Experimental necrotizing enterocolitis using oral lipopolysaccharide and protective role of breastmilk

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ABSTRACT

Introduction. Necrotizing enterocolitis (NEC) is a life-threatening condition that afflicts neonates. Breastfeeding has demonstrated to play a protective role against it. By administering lipopolysaccharides (LPS) orally in newborn rats (NBR), we have developed an experimental model to induce NEC-like gut damage. Our aim was to assess the macroscopic and microscopic appearance of the gut, to evaluate the presence of NEC and study the role of breast milk (BM).

Material and methods. NBR were divided into 3 groups: Group A (control, n= 10) remained with the mother, group B (LPS, n= 25) was isolated after birth, gavage-fed with special rat formula and oral LPS, then submitted to stress (hypoxia after gavage) and group c (BM, n= 12) was breastfed once after birth, then isolated, and submitted to stress like group B. On day 4, NBR were sacrificed, and intestine was harvested and assessed.

Results. In the control group NEC was not present either macroscopically or histologically. Both groups submitted to stress (B and C) presented a global incidence of NEC of 73%. Most of group B developed histologic signs of NEC (85%) and group C showed a statistically lower incidence of NEC (50%, p= 0.04), playing the BM a protective role against NEC (OR= 0.19; 95% CI: 0.40- 0.904)

Conclusion. Our model showed a significant incidence of NEC in NBR (73%) with the same protective role of BM as in newborn humans, achieving a reliable and reproducible experimental NEC model. This will allow us to investigate new potential therapeutic targets for a devastating disease that currently lacks treatment.

KEY WORDS: Enterocolitis, necrotizing; Animal experimentation; Animals, newborn; Breastfeeding.

ENTEROCOLITIS NECROTIZANTE EXPERIMENTAL CON LIPOPOLISACÁRIDO ORAL Y FUNCIÓN PROTECTORA DE LA LECHE MATERNA

RESUMEN

Introducción. La enterocolitis necrotizante (ECN) es una enfermedad potencialmente mortal que afecta a los neonatos, y frente a la que la leche materna ha demostrado tener un papel protector. Administrando lipopolisacáridos (LPS) por vía oral en ratas recién nacidas (RRN), hemos desarrollado un modelo experimental para inducir un daño intestinal similar al que provoca la ECN con objeto de evaluar el aspecto macroscópico y microscópico del intestino, y de ese modo, analizar la presencia de ECN y estudiar el papel que desempeña la leche materna (LM).

Material y métodos. Las RRN se dividieron en tres grupos: el grupo A (control, n= 10) permaneció con su madre; el grupo B (LPS, n= 25) fue aislado tras el nacimiento, alimentado por sonda con una fórmula especial para ratas y LPS oral, y sometido a estrés (hipoxia tras sonda); y el grupo C (LM, n= 12) fue alimentado con leche materna tras el nacimiento y posteriormente aislado y sometido a estrés al igual que el grupo B. El día 4 se sacrificó a las RRN y se recuperaron sus intestinos para su posterior evaluación.

Resultados. En el grupo de control, no se observó ECN ni macroscópica ni histológicamente, mientras que los dos grupos sometidos a estrés (B y C) presentaron una incidencia global de la ECN del 73%. La mayoría de los sujetos del grupo B desarrollaron signos histológicos de ECN (85%), y los del grupo C registraron una incidencia de la ECN estadísticamente menor (50%, p= 0,04), lo que significa que la LM desempeña una función protectora frente a la ECN (OR= 0,19; IC 95%: 0,40-0,904).

Conclusión. Nuestro modelo reveló una incidencia significativa de la ECN en RRN (73%), desempeñando la LM la misma función protectora que en el caso de los humanos recién nacidos, lo que significa que este modelo experimental de ECN es fiable y reproducible. Gracias a dicho logro, podremos investigar nuevos y potenciales objetivos terapéuticos para una peligrosa enfermedad que, a día de hoy, carece de tratamiento.

PALABRAS CLAVE: Enterocolitis necrotizante; Experimentación animal; Animales; Recién nacido; Leche materna.

INTRODUCTION

Necrotizing enterocolitis (NEC) is a common life-threatening condition that usually afflicts neonates born prematurely and weighing less than 1,500 g[1,2]. It can have clinically significant long- and short-term consequences, with high mortality rates ranging from 15% to 30%[3].

