

—Original—

Development of a quantitative method for evaluating small intestinal motility using ultrasonography in mice

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Abstract: Upper gastrointestinal (GI) motility is affected by various drugs and diseases. However, changes in upper GI motility during these conditions are not well understood, as there are few quantitative *in vivo* methods that assess small intestinal motility in mice. Ultrasonography is a noninvasive method for imaging and evaluating the condition of the abdominal organs. The aim of the present study was to establish a novel method for evaluating small intestinal motility by using ultrasonography in mice. We measured GI motility with and without loperamide, an antidiarrheal medication, by intestinal transit using an orally administered dye, a ¹³C-octanoic acid breath test, and ultrasonography. Locomotion activity of the duodenal wall was used for quantifying the GI motility observed via ultrasonography. Our results showed that upper GI transit was significantly delayed by loperamide. The ¹³C-octanoic acid breath test revealed decreased gastric emptying in loperamide-treated mice. Through ultrasonography, large peristaltic movements were observed in the duodenum of the control mice. In contrast, after treatment with loperamide, these peristaltic movements were suppressed, and the duodenal lumen was enlarged, suggesting decreased duodenal motility. In accordance with these results, quantifiable locomotion activity was also significantly decreased. In conclusion, ultrasonography is an effective *in vivo* method to quantify small intestinal motility in mice.

Key words: gastrointestinal motility, ultrasonography

Introduction

Gastrointestinal (GI) motility is a biological function that is responsible for food digestion, nutrient absorption and waste excretion [26]. Disorders of GI motility are a frequent clinical problem, which can be caused by vari-

ous diseases, drugs, or aging. GI motility disorders cause clinical symptoms, such as constipation, vomiting, nausea, abdominal pain and anorexia, and they can greatly reduce the quality of life [18, 21, 22, 27, 29, 34]. Disorders of upper GI motility, including both the stomach and small intestine, are frequently seen in both human

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Supplementary Figure and Videos: refer to J-STAGE: <https://www.jstage.jst.go.jp/browse/exanim>



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and veterinary medicine. However, there are few studies that focus on small intestinal (SI) motility in comparison to studies on gastric motility. Therefore, changes in SI motility during disease or through the action of drugs are not well understood. One reason for this gap in knowledge may have to do with the lack of techniques available to assess SI motility *in vivo*. Therefore, establishing a noninvasive method for evaluating SI motility is required.

In the human medicine, there are methods for evaluating SI motility, including the lactulose H₂ breath test [3], the wireless motility capsule (WMC) test [15, 25], and scintigraphy [25]. For rodents, assessing SI motility is usually accomplished by measuring SI transit, as estimated by the migration distance of an orally administered dye in the GI tract [11, 20, 21, 31]. However, this method cannot distinguish between gastric and SI motility. Moreover, it requires sacrificing mice, which results in an increased number of animals used in this research. Thus, there are limitations to this method. A method that enables a noninvasive assessment of SI motility is needed for use in fundamental medical research.

Ultrasonography is routinely used in human and veterinary medicine as a noninvasive method for assessing the condition of abdominal organs. Ultrasonographic evaluation of GI motility is mainly conducted on gastric emptying [5, 19, 28, 30, 33]. On the other hand, there are few studies that use ultrasonography for evaluating SI motility. The reason for this lack of studies appears to be that simple ultrasonography has poor quantitative measuring potential for SI motility; thus, evaluation of this data is highly dependent on the operator [9, 13].

Therefore, we aimed to establish a novel quantification method for assessing SI motility by using ultrasonography in mice. In this study, we validated ultrasonography by investigating a mouse model of GI hypomotility induced by loperamide, an antidiarrheal.

Materials and Methods

Animals

All animal care and experimental procedures complied with the Guide for Animal Use and Care published by the University of Tokyo and was approved by the Institutional Review Board of the University of Tokyo (P18-131). Female C57BL/6J mice weighing 18–23 g were used in this study. The animals were kept at 22 ± 2°C on a 12 h light/dark cycle.

