



Altered Intestinal Permeability and Drug Repositioning in a Post-operative Ileus Guinea Pig Model

Young Min Kim, Zahid Hussain, Young Ju Lee, and Hyojin Park*

Department of Internal Medicine, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea

Background/Aims

The aim of this study is to identify the alteration in intestinal permeability with regard to the development of post-operative ileus (POI). Moreover, we investigated drug repositioning in the treatment of POI.

Methods

An experimental POI model was developed using guinea pigs. To measure intestinal permeability, harvested intestinal membranes of the ileum and proximal colon was used in an Ussing chamber. To identify the mechanisms associated with altered permeability, we measured leukocyte count and expression of calprotectin, claudin-1, claudin-2, and mast cell tryptase. We compared control, POI, and drug groups (mosapride [0.3 mg/kg and 1 mg/kg, orally], glutamine [500 mg/kg, orally], or ketotifen [1 mg/kg, orally] with regard to these parameters.

Results

Increased permeability after surgery significantly decreased after administration of mosapride, glutamine, or ketotifen. Leukocyte counts increased in the POI group and decreased significantly after administration of mosapride (0.3 mg/kg) in the ileum, and mosapride (0.3 mg/kg and 1 mg/kg), glutamine, or ketotifen in the proximal colon. Increased expression of calprotectin after surgery decreased after administration of mosapride (0.3 mg/kg), glutamine, or ketotifen in the ileum and proximal colon, and mosapride (1 mg/kg) in the ileum. The expression of claudin-1 decreased significantly and that of claudin-2 increased after operation. After administration of glutamine, the expression of both proteins was restored. Finally, mast cell tryptase levels increased in the POI group and decreased significantly after administration of ketotifen.

Conclusions

The alteration in intestinal permeability is one of the factors involved in the pathogenesis of POI. We repositioned 3 drugs (mosapride, glutamine, and ketotifen) as novel therapeutic agents for POI.

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Key Words

Glutamine; Ileus; Ketotifen; Mosapride; Permeability

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*Correspondence: Hyojin Park, MD, PhD

Department of Internal Medicine, Gangnam Severance Hospital, Yonsei University College of Medicine, 211 Eonjuro, Gangnam-gu, 06273, Seoul, Korea
Tel: +82-2-2019-3310, Fax: +82-2-3463-3882, E-mail: hjpark21@yuhs.ac

Introduction

Post-operative ileus (POI) is the temporary cessation of coordinated propulsive movement after most surgical procedures.^{1,2} Because of this iatrogenic condition, patients present with various symptoms such as nausea, vomiting, abdominal discomfort, and inability to pass stools or tolerate a solid diet. In addition to these symptoms, decreased quality of life, prolonged length of stay in a hospital, and socioeconomic costs decrease patients' satisfaction with surgery. Although there have been attempts to improve POI, such as the conversion of the surgical method from open to laparoscopic surgery and the chewing of gum, POI is still one of the main problems faced by patients who undergo surgical procedures.^{3,4} Moreover, there is currently no definite method for the prevention and treatment of POI. To suggest effective management, the precise mechanisms underlying POI need to be investigated.

Meanwhile, there are several diseases associated with the alteration in gut permeability. These diseases include inflammatory bowel disease, malignancies, irritable bowel syndrome, rheumatic disorders, obesity-related metabolic diseases, and allergic diseases.⁵⁻⁹ In these diseases, several factors such as inflammation, tight junction (TJ) proteins, and gut dysbiosis have an effect on the alteration in intestinal permeability. For instance, in inflammatory bowel disease, the permeation of luminal noxious materials via the disruption of intestinal TJ proteins such as claudin causes defects in the mucosal immune system and inflammation.^{10,11}

A previous study reported that intestinal inflammation and TJ proteins are associated with POI.¹² In the study, inflammatory markers significantly increased in the POI group, compared with that in the control group. Moreover, of the TJ proteins, the levels of barrier-forming protein (claudin-1) decreased and pore-forming protein (claudin-2) increased in the POI group. Therefore, we hypothesized that the alteration in intestinal permeability is also associated with POI. However, studies investigating the association between POI and intestinal permeability are limited.

Therefore, in this study, we aim to characterize the alteration in intestinal permeability as a pathophysiologic mechanism of POI. Furthermore, we investigated drug repositioning in the treatment of POI.

Materials and Methods

Animals, Abdominal Operation, and Drugs

In this study, we used adult male Hartley guinea pigs (Orient Bio Inc, Seoul, Korea) weighing 300-350 g. The guinea pigs were housed under controlled breeding conditions (temperature of $22 \pm 2^\circ\text{C}$, humidity of $50 \pm 10\%$, and 12-hour light/dark cycle starting at 7 AM). All the experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, Department of Laboratory Animal Resources, Yonsei Biomedical Research Institute, Yonsei University College of Medicine (IRB No. 2017-0344).

The guinea pigs were randomly assigned into 3 groups. The first group was the "control group," which did not receive any manipulation or drugs. The second was the "POI group," which was subjected to surgical procedures as follows: an incision, followed by evisceration, gentle manipulation of the cecum using wet gauze for 60 seconds, and closing of the incision by suturing. The third was the "drug group," which received drugs, and this group was divided into the mosapride, glutamine, and ketotifen groups. The mosapride group was administered 0.3 mg/kg or 1 mg/kg of mosapride via the oral route 1 hour before and after the operation. The glutamine group received 500 mg/kg of glutamine orally 4, 3, 2, and 1 day before the operation. Finally, the ketotifen group was administered 1 mg/kg of ketotifen orally 4, 3, 2, and 1 day before the operation. We determined the dosages of these drugs as per previous animal studies.¹³⁻¹⁵ The number of guinea pigs for each group was 7. We harvested the ileum and proximal colon from each guinea pig. In the POI and drug groups, we harvested tissues 3 hours (POI 3H) and 6 hours (POI 6H) after surgery.