With the current lines of treatment (bowel rest, iv broad-spectrum antibiotics, surgery), the evolution of the

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disease remains uncertain most of the time. Therefore, the prevention of such challenging disease is a key element. Breastfeeding has demonstrated to play a protective role against NEC, thanks to the human milk that provides oligosaccharides, mucosal immune components, lowers the gastric pH, increases motility, and decreases epithelial permeability. Hence, the use of human milk (even donor human milk) is nowadays supported and recommended for several international pediatrics guidelines as first option as enteral nutrition for newborn babies, especially for those born prematurely and with low birth weight[4-6].

To understand the pathophysiology and to look for new pathways than can lead us to find new therapeutic targets, the development of valid experimental models can be a productive tool[7,8].

Several models have been designed to replicate the NEC occurred in newborn babies, one of them using newborn rats (NBR)[9-13]. Within this model, the utilization of hypoxia and hyperosmolar formulas lead to an ischemic situation, where a NEC-like gut damage can be seen[14]. However, the reproducibility of this disease using these model still varies among different studies, with a prevalence ranging from 35% to 71%[9,10]. To overcome this problem and reduce the variability, oral lipopolysaccharide (LPS) has been used to increase the incidence of NEC and its severity[2,9]. It also mimics the bacterial load that occurs in human babies affected with NEC[15].

In our experience, the use of hypoxia and hyperosmolar formulas themselves have not been enough to develop NEC in NBR. Therefore, the study aimed to: evaluate the efficacy of the oral LPS, in addition to the hypoxia and hyperosmolar formula, to induce NEC-like gut damage by evaluating the macroscopic (dilation, pneumatosis, necrosis…) and microscopic appearance of the gut, and assess the role of the breast milk in NBR submitted to stress with a NEC-like gut damage.

MATERIAL AND METHODS

The experimental protocol followed the ethical guidelines for research in animal science (ARRIVE guidelines and European regulations: Directive 2010/63/EU). The Institutional Animal Care and Use Committee of Hospital Clínico San Carlos approved the project (#280790000088; PROEX 196.4/21; CEEA 21/0 04-III).

Wistar NBR were delivered on term, immediately after birth, the neonates were weighed and assigned into one of the 3 groups: group A (control, n= 10) where NBR left with their mother to be breast-fed ad libitum, and not submitted to stress. Group B (LPS, n= 25), NBR were isolated just after birth from their breeding mother and NEC-like gut damage was induced by gavage feeding with special rat formula, followed of 10 minutes of hypoxia (5% O_2, 95% N_2) every 6 hours. Oral LPS was added to the first intake on day 1 and day 2, mixed with the formula, and finally group C (BM, n= 12) where NBR were left with their breeding mother to be breast-fed once after birth, so the first oral intake would be breast milk. Afterwards, they were set apart from their mother and NEC was induced in a similar fashion to those in group B.

NBR were gavage fed using a 2-Fr silicon catheter four times a day with special rat milk substitute prepared with 10 ml of human milk formula 60:40 (Blemil plus 1®, 60:40 ratio of whey to casein) in 30 ml of Esbilac® dog supplement, as described by Barlow et al.[17]. On the first and the second day of life, 4 mg/kg/day of oral LPS (lipopolysaccharide from *Escherichia coli*, 0127: B8-Bioxtra –1 mg/ml–) were given mixed with the formula feeding, to both groups LPS and BM.

The groups submitted to stress (LPS and BM) were exposed for 10 minutes after each feeding to systemic hypoxic stress, from breathing a gas mixture of 5% O_2 and 95% N_2 as described by Nadler et al.[16]. The fraction of inspired oxygen was monitored using an oxygen monitor.

NBR isolated from their mother remained in an incubator provided with an adequate space for enrichment and with a controlled thermometer temperature of 30°C[18].

Every six hours, rats in group B and C were checked before gavage feeding using a scoring system based on physical examination (skin aspect, weight…) and behavior to verify their welfare (Table 1), following the severity assessment framework of the European union[19]. If the NBR get a score higher than 2, a closer and detailed examination is needed, and finally if the score is higher than 5 the animal will be sacrificed, to avoid any suffering or pain.