Experimental models

Loperamide (FUJIFILM Wako Pure Chemical, Tokyo, Japan) was used to induce GI hypomotility. Animals were subcutaneously administered loperamide dissolved in physiological saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) at a dose of 5 mg/kg, which was determined based on a previous report [17].

Determination of small intestinal transit

Small intestinal transit was measured according to previous reports [11, 20, 21]. Briefly, mice were fasted for 12–16 h and administered loperamide 15 min before testing. Animals were orally administered 100 µl of 0.5% (w/v) fluorescein isothiocyanate (FITC)-dextran in physiological saline using a feeding tube. One hour after administration of FITC-dextran, the entire GI tract was isolated and separated into the following segments: stomach (Sto), 10 equally sized segments of the small intestine, cecum (Cec), and 3 equally sized segments of the colon. The fractionated GI tract was collected in 1 ml of phosphate-buffered saline to extract the contents of the lumen and then centrifuged at 1,500 × g, 4°C for 15 min. The supernatant was centrifuged at 12,000 × g for 10 min, and the fluorescent intensity of the obtained supernatant was measured under the following conditions: excitation: 485 nm and fluorescence: 535 nm. The geometric center (GC) value of the distribution, an index of GI motility, was then calculated by the following calculation formula.

$$GC = \frac{\sum (\% \text{ of total fluorescent signal per segment} \times \text{segment number})}{100}$$

¹³C-octanoic acid breath test

Gastric emptying was evaluated by the ¹³C-octanoic acid breath test as previously reported [6, 7, 30]. The animals were fasted for 12–16 h and placed in a chamber, which was large enough for the mice to move freely. After the administration of 200 mg of a test meal consisting of heated egg yolk and 0.2 µl ¹³C-octanoic acid (Cambridge Isotope Laboratories, Inc., MA, USA); a blow pump device (Thermo Fisher Scientific Inc., Tokyo, Japan) collected breath samples that had accumulated in the chamber at a flow rate of 70 ml/min for a duration of 1 min, which were directed into a breath collection bag (Otsuka Pharmaceutical). After administration of the test meal, both with and without loperamide, breath samples were collected every 10 min until 120 min had passed, and breath collection then occurred at 140, 160,

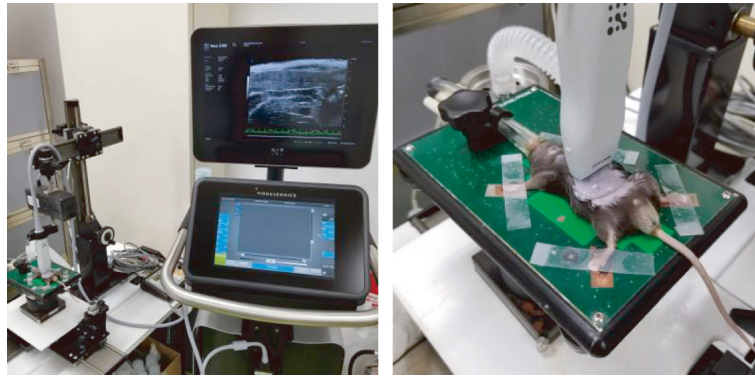


Fig. 1. The diagnostic ultrasound imaging systems used in this study. Abdominal ultrasonography was performed using a digital micro-ultrasound system (Vevo 3100, FujiFilm VisualSonics, Toronto, Canada) with a 55 MHz linear array transducer (MS-550S, FujiFilm VisualSonics). All mice were kept under anesthesia with 2% isoflurane in air (1 l/min) during the entire scanning procedure.

180, 210 and 240 min after administration of the test meal. The $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratio in the breath samples was analyzed using an infrared spectroscopic analyzer (Otsuka Electronics Co., Ltd., Osaka, Japan), and changes in $^{13}\text{CO}_2$ ($\Delta^{13}\text{C}$, ‰) were calculated from the $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratio. A mixed gas composed of 5% $^{12}\text{CO}_2$ and 95% O_2 was used as a standard. The maximum concentration (C_{max} ; ‰), the time to reach maximum concentration (T_{max} ; min) and the area under the exhalation concentration-time curve ($\text{AUC}_{240\text{min}}$; ‰ · min) were calculated using the value of $\Delta^{13}\text{C}$. The half-life ($T_{1/2}$; min) was calculated from the slope of the elimination phase in the $\Delta^{13}\text{C}$ curve [4, 17, 24].

