Ghrelin improves intestinal mucosal atrophy during parenteral nutrition: An experimental study

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ABSTRACT

Background/purpose: Total parenteral nutrition (TPN) has been reported to be associated with mucosal atrophy of the small intestine. Ghrelin has hormonal, orexigenic, and metabolic activities. We investigated whether ghrelin improved intestinal mucosal atrophy using a TPN-supported rat model.
Methods: Rats underwent jugular vein catheterization and were divided into four groups: TPN alone (TPN), TPN plus low-dose ghrelin (TPNLG), TPN plus high-dose ghrelin (TPNHG), and oral feeding with normal chow (OF). Ghrelin was administered continuously at dosages of 10 or 50 μg/kg/day. On day 6 rats were euthanized, and the small intestine was harvested and divided into the jejunum and ileum. Then the villus height (VH) and crypt depth (CD) were evaluated.
Results: The jejunal and ileal VH and CD in the TPN group were significantly decreased compared with those in the OF group. TPNHG improved only VH of the jejunum. TPNLG improved VH and CD of the jejunum and CD of the ileum. The improvement of TPNLG was significantly stronger than that in CD of the jejunum and ileum.
Conclusions: TPN was more strongly associated with mucosal atrophy in the jejunum than in the ileum. Low-dose intravenous administration of ghrelin improved TPN-associated intestinal mucosal atrophy more effectively than high-dose administration.

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Total parenteral nutrition (TPN) provides crucial therapeutic support for patients with gastrointestinal disorders, such as short bowel syndrome owing to malrotation with midgut volvulus, necrotizing enterocolitis, gastroschisis and inflammatory bowel disease. However, long-term TPN leads to intestinal mucosal atrophy because of the loss of luminal nutrient stimulation, which is provided by food [1,2]. The lack of luminal nutrient stimulation strongly promotes intestinal barrier dysfunction and bacterial translocation [3] and contributes to an increase in the risk of infectious complications, including catheter-related blood stream infections (CRBSIs) [4]. Because CRBSIs are life-threatening, the development of an efficient therapeutic strategy to improve TPN associated intestinal mucosal atrophy would be pivotal. Previous studies have demonstrated that several growth factors, including keratinocyte growth factor [5], epidermal growth factor [6], glucagon-like peptide-2 [7,8], insulin like growth factor-1 (IGF-1) [9] and GH [10], might improve TPN associated intestinal mucosal atrophy

by augmenting crypt cell proliferation. We have focused on the ghrelin, which has a strong effect on the secretion of growth hormone and IGF-1 [11,12].

Ghrelin is a 28-amino acid peptide with an n-octanoylation modification on serine 3, which is produced mainly by the X/A-like endocrine cells, which are located in the gastric body. Ghrelin has a vast range of physiological functions, including orexigenic, metabolic and hormonal functions [13].

Recent studies have suggested the involvement of ghrelin in pathological gastrointestinal conditions and immune system regulation [14,15]. We therefore hypothesized that ghrelin might have a therapeutic potential in TPN associated intestinal mucosal atrophy. In the present study, we investigated the effects of ghrelin administration on TPN associated intestinal mucosal atrophy in a rat model.

1. Materials and methods

1.1. Animals

Seven-week-old male Sprague–Dawley (SD) rats of 200 to 240 g in body weight (Kyudo Co., Ltd., Saga, Japan) were used in this experiment. The animals were individually housed in metabolic caging with ad

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libitum access to standard rat chow and water, and were acclimatized to their environment for 7 days before the experiments. The animals were maintained under standardized temperature (23 °C ± 1 °C), humidity (50 ± 10%), and a 12-h light–dark cycle (lights on at 7:00 a.m.). All of the experimental procedures were approved by the Laboratory Animal Committees of Kagoshima University Graduate School and were performed in accordance with the "Guidelines for the Care and Use of Laboratory Animals."

1.2. Study design

The animals were fasted overnight. After the placement of a central venous catheter, they were randomly assigned to one of the following four treatment groups (n = 10 in every group): TPN-alone (TPN), TPN plus low-dose ghrelin (TPNLG) (10 μg/kg/day; continuous infusion), TPN plus high-dose ghrelin (TPNHG) (50 μg/kg/day; continuous infusion), and oral feeding with ad libitum access to normal chow plus vehicle (OF). Ghrelin (Peptide Institute Inc., Osaka, Japan) was dissolved in distilled water and administered intravenously. On day 6, the animals were anesthetized, weighed, and sacrificed. The gross intestinal morphology was assessed and tissue was harvested for the subsequent analyses.