All NBR (Group A, B and C) were sacrificed on day 4, except for those that died or were sacrificed from NEC-related signs before endpoint. Immediately after sacrifice, the gastrointestinal tract was harvest and visually evaluated for typical signs of NEC. A macroscopic assessment of the small bowel and colon was performed using a scoring system based on the color of the gut, the degree of dilation and the consistency of the bowel, as suggested and validated by Zani et al.[9] (Table 2).

For the microscopic evaluation, the whole gut was fixed in formaldehyde for 3 days and the tissue was processed by dehydration until it was embedded in paraffin. Histological sections were made with a microtome (Leica Biosystems, Nussloch, Germany) at 4 microns and stained with haematoxylin – eosin. Then, the modified scoring system from Nadler et al.[9,16] was used to classify the histological damage (as seen on figure 1).

The data was analyzed using IBM SpSS Statistics V.29.0. The macroscopic appearance among groups was compared using a Mann-Whitney test. Groups B and C were compared for the presence of NEC using Fisher's exact test. In addition, the Odds ratio was calculated to evaluate if the breastmilk works as a protective factor against NEC, such as it does in human neonates.

Table 1. Clinical sickness score for the assessment of neonatal rat clinical status.

Appearance
0 = Tonic and well-hydrated
1 = Slimmer, but still tonic and hydrated
2 = Skinny, floppy and dehydrated
3 = Gasping and in agony
Natural activity
0 = Moving normally
1 = Able to wriggle if put supine
2 = Not able to wriggle if put supine
3 = Not moving limbs and lying still
Response to touch
0 = Alert
1 = Responding to mid stimulation
2 = Responding to vigorous stimulation
3 = Unresponsive notwithstanding vigorous stimulation
Body color
0 = Pink
1 = Pale (just the extremities)
2 = Pale (whole body)
3 = Grey

Table 2. Scoring system for macroscopic gut assessment validated by Zani et al.[9].

Gut consistency
0 = normal
1 = moderately friable
2 = extremely friable (jelly like)
Gut colour
0 = normal
1 = patchy discoloration
2 = extensive discoloration
Gut dilatation
0 = no dilatation
1 = patchy dilatation
2 = extensive dilatation

RESULTS

Regarding clinical situation, NBR in groups LPS and BM began exhibiting symptoms and signs of NEC at 48 hours, such as pale color, dehydration, and abdominal distension. Both groups submitted to stress exhibited a global mortality rate of 89%. The mortality rate in group LPS was

