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The protective and anti-inflammatory effects of glucagon-like peptide-2 in an experimental rat model of necrotizing enterocolitis

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ABSTRACT

Necrotizing enterocolitis (NEC) is a devastating gastrointestinal disease, that affects premature infants. Glucagon-like peptide-2 (GLP-2) is an intestinotrophic hormone and reduces the inflammation. We suspected that GLP-2 would have protective and anti-inflammatory effects in an experimental rat model of NEC. NEC was induced in newborn rats by enteral feeding with hyperosmolar formula, asphyxial stress and enteral administration of lipopolysaccharide (LPS). Rats were randomly divided into the following four groups: dam-fed, NEC, NEC + GLP-2(L) given 80 μg/kg/day of GLP-2, and NEC + GLP-2(H) given 800 μg/kg/day of GLP-2. GLP-2 was administered subcutaneously every 6 h before stress. All animals surviving beyond 96 h or any that developed signs of distress were euthanized. The clinical sickness score in the NEC + GLP-2(H) group was significantly lower than that in the NEC group. The NEC score and the survival rate in the NEC + GLP-2(H) group was significantly improved compared with those in the NEC and the NEC + GLP-2(L) groups. Villous height and crypt depth in both the GLP-2 treatment groups were significantly increased compared with those in the NEC group. There were no significant differences in the crypt cell proliferation indices among the groups. Ileal interstitial TNF-α and IL-6 level in the NEC + GLP-2(H) group was decreased to the same levels in the dam-fed group. High dose GLP-2 administration improved the incidence and survival rate for NEC. It also decreased mucosal inflammatory cytokine production. These results support a potential therapeutic role for GLP-2 in the treatment of NEC.

1. Introduction

Necrotizing enterocolitis (NEC) is the most common surgical emergency and fatal gastrointestinal disorder of premature infants [6,19]. Despite aggressive management leading to the salvage of premature infants, the incidence of NEC continues to increase, presenting at a rate of 0.5–5 cases per 1000 live births each year [22,39]. Mortality rates from NEC range from 15% to 30% and are inversely related to gestational age and birth weight [18,23,35]. We need a novel therapy to improve the mortality in NEC patients.

Epidemiological studies have identified multiple factors that increase the risk of NEC, with prematurity, enteral formula feeding except breast milk, intestinal hypoxia/ischemia, and bacterial colonization thought to play important roles in its pathogenesis [7,8]. Thus, NEC has a multifactorial etiology, with a common final pathway of intestinal inflammation and necrosis [30]. The histology of NEC is characterized by widespread mucosal loss in the gastrointestinal tract, especially the ileum.

Intestinal mucosal surface repair against the injury occurs in two phases [9,12], the first of which involves adjacent epithelial cells migrating into the injury site and the second of which involves cell proliferation. New enterocytes arise from stem cells in the crypts in the cell proliferation. Wang J. presented that putrescine and proline improved epithelial restitution in piglets [41]. However, Richter et al. showed that lipopolysaccharide binding protein, the novel candidate agent for the rat model of NEC, improve the intestinal epithelial restitution, but did not decrease the degree of intestinal damage [33]. Therefore, cell proliferation would be a requisite for epithelial repair and recovery of intestinal function in NEC. We focused on the mechanism of the cell proliferation after mucosal injury. Additionally, Impaired gut barrier integrity allows bacterial translocation and systemic circulation, which leads to NEC via perinatal hypoxia and mucosal damage. Formula feeding and bacterial proliferation allow the attachment of bacteria to the damaged

Abbreviations: GLP-2, glucagon-like peptide-2; NEC, necrotizing enterocolitis; TNF-α, tumor necrosis factor; IL-6, interleukine-6.

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and immature mucosal barrier, which in turn triggers the release of some cytokines, especially tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). Consequently, increased epithelial permeability enables the translocation of bacteria and bacterial products from the intestinal lumen [14,27,32].

