Journal of Visualized Experiments

A neonatal BALB/c mouse model of necrotizing enterocolitis

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63252R3
Full Title:	A neonatal BALB/c mouse model of necrotizing enterocolitis
Corresponding Author:	Yan Tian Guangzhou Women and Children's Medical Center Guangdong, Guangzhou CHINA
Corresponding Author's Institution:	Guangzhou Women and Children's Medical Center
Corresponding Author E-Mail:	18070504107@163.com
Order of Authors:	Yan Tian
	Junyu Huang
	Ming Fu
	Qiuming He
	Jiale Chen
	Yan Chen
	Ruizhong Zhang
	Wei Zhong
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Medicine
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Guangzhou, Guangdong, China
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release
Please provide any comments to the journal here.	Very good!

TITLE:

A Neonatal BALB/c Mouse Model of Necrotizing Enterocolitis

AUTHORS AND AFFILIATIONS:

Yan Tian[1*], Junyu Huang[1*], Ming Fu[1], Qiuming He[1], Jiale Chen[1], Yan Chen[1], Ruizhong Zhang[1], Wei Zhong[1]

[1]Provincial Key Laboratory of Research in Structure Birth Defect Disease and Department of Pediatric Surgery, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, Guangdong, China

[*]These authors contributed equally.

Email addresses of co-authors:

Yan Tian	(18070504107@163.com)
Junyu Huang	(HuangJY@stu.gzhmu.edu.cn)
Ming Fu	(mingfu1993@foxmail.com)
Qiuming He	(qiuminghe@foxmail.com)
Jiale Chen	(jialechenletter@163.com)
Yan Chen	(ychenc@hku.hk)

Corresponding authors:

Ruizhong Zhang	(zhangruizhong@gwcmc.org)
Wei Zhong	(zhongwei@gwcmc.org)

KEYWORDS:

Necrotizing enterocolitis; BALB/c mice; Immunology; T helper type 2 cells

ABSTRACT:

Necrotizing enterocolitis (NEC) is the most severe gastrointestinal (GI) disease that often occurs in premature infants, especially very low birth weight infants, with high mortality and unclear pathogenesis. The cause of NEC may be related to inflammatory immune regulatory system abnormalities. An NEC animal model is an indispensable tool for NEC disease immune research. NEC animal models usually use C57BL/6J neonatal mice; BALB/c neonatal mice are rarely used. Related studies have shown that when mice are infected, Th2 cell differentiation is predominant in BALB/c mice compared to C57BL/6J mice. Studies have suggested that the occurrence and development of NEC are associated with an increase in T helper type 2 (Th2) cells and are generally accompanied by infection. Therefore, this study used neonatal BALB/c mice to induce an NEC model with similar clinical characteristics and intestinal pathological changes as those observed in children with NEC. Further study is warranted to determine whether this animal model could be used to study Th2 cell responses in NEC.

INTRODUCTION:

Necrotizing enterocolitis (NEC), the most severe gastrointestinal (GI) disease, occurs in most

premature infants (>90%), especially those with very low birth weight (VLBW)[1]. In VLBW infants, the incidence of the disease ranges from 10% to 12%, and the mortality of children diagnosed with NEC is between 20% and 30%[2,3]. The cause of NEC may be related to mucosal injuries, invasion by pathogenic bacteria, and intestinal feeding, which can lead to inflammatory responses and the induction of intestinal injuries in susceptible hosts[3]. The pathogenesis of NEC is unclear. Relevant research shows that the affected infant's immune response is abnormal, and genetic susceptibility, microvascular tension, and intestinal bacterial changes may play important roles in the disease[3].

The NEC animal model is an indispensable tool for research on the pathogenesis of NEC. The animal species used for NEC models are pigs, rats, and mice. However, due to the long gestation period, growth cycles, and high costs, in recent years, pigs have not been the first choice for NEC models and have been replaced with rats or mice[4]. As there are differences in the immune background of different mouse strains[5], different studies need to use different strains of mice to establish NEC animal models. BALB/c mice have an important feature; when they are infected or cope with external damage, the polarization of TH2 cells during infection in mice is significantly stronger than that in other strains of mice[6-8]. T helper cells play a crucial role in the occurrence and progression of NEC, especially the development of TH2 cells[3,9-11]. Therefore, this study used BALB/c mice to establish the NEC model, which might be helpful for NEC disease research on T cells.

PROTOCOL:

This research was approved by the Medical Ethics Committee of Guangzhou Women and Children's Medical Center (NO. 174A01) and the Animal Ethical Committee of the Guangzhou Forevergen Biosciences Laboratory Animal Center (IACUC-G160100). All animals were bred in the same room in a specific pathogen-free (SPF) environment, and experiments were carried out in a conventional environment. The mice used for breeding were 7–8 weeks old; the mice for inducing NEC were separated from the dam on Day 4, and the control (Cont.) group mice were nursed and fed by the dam.

1. Preparation of reagents and devices

1.1. Prepare the milk substitute for the BALB/c mice in the corresponding ratio (premature baby milk powder: goat milk powder = 2:1).

NOTE: The final nutritional compositions of formula milk[12] are shown in **Table 1**.

1.2. LPS solution (2.5 mg/mL)

1.2.1. Dissolve a total of 10 mg of LPS powder in 4 mL of sterilized double-distilled water, mix well, and store in a refrigerator at -20 °C after aliquoting.

NOTE: The LPS solution is stored in the dark at 2–8 °C for immediate use or at -20 °C for long-term storage.

2. Induce necrotizing enterocolitis in neonatal BALB/c mice

2.1. Feed the neonatal mice.

NOTE: NEC-inducing experiments were started when the mice were 4 days old, and the mice were divided into a Cont. group (n = 24) and the NEC group (n = 72).

2.1.1. Keep the neonatal mice in the same cage with the dam, nursed by the dam on Days 0–4.

2.1.2. On the night of Day 4 (when the neonatal mice weigh 2.5–3 g), separate the neonatal mice in the NEC group from the dam to induce NEC, keep them in an animal incubator, and feed them with formula. However, the Cont. group is allowed to stay with and be fed by the dam.

NOTE: Neonatal mice that are separated from the dam must be raised in an incubator because of their weak body temperature regulation.

2.2. Prepare the gavage tube by soaking it in 75% alcohol containers for 1–2 min and wash them twice in clean, double-distilled water.

NOTE: To avoid cross-contamination among the mice, the above process must be performed after feeding each mouse.

2.3 Induce the NEC model.

2.3.1. Take the neonatal mice from the dam on Day 4 and fast them for one night.

2.3.2. Gavage the mice with LPS (20–30 µL at a time) and feed them with formula on Day 5 (40–50 µL at a time).

2.3.3. From Day 5 onwards, subject the mice to a hypoxia-reoxygenation-cold-shock cycle twice a day for 5 days. Place the mice in a hypoxia device at 5% O_2 for 90 s and reoxygenate them for 3 min; repeat this process five times. Next, place the mice in a 4 °C environment for 15 min and then transfer them to an incubator. See **Figure 1A,B** for the induction process.

NOTE: A cycle of hypoxia–reoxygenation–cold stimulation was performed once in the morning and once in the afternoon. A mixture of 5% O_2 with 95% N_2 was prepared in the container, and the concentration was measured with an oxygen detector.

