solution (PBS; Wako Pure Chemical, Osaka, Japan) for 3 days. Fixed tissues were embedded in paraffin, cut into 5- $\mu$ m sections, mounted on MAS-coated slides, and deparaffinized for standard histological staining with hematoxylin and eosin (HE).

For immunohistochemistry staining, sections were heated in 10 mM Tris-HCl buffer (pH 9.0) containing 1 mM EDTA using an autoclave for antigen retrieval, incubated in 3% hydrogen peroxide solution for 10 min to quench the endogenous peroxidase activity, and then blocked in Tris-buffered saline (TBS) containing 5% bovine serum albumin (BSA) and 0.1% Tween 20 for 1 h at room temperature. Sections of immunofluorescence incubated in 0.1% Triton X-100 (Sigma-Aldrich Japan, Tokyo) in PBS for 10 min. Blocked sections were incubated overnight at 4°C in TBS + 5% BSA, 0.1% Tween 20 and primary antibodies (Supplementary table s1). Sections were then incubated for 1 h at room temperature in secondary antibodies (Supplementary table s1). HRP-conjugated secondary binding was visualized using the Dako liquid DAB + substrate chromogen system (Dako Japan, Kyoto, Japan). Nuclei were stained with hematoxylin or 4',6-diamidino-2-phenylindole (DAPI) (Dojindo Laboratories, Kumamoto, Japan) for 20 min. Bright-field images were captured using an optical microscope (BX53; Olympus, Tokyo, Japan).

## **Quantitative real-time polymerase chain reaction (qRT-PCR)**

Tissue samples were acquired at defined time point for mRNA extraction using spin columns according to the manufacturer's instructions (NucleoSpin RNA II; Macherey-Nagel, Duren, Germany). cDNA was synthesized from total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). The samples were stored at -20°C until use. PCR was performed on an Applied Biosystems Step OnePlus Real-time PCR system using a TaqMan Gene Expression Assay Kit (Applied Biosystems) as previously reported. In brief, PCR amplification was performed using a reaction mixture containing 1  $\mu$ L of sample cDNA, 1  $\mu$ L of TaqMan Gene Expression Assay probe, 5  $\mu$ L of TaqMan Fast Universal PCR Master Mix solution (Applied Biosystems; Supplementary Table s2), and 13.5  $\mu$ L of nuclease-free water. Each amplification cycle consisted of 1 second at 95°C and 20 seconds at 60°C. A total of 40 amplification cycles were performed. The gene expression was normalized to that of GAPDH (control intestinal tissue was set as 1.0), and the mRNA expression was determined using the comparative cycle time ( $\Delta\Delta$ Ct) method.

## **Determination of plasma diamine oxidase (DAO) activity by an enzyme-linked immunosorbent assay (ELISA)**

Plasma of rats was separated in a refrigerated centrifuge and stored at -20°C before determination of the DAO activity using the rat diamine oxidase ELISA Kit (FineTest; ER0895, Wuhan, China) according to the manufacturer's instructions. The DAO activity was calculated according to a standard curve and presented in units/L.

## **Statistical analyses**

Data are provided as the mean  $\pm$  standard deviation (SD) unless otherwise stated. Statistical analyses were carried out with the GraphPad Prism software program (GraphPad Software, Inc., California, USA)

using a one-way analysis of variance, t-test, or an analysis of variance with repeated measures when appropriate. A probability (p) value < 0.05 was considered statistically significant.

## Declarations

### Acknowledgements

None

### Authors contributions

PL designed the research, performed the experiments and interpretation of the data, and wrote the manuscript. SI and SM contributed to the DAO enzyme-linked immunosorbent assay. YI and DM assisted in the experimental design, data analysis, and manuscript revision. All authors contributed to the discussion of the results and the critical revision of the manuscript. SE gave the final approval of the manuscript. All authors have read and approved the final manuscript.

### Funding

This experiment was supported by Tsumura Kampo Research Laboratories, Tsumura & Co.

### Conflict of interest

The authors of Peilin Li, Yusuke Inoue Daisuke Miyamoto Toshiyuki Adachi, Satomi Okada, Tomohiko Adachi, Akihiko Soyama, Masaaki Hidaka, Kengo Kanetaka, Shinichiro Ito and Susumu Eguchi received a research grant from Tsumura & Co. Daichi Sadatomi, Naoki Fujitsuka and Sachiko Mogami are employed by Tsumura & Co. All authors who took part in this study promised to avoid conflicts of interest (even superficial ones) with the company and the research process, and the results conformed to the research specification. All authors undertook to ensure that the personal conduct of all participants was in accordance with the guidelines and to report appropriately when there is a potential for any actual or potential conflict. All the other authors declared no competing interests.