We speculate that the novel candidate agents of treatments for NEC should have the anti-inflammatory and epithelial proliferation effects. Glucagon-like peptide-1 stimulates the insulin secretion and decreased the blood glucose level, and reduce the pro-inflammatory cytokines, although, in the only pancreatic islets [17]. Ghrelin is secreted by the X/A-like cells of the stomach and the proximal small intestine. It exerts positive effects on food intake, growth hormone secretory action, and intestinal cell proliferation and inhibits pro-inflammatory cytokines [20]. However, the effectiveness of ghrelin for the improvements of intestinal mucosal damage is not apparent. In contrast, Glucagon-like peptide-2 (GLP-2) is derived from the L cells of the small and large intestine in response to both proximal enteric neuronal signaling and the presence of luminal nutrients [34,36]. Previous studies have demonstrated that GLP-2 administration results in intestinal hypertrophy by increasing the crypt cell proliferation rate, which results in increased villous height, crypt depth, and an overall increase in small intestinal length and weight [10,25,37]. GLP-2 is primarily viewed as a trophic regulator of mucosal function in the small intestine [24]. The peptide has been shown to reduce inflammatory mucosal lesions in a rat model of inflammatory bowel disease by decreasing the expression of the inflammatory mediator TNF-α [1]. Furthermore, in our previous experiment, we identified that GLP-2 administration increased the crypt cell proliferation rate and decreased the level of inflammatory cytokines in a rat model of inflammatory bowel disease [38].

We hypothesized that GLP-2 would work as an intestinotrophic agent, maintaining mucosal health and reducing inflammatory cytokine production in the small intestinal mucosa in an experimental rat model of NEC.

2. Materials and methods

2.1. Animal model and study design

This study followed guidelines approved by the Institutional Animal Care and Use Committee of Kagoshima University (MD08069). Time-mated pregnant Sprague-Dawley rats were delivered by cesarean section under anesthesia on day 21 of gestation. Neonatal rats were placed into a neonatal incubator for control of temperature (30 °C), humidity (50 %) and 12 h light-dark cycles. The dose of GLP-2 was decided referring the previous study for NEC experiment [5]. Their study showed that 100 μg/kg/day of GLP-2 was not enough to prevent the onset of NEC. Therefore, we determined the 80 μg/kg/day as low dose and 800 μg/kg/day of GLP-2 as high dose. Rats were assigned to four groups: (1) Dam-fed (n = 18) rats left with their mother, breast fed ad libitum, and not exposed to stress; (2) NEC rats (n = 22) given 5 mL/kg of normal saline (NS) without GLP-2 subcutaneously every 6 h before stress; (3) NEC + GLP-2(L) rats (n = 16) given 20 μg of GLP-2 in 5 mL/kg NS subcutaneously every 6 h before stress; and (4) NEC + GLP-2(H) rats (n = 19) given 200 μg of GLP-2 in 5 mL/kg NS subcutaneously every 6 h before stress.The method initially described by Barlow et al. [4] and modified by Zani et al [42] was used to induce the rat model of NEC. Briefly, four hours after caesarian section, newborn rats from groups (excluding those from the dam-fed group) were gavaged with hyperosmolar rat milk every 6 h and exposed to asphyxia stress for 5-min induced by 100% nitrogen. In addition, on the first and the second days of life, the NEC rats were force-fed with 4 mg/kg/d of *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) (Sigma–Aldrich Company Ltd, UK). A special rodent formula was prepared using 15 g of Balance milk (ICREO CO. LTD, Tokyo, Japan) in a 75-mL Esbilac canine supplement (Pet-Ag Inc, Hampshire, Ill), based on the method described by Feng et al. [13], and rats ware force-fed using a 1.7 French catheter (Excelsior microcatheter, Boston Scientific Target, Bayside Parkway, USA). Human synthetic GLP-2 (1-33, Bachem Ag, Bubendorf, Switzerland) was administered subcutaneously every 6 h before milk in each of the GLP-2 treatment groups.

2.2. In vivo assessment

All rats were inspected and evaluated at each feeding point for the presence or absence of huddling. We evaluated behavior using a clinical sickness score for neonatal rats (Table 1) [42]. This evaluated rats by appearance, response to touch, natural activity and colour. Every endpoint has score from zero to three per measure. Each score was added to calculate to the total score, with lower scores being favorable. Animals that developed distress (lethargy, abdominal distention, bloody diarrhea) or imminent death before 96 h were euthanized by cervical dislocation. After 96 h, all surviving animals were euthanized in same manner. The number of deaths and approximate time of death were recorded daily for all groups and calculated as the survival rate.