Methods

We measured various markers in the harvested ileum and proximal colon. An Ussing chamber (EM-CSYS-2; Physiologic Instruments, San Diego, CA, USA) was used to analyze intestinal permeability, and we measured the leukocyte count and the expression of calprotectin to evaluate intestinal inflammation. The expression of mast cell tryptase was evaluated to identify the effect of ketotifen as a mast cell stabilizer. Moreover, we measured the expression of claudin-1 and claudin-2 to evaluate alterations in TJ proteins. We compared the measurements between the control and POI groups and between the POI and drug groups. The detailed methods of measurement are described as follows.

Intestinal Permeability

To evaluate intestinal permeability, the harvested tissues were placed in a modified Ussing chamber. Two mL Krebs-Ringer bicarbonate (KRB) solution were filled in each half chamber, and the specimen's mucosal and serosal sides were bathed. At the maintained temperature of 37°C, a gas mixture of 95% O₂ and 5% CO₂ was given to both sides. After an equilibration period of 30 minutes, the KRB containing horseradish peroxidase (HRP) at a final concentration of 0.4 mg/mL was substituted for the KRB in the chamber of the mucosal side. The KRB on the serosal side was replaced with fresh KRB, and a 0.3 mL sample was gathered and replaced with 0.3 mL KRB of the serosal side. The samples from the chamber of serosal side were enzymatically analyzed via the modified Worthington method. We used o-dianisidine dihydrochloride (OPD; Sigma Chemical Co, St Louis, MO, USA) as a substrate.

Samples (50 µL) were transferred to microtiter, and OPD working solution (100 µL) as a stable peroxide buffer, which was diluted 1:10 in OPD, was added to each well. Subsequently, the plates were incubated with shaking at 300 rpm and room temperature. Thirty minutes later, 2.5 M sulfuric acid (100 µL) was added. After 10 minutes, using a microplate reader (Model 680; Bio-Rad Laboratories, Inc, Hercules, CA, USA), the decolorized reaction product's absorbance was determined at a wavelength of 492 nm. All samples were duplicated and measured with reference to a standard curve. HRP flux was represented as ng/2 hr/mm² during steady-state permeation. Intestinal permeability via the Ussing chamber was expressed as the percentage change compared with the control group.

Leukocyte Count

Histological sections were obtained from the muscle layer of the harvested ileum and proximal colon. These sections were fixed

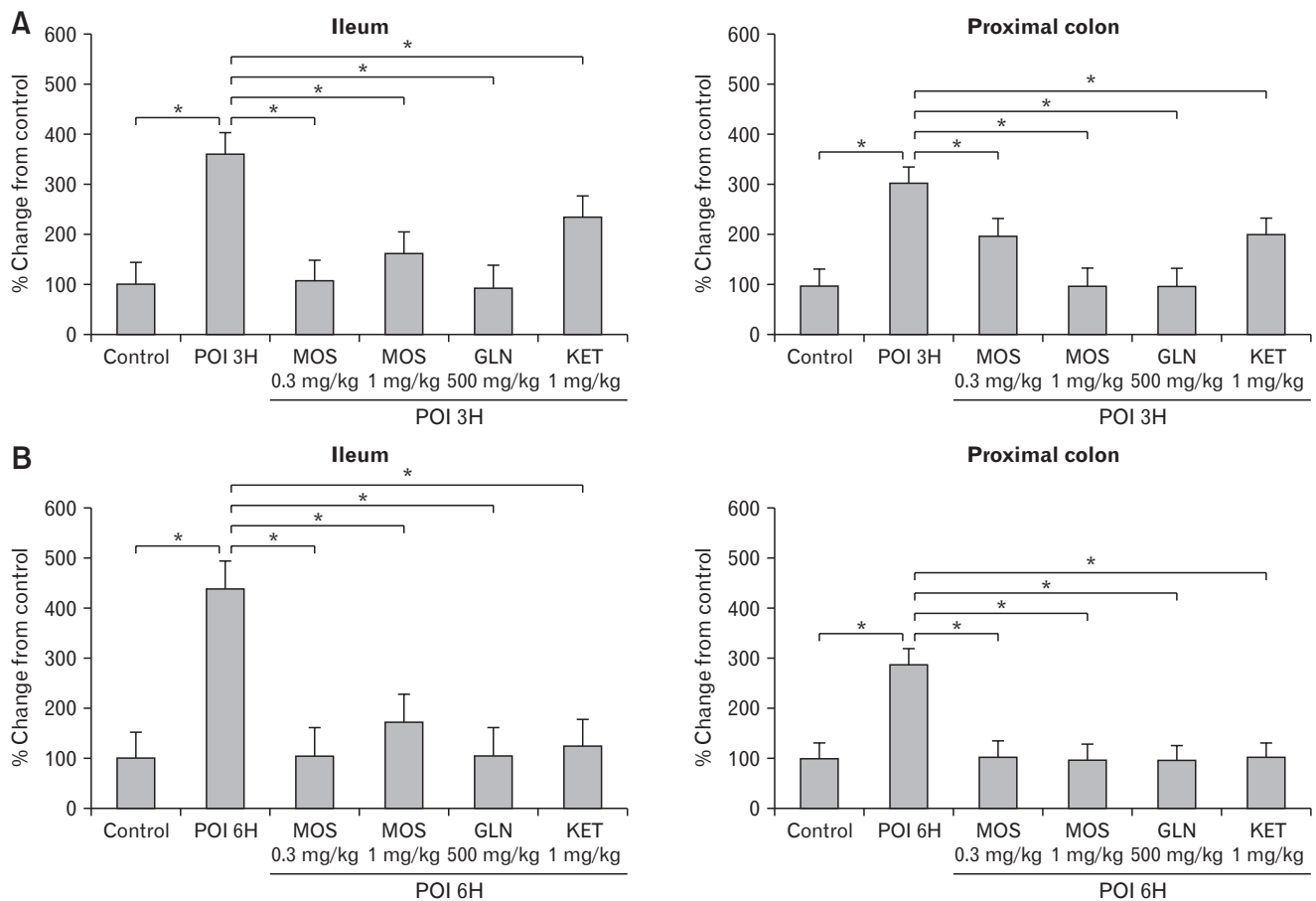


Figure 1. Intestinal permeability in the control, post-operative ileus (POI), and drug groups. (A) Three hours after the operation and (B) 6 hours after the operation. The data are expressed as a percentage change relative to the control group. Bars indicate the mean \pm SEM (control group, $n = 7$; POI group, $n = 7$; each drug group, $n = 7$). * $P < 0.05$. POI 3H, post-operative ileus at 3 hours after the operation; POI 6H, post-operative ileus at 6 hours after the operation; MOS, mosapride; GLN, glutamine; KET, ketotifen.