Ultrasonography

All mice were kept under anesthesia with 2% isoflurane in air at a flow rate of 1 l/min during the entire scanning procedure. The animals were placed in a supine position on a heated imaging platform to maintain constant body temperature. Body hair was removed from the abdominal skin using a commercially available depilatory cream. Figure 1 shows the diagnostic ultrasound imaging systems used in this study. Abdominal ultrasonography was performed using a digital micro-ultrasound system (Vevo 3100, FujiFilm VisualSonics, Toronto, Canada) with a 55 MHz linear array transducer (MS-550S, FujiFilm VisualSonics). The transducer and heated imaging platform were integrated into an imaging station with a vital monitoring system (Vevo Imaging Station, FujiFilm VisualSonics) to hold the mice in posi-

tion and support the scanning with the manipulation techniques. B-mode imaging was performed in a transverse orientation for a 700-frame cine-loop with a 70 fps sampling rate. The cine-loop dataset was imported into speckle tracking software (VevoStrain, FujiFilm VisualSonics). A trace line was placed on the boundary between the proximal wall of the duodenum and the inner cavity. We defined locomotion activity of the duodenal wall as the average longitudinal displacement between any number of speckle points on the trace line for all time frames.

Statistical analyses

All results were expressed as the mean \pm SE (SEM). The data was analyzed using an unpaired Student's *t*-test for comparisons between two groups, a one-way repeated measures analysis of variance (ANOVA), and Dunnett's test for comparisons among time-series data. A value of $P < 0.05$ was regarded as significantly different.

Results

Upper GI motility was decreased in loperamide-treated mice

To confirm the inhibitory effect of loperamide on upper GI motility, GI transit was measured. Figure 2A shows the distribution of FITC-dextran along the GI tract 60 min after its administration. In control mice, FITC-dextran was distributed mainly in the distal small intestine (6–10). In contrast, in loperamide-treated mice, FITC-dextran was distributed mainly in the stomach and

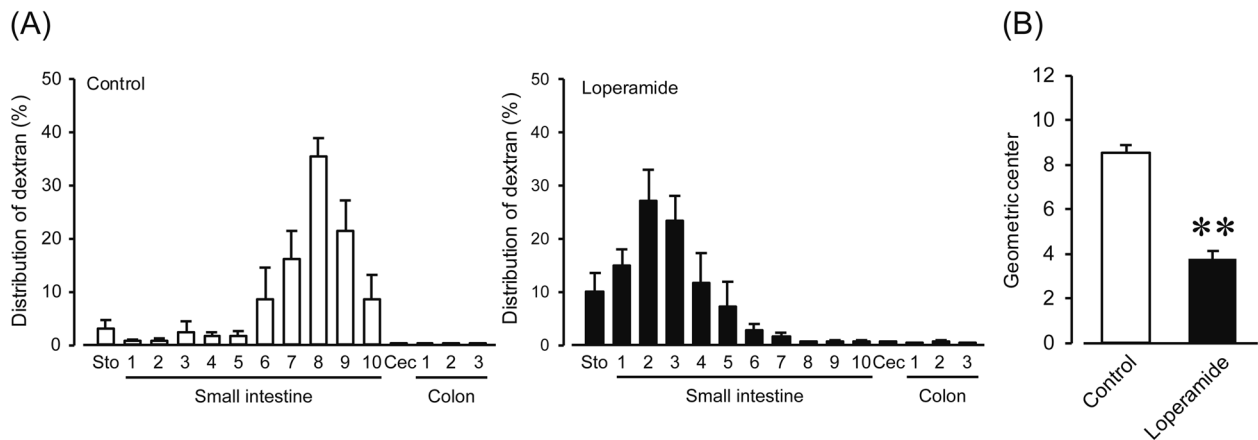


Fig. 2. Decrease of upper gastrointestinal transit by loperamide. (A) Distribution of administered fluorescein isothiocyanate (FITC)-dextran in control mice and loperamide-induced gastrointestinal motility disorder in a mouse model. Each column shows the mean \pm SEM (n=5). (B) Geometric center (GC) of each group calculated from (A). Each column shows the mean \pm SEM (n=5). ** P <0.01; significantly different from control.