1.3. The surgical procedure and maintenance methods

The animals were anesthetized with isoflurane (1.5% inhalation by mask), and an intravenous catheter was inserted into the right jugular vein. A Silastic catheter (outside diameter, 1.2 mm: NIPRO Co., Ltd., Osaka, Japan) was tunneled out of the back and attached to a standard swivel device (LOMIR BIOMEDICAL INC., Quebec, Canada). The procedures were performed with the aid of an operating microscope. All of the animals received cefazolin (50 mg/kg per dose subcutaneously; Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) to prevent postoperative infection and buprenorphine (0.01 mg/kg per dose subcutaneously; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) for analgesia. The animals were allowed ad libitum access to water immediately after surgery. TPN was delivered by a multichannel syringe pump (KDS Legato 200 Series Syringe Pump Series, KD Scientific, Inc., Holliston, MA, USA). After catheterization, the animals of the OF group were maintained with saline (60 ml/day; OTSUKA NORMAL SALINE: Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and the animals of the other three groups were maintained with low-concentration NEOPAREN® No. 2 (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) TPN solution (60 ml/day); into which 20% Intralipos® (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was added. The composition of the TPN solution was as follows (in g/L): amino acids 25, dextrose 145, and soy bean oil 33.3. The solution also contained the following electrolytes (final mmol/L): 41.6 Na$^+$, 22.5 K$^+$, 41.6 Cl$^-$, 4.1 Ca^{2+} and 4.1 Mg^{2+}. After 24 h the composition of the TPN solution was switched to amino acids 31.6, glucose 203, and soy bean oil 33.3 with similar electrolyte additives. The TPN solution was delivered at the rate of 60 ml/day. This provided equivalent isocaloric/isonitrogenous nutritional support to all of the TPN-fed animals, consisting of 76.4 kcal/rat/day (1.9 g protein, 2.0 g fat, and 12.2 g carbohydrate).

1.4. The gross intestinal morphology and histology

The rats were anesthetized by isoflurane inhalation after an overnight fasting. On day 6, blood was obtained from the heart then immediately centrifuged at 1500×g for 15 min at 4 °C. All of the plasma samples were stored at −80 °C until use. After the collection of blood, the animals were euthanized by exsanguination. The total small intestine was harvested for the gross and microscopic morphological analyses. The mesentery was removed, and the total length of the small bowel was measured from the ligament of Treitz to the ileocecal valve along the antimesenteric border. The harvested small intestine was

quickly opened along the mesenteric border, rinsed in cold saline, and weighed. The bowel width was measured at the middle point of the opened jejunum and ileum. Samples for the microscopic analysis were harvested from the jejunum (2.5 cm below the ligament of Treitz), and the distal ileum (2.5 cm above the ileocecal valve) and fixed in a 10% formaldehyde neutral buffer solution for 24 h. Paraffin sections of formalin-fixed tissue were cut at a thickness of 3 μm and stained with hematoxylin and eosin. For each sample slide, microscopic measurements of the villus height, villus width, crypt depth, muscle layer thickness, and the number of villi per 1 mm were recorded from 10 well-oriented villi/crypt units. The quantification was performed with the help of an expert pathologist. The absorptive mucosal surface area per 1 cm^2 of intestine was calculated using previously described methods. Briefly, the mucosal surface area was calculated by first considering the intestine as a cylinder and then multiplying the additional mucosal surface area contributed by the villi, with each villus considered as a cone [16].

1.5. Crypt cell proliferation

The crypt cell proliferation rate (CCPR) was quantified by immunohistochemistry using Ki67 as a marker of active cell division, as previously described [17]. In brief, antigen retrieval was performed by boiling the tissue sections in 0.01 M citrate buffer at pH 6. After the blocking of endogenous peroxidase activity and nonspecific antigen binding, the tissue sections were incubated with anti-histone Ki-67 overnight in a moist chamber. The appropriate dilution of Ki67 (Cell Signaling Technology, Inc., USA) was 1:400. After washing in Tris buffer saline, the tissue sections were incubated with universal secondary antibody (Signal Stain® Boost IHC Detection Reagent, Cell Signaling Technology, Inc., USA). Immune detection was performed using diaminobenzidine as chromogen and hydrogen peroxidubstrate, followed by counterstaining with hematoxylin. The proliferation index (PI) was calculated as the number of Ki-67 positive cells present among ten consecutive well-oriented crypts per slide.