2.3. Intestinal morphology and histology

After rats had been euthanized, their intestines were removed, washed by phosphate buffered saline (PBS). A 3 cm section of the distal ileum was harvested and fixed in 10% formalin, embedded in paraffin, sectioned at 4-μm thickness, and stained with Hematoxylin and eosin for histological evaluation of NEC, using the modified histologic scoring system described by Nadler et al. [29] and Dvorak et al. [11]. Histological changes in the intestines were graded as follows: grade 0, no damage; grade 1, slight submucosal and/or lamina propria separation without villous core separation; grade 2, moderate submucosal and/or lamina propria separation with villous core separation; grade 3, severe submucosal and/or lamina propria separation and epithelial sloughing of the villi; and grade 4, loss of villi with transmural necrosis. To determine the incidence of NEC, tissue damage with histological injury scores of grade 2 or greater were considered positive for NEC. Microscopy measurements of villous height, and crypt depth were recorded from

Table 1
Clinical sickness score for neonatal rats.

Appearance	
0	The pup rat is tonic and well hydrated
1	The pup rat is slimmer but still tonic and hydrated
2	The pup rat is skinny, floppy, and dehydrated
3	The pup rat is gasping and in agony
Response to touch	
0	The pup rat is alert (without stimulation)
1	The pup rat responds to mild stimulation
2	The pup rat responds to vigorous stimulation
3	The pup rat is unresponsive notwithstanding a vigorous stimulation
Natural activity	
0	The pup rat moves normally in the cage
1	The pup rat, if put supine, is able to wriggle
2	The pup rat, if put supine, is not able to wriggle
3	The pup rat does not move its limbs and lays still
Colour	
0	The pup rat skin colour is pink
1	The pup rat skin colour is pale (just at the extremities)
2	The pup rat skin colour is pale (whole body)
3	The pup rat skin colour is grey

Excerpted from ref Zani.

well-oriented villi/crypt units. The residual ileums were stored at −80 °C for cytokine assays.

2.4. Crypt cell proliferation for Ki-67

Proliferating cells were detected with a monoclonal mouse anti-Rat Ki-67 antigen (clone MIB-5, code no. M7248; Dako, Glostrup, Denmark). Paraffin-embedded tissue sections were deparaffinized, and the antigen retrieved by autoclave treatment in target retrieval solution, (Dako, Glostrup, Denmark) for 15 min at 121 °C. Then the sections were incubated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, before being incubated with primary antibody for 1 h and further processed for immunohistochemistry using an LSAB® 2 kit (code no.M0609; Dako, Glostrup, Denmark). Peroxidase activity was developed in diaminobenzidine as a chromogen.

2.5. Cytokine enzyme-linked immunosorbent assay

The remaining ileal tissue was homogenized in phosphate buffer using a polytron type homogenizer.The homogenized tissue was centrifuged at 2000 × *g* for 20 min at 4 °C to obtain the supernatant. The total protein concentration of the tissue supernatant was measured using a Bio-Rad, Quick Start™ Bradford protein assay kit (Bio-Rad Laboratories) for calibration.Protein concentrations of TNF-α, and IL-6 in the tissue supernatants were determined using enzyme-linked immunosorbent assay (ELISA) kits (TNF-α: Quantikine Rat TNF-alpha immunoassay, R&D Systems Inc. Minneapolis, USA; IL-6: Rat IL-6 ELISA kit, Thermo Fisher scientific Inc. Rockford, USA) according to the manufacturer's instructions.

2.6. Statistical analysis

All data are expressed as mean ± standard deviation.Statistical differences between mean values were analyzed by analysis of variance (ANOVA) with post-hoc analysis by Holm's method or c^2 analysis. Survival time was analyzed by log-rank test. Statistical significance was defined as a p-value less than 0.05.

3. Results

3.1. In vivo assessment of animal and survival rate

All rats in the dam-fed group were handled normally during the experiment period, and had a clinical sickness score of zero, with significant differences in sickness scores among the groups ($p < 0.01$). Among the three groups with induced NEC, the clinical sickness score in the NEC + GLP-2(H) group was significantly lower than that in the NEC group ($p < 0.05$) (Fig. 1).