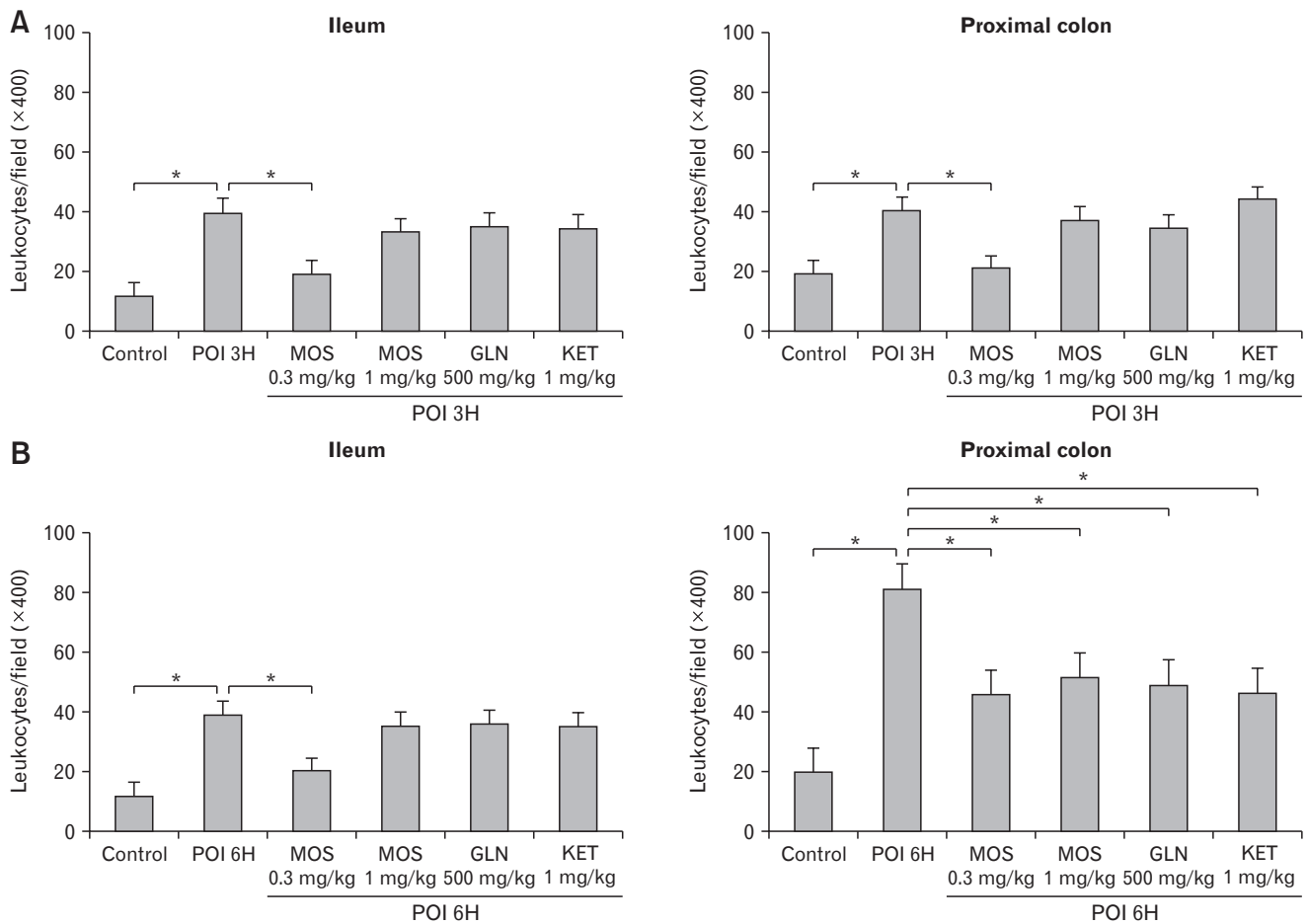


Figure 2. Leukocyte counts in the control, post-operative ileus (POI), and drug groups. (A) Three hours after the operation and (B) 6 hours after the operation. The data were analyzed using a semi-quantitative scoring system. Bars indicate the mean \pm SEM (control group, n = 7; POI group, n = 7; each drug group, n = 7). * $P < 0.05$. POI 3H, post-operative ileus at 3 hours after the operation; POI 6H, post-operative ileus at 6 hours after the operation; MOS, mosapride; GLN, glutamine; KET, ketotifen.

in 10% neutral buffered formalin solution and then embedded with paraffin. Embedded sections were sliced into 4 μ m thickness and then hematoxylin and eosin staining. We compared leukocyte counts between the control and POI groups and between POI and drug groups via a semi-quantitative scoring system.

Expression of Calprotectin and Mast Cell Tryptase

The expression of calprotectin and mast cell tryptase was determined via immunohistochemical analysis. The paraffin-embedded histological tissue sections from the ileum and proximal colon were deparaffinized. Tissue sections were incubated in 3% hydrogen peroxide for 10 minutes to block activity of endogenous peroxidase. The tissue sections were then incubated overnight with primary antibodies, anti-calprotectin (1:250; ThermoFisher, Waltham, MA, USA) and anti-mast cell tryptase (1:2000; ThermoFisher), 4°C.