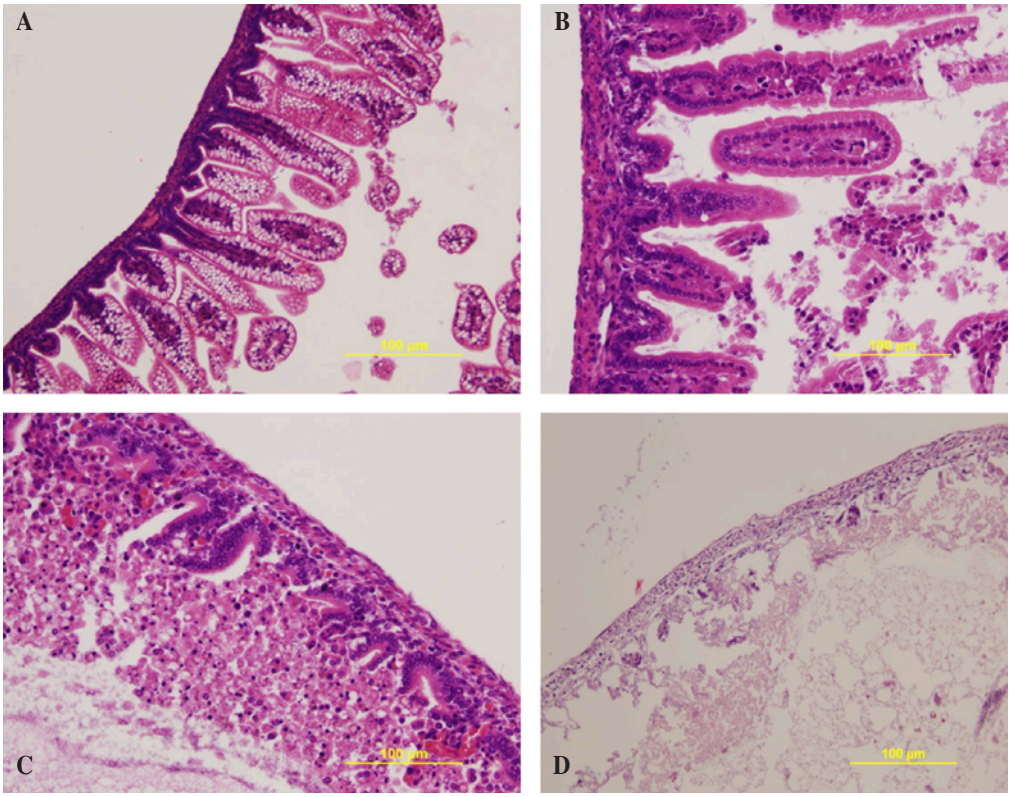

Figure 1. Histological findings of the small bowel in NBR according to Nadler score[16], being diagnosis of NEC a grade ≥ 2 (Hematoxilin & Eosin staining): A) Grade 0-1: normal intestine, disarrangement of enterocyte villi can be seen, B) grade 2: disarrangement of villus enterocytes and severe villus core separation, C) grade 3: epithelial sloughing of the villi, D) grade 4: bowel necrosis/perforation.

Table 3. **Microscopic findings among groups according to Nadler score[16]: Group LPS showed a higher incidence and severe lesions of necrotizing enterocolitis (NEC).**

		Group A (Control)	*Group B (LPS)*	*Group C (BM)*
NEC	*Grade 0*	9	0	0
	Grade 1	1	7	6
	Grade 2	0	3	1
	Grade 3	0	10	4
	Grade 4	0	5	1
	Total	10	25	12
	p Value	**0.001**		
			0.04 (OR= 0.19)	
		0.001		

p-value < 0.05 statistically significant.

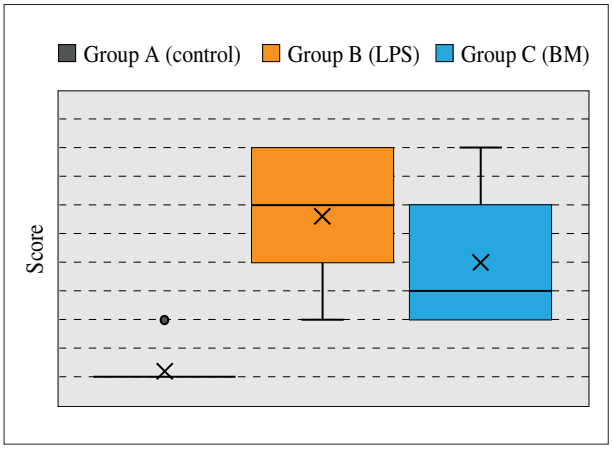

Figure 2. Macroscopic findings using the validated score proposed by Zani et al.[9], assessing gut appearance, color and dilation (as seen on table 2): NBR in the group LPS had a significantly higher score compared to the group that received breastmilk.

92%, including NBR that were euthanized and those who had died before the last intake on day 4. In group BM, the mortality rate was approximately 83%, which was lower than that of group LPS, but not statistically significant.

Macroscopically, in the group control there were no signs of bowel damage. Within the LPS group, 72% exhibited changes in bowel appearance, consistency, and dilation (score ≥ 3). The group BM presented less macroscopic damage (58%,score < 3), and the observed differences were statistically significant (p= 0.002). The LPS group had the most macroscopic findings which could be associated with the development of NEC.

Upon histological examination, the group control showed no changes or abnormalities in intestinal histology, except for one specimen that showed grade 1 in both the small and large intestine. However, in both groups subjected to stress (group LPS and BM), we found a global incidence of NEC (graded as 2 or higher) of 72.9%. Most of the lesions were found in the small bowel, but signs of NEC were also present in the colon and rectum.

Comparing the results of the LPS and BM groups, the incidence of NEC was significantly lower in the breastfeeding group (p= 0.04), and breastmilk acted as a protective factor (OR= 0.19; 95% CI: 0.40-0.904) against NEC (Table 3).

DISCUSSION

An initial insult such as hypoxia result in mild mucosal damage with the subsequent variation in mucosal permeability. This increased permeability is thought to be a key factor in the development of NEC, as it allows for bacterial translocation, the recruitment of polymorphonuclear leukocytes, and subsequent tissue damage.