### Data availability

The datasets obtained and analyzed in the current study are available from the corresponding authors on reasonable request.

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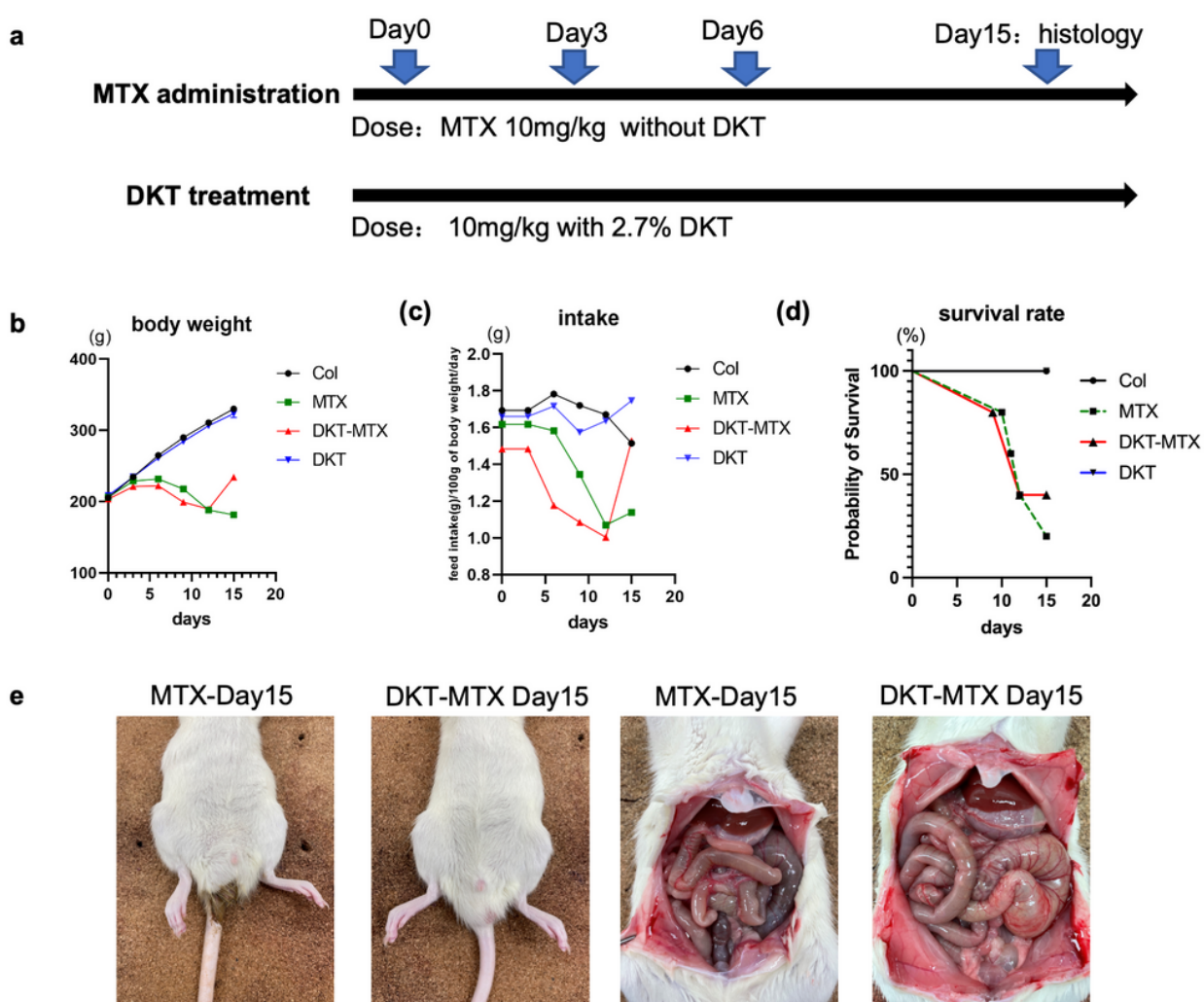
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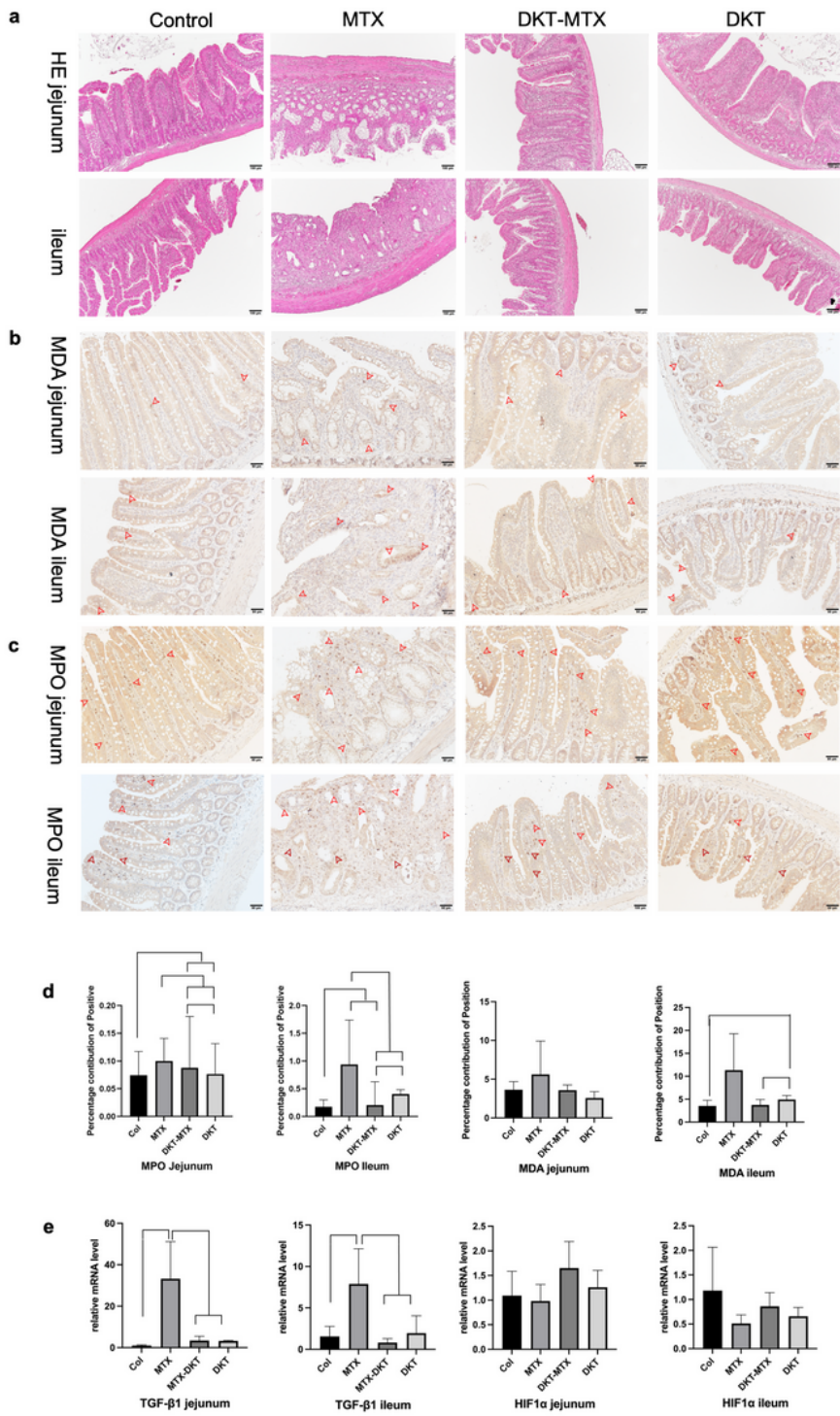
## Figures