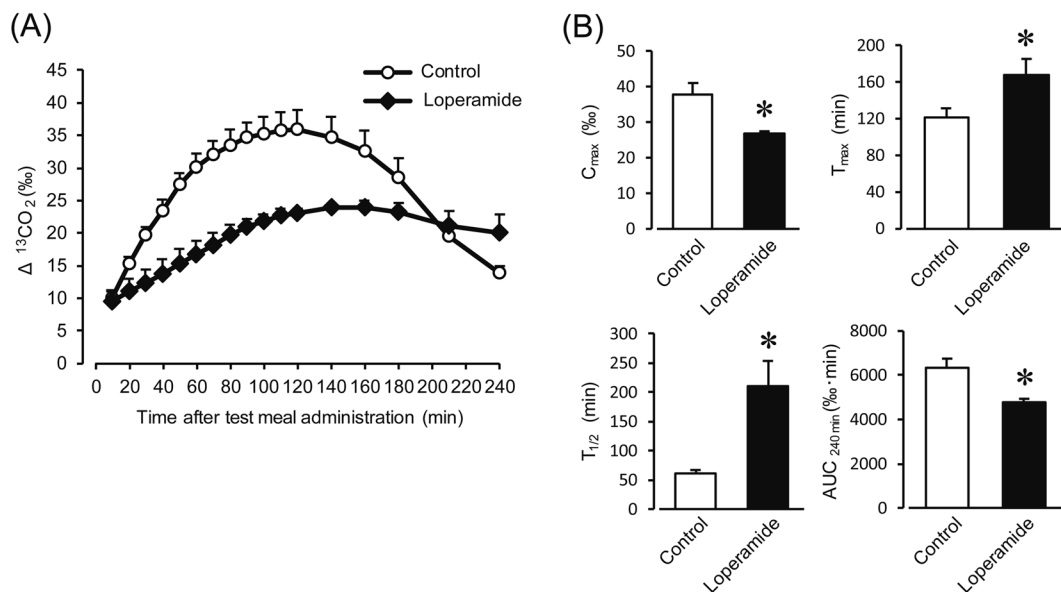


Fig. 3. Delayed gastric emptying by loperamide. (A) Time-course of gastric emptying measured by the ^{13}C -octanoic acid breath test in control mice and loperamide-induced gastrointestinal motility disorder in a mouse model. Each column shows the mean \pm SEM (n=6). (B) Quantification of C_{\max} , T_{\max} , $\text{AUC}_{240\text{min}}$, and $T_{1/2}$ are calculated from (A). Each column shows the mean \pm SEM (n=6). * P <0.05; significantly different from control.

proximal small intestine (1–5). Figure 2B shows the calculated GC value. The GC value was significantly decreased in loperamide-treated mice compared to that of the control mice (control: 8.5 ± 0.3 , loperamide: 3.8 ± 0.4 ; P <0.01 control vs loperamide, n=5). These results indicate that loperamide inhibits upper GI motility.

Loperamide induces delayed gastric emptying

To investigate the inhibitory effect of loperamide on gastric motility, gastric emptying was measured using the ^{13}C -octanoic acid breath test. Figure 3A shows the $^{13}\text{CO}_2$ excretion curve. In control mice, $^{13}\text{CO}_2$ concentration increased rapidly and showed a peak at 120 min and then decreased to near base value at 240 min. Conversely, the $^{13}\text{CO}_2$ concentration of loperamide-treated mice