2.4. Closely observe all the mice, weigh them every day, record the survival of the mice during

the induction period, and record the stool characteristics (with or without sticky stools/bloody stools).

NOTE: The established NEC model lasts for 5 days.

2.5. On Day 10 or earlier, when the mice show NEC symptoms (ileus, hematochezia, diarrhea)[13], euthanize the mice by inhalation anesthesia with isoflurane, and collect the intestinal tissue.

NOTE: In this study, tissues were taken immediately after euthanasia after the appearance of NEC symptoms. Tissues were not collected from mice that died spontaneously.

3. Gavage the mouse.

3.1. Fix the mouse head, holding the gastric tube in the right hand. Insert the gastric tube from the left corner of the mouth of the mouse.

NOTE: The head was fixed with the index finger on the mouse's head and gently pressed backward and downwards to prevent the mouse from bending forwards during the operation and affecting the insertion of the gastric tube.

3.2. Slowly move the tube to the center of the mouth. After inserting the tube approximately 2–3 cm, push 40–50 µL of formula or 20–30 µL of LPS into the digestive tract. See **Figure 2A,B** for the gavage.

NOTE: Under normal circumstances, the gastric tube is inserted into the digestive tract smoothly. If the mouse has a strong vomiting reflex, the gastric tube has been inserted into the trachea by mistake. The gastric tube must be pulled out gently and the mouse allowed to rest for a while before attempting gavage again.

4. Collect fresh intestinal tissue specimens for hematoxylin and eosin (H&E) staining

4.1. Immerse the fresh ileum tissue from the mouse in 10% formalin for 24 h.

4.2. Embed the tissues in paraffin and slice them into 4 µm sections.

4.3. Deparaffinize the sections in xylene and rehydrate them successively in absolute ethanol, 95% ethanol, 80% ethanol, 70% ethanol, and distilled water, soaking for 5 min in each step. Stain the sections with hematoxylin solution for 5 min and differentiate them in 1% hydrochloric acid in 75% alcohol for 5 s. Finally, stain them with eosin solution for 1 min.

NOTE: After staining with hematoxylin solution, it must be differentiated with 1% hydrochloric acid in ethanol to remove excessively bound hematoxylin solution and cytoplasmic hematoxylin dye. The concentration of 1% hydrochloric acid is suitable for intestinal tissue.

4.4. Examine the histopathology of the intestinal tissue at 40x magnification.

REPRESENTATIVE RESULTS:

The BALB/c mouse NEC model was induced by formula feeding, LPS feeding, hypoxia, and cold stimulation. During the induction period, the mice were observed for intestinal pathology, stool characteristics, body weight changes, and daily survival. Representative images of the small intestine during NEC induction; the numbers in the picture represent the intestinal pathology score from 0 (normal epithelium) to 4 (the most severe) (**Figure 3A**). The intestinal pathology score was significantly higher in the NEC group than in the Cont. group (**Figure 3B**). The numbers in the picture represent stool scores from 0 (well-formed pellets) to 3 (liquid stools) (**Figure 3C**). On Day 10, the stool scores of the NEC groups were significantly higher in the NEC group, indicating that intestinal dysfunction in the NEC group was more serious (**Figure 3D**). On Day 5, the first day of induction of the NEC model, there was no significant difference in body size between the two groups. However, on Day 10, the mice in the NEC group were significantly thinner than the mice in the Cont. group (**Figure 4A**).

During the 5 days during which the model was established, the weight of the mice in the NEC group increased slowly or even showed negative growth, and the survival rate of the mice in the NEC group gradually decreased compared with the Cont. group (**Figure 4B,C**). In addition, another batch of mice was used to induce the NEC model but without collecting the tissues; by Day 13, all the mice in this NEC group had died, and the survival curve was significantly reduced **(Supplemental Figure S1**). **Figure 5A** shows the morphology and pathological results (necrosis of intestinal mucosal tissue) of the resected ileocecal area of the intestinal tissue from NEC patients in this hospital. In this study, the mice in the NEC group (1/13) developed ileocecal hemorrhage and necrosis (**Figure 5B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Induction of the BALB/c NEC model process. (**A**) The mice in the NEC group were separated from the dam at birth until they were 4 days old (on Day 4) and fasted that night. The NEC model was induced from Day 5 onwards after birth and lasted for 5 days. Intestinal tissue specimens were collected on Day 10 or earlier. The mice in the Cont. group were housed with and nursed by the dam. (**B**) The sequence of operations for each day after inducing the NEC model. Abbreviations: Cont. = control; NEC = necrotizing enterocolitis; LPS = lipopolysaccharide.

Figure 2: Gastric gavage. (**A**) A specialized gavage device was used in this study, which was combined with a plastic tube and syringe. (**B**) The gavage tube entered from the corner of the mouth at a 45° angle to the vertical line. (**C**) The tube was slowly moved to the center of the mouse's mouth to ensure that the gastric tube and the esophagus were at the same vertical level. Abbreviations: D= diameter.

Figure 3: BALB/c mouse NEC model. (**A**) Photomicrographs of the intestinal pathology score from the two groups, e.g., showing intact and normal mucosa in the Cont. group (score 0),

mild submucosal or lamina propria swelling separation in two groups (score 1), moderate submucosal and/or lamina propria separation in the NEC group (score 2), severe submucosal and/or lamina propria separation in the NEC group (score 3), intestinal villi disappearance with intestinal necrosis in the NEC group (score 4). (**B**) The intestinal pathology scores in the mice after NEC induction were higher than that of the Cont. group (n = 9 in the Cont. group, n = 35 in the NEC group, *** $P < 0.001$ with Student's *t*-test). (**C**) Photomicrographs of stool scores from two groups, e.g., showing well-formed pellets in the Cont. group (score 0), formed stools in two groups (score 1), semiformed stools in the NEC group (score 2), and liquid stools in the NEC group (score 3). (**D**) The stool scores in the NEC group were significantly higher than that in the Cont. group (n = 6 in the Cont. group, n = 13 in the NEC group, *** $P < 0.001$ with Student's *t*-test). The red triangle represents the separation of mucosa and lamina propria, and the black arrow points to mouse feces. Scale bars = 50 μm. Abbreviations: Cont. = control; NEC = necrotizing enterocolitis; HE = hematoxylin and eosin.

Figure 4: Comparison of body shape and the survival of mice between the Cont. group and the NEC group. (**A**) The appearance of the two groups of mice on Day 5 and Day 10. (**B**) This section shows the weight changes of the mice in two groups over time; the x-axis represents the number of days after the mice were born, and the y-axis represents the weight changes of the mice; **P < 0.01, ***P < 0.001 with Student's *t*-test to compare the Cont. group (n = 10) and the NEC group (n = 27) (**C**) This section shows the survival curves of mice in the control group (n = 10) and the NEC group (n = 25). Abbreviations: Cont. = control; NEC = necrotizing enterocolitis.