1.6. Statistical analysis

The data are presented as the mean values ± standard error (SE). The statistical analyses between groups and time courses were performed using a 2-factor factorial analysis of variance (ANOVA) followed by Tukey's multiple-comparison post-hoc test. P values of <0.05 were considered to indicate statistical significance.

2. Results

2.1. The changes in the daily assessment data

The daily urine volume was measured, however, there were no significant differences among the three TPN or coadministered ghrelin groups.

2.2. The gross intestinal morphology

The gross intestinal morphology results are shown in Table 1. There was no significant difference in the total length of small intestine among the four groups. In the OF group, the bowel weight per 1 cm was significantly greater and the widths of the jejunum and ileum were significantly wider than in the TPN, TPNLG and TPNHG groups.

2.3. The microscopic intestinal morphology of the jejunum

Fig. 1 shows a representative image of the histological morphology of the jejunum. The TPN group showed severe mucosal atrophy (Fig. 1a). The administration of ghrelin inhibited mucosal atrophic change in the TPNLG (Fig. 1b) and TPNHG groups (Fig. 1c). The OF

Table 1
Gross intestinal morphology.

	Total small bowel		Bowel width	
	Length(cm)	Weight(mg/cm)	Jejunum	Ileum
TPN	61.9 ± 2.5	92.0 ± 5.0	7.0 ± 0.5	7.3 ± 0.3
TPN + ghrelin (Low)	61.2 ± 2.3	104.0 ± 4.6	7.2 ± 0.5	8.0 ± 0.4
TPN + ghrelin (High)	59.3 ± 2.5	102.5 ± 5.9	7.3 ± 0.6	8.0 ± 0.5
Oral Feeding	64.9 ± 2.1	121.3 ± 5.8[a]	9.8 ± 0.6[a,b,c]	10.4 ± 1.1[a,b,c]

Values: means ± S.E., n = 10 per group.
  [a] P < 0.05 vs. TPN.
  [b] P < 0.05 vs. TPN + ghrelin (Low).
  [c] P < 0.05 vs. TPN + ghrelin (High).

group showed no mucosal atrophy (Fig. 1d). Based on these morphological results, the villus height, the crypt depth, the thickness of muscle layer, the villus density and the absorptive mucosal surface area were measured (Table 2). In the TPN group, the villus height and crypt depth in the jejunum were significantly decreased in comparison to the OF group, which means that TPN was associated with mucosal atrophy (Table 2). The villus height was significantly maintained in the jejunum of both the TPNLG and TPNHG groups in comparison to the TPN group (Table 2). However, the crypt depth was only significantly affected in the TPNLG group. The thickness of the muscle layer in the TPNLG group was significantly increased in comparison to the other three groups. The villus density was significantly higher in the TPNLG and OF groups than it was in the TPN group. The absorptive mucosal surface area in the TPNLG group was higher than that in the TPN and TPNHG groups, but the effect was not statistically significant. The administration of ghrelin did not inhibit the decrease in the absorptive mucosal surface area.

2.4. The microscopic intestinal morphology of the ileum

Fig. 2 shows a representative image of the histological morphology of the ileum. The TPN and TPNHG groups showed mild atrophic changes (Fig. 2a and c). The TPNLG and OF groups showed no atrophic changes (Fig. 2b and d). The measurements and analyses followed the same procedures as those that were applied in the jejunum (Table 3). The crypt depth of the TPNLG group was significantly different to that in the TPN and TPNHG groups. The thickness of the muscle layer in the TPNLG group was significantly increased in comparison to the other three groups. The villus density did not differ to a statistically significant extent among the four groups. The results regarding the absorptive mucosal surface area were the same as those observed in the jejunum.

2.5. The crypt cell proliferation rates

In the TPNLG group, the CCPRs in the jejunum and ileum were significantly increased in comparison to the other three groups. Furthermore, in the TPNHG group the CCPR in the ileum was significantly increased in comparison to the TPN group (Tables 2 and 3).

