## Original Article
# Characterization of a necrotizing enterocolitis model in newborn mice

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**Abstract:** Background: Necrotizing enterocolitis (NEC) is a major health concern for premature infants and its pathogenesis remains poorly understood. The current mouse NEC model has not well been characterized. Objectives: In this study, we develop a simple mouse model of NEC and determine the role of several factors modulating human NEC (i.e., breast milk, birth weight, cesarean section and bacteria) on intestinal injury. Methods: In a first experiment, pups born naturally and dam fed for <12 hours were gavaged with adult commensal bacteria or *E. Fecalis*, and exposed to hypoxia-cold stress-formula feeding, and compared with controls without bacteria inoculation. 72-hour mortality was recorded, and small intestines were examined histologically. In a second experiment, we compared the incidence of NEC in mice dam fed for <12 hours to those dam fed for 12 to 24 hours or delivered by cesarean section prior to being submitted to the NEC protocol. Results: In pups inoculated with $10^7$ CFU of a standardized preparation of adult commensal bacteria or $10^5$ CFU of *E. Fecalis*, the incidence of severe NEC (≥grade 2) was 70% and 37% respectively vs 6% in the controls (no bacteria)(p<0.05). In pups dam fed for 12 to 24 hours, NEC incidence was 44(±12)% lower vs those dam fed less than 12 hours (p<0.05). We did not find any difference in the NEC incidence between naturally-born pups dam fed for less than 12 hours and these born by cesarean section. The incidence of severe NEC was higher in pups with low birth weight. Conclusions: we have simplified and characterized a neonatal mouse NEC model that shares several risk factors with human NEC. Now that transgenic mice are available, this model will be useful to study the role played by specific proteins in vivo in NEC development.

**Keywords:** Necrotizing enterocolitis, animal model, mouse, intestine

## Introduction

Necrotizing enterocolitis (NEC) is a common disease affecting about 7-9 % of very low birth weight infants (<1500 g) [1,2], and it is associated with great morbidity and mortality [3, 4]. Major risk factors have been shown to contribute to the development of human NEC such as prematurity, abnormal intestinal bacterial colonization, formula feeding and intestinal ischemia [3]. Due to incomplete understanding of the pathogenesis of NEC, no specific treatment is currently available, and further research is desperately needed. Studies based on surgical specimens from infants with NEC have been of limited value, because these resected tissues are necrotic and show non-specific inflammatory changes. Therefore, they are not suited for

studying the early events leading to NEC. For this reason, *in vivo* animal models must be employed to study the disease.

Several animal models have been used to study NEC, such as a model of acute bowel injury induced by the intravascular administration to young adult rats [5] and mice [6] of platelet-activating factor (PAF), a potent pro-inflammatory phospholipid that has been shown to play a role in NEC, or the intestinal injury induced by the perfusion of isolated rabbit loops with fatty acids solution [7]. However, these models do not take into account the developmental differences specific to the neonatal period and to prematurity. In an attempt to do so, other models were developed in neonatal piglets such as a model of NEC induced by hypoxia

[8] or by mesenteric ischemia [9,10].

A neonatal rat NEC model induced by hypoxia, cold stress and formula feeding, three contributing factors of the human disease, is currently widely used by investigators to gain insight into the pathogenesis of NEC [11-15]. Compared to rabbits and pigs, rats are easily available and easy to breed. However, while the rat NEC model allows studying the effects of pharmacological interventions [13,16], its use is of limited value because it does not allow "in-depth" mechanistic studies due to the lack of genetically-manipulated rats. Recently, a neonatal mouse NEC model in which neonatal mice are delivered by cesarean section, formula fed and exposed to cold stress-hypoxia twice daily has been developed [17]. However, cesarean section is associated with a higher mortality not related to NEC amongst neonatal mice. Therefore, our laboratory has optimized the model by using naturally delivered mouse pups. Our model consists of inoculating the pups one time with $10^7$ CFU of a standardized preparation of adult commensal bacteria at the entry into the NEC protocol, gavaging them with formula every three hours and exposing them to hypoxia-cold stress twice a day. We found that this modified model produces a consistent rate of NEC while allowing control for genetic background in biochemical studies. Using this neonatal mouse NEC model that our lab developed, we investigated the effect of breast milk exposure and birth weight on the incidence of histological bowel injury. One shortcoming of this model is that a mixed commensal bacterial preparation derived from adult mice might not be consistently reproducible in causing NEC. Thus, we attempted to further simplify the model by replacing the mixed bacterial preparation with *E. fecalis* alone.