Figure. 5: Ileocecal hemorrhage in children with NEC and mice with NEC. (**A**) Hemorrhage and necrosis of the ileocecal area in children with NEC. (**B**) Hemorrhage and necrosis in the ileocecal area of mice with NEC (1/13); however, the intestines of the mice in the Cont. group were normal, without hemorrhage and necrosis. The black triangle refers to intestinal hemorrhage and necrosis, and the red arrow shows hemorrhage and necrosis of the ileocecal area. Scale bars = 50 μm. Abbreviations: Cont. = control; NEC = necrotizing enterocolitis.

Table 1: Dairy formula milk ingredients.

Supplemental Figure S1: The survival curve was significantly reduced in the NEC group so that all mice died spontaneously (n = 5 in the Cont. group, n = 10 in the NEC group). Abbreviations: Cont. = control; NEC = necrotizing enterocolitis.

DISCUSSION:

NEC is the most common gastrointestinal system emergency for neonates, with a high incidence and mortality, especially in premature infants[1-3]. However, its pathogenesis is still unclear. It is currently believed that mucosal damage, pathogen invasion, and enteral feeding are high-risk factors for NEC[3]. To date, the animals used for the NEC model are mainly pigs, rats, and mice. Most studies have used neonatal C57BL/6 mice to induce NEC[13-16], and very

few studies have used BALB/c neonatal mice to induce NEC. However, BALB/c mice have the advantage of Th cell polarization[6-8], which warrants further study to determine whether they can be a good NEC model for Th cell research of the disease.

We referred to the Nadler pathological score standard of NEC model[17] and found that the score of the NEC group was significantly higher than that of the control group. A score ≥ 2 indicates NEC, and the success rate of inducing NEC is 38–50%. We also evaluated the stool scores[18] of the two groups of mice and found that the scores of the NEC group were higher than those of the control group. The higher the score, the more serious the intestinal dysfunction. All these data show that the establishment of the NEC model was successful. In addition, it is encouraging that the neonatal BALB/c mouse model of NEC can simulate human NEC to a certain extent. Hemorrhage and necrosis occur in the intestines of children with NEC[3,19]; similar pathological conditions were observed in this model.

Gavage is the key step in inducing NEC in the mouse model. If the gavage operation was not proficiently mastered, it was easy to mistakenly place the gastric tube into the trachea and cause the mouse to die. During gastric gavage, the left thumb, middle finger, and ring finger were used to clamp both sides of the mouse's torso, and the index finger was placed on the head to fix the mouse in place. This was to prevent the mouse from moving around and causing the gastric tube to damage the esophagus. The gastric tube was inserted from the left corner of the mouse's mouth. It is only when the gastric tube enters the esophagus smoothly without resistance could we continue inserting it. LPS or formula milk should be injected only after inserting the gastric tube 2–3 cm from the mouse's lower lip.

The length of hypoxia should be carefully monitored. In this study, hypoxia lasted for 90 s each time. If hypoxia is too long, the mice will not be able to tolerate it and will die. This model may be used to research NEC-related immune cells, especially TH1 and TH2 cells[3,9-11,20]. In the future, we plan to investigate whether this model is useful to study Th2 cell responses in NEC. In addition, this study also introduced a new method of stool scoring to evaluate intestinal dysfunction in mice with NEC[18]. However, there are some limitations to this study. For example, the success rate of the model was not very high. Efforts are ongoing to improve the method of BALB/c NEC modeling to increase the success rate by adjusting the hypoxia level from 5% O_2 to 1% O_2, as described previously[15].

ACKNOWLEDGMENT:

The authors thank the Clinical Biological Resource Bank of Guangzhou Women and Children's Medical Center for providing the clinical sample and Guangzhou Forevergen Biosciences Laboratory Animal Center for providing mice. This research was supported by the National Natural Science Foundation of China grant 81770510 (R.Z.).

DISCLOSURES:

The authors have no conflicts of interest to disclose.

REFERENCES:

1 Horbar, J. D. et al. Mortality and neonatal morbidity among infants 501 to 1500 grams from 2000 to 2009. *Pediatrics.* **129** (6), 1019–1026 (2012).
2 Stoll, B. J. et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics.* **126** (3), 443–456 (2010).
3 Neu, J., Walker, W. A. Necrotizing enterocolitis. *New England Journal of Medicine.* **364** (3), 255–264 (2011).
4 Sangild, P. T. et al. Invited Review: The preterm pig as a model in pediatric gastroenterology. *Journal of Animal Science.* **91** (10), 4713–4729 (2013).
5 Cancro, M. P., Sigal, N. H., Klinman, N. R. Differential expression of an equivalent clonotype among BALB/c and C57BL/6 mice. *Journal of Experimental Medicine.* **147** (1), 1–12 (1978).
6 Kuroda, E., Yamashita, U. Mechanisms of enhanced macrophage-mediated prostaglandin E2 production and its suppressive role in Th1 activation in Th2-dominant BALB/c mice. *Journal of Immunology.* **170** (2), 757–764 (2003).
7 Fornefett, J. et al. Comparative analysis of clinics, pathologies and immune responses in BALB/c and C57BL/6 mice infected with *Streptobacillus moniliformis*. *Microbes and Infection.* **20** (2), 101–110 (2018).
8 Rosas, L. E. et al. Genetic background influences immune responses and disease outcome of cutaneous *L. mexicana* infection in mice. *International Immunology.* **17** (10), 1347–1357 (2005).
9 Sproat, T., Payne, R. P., Embleton, N. D., Berrington, J., Hambleton, S. T cells in preterm infants and the influence of milk diet. *Frontiers in Immunology.* **11**, 1035 (2020).
10 Nanthakumar, N. et al. The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. *PLoS One.* **6** (3), e17776 (2011).
11 Afrazi, A. et al. New insights into the pathogenesis and treatment of necrotizing enterocolitis: Toll-like receptors and beyond. *Pediatric Research.* **69** (3), 183–188 (2011).
12 Auestad, N., Korsak, R. A., Bergstrom, J. D., Edmond, J. Milk-substitutes comparable to rat's milk; their preparation, composition and impact on development and metabolism in the artificially reared rat. *British Journal of Nutrition.* **61** (3), 495–518 (1989).
13 Liu, Y. et al. Lactoferrin-induced myeloid-derived suppressor cell therapy attenuates pathologic inflammatory conditions in newborn mice. *Journal of Clinical Investigation.* **129** (10), 4261–4275 (2019).
14 MohanKumar, K. et al. A murine neonatal model of necrotizing enterocolitis caused by anemia and red blood cell transfusions. *Nature Communications.* **10** (1), 3494 (2019).
15 He, Y. M. et al. Transitory presence of myeloid-derived suppressor cells in neonates is critical for control of inflammation. *Nature Medicine.* **24** (2), 224–231 (2018).
16 Cho, S. X. et al. Characterization of the pathoimmunology of necrotizing enterocolitis reveals novel therapeutic opportunities. *Nature Communications.* **11** (1), 5794 (2020).
17 Halpern, M. D. et al. Decreased development of necrotizing enterocolitis in IL-18-deficient mice. *American Journal of Physiology. Gastrointestinal and Liver Physiology.* 294 (1), G20–G26 (2007).
18 Wu, N. et al. MAP3K2-regulated intestinal stromal cells define a distinct stem cell niche. *Nature.* **592** (7855), 606–610 (2021).
19 Nino, D. F., Sodhi, C. P., Hackam, D. J. Necrotizing enterocolitis: new insights into pathogenesis and mechanisms. *Nature Reviews. Gastroenterology & Hepatology.* **13** (10),

590–600 (2016).
20 Chuang, S. L. et al. Cow's milk protein-specific T-helper type I/II cytokine responses in infants with necrotizing enterocolitis. *Pediatric Allergy & Immunology.* **20** (1), 45–52 (2009).