## Figure 1

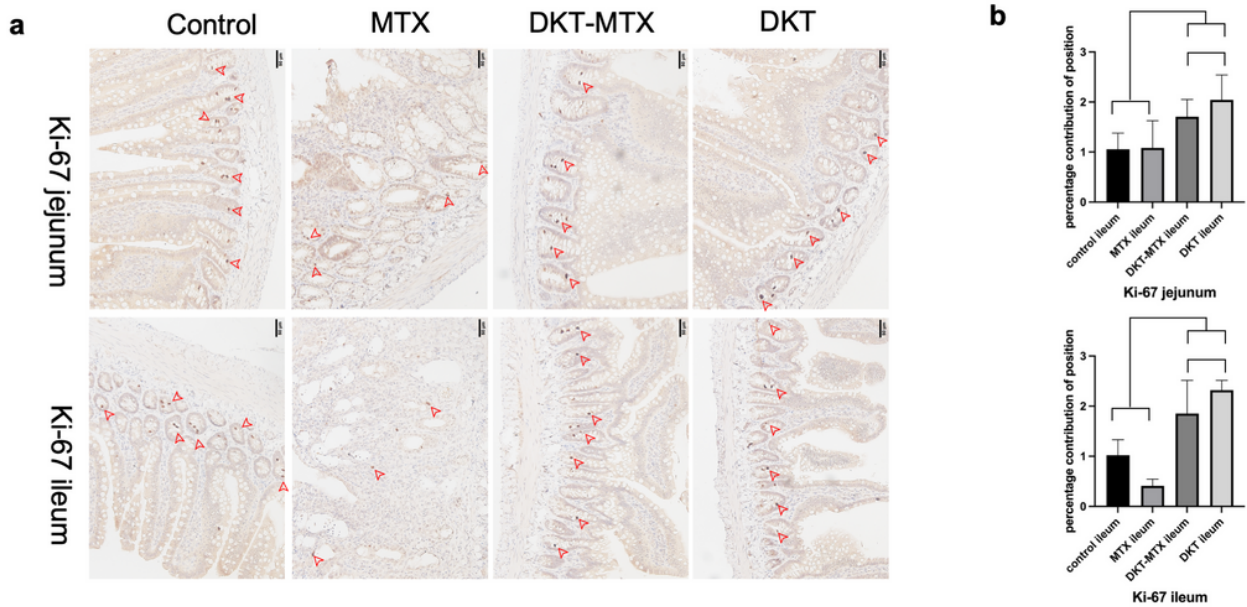
**DKT enhanced the body weight recovery and ameliorated the symptoms of IM induced by MTX. (a)** The experiment was performed according to the diagram. **(b)** Compared with the control group, the body weight of the rats in the MTX and DKT-MTX groups decreased, but that of the DKT-MTX group increased again after day 12. **(c)** The food intake decreased in both the MTX and DKT-MTX groups but gradually increased again after day 12 in the DKT-MTX group. **(d)** There was no significant difference in the survival rate between the MTX group and the DKT-MTX group at day 15. Log-rank (Mantel-Cox) test, value=0.5890. **(e)** The rats in the DKT-MTX group had no obvious diarrhea, and the intestinal walls of the rats in the MTX group were thinned.



**Figure 2**

**DKT attenuated MTX-induced inflammation and mucosal damage in the small intestinal mucosa. (a)** HE staining showed the structural changes in all groups. Scale bar=100 μm. **(b, c, d)** The immunohistochemistry results showed a lower percentage contribution of positivity for **(d)** MDA **(b)** and MPO **(c)** in the jejunum and ileum of the DKT-MTX group than in those of the MTX group. For each criterion, the percentage contribution of positivity was calculated for at least 10 positions. Scale bar=50

