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The novel preventive effect of Daikenchuto (TJ-100), a Japanese herbal drug, against neonatal necrotizing enterocolitis in rats

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Abstract

Purpose: Necrotizing enterocolitis (NEC) is a devastating gastrointestinal disease of premature infants. Daikenchuto, a Japanese herbal drug has several effects on the digestive system, so we investigated its preventive effects in a rat model of NEC.

Methods: NEC was induced in newborn rats via asphyxia (100% N_2 for 90 s; every 4 h) + LPS (4 mg/kg/day [administered orally on Days 0 and 1]). The effects of Daikenchuto were evaluated in 4 groups (control: 0 g/kg/day, I: 0.3 g/kg/day, II: 0.6 g/kg/day, III: 1.0 g/kg/day). Daikenchuto was administered into the stomach through a microcatheter. The incidence and severity of NEC were pathologically assessed using the NEC grade in accordance with Dovorak's previous report. Cell positivity for inflammatory cytokine (IL-6) was also evaluated.

Results: Daikenchuto reduced the incidence of NEC in control, Groups I, II, and III to 68.7%, 30.0%, 30.7%, and 13.3%, respectively. High dose Daikenchuto significantly improved the incidence of NEC, and the rate of IL-6 positive cells in group III was significantly lower than in the control group ($p=0.04$).

Conclusion: We evaluated the effect of Daikenchuto against NEC and found that it reduced the incidence rate of NEC due to a decrease in the IL-6 production.

(200 words/Limits 200)

Key words: necrotizing enterocolitis, rat model, Daikenchuto, NEC grade, IL-6

Introduction

Necrotizing enterocolitis (NEC) is the most severe surgical emergency and life-threating gastrointestinal disorder in premature infants. Despite advances in management techniques improving the survival of premature infants, the incidence of NEC is increasing [1-3]. While the outcomes in low-birth-weight infants have been improved by perinatal medical advances, the death rate after NEC onset remains high [4]. Indeed, one-third of infants who develop NEC in North America reportedly die of the disease [5]. NEC has a broad spectrum of severity, from mild to critically serious, and affects the whole intestine. In Japan, we often experience such severe cases.

Previous pathophysiological studies of NEC have reported that prematurity, enteral artificial formula feeding, intestinal hypoxia/ischemia, intestinal dysmotility, and bacterial overgrowth are associated with an increased risk of NEC [6, 7]. Experimental animal models of NEC have been established by the combination of hypoxic, cold stress, and bacterial infection in order to clarify the pathogenesis of NEC and develop more effective treatments.

Several studies have shown that the inflammatory cascade, especially inflammatory cytokines such as tumor necrotic factor-α（TNF-α）, interleukin (IL)-6, IL-12, IL-18, is strongly associated with the onset and progression of NEC, and controlling this inflammation may be key to treating the disease [8]. On the basis of these basic, clinical, and etiological studies, we developed an animal model of NEC to help identify novel effective agents.

The traditional Japanese herbal medicine Daikenchuto (TJ-100) is prepared as a dried extract powder of dried ginger root, ginseng, and zanthoxylum fruit in the ratio of 5:3:2, respectively. And it has been shown to have several pharmacological effects on the gastrointestinal tract. Daikenchuto has been used for the treatment of various visceral diseases, such as constipation, motility disorder, and adhesive bowel obstruction. Its well-known major effects are as follows: promoting lower gastrointestinal tract motility, increasing the blood flow of the small intestine, and inhibiting the production of inflammatory cytokines [9-11]. In addition, our previous study showed that inflammatory cytokines, especially, IL-6, were associated with the development of NEC [12]. We therefore focused on these effects of Daikenchuto as a therapy for NEC.

The aim of this study was to evaluate the effects of Daikenchuto against NEC using our rat model of NEC.

Methods

This study complied with the guidelines set by the Institutional Animal Care and Use Committee of Kagoshima University (MD09090, MD16119).