After 3 washes with phosphate-buffered saline, the tissue sections were incubated with secondary antibody anti-mouse IgG (1:200; Vector Laboratories). Next, the sections were incubated with streptavidin-HRP for 30 minutes and then treated with AB-peroxidase solution and counterstaining with hematoxylin. Images were analyzed via MetaMorph (MDS Analytical Technologies, Sunnyvale, CA, USA).

Expression of Claudin-1 and Claudin-2

The expression of claudin-1 and claudin-2 was determined via immunofluorescence analysis. At 6 hours after the operation, the histological sections from the ileum and proximal colon were fixed in 4% paraformaldehyde, embedded with paraffin, and sliced into 4 μ m thick sections. Tissues then were deparaffinized, rehydrated, and rinsed using standard methods. The sections of slide were in-

cubated overnight with the primary antibody for claudin-1 (1:50; Invitrogen, South San Francisco, CA, USA) or claudin-2 (1:200; Invitrogen) at 4°C, followed by washing and incubation with the secondary antibody goat anti-rabbit IgG-fluorescein isothiocyanate (1:200; Santa Cruz Biotechnology) for 30 minutes at 37°C. The stained samples were evaluated under a fluorescence microscope (Zeiss Axio Imager Z1; Carl Zeiss, Jena, Germany), and the images were analyzed via MetaMorph.

Statistical Methods

The data were expressed as the mean \pm SE, and statistical analysis was performed using a *t* test or Wilcoxon rank-sum test between the 2 groups and a one-way ANOVA for multiple comparisons. For statistical analysis, we used SPSS version 23.0 (IBM Corp, Armonk, NY, USA). A two-tailed *P*-value of < 0.05 indicated statistical significance.

Results

Intestinal Permeability

Figure 1 shows the result of intestinal permeability. The intestinal permeability in the POI 3H samples was significantly higher than that in the control group of the ileum and proximal colon ($P < 0.05$). The administration of mosapride, glutamine, or ketotifen significantly decreased the permeability ($P < 0.05$) (Fig. 1A). There was a significant increase in the permeability of the POI 6H ileum and proximal colon, compared with that of the control group ($P < 0.05$) (Fig. 1B). This increase was significantly reversed after the administration of mosapride, glutamine, or ketotifen ($P < 0.05$).

Leukocyte Count

The leukocyte counts in the POI 3H ileum and proximal colon were significantly higher than that in the control group ($P < 0.05$). The leukocyte count decreased significantly after the administration of mosapride (0.3 mg/kg) ($P < 0.05$) (Fig. 2A). The leukocyte counts in the POI 6H ileum and proximal colon were significantly higher than that in the control group ($P < 0.05$). After the administration of mosapride (0.3 mg/kg), the leukocyte count decreased significantly in the ileum ($P < 0.05$). Moreover, the leukocyte count decreased significantly after the administration of mosapride, glutamine, or ketotifen in the proximal colon ($P < 0.05$) (Fig. 2B).

Expression of Calprotectin

There was a significant increase for the expression of calpro-

tecin in the POI 3H of the ileum and proximal colon, compared with that in the control group ($P < 0.05$) (Fig. 3A). In the POI 3H group of the ileum, the expression of calprotectin significantly decreased after the administration of mosapride, glutamine, or ketotifen ($P < 0.05$). The expression of calprotectin in the POI 6H group ileum and proximal colon was significantly higher than that in the control group ($P < 0.05$), and it decreased significantly after the administration of mosapride (0.3 mg/kg), glutamine or ketotifen ($P < 0.05$) (Fig. 3B).

Expression of Mast Cell Tryptase

There was no significant change for the expression of mast cell tryptase in the POI 3H and 6H ileum (Fig. 4A). There was a significant increase for the expression of mast cell tryptase in the POI 3H and POI 6H proximal colon compared with that in the control group ($P < 0.05$). Moreover, the expression of mast cell tryptase in the proximal colon decreased significantly after the administration of ketotifen ($P < 0.05$). Figure 4B and 4C show representative immunohistochemical images of mast cell tryptase in the control, POI, and ketotifen groups.

Expression of Claudin-1 and Claudin-2

The expression of claudin-1 in the POI 3H group ileum and proximal colon decreased significantly, compared with that in the control group ($P < 0.05$). After the administration of glutamine, the expression of claudin-1 in the ileum and proximal colon improved significantly ($P < 0.05$) (Fig. 5A). The expression of claudin-1 was significantly downregulated in the ileum and proximal colon of the POI 6H group ($P < 0.05$), which improved significantly ($P < 0.05$) after the administration of glutamine (Fig. 5B). Representative images of claudin-1 via immunofluorescence staining in the control, POI, and glutamine groups are shown in Supplementary Figure 1A (POI 3H) and 1B (POI 6H).

The expression of claudin-2 in the ileum and proximal colon of the POI 3H and 6H groups increased significantly compared with that in the control group ($P < 0.05$) and was significantly restored after the administration of glutamine in the ileum and proximal colon (Fig. 5C and 5D). Representative immunofluorescence images of claudin-2 in the control, POI, and glutamine groups are shown in Supplementary Figure 2A (POI 3H) and 2B (POI 6H).