Fig. 2 shows the results of the Kaplan–Meier analysis. All rats in the dam-fed group survived during the experimental period. The survival rate in dam-fed group was significantly higher than those in the NEC and both GLP-2 treated groups. The survival rate in the NEC + GLP-2 (H) group was significantly higher than those in the NEC and NEC + GLP-2 (L) groups

3.2. NEC score and the incidence of NEC (Fig. 3)

We used standard NEC scoring system and evaluated histological intestinal damage among the groups. The NEC score in the dam-fed group was 0–1, with no incidence of NEC during the experiment period. The NEC score in the NEC + GLP-2 (H) group was significantly lower than in either the NEC or NEC + GLP-2 (L) groups [NEC + GLP-2 (H) 1.3 ± 0.5 vs NEC 2.3 ± 1.1, NEC + GLP-2 (L) 2.2 ± 1.1 ($p < 0.01$), respectively]. The incidence of NEC, the NEC score is grade 2 or greater, in NEC + GLP-2(H) group was significantly lower than

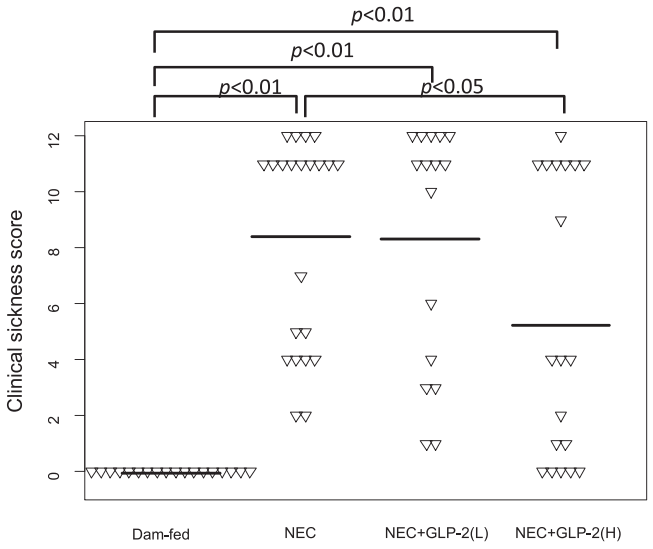

Fig. 1. Rat behaviour assessed using the clinical sickness score.

in either the NEC or NEC + GLP-2 (L) [NEC + GLP-2 (H) 26.3% vs NEC 72.7%, NEC + GLP-2 (L) 68.8% ($p < 0.05$), respectively]. The administration of high dose GLP-2 improved the NEC score and reduced the incidence of NEC .

3.3. Microscopic evaluation

The villous height in the dam-fed, and GLP-2 treated rats was significantly increased compared with that in control vehicle treated NEC [dam-fed 201.3 ± 61.7 μm, NEC + GLP-2 (L) 195.7 ± 75.8 μm, NEC + GLP-2 (H) 209.8 ± 75.6 μm vs NEC 67.1 ± 69.3 μm, ($p < 0.01$)]. The crypt depths in the dam-fed and GLP-2 treated rats were also significantly increased compared with untreated NEC [dam-fed 13.3 ± 5.1 μm, NEC + GLP-2 (L) 14.1 ± 5.6 μm, NEC + GLP-2 (H) 12.9 ± 5.1 μm vs NEC 8.0 ± 3.7 μm ($p < 0.01$)]. There was no significant difference in the crypt cell proliferation index among all groups, even in the dam-fed and NEC group (Table 2).

3.4. Ileal interstitial cytokines

The ileal interstitial TNF-α level in the NEC + GLP-2(H) group was significantly lower than that in the NEC group [NEC + GLP-2 (H) 1.36 ± 0.65 pg/mg protein vs NEC 5.30 ± 5.48 pg/mg protein, ($p < 0.01$)] (Fig. 4A). Although the ileal interstitial TNF-α level in the NEC + GLP-2(L) group was higher than that in NEC + GLP-2 (H) group, the result was not statistically significant [NEC + GLP-2 (L) (3.85 ± 2.51 pg/mg protein)]. The ileal interstitial IL-6 level in the NEC + GLP-2(H) group was significantly lower than that in the NEC + GLP-2(L) group [NEC + GLP-2(H) 70.92 ± 30.13 pg/mg protein vs NEC + GLP-2(L) 290.3 ± 347.9 pg/mg protein, ($p < 0.01$)] (Fig. 4B), but there was no significant difference between the NEC + GLP-2(H) and dam-fed groups [dam-fed 68.94 ± 23.28 pg/mg protein]. It is striking that the ileal interstitial TNF-α and IL-6 levels in the NEC + GLP-2(H) group were decreased to the same level as were present in the dam-fed group. The administration of high-dose GLP-2 therefore inhibited the production of induced inflammatory cytokines in this model.