3. Discussion

Growth hormone plays a major role in somatic growth, stimulates intestinal growth, and has been used in the intestinal rehabilitation therapy for pediatric patients with short bowel syndrome. We focused on ghrelin, which has a strong effect on the secretion of growth hormone, and investigated the effects of the administration of ghrelin on TPN associated intestinal mucosal atrophy using a rat model.

The major findings of this study were as follows. (1) TPN for 6 days induced mucosal atrophy; the mucosal atrophy in the jejunum was more severe than that in the ileum. (2) The administration of ghrelin improved mucosal atrophy in our rat model of TPN. (3) Low-dose ghrelin had a significantly greater effect on the crypt depth, the thickness of the muscle layer, and the CCPR than high-dose ghrelin.

Diaphoresis has been reported as an adverse event owing to the administration of ghrelin in postoperative patients [18]. However, given that there were no significant differences in the daily urine volume among the three TPN groups, the doses used in the present study are not expected to cause any adverse events.

In line with previous studies, TPN was shown to induce a reduction in the villus height, crypt depth and CCPR [6]. Ghrelin tended to increase the villus height and crypt depth in the jejunum and ileum. Notably, there were significant differences in the villus height in the jejunum in both of the ghrelin-treated groups and the crypt depth in the ileum of
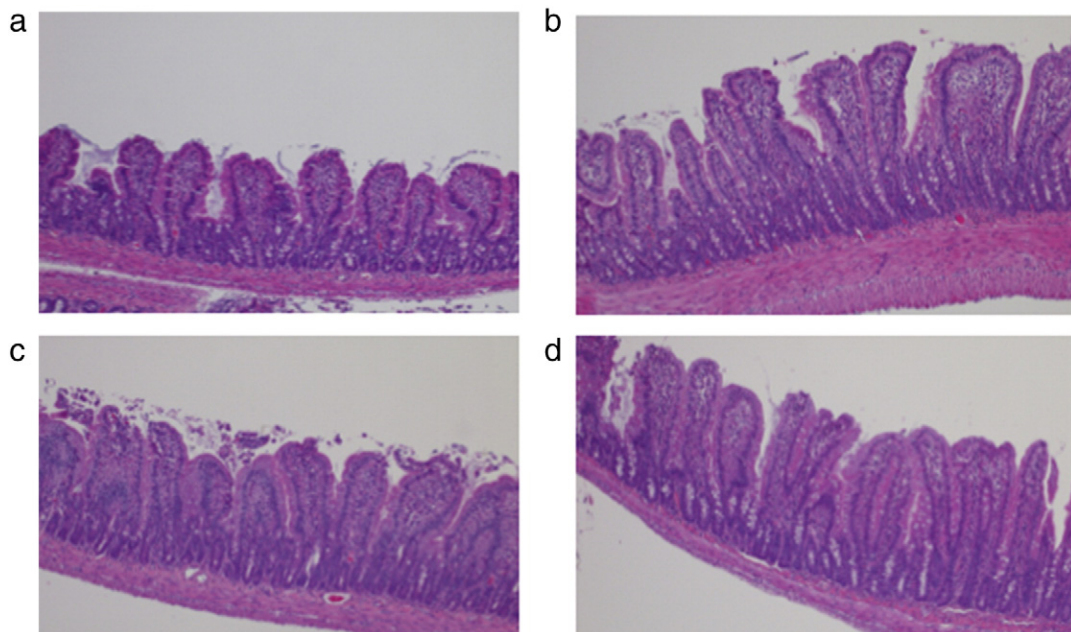

Fig. 1. Histological morphology of the jejunum. Light micrographs of the jejunum stained by hematoxylin and eosin. (a) TPN- alone, (b) TPN plus low-dose ghrelin (TPNLG), (c) TPN plus high-dose ghrelin (TPNHG), and (d) oral feeding(OF).

Table 2
Microscopic intestinal morphology of the jejunum.