Figure

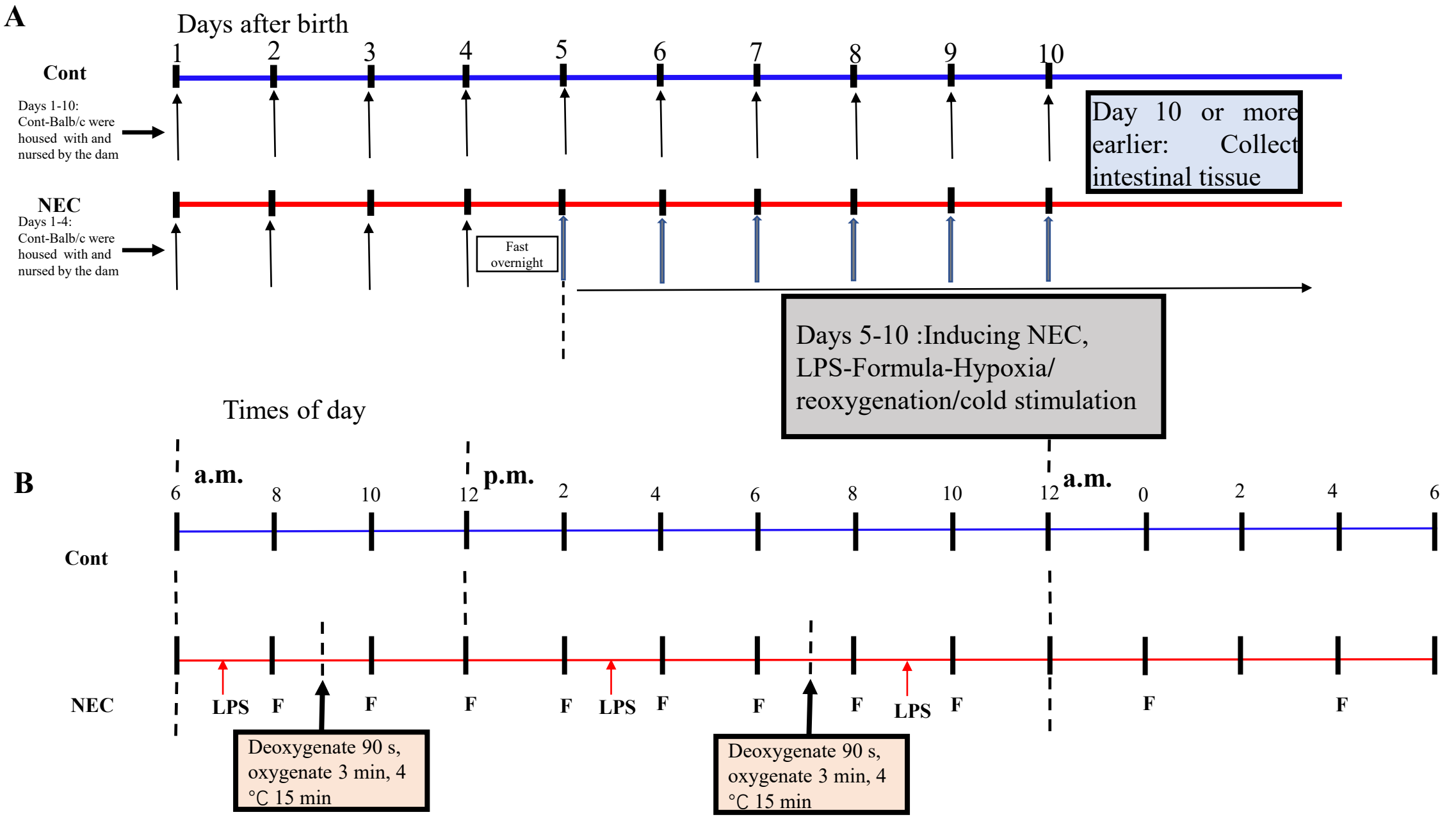


Figure 2

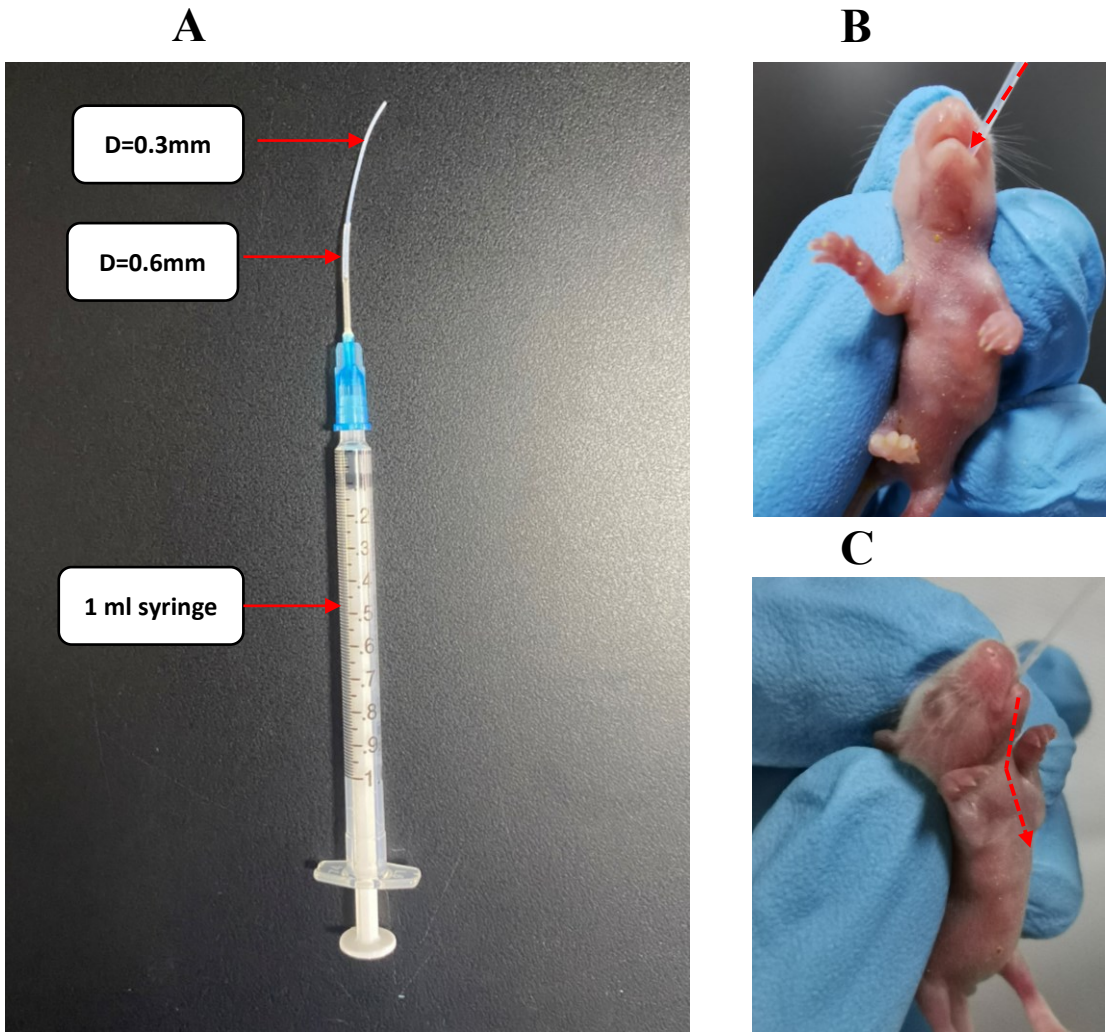


Figure 3

Click here to access/download;Figure;Fig 3.pdf

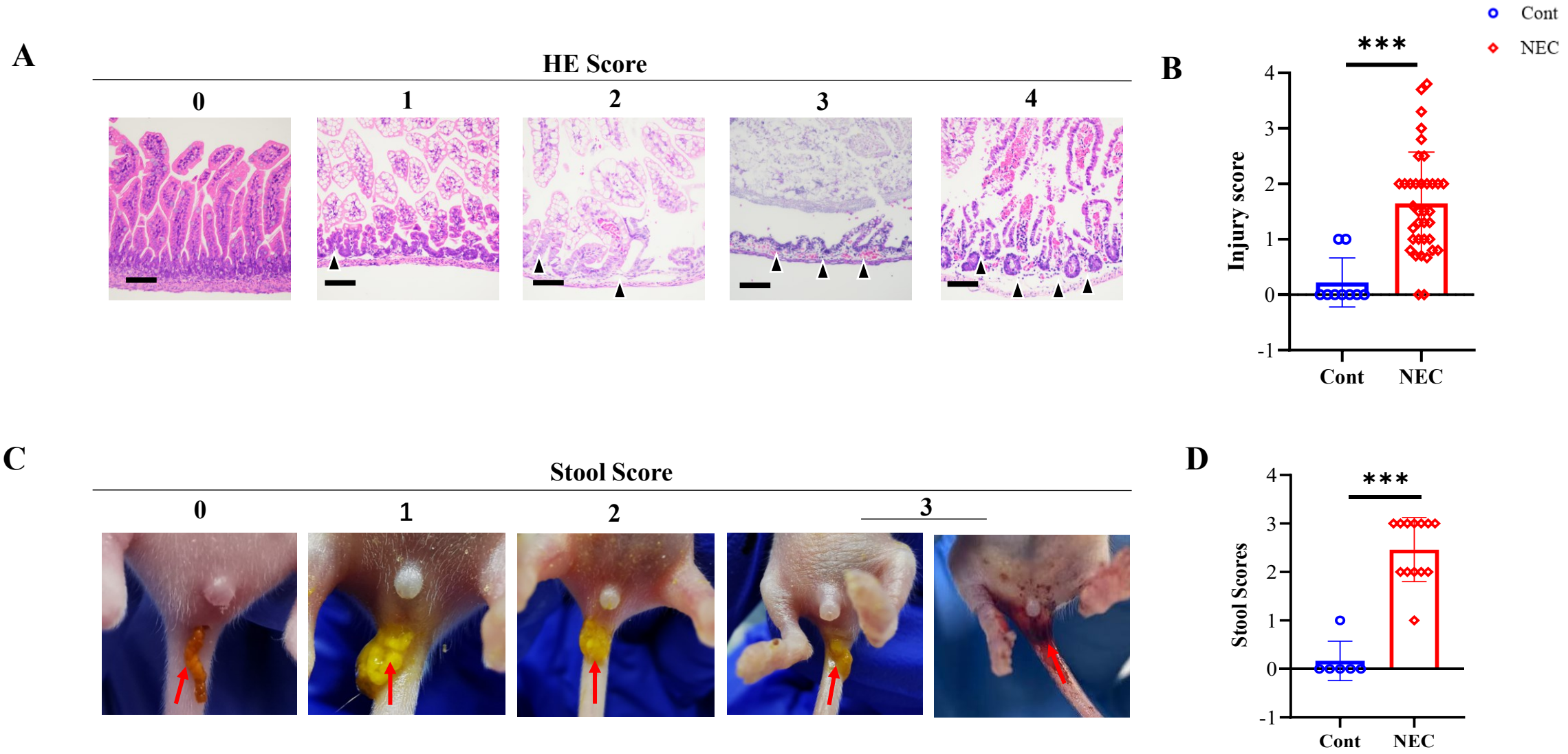


Figure 4

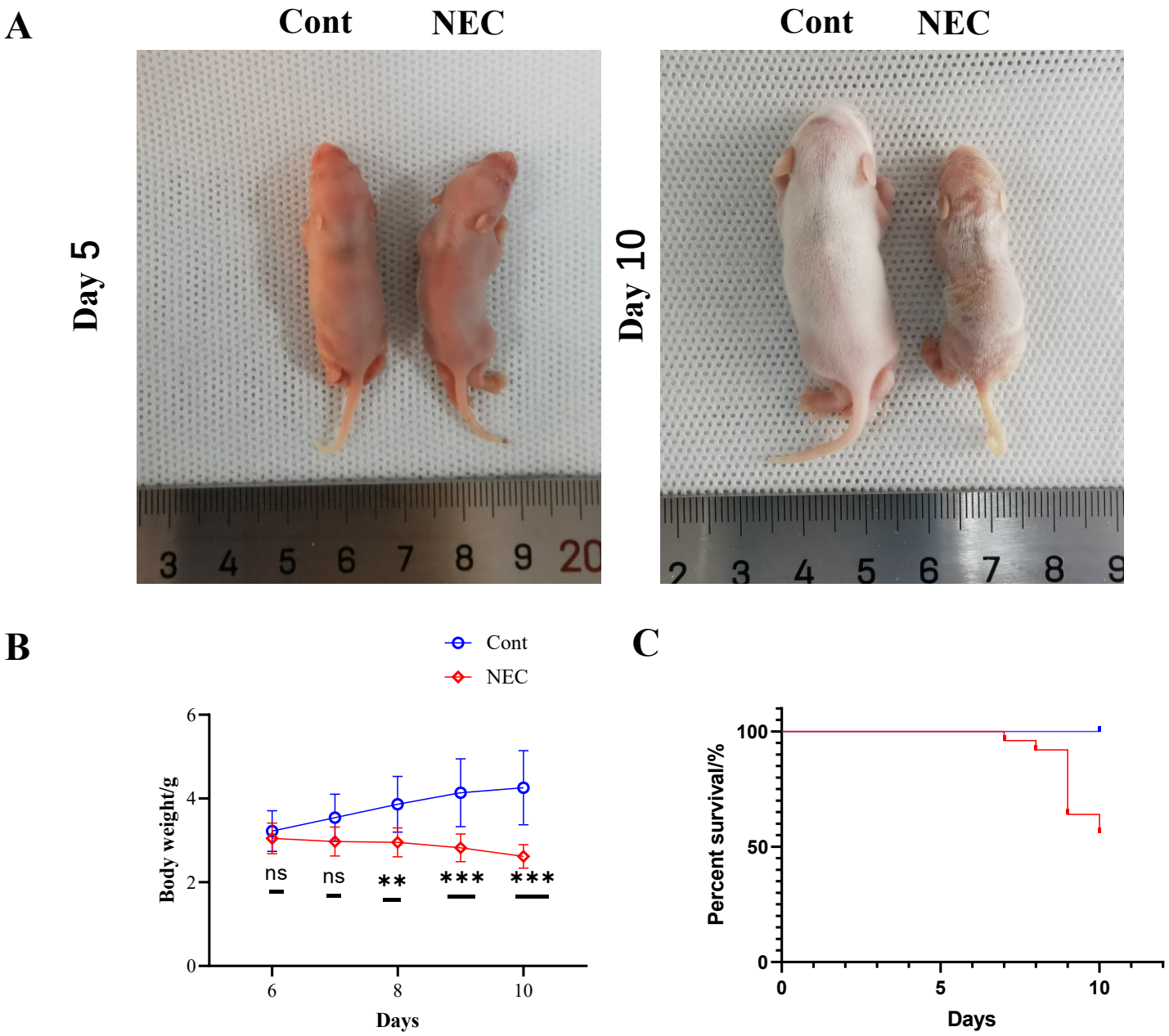


Figure 5

Click here to access/download;Figure;Fig 5.pdf

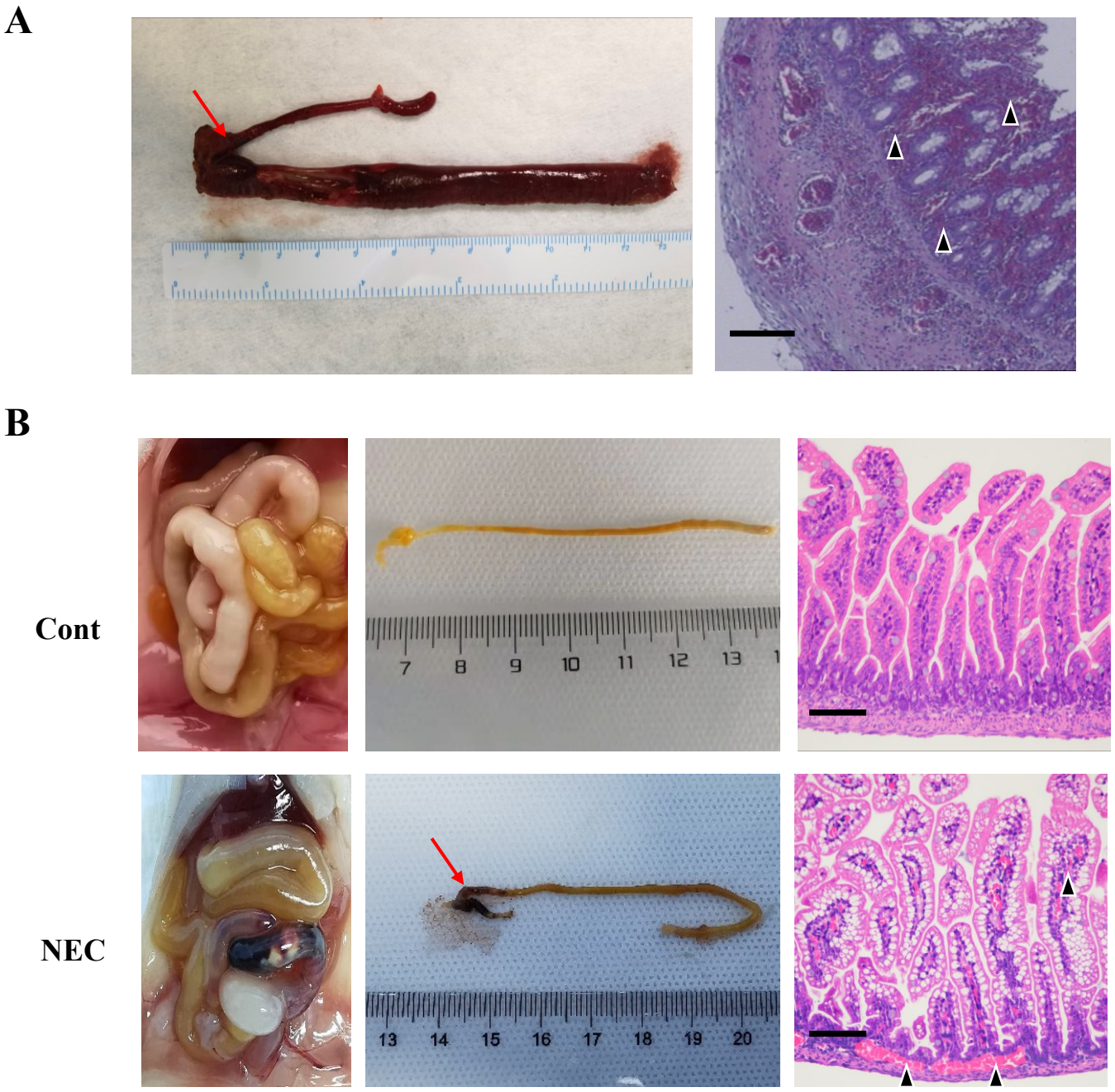


Table 1

Composition	Mouse (g/L)	Milk substitute (g/L)
protein	69-118	100
fat	93-175	100
carbohydrate	28-37	50
calcium	0.97-6.2	2.84
phosphorus	1.6-2.72	1.62
sodium	0.66-1.4	1
potassium	1.08-1.7	1.2
chloride	1.17-1.76	1.76
magnesium	0.0001-0.3	>0.12
zinc	0.009-0.055	0.018
iron	0.004-0.007	0.017
copper	0.0017-0.007	0.0018

Click here to access/download

Table of Materials

Table 2 (2).xlsx

We thank our reviewers and Editor for their thoughtful comments and suggestions about our manuscript entitled " *A neonatal BALB/c mouse model of necrotizing enterocolitis*" by Tian et al. (Number: JoVE63252R1). We have addressed all the concerns and questions by performing further detailed analysis of our data and providing answers to specific questions. We think the revised manuscript has been substantially improved and we hope it is suitable for publication on *JoVE*. Our point-by-point responses (blue) to editor' and reviewers' comments are provided below. comments are provided below and we indicate text changes made in response to the reviewer comments by font color (red) in the revised manuscript.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have carefully checked throughout the manuscript and corrected the errors in spelling and grammar.

2. Please revise the following lines to avoid previously published work: 119-123. Please refer to the iThenticate report attached.

Thanks for the editor's suggestion, we have revised it in the manuscript.

3. Please provide an institutional email address for each author.

We have provided the corresponding author and co-corresponding author's institutional email address.

4. Please include a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

We have added a summary that "Here, we present a protocol to use neonatal BALB/c mice to establish an NEC model" at the end of the introduction.

5. Please rephrase the Abstract to more clearly state the goal of the protocol in 150-300 words.

Thanks to the editor's suggestion. We have revised the abstract again according to the suggestion.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and

use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: ESBILAC's, Abbott, etc

We have changed the vocabulary of commercial language into generic terms in accordance with the editor's suggestion.