Discussion

This study demonstrated that intestinal permeability increased significantly in POI guinea pig models, compared with that in con-

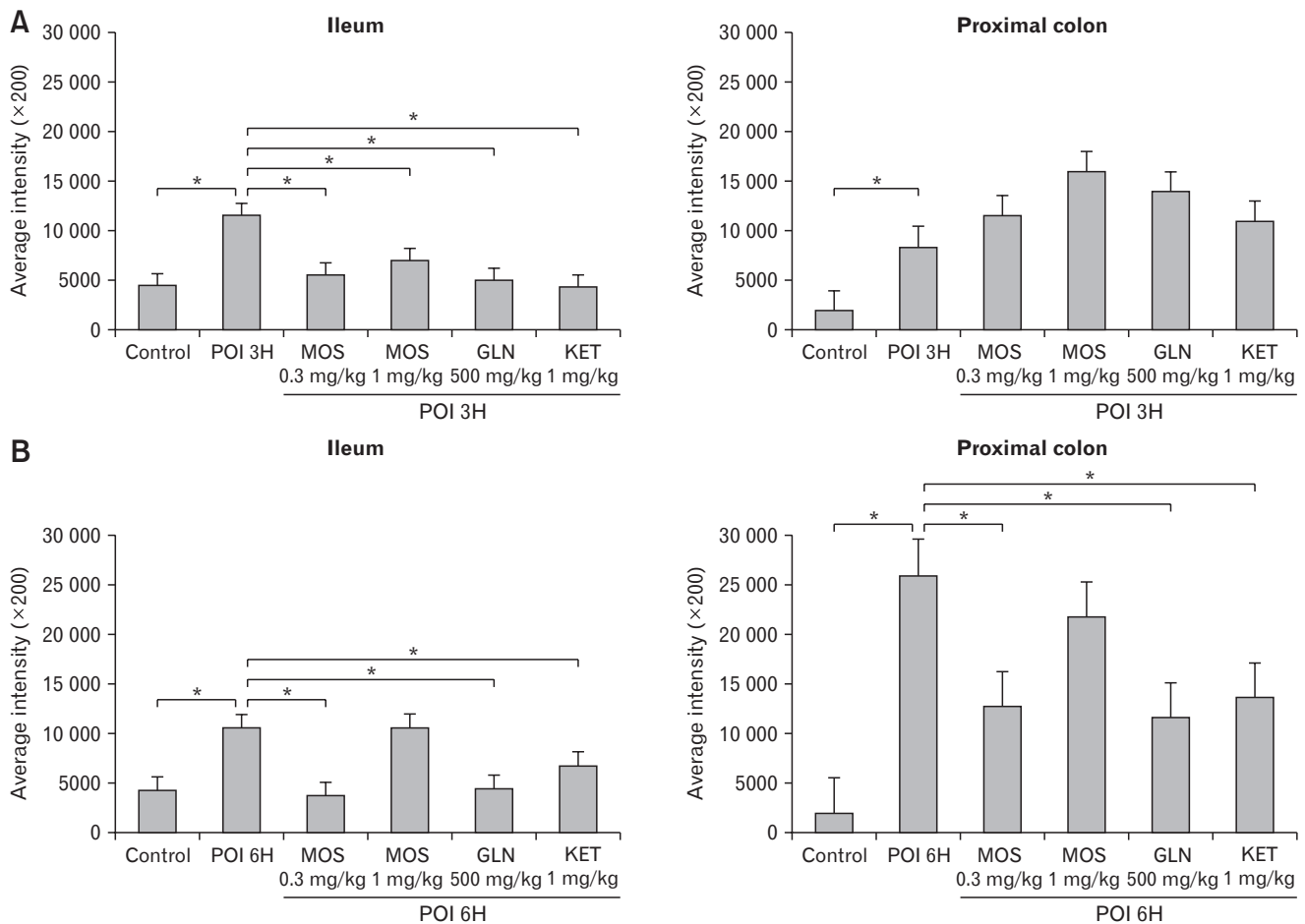


Figure 3. Expression of calprotectin in the control, post-operative ileus (POI), and drug groups. (A) Three hours after the operation and (B) 6 hours after the operation. The average intensity of the expression of calprotectin was analyzed via MetaMorph microscopy automation. Bars indicate the mean \pm SEM (control group, n = 7; POI group, n = 7; each drug group, n = 7). * $P < 0.05$. POI 3H, post-operative ileus at 3 hours after the operation; POI 6H, post-operative ileus at 6 hours after the operation; MOS, mosapride; GLN, glutamine; KET, ketotifen.

control pigs. Moreover, mosapride, glutamine, and ketotifen effectively reduced the observed permeability. To the best of our knowledge, this is the first study to identify alterations in intestinal permeability as an important mechanism associated with POI. Moreover, this study evaluated not only intestinal permeability but also possible factors contributing to the alteration in permeability.

We created POI guinea pig models at 3 hours and 6 hours post operation. In a previous study, the degree of POI, which was evaluated by the level of gas distention, peaked at 3 hours and resolved at 6 hours after the operation.¹⁶ Therefore, these 2 time points are appropriate for evaluating the early stage of POI. Although the recovery time of POI differs with the different portions of the gastrointestinal tract,^{12,17} the permeability of the ileum and proximal colon increased at both 3 hours and 6 hours post operation in the present study. These findings suggest that the alteration in permeability lasts

longer than the contractile activity. Because increased permeability leads to the translocation of bacteria and toxins through the intestinal mucosa, it is important to regulate the alteration in gut permeability in post-operative conditions.

The most prominent increase in permeability was observed in the POI 6H ileum. Among factors such as leukocytes, calprotectin, claudin, and mast cell tryptase, the expression of claudin showed the most significant change in the POI 6H ileum. Thus, TJ proteins may be the most important factor involved in the alteration in permeability.

After the administration of mosapride, glutamine, or ketotifen, the intestinal permeability decreased significantly. The dosages of the drugs were determined based on previous animal studies. Mosapride (0.3 mg/kg and 1 mg/kg) attenuated intestinal inflammation and motility dysfunction in a POI rat model.¹³ In the case