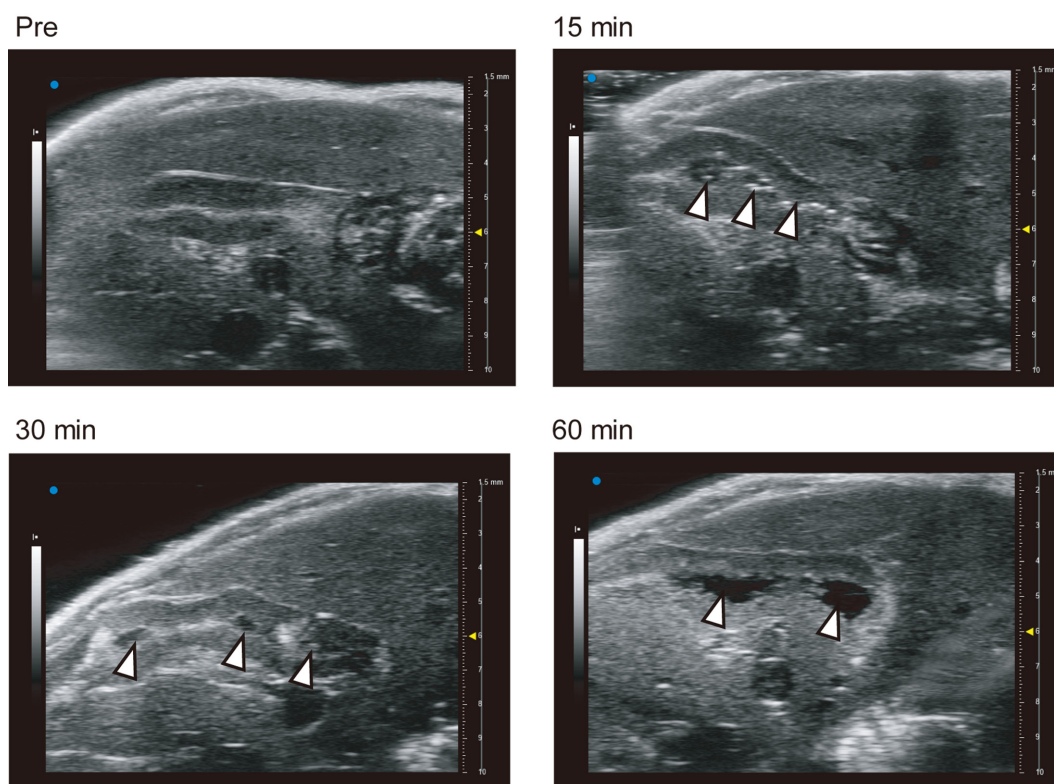


Fig. 4. Representative ultrasonographic images of a longitudinal cross-section of the duodenum. These images show changes in the duodenal lumen in control mice (Pre) and at 15, 30 and 60 min after administration of loperamide. Original B-mode imaging are shown in Supplementary Videos 1–4. White triangles indicate the expansion site of the duodenal lumen. B-mode images were acquired in transverse orientation using a 700-frame cine-loop with a 70 fps sampling rate.

was lower than that of control mice, and the time-to-peak was delayed to 240 min. Figure 3B shows C_{\max} , T_{\max} , $AUC_{240\text{min}}$, and $T_{1/2}$, which were calculated from the $^{13}\text{CO}_2$ excretion curve. As gastric emptying was delayed, the value of C_{\max} and $AUC_{240\text{min}}$ decreased, but T_{\max} and $T_{1/2}$ increased. C_{\max} and $AUC_{240\text{min}}$ were significantly decreased in loperamide-treated mice (control C_{\max} : 37.7 ± 3.2 ‰, $AUC_{240\text{min}}$: $6,321 \pm 422$ ‰·min; loperamide C_{\max} : 26.9 ± 0.7 ‰, $AUC_{240\text{min}}$: $4,777 \pm 141$ ‰·min; $P < 0.05$ control vs loperamide, $n=6$). T_{\max} and $T_{1/2}$ were significantly elevated in loperamide-treated mice (control T_{\max} : 121.7 ± 9.3 min, $T_{1/2}$: 61.9 ± 4.3 min; loperamide T_{\max} : 167.5 ± 18.1 min, $T_{1/2}$: 209.8 ± 43.6 min; $P < 0.05$ control vs loperamide, $n=6$). These results indicate that loperamide has a sustained inhibitory action on gastric emptying, and the delayed GI transit induced by loperamide is caused, at least in part, by delayed gastric emptying.