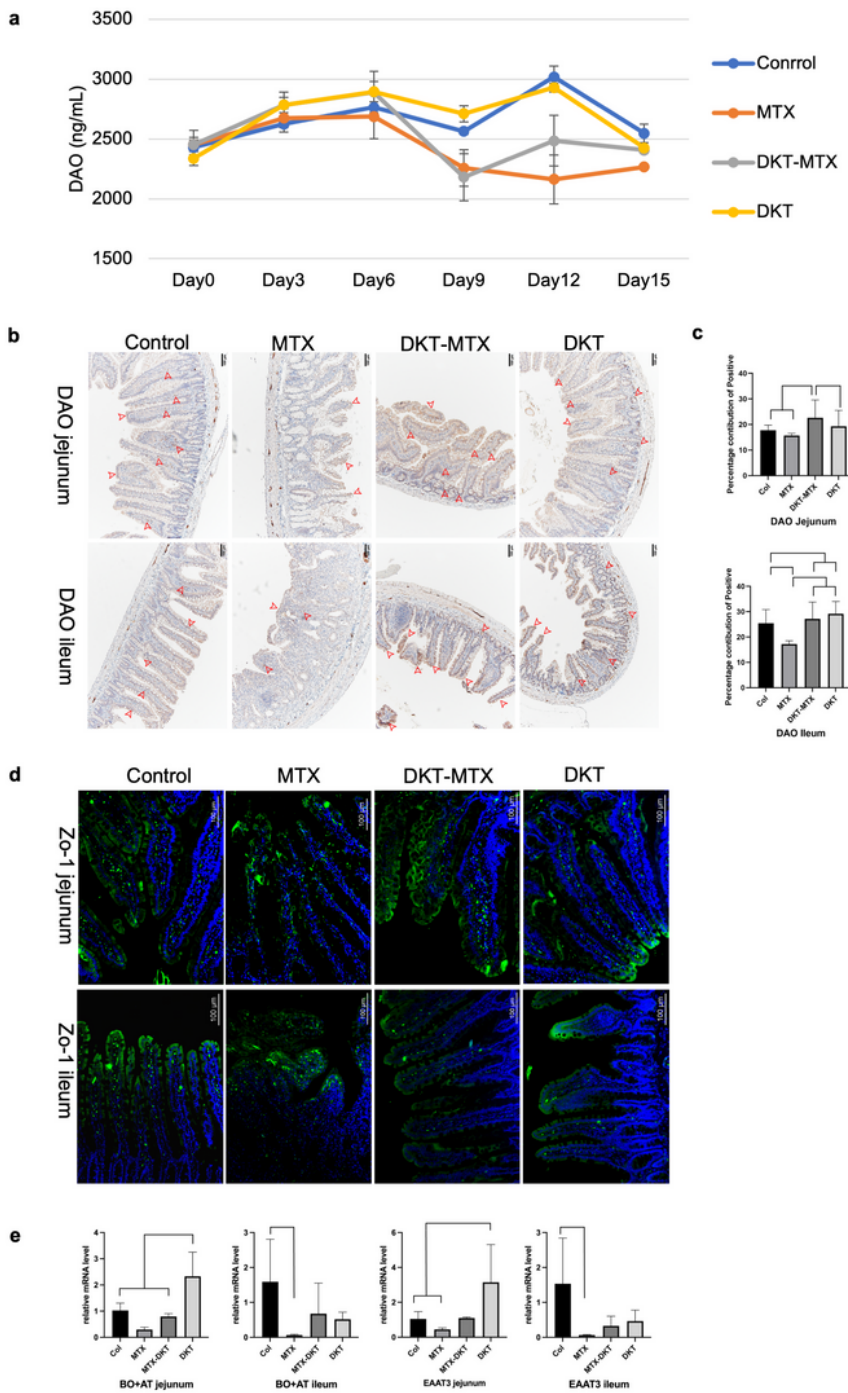
$\mu\text{m}$ , One-way ANOVA: ns >0.05, \*p <0.05. **(e)** RT-qPCR showed the expression of inflammation-related mRNA of TGF- $\beta$ 1 and HIF-1 $\alpha$  in the four groups. Values were determined relative to GAPDH and presented as fold-change relative to the control group. One-way ANOVA: ns >0.05, \*p <0.05.



**Figure 3**

**DKT enhanced the small intestinal crypt cell proliferation after the administration of MTX. (a)**

Immunohistochemical results showed that the small intestinal mucosal crypts in the DKT-MTX group contained more Ki-67-positive cells than the MTX group. **(b)** Cell counting showed that the DKT-MTX group had a higher percentage contribution of Ki-67 positivity than the MTX group. Scale bar=50  $\mu\text{m}$ . For each criterion, the percentage contribution of Ki-67 positivity was calculated for at least 10 positions. One-way ANOVA: ns >0.05, \*p <0.05.



**Figure 4**

**DKT promoted the repair of the mucosal barrier and nutrient absorption functions. (a)** The plasma DAO level in the control, MTX, DKT-MTX, and DKT groups. The DAO level decreased in both the MTX and DKT-MTX groups compared with the control group but gradually recovered after day 9 in the DKT-MTX group. Units: ng/ml; DAO, diamine oxidase. **(b)** Immunohistochemical staining shows the positions and relative level of DAO in the jejunum and ileum of the control, MTX, DKT-MTX, and DKT groups, and **(c)** the

percentage contributions of positivity are shown. Scale bar=100  $\mu$ m. For each criterion, the percentage contribution of positivity was calculated for at least 10 positions. One-way ANOVA tests: ns>0.05, \*p <0.05. **(d)** Immunofluorescence staining with the anti-Zo-1 antibody showed the expression and positions of the tight junction protein Zo-1. Bar=100  $\mu$ m, Zo-1, zonula occludens-1. **(e)** RT-qPCR showed the relative mRNA levels of BO+AT and EAAT3 in the jejunum and ileum of the control, MTX, DKT-MTX, and DKT groups. Values were determined relative to GAPDH and presented as fold-change relative to the control group. One-way ANOVA: ns>0.05, \*p <0.05, (*n* = 3). EAAT3: excitatory amino acid transporter 3; BO+AT: broad neutral amino acid transporter.

## Supplementary Files

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