Jejunum	Villus height (μm)	Crypt depth (μm)	Thickness of muscle layer (μm)	Villus density (n/mm)	Absorptive mucosal surface (cm^2/1 cm^2)	CCPR*
TPN	247.4 ± 6.9	139.3 ± 5.2	43.5 ± 3.4	6.2 ± 0.1	7.6 ± 0.6	0.77 ± 0.01
TPN + ghrelin (Low)	286.2 ± 13.2[a]	166.2 ± 5.7[a,c]	56.3 ± 6.1[a,c,d]	7.3 ± 0.3[a]	10.5 ± 0.9	0.89 ± 0.007[a,c,d]
TPN + ghrelin (High)	279.2 ± 11.7[a]	141.9 ± 6.9	46.5 ± 3.6	6.9 ± 0.1	8.9 ± 0.7	0.77 ± 0.01
Oral Feeding	387.4 ± 19.8[a,b,c]	170.6 ± 10.0[a,c]	48.7 ± 7.1	7.4 ± 0.2[a]	20.3 ± 1.1[a,b,c]	0.83 ± 0.01[a,c]

Values means ± S.E., n = 10 per group.
* Ki67 positive cells/1 crypt cells.
[a] P < 0.05 vs. TPN.
[b] P < 0.05 vs. TPN + ghrelin (Low).
[c] P < 0.05 vs. TPN + ghrelin (High).
[d] P < 0.05 vs. OF.

the rats in the TPNLG group. Previous studies have showed that ghrelin induced epithelial cell proliferation under the conditions of 48-h fasting or elemental diet-induced mucosal damage [19]. Ghrelin was able to improve TPN associated mucosal atrophy by stimulating intestinal epithelial cell proliferation.

In this study, the administration of ghrelin did not increase the intestinal length. A previous study also showed that ghrelin did not alter the length of the ileum or increase the villus height of the ileum [20]. In terms of the absorptive mucosal surface area, which was calculated based on the microscopic morphological parameters, there were no significant differences among the TPN-treated groups in this study. However, as the villus height and crypt depth of the small intestine in the TPNLG group were significantly increased in comparison to those in the TPN group, long-term TPN, more than 6 days, might result in a significant increase in mucosal surface area.

The dose of exogenous ghrelin in our study was determined based on the experiments of previous studies. In Wistar rats, the effects of ghrelin on the gastrointestinal mucosa were strongest at a dose of 40 μg/kg/day (intraperitoneal) [21]. In piglets, the effects were strongest at a dose of 45 μg/kg/day (enteral) rather than 20 μg/kg/day [20]. We decided on doses of 10 and 50 μg/kg/day expecting a dose-dependent response, with reference to previous studies. In our study, the administration of low-dose ghrelin remarkably improved mucosal atrophy. On the other hand, the administration of high-dose ghrelin

reduced the improvement observed in the villus height and CCPR. In an in vitro study, ghrelin was found to stimulate intestinal cell proliferation in FHs74Int and Caco-2 cells in a dose-dependent fashion with a maximal effect at 10 nmol, while high-dose ghrelin (100 nmol) decreased the CCPR [15]. The authors were not able to elucidate the mechanism underlying the decreased cell proliferation that was observed when an excessive higher dose of ghrelin was administered. In the present study we were not able to show the dose-dependent effects of ghrelin because only two doses were used (10 and 50 μg/kg/day). We believe that the dose of 50 μg/kg/day induced a response beyond dose-dependence, indicating that this high was excessive; inter-species differences (e.g. piglets, Wistar rats, and SD rats) and differences in the manner of administration (intraperitoneal one-shot infusion, enteral administration and continuous intravenous infusion) may explain these results. We hypothesize that a specific dose of ghrelin is needed to achieve the optimal improvement of TPN associated intestinal mucosal atrophy. Further studies are required to elucidate the appropriate dose and route of administration.