Animal NEC model

Time-mated pregnant Sprague-Dawley (SD) rats were delivered by Caesarean section under anesthesia on Day 21 of gestation. Newborn SD rats were taken from the dam in order to avoid breast feeding and placed into a neonatal

incubator with a controlled temperature (30 °C) and humidity (60%) and a 12-h light-dark cycles.

Esbilac®, an artificial milk product including 0.82 kcal/ml (Pet-Ag, Inc., Hampshire, IL, USA) was forced-fed through a 1.7-Fr microcatheter (Boston Scientific, Marlborough, MA, USA) every 4 h at 0.1 ml (100 Cal/kg/day). Our NEC model was developed in accordance with the methods of previous studies [13-15]. We modified these methods in order to obtain a model with a high incidence and severity of NEC. Briefly, stimulation of the newborn SD rats was started 1 h after birth via asphyxia ([100% N_2 90 s] every 4 h) + Lipopolysaccharide (LPS) (Sigma-Aldrich Co., Ltd., Dorset, UK) (4 mg/kg/day [administered orally on Days 0 and 1]).

Daikenchuto administration

Daikenchuto (15 g) (Tsumura & CO., Tokyo, Japan) has been used to treat intestinal dysmotility due to paralytic ileus in adult patients at an estimated daily dosage of 0.3 g/kg/day [9]. It was easily dissolved by distilled lukewarm water at a concentration of 0.015g/ml and administered through previous afore-mentioned microcatheter. The effects of the administration of Daikenchuto in our severe NEC model were evaluated in 4 dose groups (control group: 0 g/kg/day. Group I: 0.3 g/kg/day, Group II: 0.6 g/kg/day, Group III: 1.0 g/kg/day). The daily dosage of Daikenchuto was equally divided and administered into the stomach with a mixture of artificial milk every 12 h through a microcatheter during stimulation for the NEC model.

NEC evaluation (incidence and severity)

All rats were inspected at each feeding point. Animals that developed distress (lethargy, abdominal distention, bloody diarrhea) or imminent death before 96 h were euthanized by cervical dislocation and the whole intestines were removed. After 96 h, all surviving animals were euthanized in the same manner and the whole intestines were removed. Intestinal tissue from 1 cm proximal to the ileocecal valve was cut, fixed in 70% ethanol, embedded in paraffin, sectioned (4-6 μm/section), and counterstained with hematoxylin and eosin. The incidence of NEC was pathologically determined; the severity was assessed as grade according to the NEC scoring system reported by Dvorak et al. [14], as follows; the histological changes in the intestinal architecture of rats with NEC were assigned an NEC grade. Grade N (normal), no separation in the submucosa or lamina propria; grade L (low), slight submucosal and lamina propria separation; grade M (moderate), increased submucosal and lamina propria separation with edema of the submucosa; grade I (intermediate), severe separation of the submucosa and lamina propria; and grade S (severe), necrosis and loss of villi structure. To determine the incidence of NEC, tissue damage with histological injury of grade M, I and S were considered positive for NEC followed by Dvorak study.

Immunohistology of IL-6

The anti-inflammatory effect of Daikenchuto on the NEC model was evaluated based on the immunohistology of inflammatory cytokine. Using the

same tissue processed as described for staining with hematoxylin and eosin, serial sections were deparaffinized in three changes of xylene and rehydrated in a graded series of ethanol dilutions. Antigen retrieval was performed by boiling the tissue sections in 0.01 M citrate buffer at pH 6 for 20 minutes. After blocking the endogenous peroxidase activity and nonspecific antigen binding, the tissue sections were incubated with IL-6 antibody for 30 minutes in a moist chamber. The appropriate dilution of these antibodies was a 1:100 dilution of IL-6 (PROTEINTECH, Rosemont, IL, USA). After washing in Tris-buffered saline, the tissue sections were incubated with universal secondary antibody (ImmPRESS Reagent; VECTOR LABORATORIES, Burlingame, CA, USA). Immunohistology of IL-6 was evaluated as follows; brown-stained cytoplasm of the crypt cell indicated IL-6 positivity. IL-6 positive cells in the obviously formed crypts were counted by assessing at least 10 randomly selected fields. The rate of IL-6 positivity was calculated as follows; the number of IL-6 positive cells was divided by the number of the total crypt cells. Stained slides were evaluated by blinded two observers.