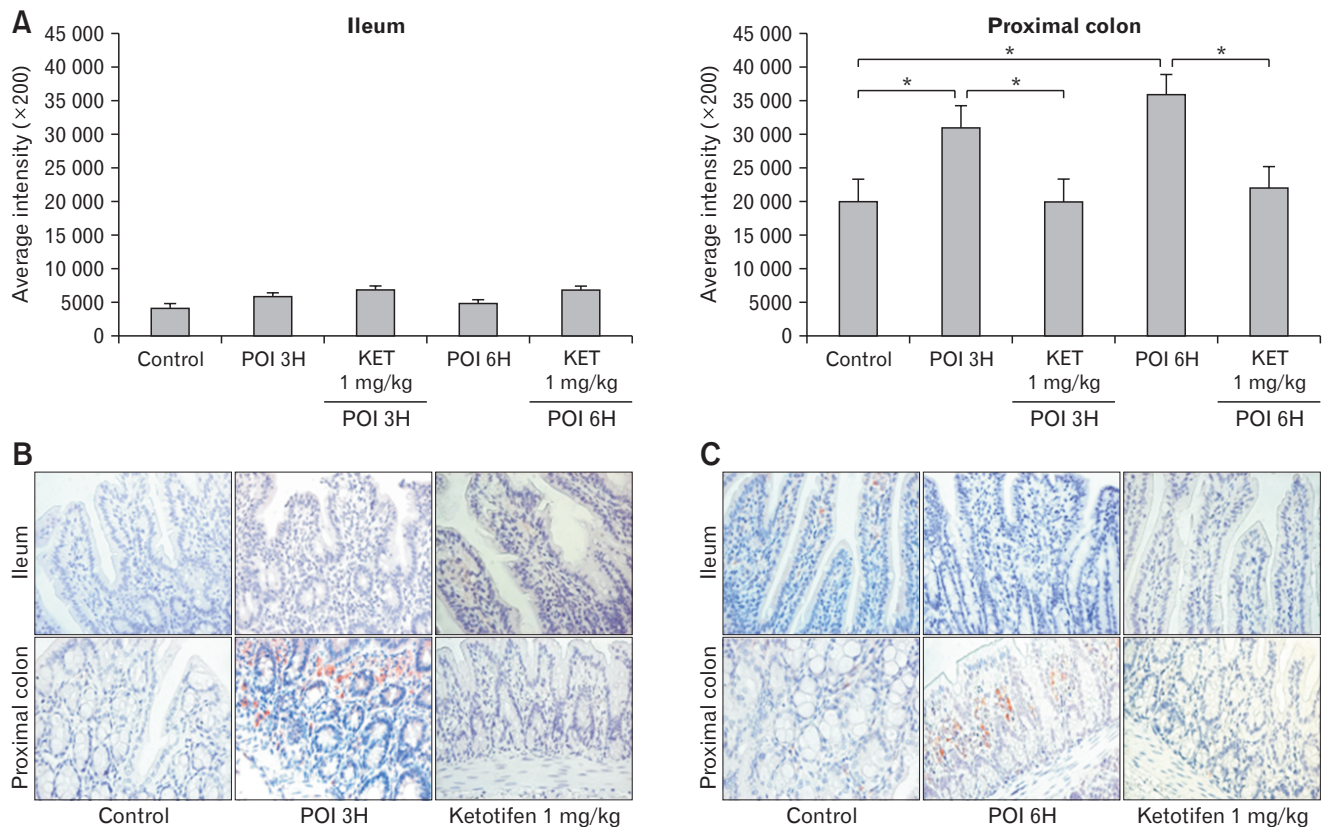


Figure 4. Expression of mast cell tryptase in the control, post-operative ileus (POI), and ketotifen groups. (A) The average intensity of the expression of calprotectin was analyzed via MetaMorph microscopy automation. Representative immunohistochemical staining of mast cell tryptase for POI at 3 hours after the operation (POI 3H) (B) and POI at 6 hours after the operation (POI 6H) (C). Bars indicate the mean \pm SEM (control group, $n = 7$; POI group, $n = 7$; ketotifen group, $n = 7$). $*P < 0.05$. KET, ketotifen.

of glutamine, a previous study evaluated the effect of glutamine (500 mg/kg) on intestinal permeability in a post-operative murine model.¹⁴ It was observed that glutamine decreased intestinal permeability and bacterial translocation, thereby preserving barrier integrity. Another study evaluated the role of ketotifen as a mast cell stabilizer for inflammation in a mouse model of POI¹⁵ and reported that 1 mg/kg of ketotifen prevented surgery-induced inflammation and gastroparesis.

To identify the mechanisms associated with the alteration in permeability, we evaluated inflammatory markers and TJ proteins, which are known to have an effect on intestinal permeability. In the present study, the leukocyte count and the expression of calprotectin, as inflammatory markers, increased significantly in the ileum and proximal colon of the POI group. The degree of increase in these inflammatory markers was prominent in the proximal colon at 6 hours post operation, and this result was consistent with that of a previous study.¹² This result may be associated with the difference in the recovery time of the ileus in that the contractile activity of the

proximal colon lasted longer than that of the ileum.

Among the 3 drugs selected, mosapride was the most effective against inflammation. Mosapride is well known as a prokinetic 5-hydroxytryptamine 4 (5-HT₄) receptor agonist. In addition to the prokinetic effect, mosapride has an anti-inflammatory effect mediated by acetylcholine release for cholinergic myenteric neurons.^{13,15,18} The released acetylcholine binds to the $\alpha 7$ nicotinic receptor on activated macrophages, thereby preventing leukocyte infiltration. Notably, the anti-inflammatory effect of mosapride was attenuated in a higher dose (1 mg/kg) compared with lower dose (0.3 mg/kg) of mosapride. The possible explanation of this result may be the desensitization of 5-HT₄ receptors located on sensory neurons in the presence of a higher concentration of 5-HT₄ receptor agonists. Previous studies reported the desensitization of tegaserod, which is another 5-HT₄ receptor agonist. One in vitro study showed that a higher dose of tegaserod did not cause an increase of velocity for fecal propulsion in the guinea pig.¹⁹ Another study demonstrated that the effect of tegaserod to elicit the desensitization