4. Discussion

The response to intestinal mucosal injury occurs in two phases; mucosal adjacent epithelial cells migrates into the damaged area in the first phase, and cell proliferation occurs from stem cells in

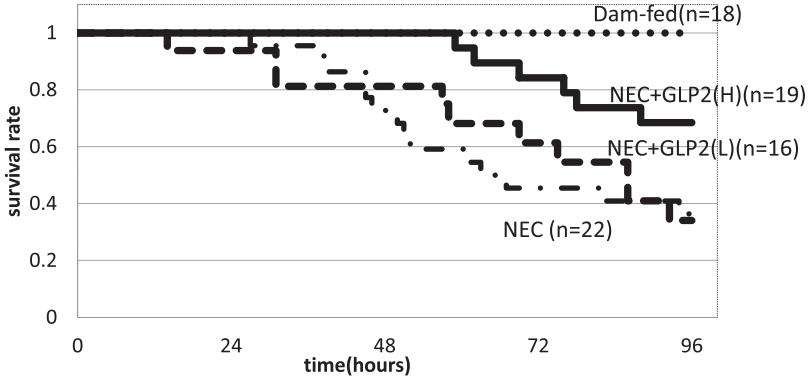

Fig. 2. Kaplan–Meier survival curve. The survival rate in the Dam-fed group was significantly well than those in the NEC and both NEC + GLP-2 treatment groups ($p < 0.01$ vs NEC, NEC + GLP-2(L), $p < 0.05$ vs NEC+GLP-2(H), respectively). The survival rate in the NEC + GLP-2 (H) group was significantly high than those in the NEC and NEC + GLP-2 (L) groups, respectively ($p < 0.05$).

the crypts in the second phase. According to the previous study, restitution effect, enterocyte migrates into the damaged space, was not enough to improve the incidence of NEC in the rat model [33]. We focused on the cell proliferation in the mucosal recovery from injury. The aim of this study was to clarify whether GLP-2 administration to preterm rats would maintain intestinal mucosal health and reduce the inflammatory cytokines production. Consequently, high dose administration of GLP-2 could reduce the severity of NEC, improve the survival rate, and decrease the production of mucosal inflammatory cytokines.

In the microscopic analysis, the villous heights and crypt depths in both GLP-2 treatment groups were significantly increased compared with the NEC group. It was striking that in both GLP-2 treatment groups, both histological endpoints were at the same levels as found in the dam-fed group. Therefore, there was no significant difference in either factor between the GLP-2 treatment groups. These findings probably resulted from the intestinotrophic effects of GLP-2, as a previous study indicated [24], with even low dose GLP-2 being sufficient to increase the villous height and crypt depth in this model.

As above mentioned, villous heights and crypt depths in both GLP-2 treatment groups were comparable to those in the dam-fed group. However, the clinical sickness and NEC scores were only significantly improved in the NEC + GLP-2(H) group when compared with the NEC + GLP-2 (L) and NEC groups. The NEC score was evaluated microscopically by the separation of the mucosa from the muscle layer, which indicated the severity of the histological damage. This was consequently expected to be associated with the clinical sickness scores and the survival rates. Survival rates in the NEC + GLP-2(H) group were significantly better compared with those in the NEC + GLP-2 (L) and NEC groups. In the clinical situation, this scoring system would be an effective, objective means of the prognosis of rats [5].

There was no significant difference in the crypt cell proliferation index among the four groups, even between the dam-fed and NEC groups. One study examining intestinal specimens from NEC