Statistical analyses

NEC grade was evaluated using Fisher's exact analysis as either positive NEC (grade M, I and S) and negative NEC (grade N and L) with post-hoc analysis by Holm's method. The IL-6 positive rate was evaluated using Student's t- test. Statistical significance was defined as a p-value less than 0.05.

Results

Evaluation of NEC grade

The incidence rate of NEC (grade M and I) in each group was as follows: control group (68.7%, 11/16), group I (30.0%, 3/10), group II (30.7%, 8/26), and group III (13.3%, 2/15) (Fig.1). There was a significant difference in incidence rate of NEC between control group and group III (p<0.019) (Fig.2). High-dose (1.0 g/kg/day) Daikenchuto in group III tended to improve the NEC grade more than lower doses (Fig.2). In terms of the clinical symptoms, almost rats classified as NEC grade M and I in all groups had bloody diarrhea.

Fig.1

Fig.2

Fig.2

Regarding the histological findings of NEC grade M and I, mucosal necrosis was not observed. Figure 3 shows the representative microscopic morphology of the intestinal samples. Figure 3a shows the severe separation of the submucosa and lamina propria that we evaluated as grade I for the NEC grade (control group). Figure 3b further shows the resolved separation of the submucosa and lamina propria and preventive findings following high-dose Daikenchuto administration (group III, NEC grade L).

Fig.3

Fig.3a

Fig.3b

Immunohistology of IL-6

Cytoplasm of crypt cell was stained brown, which was taken to indicate positive cells. The IL-6-positive cell rate in the crypt cells in group III was significantly lower than that in control (group III 0.61 ± 0.19, control 0.79 ± 0.02, p=0.04) (Fig. 4). High-dose administration of Daikenchuto suppressed IL-6, a typical inflammatory cytokine.

Fig.4

Discussion

In this study, we investigated the effect of Daikenchuto using NEC model in rats. The major new findings of this study were as follows: (1) administration of Daikenchuto tended to decrease the NEC grade on the basis of a histological evaluation, and high-dose (1.0 g/kg/day) administration of Daikenchuto (2) significantly improved the incidence rate of NEC and (3) significantly reduced the rate of IL-6-positive cells.

Pathophysiological studies of NEC have revealed associations with prematurity, enteral formula feeding, intestinal hypoxia/ischemia, intestinal dysmotility, and bacterial colonization. Most animal NEC models are developed by a combination of artificial formula milk with intermittent stresses, such as hypoxia, hypothermia, or the administration of LPS, which induces inflammation.

In our pilot study, we referred to the Dvorak study [14] and gave rats artificial formula milk while subjecting them to asphyxia and cold stress twice a day. As the incidence rate of NEC in our pilot study was only 16%, this model was not efficient for evaluating the efficacy of a new therapeutic agent against NEC. We speculate that the low incidence of NEC using Dvorak's methods in our pilot study resulted from the differences of temperature and humidity in the incubator and the outside environment of incubator during asphyxic stress. We therefore increased the frequency of asphyxia and administered 4 mg/kg/day of LPS as in Zani's study [16]. The incidence rate of NEC in controls without Daikenchuto was

68.7%, a result that was sufficient for evaluating the efficacy of a new agent as a treatment against NEC.

Daikenchuto is a traditional Japanese herbal medicine, prepared as a dried extract powder of dried ginger root, ginseng, and zanthoxylum fruit and is generally used to treat adhesive bowel obstruction and feelings of coldness in the abdomen [9]. Many studies have shown that Daikenchuto promotes lower gastrointestinal tract motility, increases the blood flow, and reduces the release of inflammatory cytokines [9-11]. Our previous study of NEC showed that TNF-α and IL-6 were associated with severity of NEC [12]. We therefore hypothesized that Daikenchuto would be similarly effective against NEC.