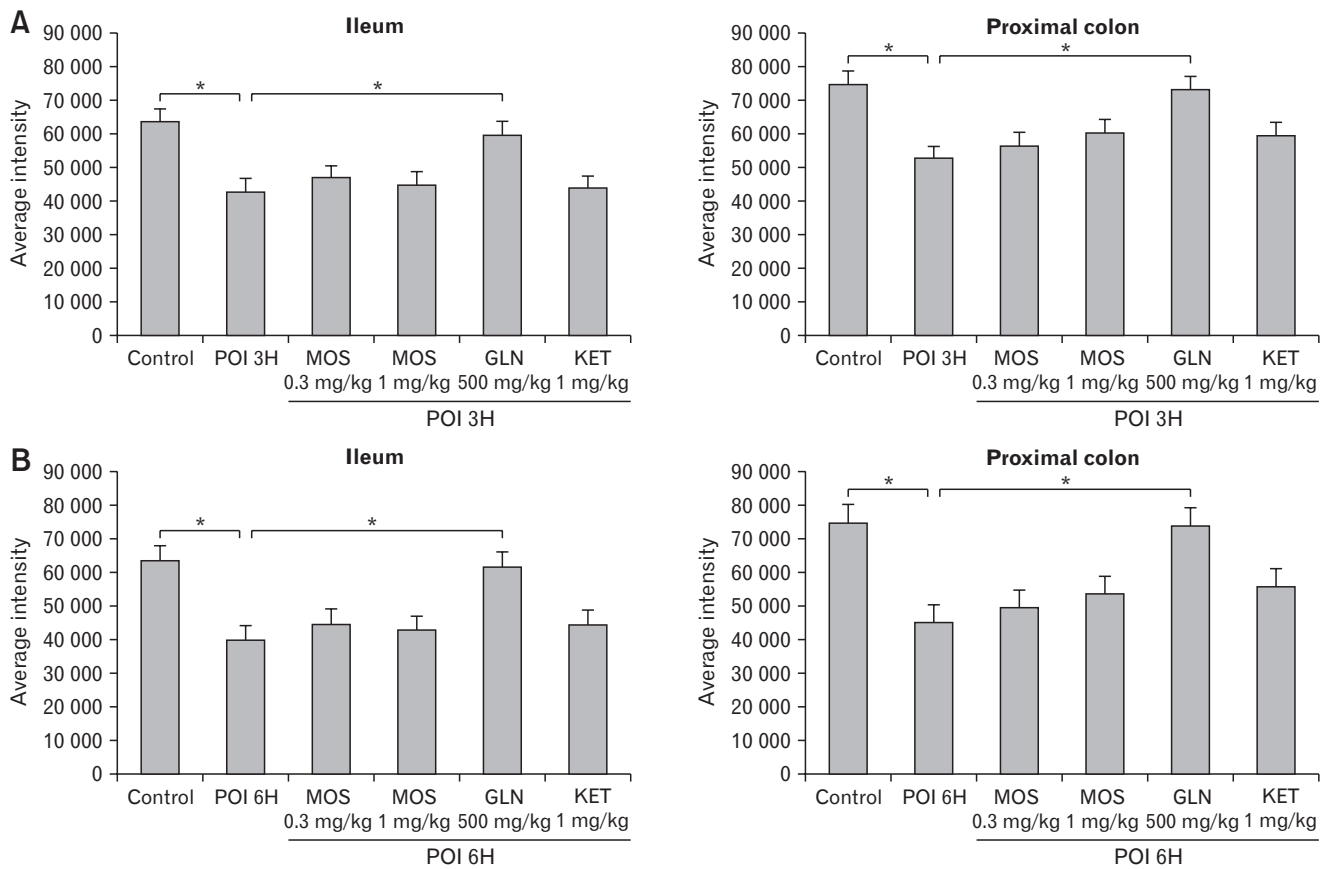


Figure 5. Expression of claudin in the control, post-operative ileus (POI), and drug groups. (A) Claudin-1 in the 3 hours after the operation, (B) claudin-1 in the 6 hours after the operation, (C) claudin-2 in the 3 hours after the operation, and (D) claudin-2 in the 6 hours after the operation. The average intensity of the expression of calprotectin was analyzed via MetaMorph microscopy automation. Bars indicate the mean \pm SEM (control group, n = 7; POI group, n = 7; each drug group, n = 7). * $P < 0.05$. POI 3H, post-operative ileus at 3 hours after the operation; POI 6H, post-operative ileus at 6 hours after the operation; MOS, mosapride; GLN, glutamine; KET, ketotifen.

was dependent on the concentration of tegaserod.²⁰ Further study is need to evaluate whether mosapride desensitizes the 5-HT₄ receptor at a higher dose in the guinea pig. In addition to mosapride, glutamine and ketotifen also showed anti-inflammatory effects in the POI model in the present study. Glutamine exhibits anti-inflammatory effects by modulating the inflammatory signaling pathways such as the nuclear factor κ B and signal transducer and activator of transcription pathways.²¹ Moreover, ketotifen exerts an anti-inflammatory effect that is mediated by its ability to decrease the production of pro-inflammatory mediators, such as nitric oxide, interleukin-1 β , and interleukin-6.^{22,23}

In addition to leukocytes and macrophages, mast cells are also associated with inflammation in the pathogenesis of POI. Intestinal manipulation induces mast cell degranulation, followed by the activation of resident intestinal macrophages. This promotes the phosphorylation of transcription factors and consequently the secre-

tion of cytokines and chemokines, which induce the upregulation of endothelial adhesion molecules and the influx of leukocytes.^{24,25} Mast cell tryptase is one of the granules (such as histamine, prostaglandin, and cytokines) released from activated mast cells.^{26,27} In our study, the expression of mast cell tryptase increased significantly in the proximal colon, which was effectively decreased by ketotifen. Ketotifen is a mast cell-stabilizing agent that blocks the release of mast cell granules.²⁸

Epithelial TJs are multiple protein complexes that maintain the intestinal barrier while regulating the permeability of ions, water, and nutrients.²⁹ Of the 4 integral transmembrane proteins—occludin, claudins, junctional adhesion molecules, and tricellulin, claudins are classified as a barrier-forming or pore-forming proteins.^{30,31} Claudin-1 and claudin-2 are known to be predominantly barrier-forming and pore-forming proteins, respectively.^{32,33} In this study, the expression of claudin-1 and -2 changed significantly in the POI