7. Please ensure that the Introduction includes all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

We have revised the introduction according to the editor's suggestion and We think the revised introduction has been substantially improved.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

We have adjusted the protocol number with reference to the latest publications according to JOVE and the JOVE instructions for authors.

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Thanks to the editor for providing so many valuable suggestions in this section, we have revised it in accordance with the suggestions.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. (e.g., lines 92-99, 103-109, etc.)

According to the editor's suggestion, we have carefully revised this part in the manuscript.

11. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have already written the details of the steps in the notes.

12. Lines 125-142: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. Please revise to actionable steps or consider moving the lines to the Discussion section.

Regarding the content of lines 125-142, we agreed with the editor's suggestion. We moved them to the appropriate paragraph in the discussion section.

13. Please move the Figure legends to the end of the Representative results section.

We have moved the Figure legends to the end of the Representative results section.

14. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Thanks to the editor for some suggestions on the discussion, we mentioned these points in the discussion section:
a) Gavage and hypoxia are key steps in the protocol, and we have emphasized in the third paragraph of the discussion section.
b) The main difficulty of this technique is the mastery of the gavage operation and the hypoxic operation. Regarding the gavage operation, our team has mastered it very well, and the hypoxic operation will be adjusted in the future. These are mentioned in the third and fourth paragraphs of this discussion.
c) The limitation of this technique is the low success rate of model building, but we will try to improve. This is mentioned in the fourth paragraphs of this discussion.
d) and e) Regarding those two points, we have mentioned in the first paragraph of the discussion section.

15. Please title case and italicize journal titles and book titles in the references. Do not

use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

Regarding the format of the reference, we have modified it in accordance with the editor's request.

16. Figure 3/5: Please include scale bars in all histology images
.
In Figure 3 and Figure 5, we have followed the editor's suggestion to insert the scale bars.

17. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

Information about equipment, reagents and materials has been sorted in table 2 in accordance with the editor's requirements.

Reviewers' comments:
Reviewer #1:
It's a pleasure review the manuscript:
A neonatal BALB/c mouse model of necrotizing enterocolitis by Tian et al.

The purpose is very interesting and the manuscript was well conducted, rich in details. The abstract and introduction are well written. The figures have high resolution. The n is appropriate.

Thanks for the reviewer's positive comments.

Minnor points:
In protocol, please provide the number of approve by Institutional Animal Care and Use Committee.

Thank you for your suggestion, in protocol, we provide the number of approve by Institutional Animal Care and Use Committee.

In Note, line number 86, the term "dam" is not more adequate, as well the term "placed". Please rewrite.

Thank you for your suggestion. About dam, in our research, it refers to the mother of the mouse. The same "dam" was used in other articles from JoVE, such as "Carini L M, Murgatroyd C A, Nephew B C. Using Chronic Social Stress to Model Postpartum Depression in Lactating Rodents[J]. Journal of Visualized Experiments,2013(76)", "Lemons A, Saré R M, Beebe Smith C. Chronic Sleep Deprivation in Mouse Pups by Means of Gentle Handling[J]. Journal of Visualized

Experiments,2018(140)" and so on.

In line 120, the term "Dewax" is not more adequate too. Please rewrite.

We agree with your suggestion. The term "Dewax" is replaced with "Deparaffinize".

Why the authors used 1% hydrochloric acid alcohol for histology?

Thank you for your suggestion. Our answer to this question is as follows:
After staining with hematoxylin, it must be differentiated with 1% hydrochloric acid and ethanol to remove excessively bound hematoxylin dye and cytoplasmic hematoxylin dye. And 1% hydrochloric acid is suitable for intestinal tissue to make H&E stain.

Reviewer #2:
Manuscript Summary:
The authors present a NEC mouse model using BALB/c mice, which have the advantage of Th cell polarization. For NEC induction the authors are present a protocol including gavage feeding, LPS, cold stimulation and hypoxia as well as separation from the mother. The protocol and handling is explained very well. Major outcomes were development of bodyweight, stool characteristics and intestinal pathology as well as survival. The results show significant higher tissue damage as well as stool pathologies in mice if the NEC induction group compared to controls. The bodyweight development shows similar findings. The authors conclude that the described protocol using BALB7c mice is useful for Th cell research in NEC.

Thanks for the reviewer's positive comments.

Major Concerns:
Why was it necessary to use such a stressful protocol for NEC induction. Many protocols for NEC induction are published using maternal separation or cold stimulation or hypoxia. For my opinion and experience it is not necessary to use such an intense protocol. Many protocols were described without maternal separation or cold stimulation but with constant NEC inductions (e.g. Welak et al., Vincent et al., Klinke et al.)

Thank you for your question. However, in different studies, the strains of mice used to induce NEC are different, and the methods for inducing NEC are different. Previously, we used neonatal BALB/c mice either hypoxia or cold stab to induce NEC, but it was difficult to successfully induce NEC. Therefore, we used hypoxia combined with cold stab to induce NEC, and our conditions for inducing NEC are similar to Steven X. Cho et al (doi.org/10.1038/s41467-020-19400-w).

More over, in my experience the newborn mice are not very stress resistant and may die because of many interventions. Therefore, for my opinion it is questionable if it is necessary and advisable to do so many gavage feedings and handlings with the animals. There are no comments of analgesic therapy for the mice pups during the NEC protocol.

Did you give them any analgesia? If yes, what and in which way?

Thank you for your question. During our experience the newborn mice are not very stress resistant and may die. Therefore, in order to prevent the mice from dying due to low body temperature, we put the mice in the incubator when not operating. Regarding gavage, our purpose is mainly to provide energy for mice. Our team has mastered the gavage operation, and we use a specialized gavage device（Fig. A）, the gastric plastic tube that enters the esophagus and stomach is only 0.3mm in diameter, and well-trained gavage does not lead to fatal harm to mice. In terms of analgesia, we use isoflurane for euthanasia when drawing materials.

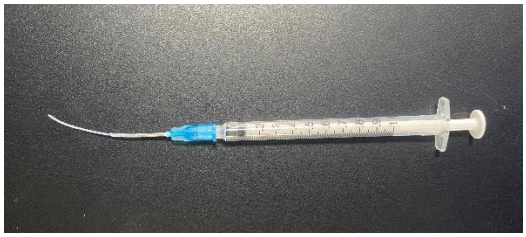


Fig. A

Did analyze any other signs of NEC severity as inflammation markers, markers of apoptosis etc? Your are recommending the described protocol for Th cell research in NEC, but you did not show what happens to the TH cells during your protocol, and discuss this with the findings of Th cell research in humans with NEC. For my opinion you should show and proof that your protocol is really useful for th cell research in NEC not just using mice with Th cell polarization.

First of all, thanks to the review experts for their questions in assessing the severity of NEC. The purpose of our research is to use BALB/c neonatal mice to induce NEC. According to other studies, whether the NEC model is successful is based on the Nadler pathological score of intestinal tissue. For example, the scoring standards used in the research of Steven X. Ch (doi.org/10.1038/s41467-020-19400-w) and Melissa D. Halpern (doi:10.1152/ajpgi. 00168.2007) et al. If the score is ≥2 scores, the model is successful. And the higher the score, the more serious the intestinal damage. In addition, our team will conduct research on TH cells in future research, and we discuss this in our Discussion section.