Sase et al. noted that hypomotility induced by hypoxia was associated with the occurrence of NEC [17]. As our NEC model was induced by hypoxic stress, hypomotility of the gastrointestinal tract was deemed likely to exist in this model. We believe that the activation of gastrointestinal tract motility by Daikenchuto improved the stagnation of the bowels contents, thereby suppressing bacterial overgrowth and helping prevent infection in the immature intestinal tract of NEC patients. As another mechanism of Daikenchuto against NEC, the effect of increasing the bowel blood flow may have played an important role in maintaining the intestinal tract wall.

Bacteria permeation of the epithelial cells due to stress and direct damage stimulates an inflammatory reaction and promotes the release of inflammatory cytokine in NEC. Rentea et al. reported that the gene expression of inflammatory cytokines, especially IL-6, in the ileum as determined by reverse

transcription polymerase chain reaction was significantly increased in rat model of NEC [18]. They speculated that this augmentation of IL-6 was strongly associated with the progression of NEC. Halpern et al. reported that inflammatory cytokine-positive cells were more frequent in crypts than in the lamina propria in severe cases of NEC [19]. As our previous study of NEC presented that IL-6 was associated with severity of NEC [12], we evaluated the rate of IL-6-positive cells in crypts. We found the rate of IL-6 positive cell was decreased in rats administered high-dose Daikenchuto compared to control animals. Daikenchuto has been shown to be effective in reducing elevated levels of inflammatory cytokine [11]. We therefore believe that the anti-inflammatory effect of Daikenchuto reduced the incidence rate and severity of NEC.

We calculated the appropriate dose for rats based on the amount (15 g) administered to a patient of 50 kg body weight for ileus and estimated the dose of Daikenchuto as 0.3 g/kg/day. As several studies have reported that Daikenchuto improves the small intestinal motility [20], intestinal blood flow [21], and inflammation [22] dose-dependently, we examined three concentrations of Daikenchuto in the present study: 0.3, 0.6, and 1.0 g/kg/day. The incidence of NEC and the NEC grade were both lower in the treated groups than in the control group, but only the 1.0 g/kg/day group showed a statistically significant reduction. As Daikenchuto was administered orally in this study, its absorption via the small intestinal mucosa may have been decreased due to the inflammatory condition of NEC. We therefore speculate that the most effective dose of Daikenchuto was 1.0 g/kg/day. However, this dose (1.0g/kg/day) was higher than that in usually used in

a clinical setting (0.3 g/kg/day). As Daikenchuto can induce diarrhea, liver dysfunction and interstitial pneumonitis as side effects, further studies are needed to establish the safety of this agent for neonates.

In our study of the administration of Daikenchuto in a rat model of NEC, the pharmacologic action of Daikenchuto helped to reduce the incidence of NEC as well as slow its development by decreasing the production of IL-6. Regarding the clinical applications, as Daikenchuto must be administered into the gastrointestinal tract, low-birth weight babies, who have a high risk of developing NEC are candidates for prophylactic administration.

“In conclusion, we described the effects of the administration of Daikenchuto in a rat model of NEC. The administration of high dose Daikenchuto improved the incidence of NEC, possibly via the suppression of IL-6. The detailed mechanisms underlying the effects of Daikenchuto are unknown and should be clarified to facilitate its clinical introduction.”

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Conflict of interest

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

References

1 Neu J, Walker WA: Necrotizing Enterocolitis. (2011) N Engl J Med. 364: 225-264

2 Samuels N, van de Graaf RA, de Jonge RCJ, et al. (2017) Risk factors for necrotizing enterocolitis in neonates: a systematic review of prognostic studies. BMC Pediatr. 17: 105

3 Yagi M, Kohno M, Asagiri K, et al. (2015) Twenty-year trends in neonatal surgery based on a nationwide Japanese surveillance program. Pediatr Surg Int. 31: 955-962.