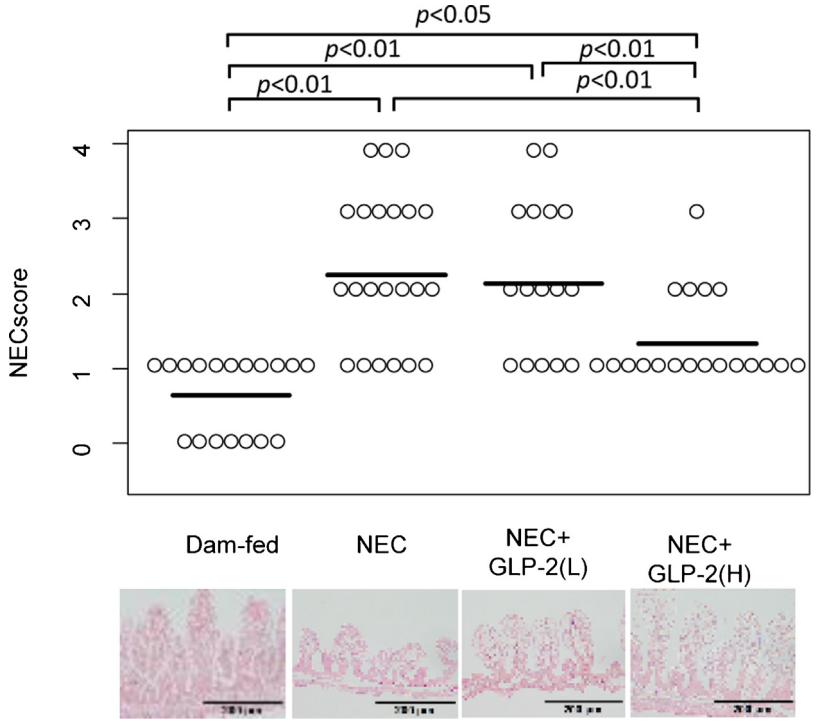

Fig. 3. NEC score in the NEC + GLP-2 (H) group was significantly lower than those in the NEC and NEC+GLP-2 (L) group [NEC + GLP-2(H) 1.3 ± 0.5 vs NEC 2.3 ± 1.1, NEC + GLP-2(L) 2.2 ± 1.1 ($p < 0.01$), respectively].

Table 2
Microscopic morphology and crypt cell proliferation.

	n	Villus height(μm)	Crypt depth(μm)	Crypt cell proliferation index
Dam-fed	18	201.3 ± 61.7*	13.3 ± 5.1*	0.47 ± 0.15
NEC	22	167.1 ± 69.3	8.0 ± 3.7	0.48 ± 0.18
NEC + GLP-2(L)	19	195.7 ± 75.8*	14.1 ± 5.6*	0.45 ± 0.16
NEC + GLP-2(H)	16	209.8 ± 75.6*	12.9 ± 5.1*	0.50 ± 0.16

* Dam-fed, NEC + GLP-2(L), NEC + GLP-2(H) vs NEC, $p < 0.01$.

patients by Ki-67 staining revealed that crypt cell proliferation was increased during the acute phase of NEC and persisted during the recovery period of NEC [40]. They speculated that the cells produced in NEC are immature, short lived, and unable to provide an adequate barrier to prevent NEC development. We speculate that the crypt cell proliferation in both GLP-2 treatment groups had been maintained the similar level to that of NEC group, which would result from GLP-2's original intestinotrophic effects of augmentation of crypt cell proliferation rather than maintaining the inflammation of NEC, according to the increase in the villous height and crypt depth in both GLP-2 treatment groups. Feng et al. showed that the apoptosis was increased in the experimental rat model of NEC [13]. And other recent study presented that GLP-2 improved the apoptosis [2]. In addition, Baldassano's study showed that villous height would be closely associated with crypt cell proliferation and apoptosis [3]. According to these studies, we speculate that apoptosis in NEC + GLP-2 treated groups would be decreased than that in control vehicle treated NEC group, and consequently villous height in the NEC + GLP-2 treated groups was significantly increased than that in control vehicle treated NEC group.

In the current study, ileal interstitial TNF-α and IL-6 were increased in the NEC group compared with the dam-fed group, consistent with the results of a previous paper.In that report, the gene expression of some inflammatory cytokines in the terminal ileum, especially, TNF-α and IL-6 were increased in a rodent NEC model [32]. We speculate that inflammatory cytokine reductions improve the morbidity and mortality of NEC. Indeed, GLP-2 was able to reduce the severity of inflammatory mucosal lesions in a rat model of inflammatory bowel disease by decreasing the expression of the inflammatory mediator TNF-α, which supports the use of GLP-2 in the treatment of this disease. The ileal interstitial TNF-α level in the NEC + GLP-2(H) group was significantly decreased compared with the NEC group, and although it was higher in the NEC + GLP-2(L) group than that in the NEC + GLP-2(H) group, the difference was not significant. There was also no significant difference in IL-6 levels between NEC + GLP-2(H) and NEC groups, however, the ileal intestinal IL-6 level in the NEC + GLP-2(H) group was significantly deceased compared with the NEC + GLP-2(L) group. According to our results, the TNF-α and IL-6 levels in the NEC + GLP-2(H) group were decreased to the same levels as observed in dam-fed group. Consistent with this, Halpern presented that anti-TNF-α therapy reduced the incidence and severity of experimental NEC [15]. TNF-α has been shown to alter intestinal permeability by acting on adhesion molecules, and inducing bacterial translocation in NEC [28]. Moran presented that GLP-2 could improve the impairment of the adhesion molecules caused by TNF-α [26]. Thus, high dose GLP-2 administration is biologically plausible as a potential treatment against NEC.