Several models of experimental NEC have been developed using hypoxia, special rat formula, or both[16], resulting in an incidence of NEC-like gut damage varying from 35-71%. However, our group has not been able to replicate these models and has observed a variable and much lower incidence of NEC. The lack of consistency has not allowed us to publish any of the observed results until now.

To increase the incidence and severity of experimental NEC, some authors[2] have proposed adding lipopolysaccharide to the enteral formula administered to the NBR (to simulate the bacterial load that occurs in neonates affected by NEC).

With this latest modification and performing a macroscopic assessment as presented by Zani et al.[9], we have accomplished a higher and severe incidence of NEC.

The mortality rate associated with NEC was significantly higher in the stress-exposed groups. Although the mortality rate was higher in the LPS group, no significant differences were found compared with the BM group, probably due to the small sample size. However, when macroscopic and microscopic data were analyzed, greater damage and a higher incidence of NEC-related damage were observed in the group receiving oral LPS. Thus, oral LPS acts as an enhancer, facilitating intestinal damage such as that seen in NEC.

NECis the perinatal acquired disease with the highest morbidity and mortality associated. Remotely affecting organs such as the brain and placing affected infants at a substantially increased risk of neurodevelopmental delays[20,21]. Despite decades of research, the pathogenesis of this disease remains unclear, and prevention strategies are limited and not entirely successful. One key strategy

has been providing human milk feedings, both mother's own milk and donor human milk, to preterm infants.

The bioactive components in breastmilk work together to provide various levels of protection against NEC, including immunomodulatory, anti-infective, antioxidant, growth-promoting, and gut-colonizing effects[22-24].

Like human neonates, NBR have a relatively weak immune system. Part of its total supply of IgG is received through the yolk sac, the remainder must be absorbed intact from breast milk through the intestine. Thus, it is not surprising that breastfeeding provided protection against the development of NEC in NBR (even in such modest quantities).

New treatments proposed for NEC should be based on specific studies rather than extrapolations from effective treatments for older children or adults. This is due to the differences in pathophysiology and pharmacodynamics in neonates. Therefore, it is important to develop specific neonatal models to propose new therapeutic targets[25-27] for a disease that remains without a cure.

The combined use of oral LPS, hypoxia and a special rat formula allowed us to replicate an experimental model of NEC, with a significant incidence of 73%. This model seems to act in a very similar way to NEC in human neonates, since BM also acts as a protective factor and reduces its incidence.

The development of this model will allow us to explore new therapeutic targets for a devastating disease where our only effective tool is prevention.