Ultrasonography revealed loperamide-induced hypomotility of small intestine

B-mode abdominal ultrasound detected all of the small intestines, and the serosa, muscularis, submucosa, and mucosa were identified by their echogenicity (Supplementary Fig. 1). In the present study, we measured duodenal motility. Before loperamide administration, the duodenum exhibited dynamic peristaltic movement (Fig. 4: Pre; Supplementary Video 1). However, after treatment with loperamide, peristaltic movement was significantly suppressed (Fig. 4: 15, 30 and 60 min; Supplementary Video 2–4). Furthermore, the duodenal lumen was enlarged after loperamide treatment, which is a signature of decreased SI motility.

Ultrasonography enables quantification of SI motility

Next, we investigated whether the quantification of SI motility using abdominal ultrasound was possible. The locomotion activity of the duodenal wall was used to quantify SI motility and was defined by the average

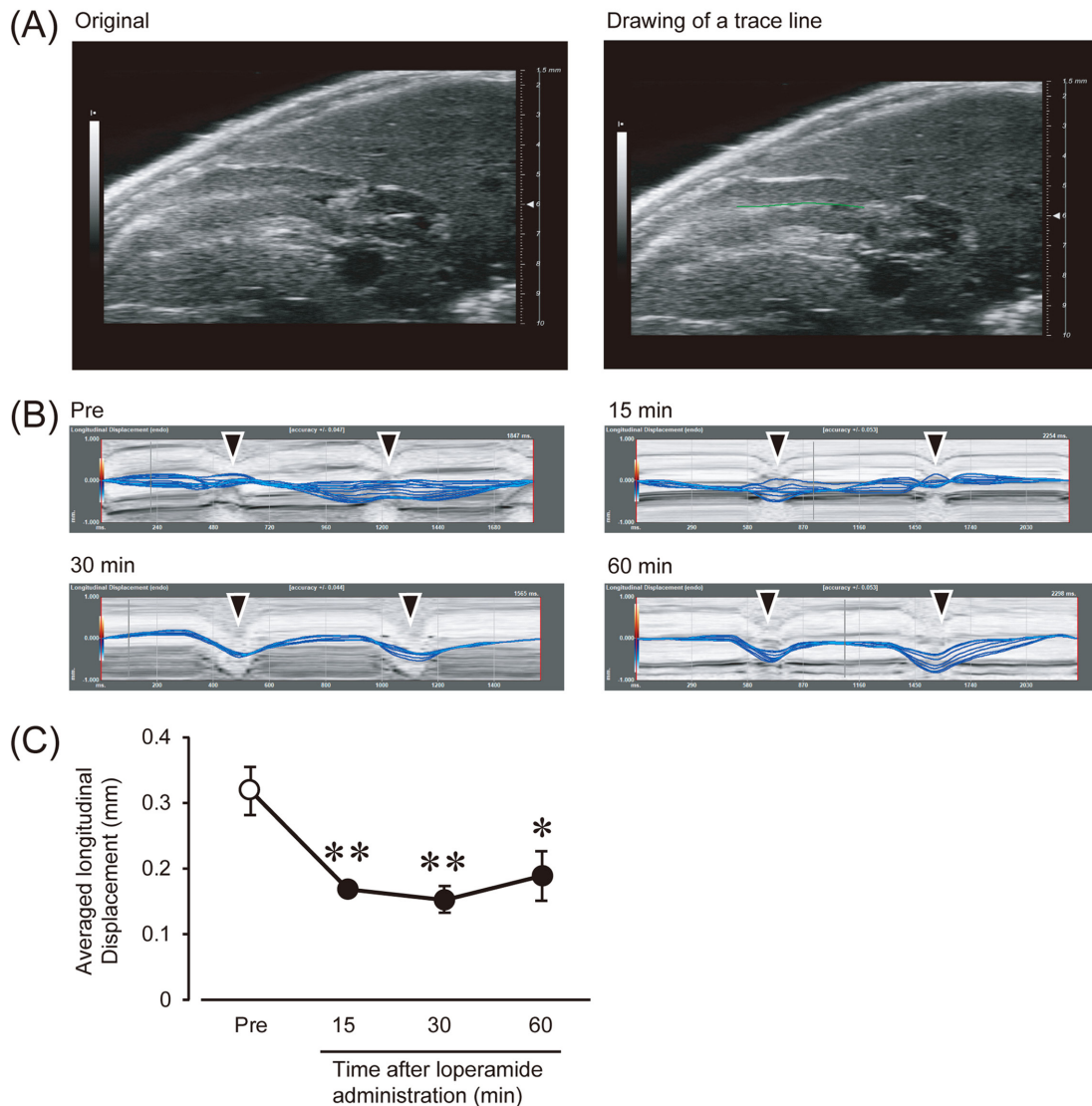


Fig. 5. Drawing of trace lines in the duodenal lumen and an analysis of the movement of the trace lines. (A) Representative images of the long axes of the duodenum with trace lines (green) drawn. (B) Representative images of the change in speckle points on the trace lines in control mice (Pre) and at 15, 30 and 60 min after administration of loperamide. Black triangles indicate the timing of respiration. Original B-mode imaging are shown in Supplementary Videos 5–8. (C) Quantification of locomotion activity is calculated from (B). Each column shows the mean \pm SEM ($n=6$). * $P<0.05$, ** $P<0.01$; significantly different from control.

maximum longitudinal displacement of speckle points on the trace line. Figure 5A shows an example of the long-axis, ultrasound images of the duodenum and the trace line drawn on the duodenal wall. Figure 5B shows the longitudinal movement of speckle points before (Pre) and 15, 30 and 60 min after loperamide administration. It is difficult to remove the artifact caused by respiration (black triangle), so we calculated the average maximum displacement during 3 breaths. Figure 5C shows the locomotion activity before and after treatment with loperamide.