Low-dose ghrelin promoted the thickening of the muscle layer in our study. Previous studies have shown that IGF-1 induces the augmentation of the intestinal muscle layer, but not of the villus height or crypt depth in parenterally-fed mice [22]. These results might confirm that ghrelin promotes the secretion of IGF-1 through GH stimulation. Thus, the improvement of TPN associated mucosal atrophy might be induced
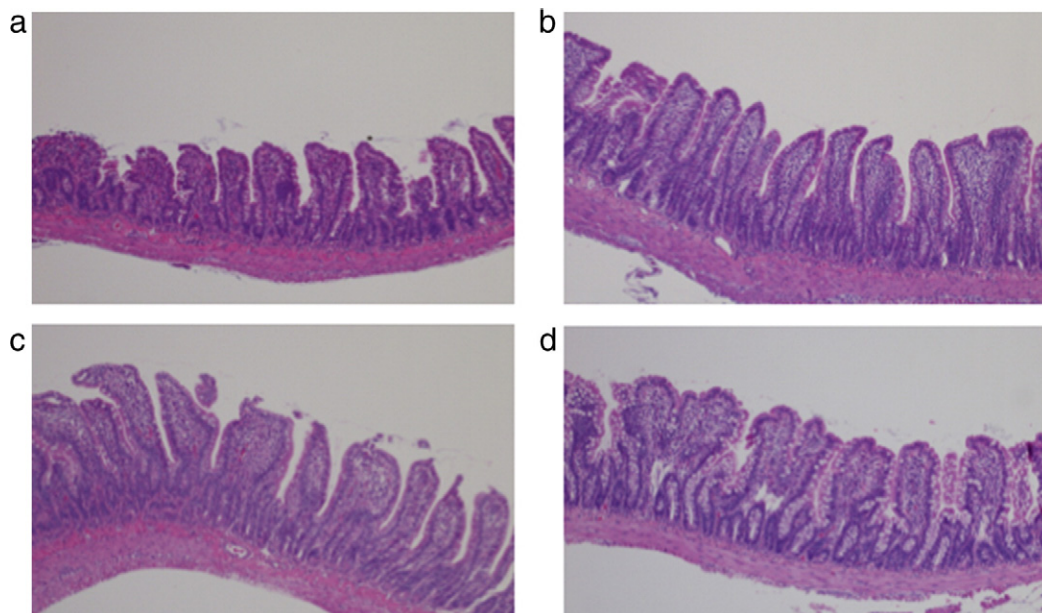

Fig. 2. Histological morphology of the ileum. Light micrographs of the ileum stained by hematoxylin and eosin. (a) TPN- alone, (b) TPN plus low-dose ghrelin (TPNLG), (c) TPN plus high-dose ghrelin (TPNHG), and (d) oral feeding(OF).

Table 3
Microscopic intestinal morphology of the ileum.

Ileum	Villus height (μm)	Crypt depth (μm)	Thickness of muscle layer (μm)	Villus density (n/mm)	Absorptive mucosal surface (cm²/1 cm²)	CCPR[*]
TPN	220.9 ± 6.6	132.0 ± 6.3	50.8 ± 4.9	6.4 ± 0.2	6.1 ± 0.5	0.82 ± 0.008
TPN + ghrelin (Low)	231.8 ± 6.3	152.8 ± 5.8[a,c]	66.2 ± 6.6[a,c,d]	6.6 ± 0.2	7.8 ± 0.2	0.89 ± 0.004[a,c,d]
TPN + ghrelin (High)	226.6 ± 7.6	129.4 ± 6.9	46.9 ± 3.9	6.6 ± 0.1	7.0 ± 0.4	0.85 ± 0.006[a]
Oral Feeding	234.9 ± 9.6[a]	145.1 ± 4.0[a,c]	50.4 ± 2.3	6.6 ± 0.1	10.6 ± 0.6[a,b,c]	0.83 ± 0.008

Values: means ± S.E., n = 10 per group.
  [*] Ki67 positive cells/1 crypt cells.
  [a] P < 0.05 vs. TPN.
  [b] P < 0.05 vs. TPN + ghrelin (Low).
  [c] P < 0.05 vs. TPN + ghrelin (High).
  [d] P < 0.05 vs. OF.

by ghrelin's direct effects on the villi and crypts, and indirect effects on the muscle layer. A further study is necessary to elucidate the detail mechanisms underlying the effects of ghrelin.

As a next step, the effects of the administration of ghrelin on nutritional absorption after TPN management must be elucidated. These further studies may reveal the clinical applications of ghrelin, which is likely to show promise in the treatment of malnutrition, particularly in pediatric patients with conditions such as short bowel syndrome and motility disorders.

In conclusion, the administration of ghrelin improved TPN-associated intestinal mucosal atrophy owing to an increased villus height and crypt depth. We clarified one of the mechanisms of these results as the increased CCPR of the intestine. However, further studies are needed to determine the appropriate dose of ghrelin and the manner in which it should be administered in order for it to be used in the clinical setting.

Conflict of interest

The authors declare no conflicts of interest in association with this study.

Acknowledgments

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