A previous study reported that, rather than preventing it, administration of 100 μg/kg/d of GLP-2 only delayed the occurrence of NEC [5]. In our study, we also found that low dose GLP-2 (80 μg/kg/day) did not improve either the NEC score or the survival rate, but that high dose GLP-2 (800 μg/kg/day) improved both outcomes. Accordingly, GLP-2 appears to have a dose dependent effect on the incidence and mortality of NEC. However, the effective GLP-2 dose in this study, 800 μg/kg/day, was higher than that in some previous studies [21,24,25]. GLP-2 has a very short half life, several minutes in rats, and 7 min in humans [16],

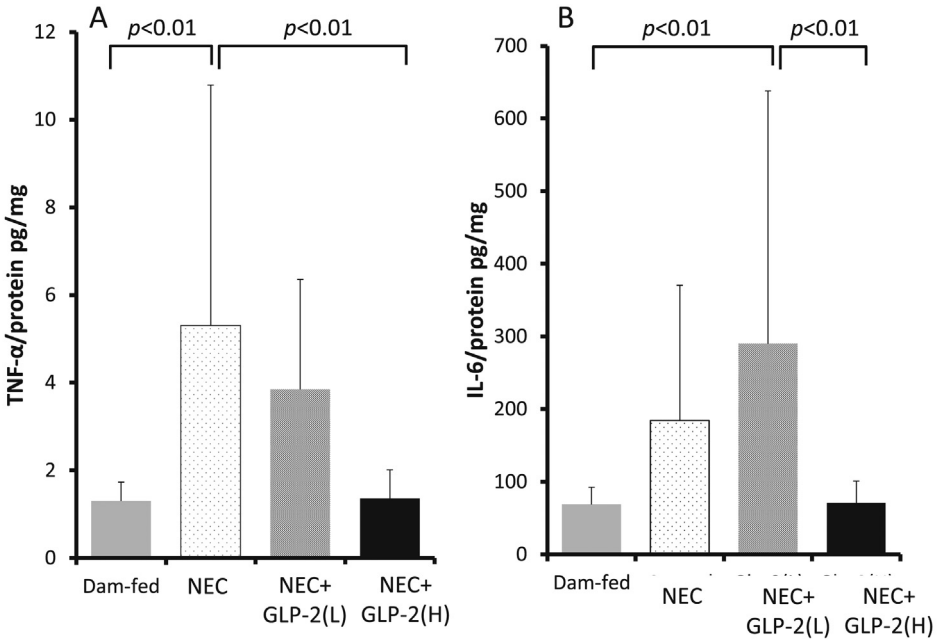

Fig. 4. The ileal interstitial TNF-α level in NEC + GLP-2 (H) group was significantly low in comparison with that in the NEC group. The ileal interstitial IL-6 level in NEC + GLP-2 (H) group was significantly low in comparison with that in NEC + GLP-2(L) group.

making it difficult to maintain effective concentrations to produce the desired intestinotrophic effect. In this study, the newborn rats received subcutaneous GLP-2, which according to a previous study, could be expected to result in normal serum GLP-2 concentrations after a few hours [16]. Thus, effective serum concentration of GLP-2 injected subcutaneously may not have been maintained in this experiment. Our previous experiment clarified that continuous intravenous administration of GLP-2 was significantly effective compared with the intermittent intravenous administration [21]. In a clinical setting with a continuous intravenous infusion, lower dose of GLP-2 may therefore be sufficient to achieve effective concentrations.

The GLP-2 receptor is expressed in enteric neurons and not in the epithelium of rat mucosa [31]. Thus, it is likely that GLP-2 ameliorates NEC by indirect mechanism. And previous study presented that GLP-2 induced the anti-inflammatory actions activating the enteric neural pathways in the rat model of drug induced inflammatory disease. We need further study in order to clarify the mechanism for neural signaling in the rat model of NEC.

5. Conclusion

Our results demonstrate that high dose GLP-2 could reduce the severity of NEC and improve the survival rate, probably by reducing the production of mucosal inflammatory cytokines. Further studies are needed to clarify the effective dose and method of injection for GLP-2 in the treatment and prevention of NEC.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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