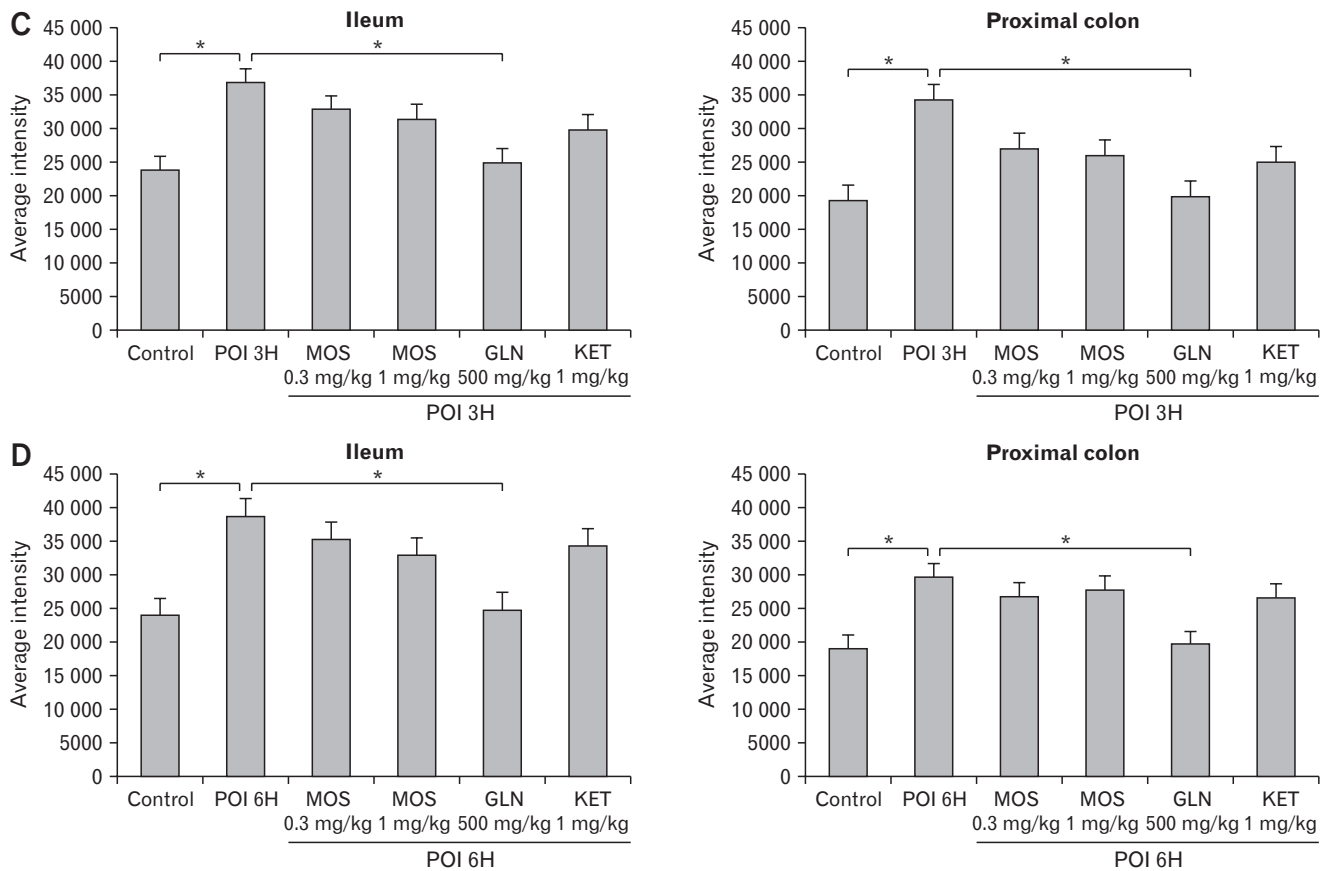


Figure 5. Continued.

3H and POI 6H ileum and proximal colon. Moreover, glutamine had a significant effect on the levels of claudin-1 and -2 in both the ileum and proximal colon. Glutamine maintains intestinal tissue integrity, and one of the several mechanisms associated with this function is the induction of the expression of TJ proteins such as claudin-1, occludin, and zonula occludens.³⁴

Based on our study results, mosapride, glutamine, and ketotifen are potential therapeutics for POI in clinical settings. Mosapride is widely prescribed as a prokinetic agent for dyspepsia and has proven safety. As mentioned earlier, in addition to its prokinetic effect, mosapride has an anti-inflammatory effect, which explains its ability to reduce intestinal permeability in this study. Therefore, it is necessary to determine whether the inflammation and symptoms of POI are improved after mosapride administration.

A recent study reported the induction of gut bacterial dysbiosis in a guinea pig POI model.³⁵ In that study, bowel movement was restored upon treatment with probiotics. Another study reported that gut dysbiosis leads to the disruption of intestinal integrity and the development of intestinal inflammation.³⁶ Therefore, in addi-

tion to inflammation and TJ proteins, other factors such as the gut microbiota may also have an effect on intestinal permeability. Future studies investigating the association between the microbiota and permeability are warranted to clarify our findings.

In conclusion, altered intestinal permeability is one of the factors involved in the pathogenesis of POI. Moreover, changes in the expression of inflammatory markers such as leukocytes, calprotectin, and mast cell tryptase, and TJ proteins were also observed in the POI model. Mosapride, glutamine, and ketotifen exhibited anti-inflammatory effects, thereby modulating intestinal permeability. Notably, glutamine had an effect on TJ proteins and ketotifen had an effect on mast cells. Therefore, we repositioned the 3 drugs as novel and potential therapeutic agents for POI.

Supplementary Materials

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

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Conflicts of interest: None.

Author contributions: Young Min Kim performed the experiment, analyzed and interpreted the data, and wrote a manuscript; Zahid Hussain and Young Ju Lee performed the experiment and interpretation of the data; and Hyojin Park designed, organized, and mediated the present study, and supervised the manuscript.

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