4 Kodaka T, Iwanaka T (2009) Difference in these diseases to seem to know it, and not to know-Necrotizing enterocolitis and Localized intestinal perforation. Japanese J Pediatr Surg. 41: 367-370

5 Sodhi C, Richardson W, Gribar S, Hackam DJ (2008) The development of animal models for the study of necrotizing enterocolitis. Dis Model Mech 1(2-3): 94-98

6 Caplan MS, MacKendrick W (1993) Necrotizing enterocolitis a review of pathogenetic mechanisms and implications for prevention. Pediatr Pathol 13: 357–369

7 Crissinger KD (1995) Animal models of necrotizing enterocolitis. J Pediatr Gastroenterol Nutr 20: 17–22

8 Frost BL, Jilling T, Caplan MS (2008) The importance of pro-inflammatory signaling in neonatal necrotizing enterocolitis. Semin Perinatol 32(2):100-106. doi: 10.1053/j.semperi.2008.01.001.

9 Fukuda H, Chen C, Mantyh C, Ludwig K, Pappas TN, Takahashi T (2006) The herbal medicine, Dai-Kenchu-to, accelerates delayed gastrointestinal transit after the operation in rats. J Surg Res 131(2): 290-295

10 Kono T, Omiya Y, Hira Y, Kaneko A, Chiba S, Suzuki T, Noguchi M, Watanabe T (2011) Daikenchuto (TU-100) ameliorates colon microvascular dysfunction via endogenous adrenomedullin in Crohn's disease rat model. J Gastroenterol 46(10): 1187-1196

11 Yoshikawa K, Kurita N, Higashijima J, Miyatani T, Miyamoto H, Nishioka M, Shimada M (2008) Kampo Medicine "Dai-Kenchu-To" prevents bacterial translocation in rats. Dig Dis Sci 53(7): 1824-1831

12 Nakame K, Kaji T, Mukai M, et al. The protective and anti-inflammatory effects of glucagon-like peptide-2 in an experimental rat model of necrotizing enterocolitis. Peptides. 2016 Jan;75:1-7.

13 Barlow B, Santulli TV (1975) Importance of multiple episodes of hypoxia or cold stress on the development of enterocolitis in an animal model. Surgery 77(5): 687-690

14 Dvorak B, Halpern MD, Holubec H, Williams CS, McWilliam DL, Dominguez JA, Stepankova R, Payne CM, McCuskey RS (2002) Epidermal growth factor reduces the development of necrotizing enterocolitis in a neonatal rat model. Am J Physiol Gastrointest Liver Physiol 282(1): G156-164

15 Caplan MS, Hedlund E, Adler L, Hsueh W (1994) Role of asphyxia and feeding in a neonatal rat model of necrotizing enterocolitis. Pediatr Pathol 14(6): 1017-1028

16 Zani A, Eaton S, Leon FF, Malerba A, Hall NJ, De Coppi P, Smith VV, Pierro A (2008) Captopril reduces the severity of bowel damage in a neonatal rat model of necrotizing enterocolitis. J Pediatr Surg 43(2): 308-314

17 Sase M, Lee JJ, Ross MG, Buchmiller-Crair TL (2001) Effect of hypoxia on fetal rabbit gastrointestinal motility. J Surg Res 99(2): 347-351

18 Rentea RM, Welak SR, Fredrich K, Donohoe D, Pritchard KA, Oldham KT, Gourlay DM, Liedel JL (2013) Early enteral stressors in newborns increase inflammatory cytokine expression in a neonatal necrotizing enterocolitis rat model. Eur J Pediatr Surg 23(1): 39-47 doi: 10.1055/s-0032-1329704

19 Halpern MD, Holubec H, Dominguez JA, Williams CS, Meza YG, McWilliam DL, Payne CM, McCuskey RS, Besselsen DG, Dvorak B (2002) Up-regulation of IL-18 and IL-12 in the ileum of neonatal rats with necrotizing enterocolitis. Pediatr Res 51(6):733-739