The locomotion activity of the duodenum was significantly decreased after loperamide administration (control: 0.32 ± 0.04 mm, loperamide 15 min: 0.17 ± 0.01 mm, loperamide 30 min: 0.15 ± 0.02 mm, loperamide 60 min: 0.19 ± 0.04 mm; $P<0.05$ control vs loperamide 15 min $P<0.01$ control vs loperamide 30 min, $P<0.05$ control vs loperamide 60 min, $n=6$). These results indicate that ultrasonography can detect and quantify decreased SI motility.

Discussion

In human medicine, several methods are used to assess SI motility for diagnosing GI motility disorders such as chronic intestinal pseudo-obstruction [12]. However, in experiments with rodents, there are no effective methods. Therefore, in the present study, we validated ultrasonography as a solution to this problem by investigating the effect of loperamide on SI motility in mice. The results of this investigation suggest that ultrasonography can identify loperamide-induced hypomotility of the duodenum. In accordance with this result, quantifiable duodenal motility was significantly decreased. This indicates that ultrasonography can be an effective method to non-invasively quantify SI motility in mice.

Loperamide activates μ -opioid receptors expressed in enteric neurons to relax intestinal smooth muscles. Indeed, measurements of GI transit indicated slow upper GI transit in loperamide-treated mice. Additionally, the ^{13}C -octanoic acid breath test revealed delayed gastric emptying. These results indicate that loperamide induces the relaxation of gastric smooth muscles, which results in slow upper GI transit. On the other hand, the effect of loperamide on SI motility was unclear from these two results. There remain two possibilities, as follows: (1) the SI motility was suppressed along with gastric motility, or (2) the SI motility was not suppressed but gastric motility was. The results of abdominal ultrasonography revealed that the large peristaltic movements of the duodenum were suppressed, with the contractile pattern becoming more irregular after loperamide treatment. Additionally, an expanded duodenal lumen was observed after loperamide treatment, which is a signature of decreased intestinal motility. In summary, abdominal ultrasonography revealed that the causes of loperamide-induced, slow upper GI transit are not only induced by delayed gastric emptying but also by the suppression of small intestinal motility. Thus, abdominal ultrasonography is an effective method to directly investigate the effects of drugs or diseases on SI motility.