Minor Concerns:
You explain that all mice died until day 13. Did you euthanize the mice at a defined endpoint or didi you use characteristics for deciding of euthanisation? Or did the mice die spontaneously, and if the do so, how many time was between dead and tissue collection? Did you also use tissue of mice, which you found dead in the incubator? Please describe this more precisely.

Thank you for your question. We explain that all mice died until day 13, which were not collected tissue and died spontaneously. This is to make the survival curve of spontaneous death of mice.

The results of this experiment are shown in Fig.4C in the manuscript submitted for the first time, but in the revised manuscript we have included Supplementary Fig. 1.
In addition, NEC was induced for 5 days. The mice were euthanized and collected sample when they appeared symptoms, such as ileus, hematochezia, diarrhea and so. The results of this experiment are shown in Fig.4C in the reworked manuscript.

Reviewer #3:
Manuscript Summary:
Thank you for inviting me to review the manuscript titled " A neonatal BALB/c mouse model of necrotizing enterocolitis by Yan Tian et al. The authors in this manuscript aimed to optimize a NEC model using neonatal BALB/c mice proposing more clinical relevance due to polarization of TH2 cells during infection in this particular strain compared to the more commonly used C57BL/6J mice. The authors used the more typical method for developing the intestinal injury with the combination of formula feeding, recurrent hypoxia, and hypothermia in association with LPS. The authors demonstrated histological injury in some animals suggestive of NEC-like intestinal injury in the model. The protocol is relatively well written, but there are some areas that need clarification especially if the goal is to provide a protocol that will be replicated by other investigators.

Thanks for the reviewer's positive comments.

Major Concerns:
1- One of the major concerns is the unclear duration and outcome of the results. The authors noted a range for volume of feeds of 40-50 ul/feed throughout the duration of the experiment. Generally the volume of feeds is increased in other similar models per day (~ 50ul/gram body weight) adjusted on daily basis. Is there a reason the authors stayed at the same volume? would this play a role in the lack of weight gain in the animals?

Thanks for your question. According to the growth energy requirements of normal mice, it is necessary to increase the feeding amount daily according to what you said. However, mice that have been induced to NEC will appear intestinal symptoms of NEC over time. At this time, their feeding volume cannot increase day by day. This is similar to the feeding of clinical children with NEC, and children with NEC will fast or reduce feeding energy when they appear symptoms of NEC.

2- The authors noted 4-5 days of experimentation and "maybe longer". In the results section, the duration was up to 8 days total where all the animals died at the end. What would the authors suggest for others to use for optimal results? 8 days of feeding is a long period of time especially with feeding throughout the day. Generally in other similar models, the duration is 4 days total after which surviving animals are euthanized.

Thank you for your questions. We explain that the duration was up to 8 days total where all the animals died at the end, w*hich were not collected tissue and died spontaneously. This is to make*

the survival curve of spontaneous death of mice. The results of this experiment are shown in Fig.4C in the manuscript submitted for the first time, but in the reworked manuscript we have included Supplementary Fig. 1.
In addition, we also induced NEC for 5 days, similar to models in other studies. When the NEC symptoms appeared, such as ileus, hematochezia, diarrhea and so on. (doi.org/10.1038/s41467-020-19400-w), the mice were euthanized and collected tissue. The results of this experiment are shown in Fig.4C in the reworked manuscript.

3- The authors show bar graph figure for histological injury. At what point where the tissues collected? If all the animals died at day 13, where the tissues collected after the animals died? Generally in the NEC model, tissues are collected from the surviving pups, otherwise the histological injury will be variable and based on the day of death. This would lead to inability to replicate the results especially when looking at preventative approaches.
Shown in Supplementary Fig 1, the survival curve that the mice in the NEC group die spontaneously, all the animals died at day 13, which were not collect tissues.

Thank you for your questions. As you asked in our answer to question 2, mice that have induced NEC for 5 days are used to collect tissues for H&E staining, *and tissues are collected from the surviving pups when they appeared NEC symptoms. In order to make the survival curve that the mice in the NEC group die spontaneously, extend the NEC induction time until all mice die, which were not collect tissues and until day13 all the mice died, shown in Supplementary Fig 1.*

4- The authors at the end mentioned "success rate is not very high" but in the results all the animal died in the experimental NEC. How do they suggest optimization? and where they noting histological injury? On that note, please provide incidence of NEC in the model.

Thanks for your question. Regarding the three points of this question, the first point has been explained in questions 2 and 3. Regarding the optimization of the induced NEC model, we plan to adjusted the oxygen concentration in the later stage, from 5% O2 in our study to 1% in Yu-Mei He's studies(doi:10.1038/nm.4467). We have provided incidence of NEC in the model in the revised manuscript, which was 38-50%.
Minor Concerns:

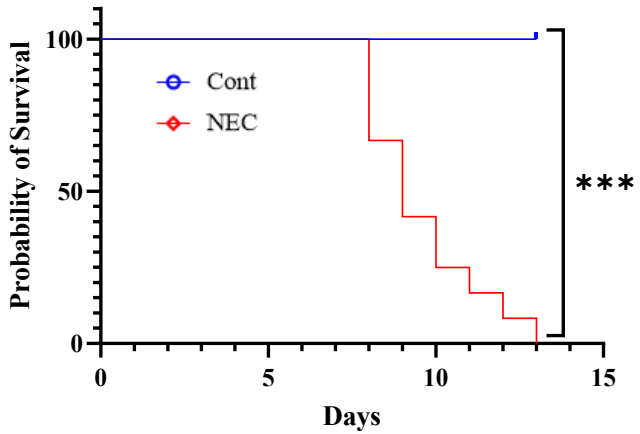
1- I'm still not convinced that using BALB/c mice is superior to C57/B6J despite the justification of the authors. If genetic modulation is needed then the later background is more relevant.

Different strains of mice have their advantages when used to induce NEC models. In our study, BALB/c mice are used to induce NEC mainly because they have an advantage in T helper cells.

2- The stool scoring will only provide more subjective data that will be difficult to standardize. It would have been more helpful if occult or frank blood was the outcome

from the stool scoring instead of their approach.

We agree with your opinion. Stool score is used to help explain intestinal dysfunction in NEC mice, and stool score is the average of three people scored independently.



Editing Certificate

This document certifies that the manuscript

A Neonatal BALB/c Mouse Model of Necrotizing Enterocolitis

prepared by the authors

Yan Tian1*, Junyu Huang1*, Ming Fu1, Qiuming He1, Jiale Chen1, Yan Chen1, Ruizhong Zhang1, Wei Zhong1

was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at AJE.

This certificate was issued on **November 9, 2021** and may be verified on the AJE website using the verification code **F8A4-86D1-C448-5539-A37C** .

Neither the research content nor the authors' intentions were altered in any way during the editing process. Documents receiving this certification should be English-ready for publication; however, the author has the ability to accept or reject our suggestions and changes. To verify the final AJE edited version, please visit our verification page at aje.com/certificate. If you have any questions or concerns about this edited document, please contact AJE at support@aje.com.