20 Satoh K, Kase Y, Hayakawa T, Murata P, Ishige A, Sasaki H (2001) Dai-kenchu-to enhances accelerated small intestinal movement. Biol Pharm Bull 24(10): 1122-1126

21 Murata P, Kase Y, Ishige A, Sasaki H, Kurosawa S, Nakamura T (2002) The herbal medicine Dai-kenchu-to and one of its active components [6]-shogaol increase intestinal blood flow in rats. Life Sci 70(17): 2061-2070

22 Endo M, Hori M, Ozaki H, Oikawa T, Hanawa T (2014) Daikenchuto, a traditional Japanese herbal medicine, ameliorates postoperative ileus by anti-inflammatory action through nicotinic acetylcholine receptors. J Gastroenterol 49(6): 1026-1039 doi: 10.1007/s00535-013-0854-6

Legends for Figure/Table

Figure 1:

The incidence rate of NEC. The incidence of NEC was determined by a histological evaluation in which tissue damage with an injury score of grade M, I and S was deemed indicative of positive NEC.

Figure 2:

NEC grade. The NEC severity in the high-dose Daikenchuto (group III) was significantly lower than in the control group (p=0.019) using Fisher's exact test.

Figure 3:

Microscopic histology (H&E staining). a: Microscopic morphology in control animals. Severe separation of the submucosa and lamina propria was observed (NEC grade I). b: Microscopic morphology in group III (high-dose Daikenchuto). The separation of the submucosa and lamina propria was resolved following Daikenchuto administration (NEC grade L).

Figure 4:

Brown-stained cytoplasm of the crypt enterocyte indicates IL-6 positivity. IL-6-positive cell rate was calculated as follows; the number of IL-6 positive cells was divided by the number of total crypt cells. The IL-6-positive cell rate in the

high-dose Daikenchuto (group III) was significantly lower than that in the control group (p=0.04). The arrows indicate IL-6-positive cells.