The small intestine is composed of the duodenum, jejunum, and ileum. However, only the duodenum motility was investigated in this ultrasound experiment. There are two reasons for this limitation. First, the duodenum is easier to distinguish from other regions of the small intestine in the ultrasound experiment. Second, the jejunum and ileum can freely move in the abdominal cavity, so it is difficult to focus on the same section during the

experiment. Intestinal motility occurs by the coordinated function of smooth muscle cells, enteric neurons, interstitial cells of Cajal (ICC) and platelet-derived growth factor receptor- α positive interstitial cells [26]. The ICC serve as intestinal pacemakers. They generate electrical activity (slow waves), which induces the contraction of smooth muscle cells. ICC form networks throughout the small intestine without any gaps. Indeed, one *in vitro* experiment has shown that slow waves generated in the duodenum propagate to the jejunum [14]. These facts may indicate that whole SI motility can be estimated by measuring duodenal motility using abdominal ultrasonography. On the other hand, the slow waves in the stomach and small intestine are largely independent, as there is a gap in the ICC network [32]. It has been reported in the literatures that patients with gastroparesis show abnormal duodenal motility [1, 8]. Therefore, focusing on duodenal motility and its mechanism and regulation is important for gastroparesis research.

During ultrasonography, the thickness of the intestinal wall is routinely measured since a thickened wall suggests inflammation. Intestinal motility is also assessed using ultrasonography; however, this assessment largely depends on the researcher's subjectivity. Therefore, in mouse experiments, it can be difficult to conclude if a small change in intestinal motility is the result of drugs or diseases. To address this problem, we calculated the locomotion activity of the duodenal wall for the quantification of SI motility. Locomotion activity was significantly decreased 15 min after loperamide treatment, and this effect continued until 60 min after treatment. The time-course of loperamide action appears to correlate with that of the ^{13}C -octanoic acid breath test. These results suggest that the calculated value is suitable for the quantification of SI motility. In the present study, we did not investigate whether locomotion activity would change depending on the dose of loperamide or of other types of drugs, including gastrointestinal prokinetic drugs. Further validation is needed to show the effectiveness and sensitivity of this value. However, our quantification method can detect decreased SI motility induced by loperamide. In addition to rodent experiments, this quantitative method may be applied to human and veterinary medicine in the future.

There are methods available to measure SI motility, or transit, in humans. The lactulose H_2 breath test estimates SI transit time by measuring H_2 exhalation after

the bacterial metabolism of lactulose in the cecum [3]. This test is easy and noninvasive; however, the results are affected by intestinal bacterial conditions, and the lactulose itself accelerates transit due to osmotic fluxes into the small intestine [23]. Moreover, gastric emptying time may also affect the results of this test. Recently, a WMC has become available, which is orally administered and records pH and pressure in the GI tract. The recorded pH is used to identify the location of the WMC. For example, a sudden and sustained increase in pH would indicate that the WMC had reached the duodenum. The recorded pressure provides detailed patterns of intestinal motility. However, this approach requires a long amount of time (11 h 30 min in healthy patients) to excrete the WMC, making it difficult to measure SI motility for short intervals. Moreover, a commercially available WMC is too large for use in rodents. Scintigraphy is also used to measure SI transit but requires a specialized facility and exposure to radiation [2, 10, 16]. Abdominal ultrasonography, however, provides noninvasive, real-time images and measures SI motility quickly. Abdominal ultrasonography can be performed at different time points in the same animal and takes only 5–10 min to obtain a set of images. These advantages may be especially useful for measuring the action time of drugs with regard to intestinal motility. In addition, using a noninvasive method reduces the number of animals used for experimental studies, which contributes to 3R animal testing compliance. On the other hand, abdominal ultrasonography has some limitations. For example, our evaluation method can only assess the duodenum. Additionally, this method should be performed under anesthesia, which can potentially affect GI motility or the pharmacokinetics of different drugs. Despite these limitations, this method is effective in measuring SI motility in mice and contributes to the development of new drugs for GI diseases. It may be effective to combine several methods for measuring SI motility and transit in small experimental animals.

In conclusion, we have presented the first evidence that abdominal ultrasonography can be applied to measure SI motility quickly, noninvasively and objectively. This method will contribute to the research field of gastroenterology.

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