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REFERENCES

1. Rich BS, Dolgin SE. Necrotizing enterocolitis. Pediatr Rev. 2017; 38: 552-9.
2. Feng J, El-Assal ON, Besner GE. Heparin-binding epidermal growth factor–like growth factor decreases the incidence of necrotizing enterocolitis in neonatal rats. J Pediatr Surg. 2006; 41: 144-9.
3. Henry MCW, Moss RL. Neonatal necrotizing enterocolitis. Semin Pediatr Surg. 2008; 17: 98-109.
4. Eidelman AI, Schanler RJ, Johnston M, Landers S, Noble L, Szucs K, et al. Breastfeeding and the use of human milk. Pediatrics. 2012; 129: e827-41.
5. Lucas A, Cole TJ. Breast milk and neonatal necrotising enterocolitis. Lancet. 1990; 336: 1519-23.
6. Kantorowska A, Wei JC, Cohen RS, Lawrence RA, Gould JB, Lee HC. Impact of donor milk availability on breast milk use and necrotizing enterocolitis rates. Pediatrics. 2016; 137: e20153123.
7. Del Pozo A, Villa M, Vargas C, Castejón D, Fernández-Valle ME, Gutiérrez-Rodríguez A, et al. Intraventricular hemorrhage induces inflammatory brain damage with blood-brain barrier dysfunction in immature rats. Pediatr Res. 2023; 93: 78-88.
8. Ceprián M, Jiménez-Sánchez L, Vargas C, Barata L, Hind W, Martínez-Orgado J. Cannabidiol reduces brain damage and improves functional recovery in a neonatal rat model of arterial ischemic stroke. Neuropharmacology. 2017; 116: 151-9.
9. Zani A, Cordischi L, Cananzi M, De Coppi P, Smith V, Eaton S, et al. Assessment of a neonatal rat model of necrotizing enterocolitis. Eur J Pediatr Surg. 2008; 18: 423-6.
10. Zamora R, Bryan NS, Boyle P, Wong C, Milsom AB, Jaffe R, et al. Nitrosative stress in an animal model of necrotizing enterocolitis. Free Radic Biol Med. 2005; 39: 1428-37.
11. Caplan MS, Sun XM, Hsueh W. Hypoxia causes ischemic bowel necrosis in rats: the role of platelet-activating factor (PAF-acether). Gastroenterology. 1990; 99: 979-986.
12. Akisu M, Küllahçioglu Girgin F, Baka M, Hüsseyinov A, Kültürsay N. The role of recombinant human erythropoietin in lipid peroxidation and platelet-activating factor generation in a rat model of necrotizing enterocolitis. Eur J Pediatr Surg. 2001; 11: 167-72.
13. Dvorak B, Halpern MD, Holubec H, Williams CS, McWilliam DL, Dominguez JA, et al. Epidermal growth factor reduces the development of necrotizing enterocolitis in a neonatal rat model. Am J Physiol Gastrointest Liver Physiol. 2002; 282: G156-64.
14. Caplan MS, Hedlund E, Adler L, Hsueh W. Role of asphyxia and feeding in a neonatal rat model of necrotizing enterocolitis. Pediatr Pathol. 1994; 14: 1017-28.
15. Bazacliu C, Neu J. Pathophysiology of necrotizing enterocolitis: An update. Curr Pediatr Rev. 2019; 15: 68-87.
16. Nadler EP, Dickinson E, Knisely A, Zhang XR, Boyle P, Beer-Stolz D, et al. Expression of inducible nitric oxide synthase and interleukin-12 in experimental necrotizing enterocolitis. J Surg Res. 2000; 92: 71-7.
17. Barlow B, Santulli TV, Heird WC, Pitt J, Blanc WA, Schullinger JN. An experimental study of acute neonatal enterocolitis--the importance of breast milk. J Pediatr Surg. 1974; 9: 587-95.
18. Van Zutphen LFM, Baumans V, Beynen AC. Principles of Laboratory Animal Science, Second ed. Amsterdam: Elsevier Science; 2001.
19. European Commission, Directorate-General for Environment, Caring for animals aiming for better science: Directive 2010/63/EU on protection of animals used for scientific purposes: severity assessment framework. Publications Office; 2018. Available from: doi/10.2779/068620.
20. Hickey M, Georgieff M, Ramel S. Neurodevelopmental outcomes following necrotizing enterocolitis. Semin Fetal Neonatal Med. 2018; 23: 426-32.
21. Moschopoulos C, Kratimenos P, Koutroulis I, Shah BV, Mowes A, Bhandari V. The neurodevelopmental perspective of surgical necrotizing enterocolitis: The role of the gut-brain axis. Mediators Inflamm. 2018; 2018: 7456857.
22. Zhou P, Li Y, Ma LY, Lin HC. The role of immunonutrients in the prevention of necrotizing enterocolitis in preterm very low birth weight infants. Nutrients. 2015; 7: 7256-70.

23. Hassiotou F, Hartmann PE. At the dawn of a new discovery: the potential of breast milk stem cells. Adv Nutr 2014; 5: 770-8.
24. Lönnerdal B. Bioactive proteins in human milk: mechanisms of action. J Pediatr. 2010; 156: S26-30.
25. Wang Y, Jaggers RM, Mar P, Galley JD, Shaffer T, Rajab A, et al. Lactobacillus reuteri in its biofilm state promotes neurodevelopment after experimental necrotizing enterocolitis in rats. Brain Behav Immun Health. 2021; 14: 100256.
26. Shinyama S, Kaji T, Mukai M, Nakame K, Matsufuji H, Takamatsu H, et al. The novel preventive effect of Daikenchuto (TJ-100), a Japanese herbal drug, against neonatal necrotizing enterocolitis in rats. Pediatr Surg Int. 2017; 33: 1109-14.
27. Nakame K, Kaji T, Mukai M, Shinyama S, Matsufuji H. The protective and anti-inflammatory effects of glucagon-like peptide-2 in an experimental rat model of necrotizing enterocolitis. Peptides. 2016; 75: 1-7.