Fig. 1 : Incidence rate of NEC

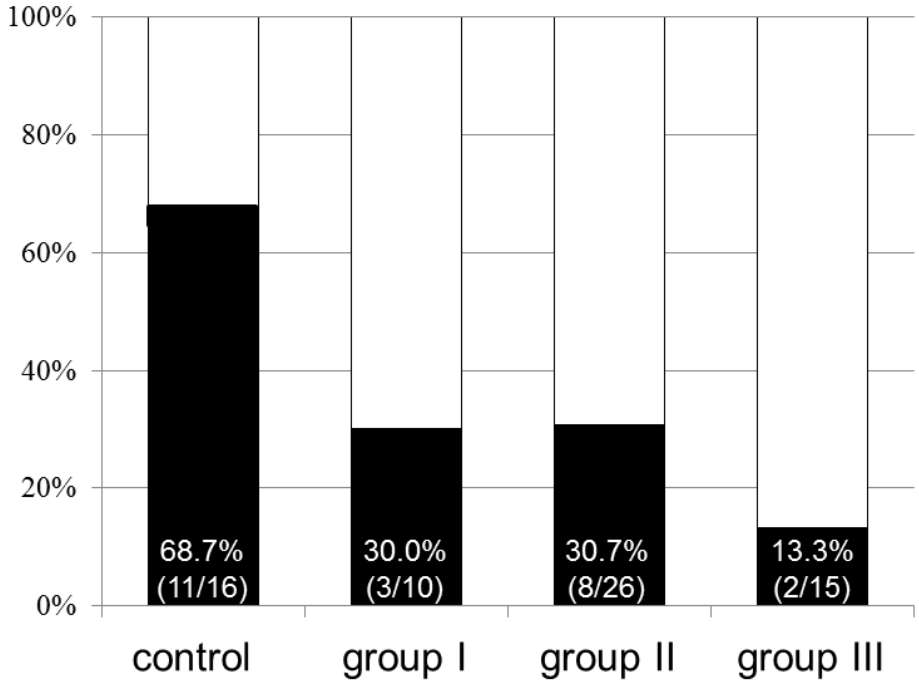
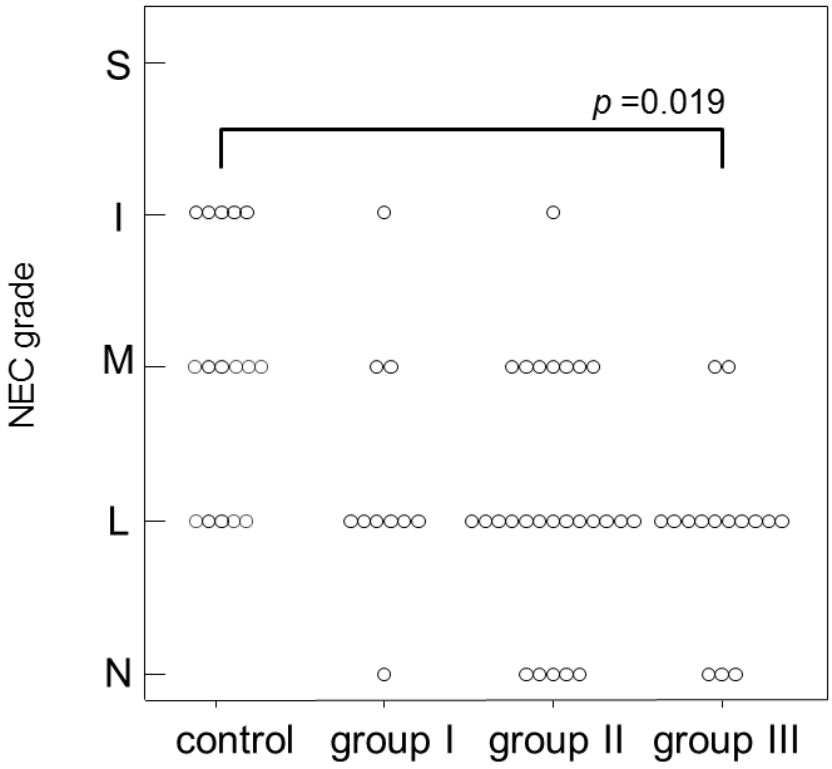
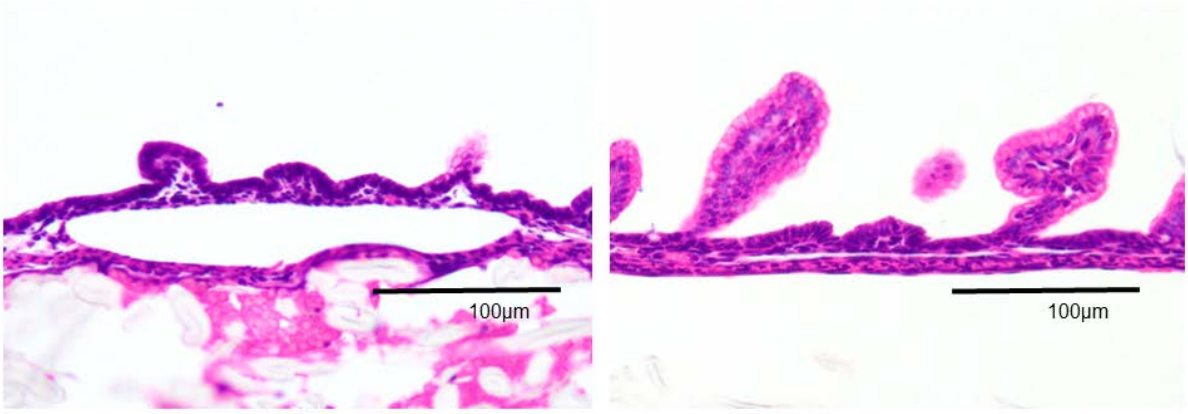


Fig. 2: NEC grade





3a:control (NEC grade I)　　3b: group III (NEC grade L)

Fig. 3 Microscopic histology (H&E)

Fig. 4: Immunohistology of IL-6