## Materials and methods

### Animal model of NEC

This study was approved by the Children's Memorial Research Center (CMRC) Institutional Animal Care and Use Committee. C57BL/6 male and female mice were housed in our barrier facility. Time-pregnancies were induced by overnight mating. 18 days later, pups were divided into 3 groups: Group 1: Pups not yet delivered were delivered by cesarean section on day 18 (term, 18-19 days); group 2 had been delivered naturally and had been with dams for less than 12 hours; group 3: had been delivered naturally and dam fed for 12 to 24 hours (group 3).

In group 1, dams were euthanized in a $CO_2$ chamber for 60 seconds followed immediately by cardiotomy and cesarean section. Neonatal mouse pups (5-8 per litter) were dried and placed in a pre-heated (37°C) and humidified neonatal incubator (air-Shield Vickers Medical, Hatboro, PA). Pups delivered by cesarean section were allowed to recover for an hour. Naturally delivered pups were placed in the incubator for an hour prior to the experiment. Pups were weighed, and at the beginning of the NEC protocol, they were gavaged once with 0, $1 \times 10^6$ or $1 \times 10^7$ colony forming units (CFU) of a standardized adult commensal bacterial mixture or $1 \times 10^5$ CFU *E. fecalis* diluted in 30 µl of 0.9% NaCl. Residual bacterial preparations were frozen at -80°C for bacterial characterization (PCR and DGGE analysis).

The standardized bacterial mixture was prepared from adult commensal bacteria as we previously described for our neonatal rat NEC model [16]: The caecum content of three healthy adult mice from our barrier facility was cultured overnight at 37°C in regular bacterial culture media (1:1)(LB broth base, GIBCO, Invitrogen). Following centrifugation, bacteria were suspended in 50% glycerol-bacterial culture medium, and multiple aliquots frozen at -80°C. The day prior to each experiment, a bacterial aliquot was thawed, cultured in standard bacteria culture medium for 16 hours at 37°C (with shaking at approximately 140 rpm), and diluted to the appropriate concentration.

The *E. fecalis* used in the study was isolated from the adult mouse commensal bacterial preparation. The phenotypic identification was performed using routine clinical microbiology testing methods for clinical samples at Children's Memorial Hospital Clinical Microbiology Laboratory. Biochemical reactions and susceptibility testing were measured with the gram positive combo panel 21 on MicroScan® Walkaway instrument (Siemens Healthcare Diagnostics, Deerfield, IL).

Neonatal mice were fed every three hours with 33% Esbilac formula (volume of 30, 40 and 50 µl on day 1, 2 and 3 respectively) by oro-gastric gavage using a 1.9F silastic catheter (**Figure 1**) beginning one hour after bacterial inoculation.
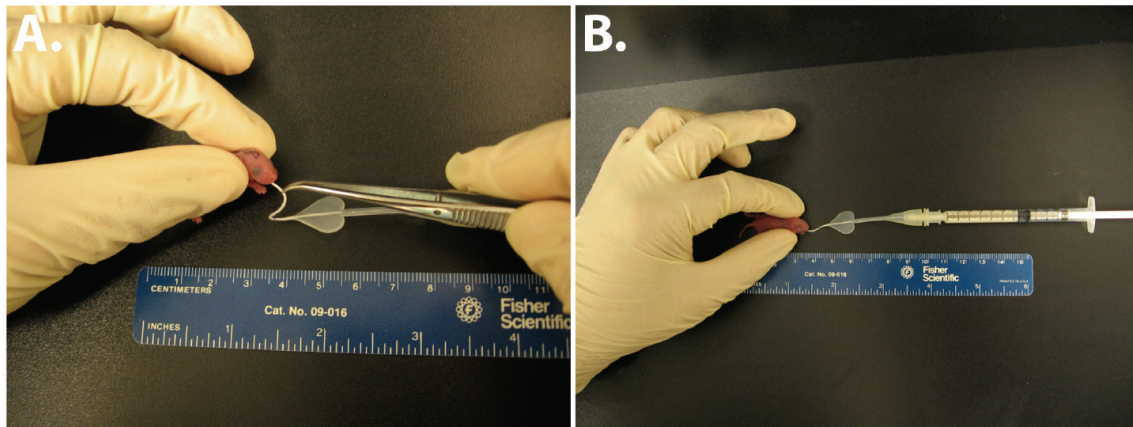
**Figure 1.** Neonatal pup gavage: Neonatal mice were fed 33% Esbilac formula by oro-gastric gavage using a 1.9F silastic catheter. Picture showing (**A**) the introduction of a silastic catheter in a neonatal mouse esophagus for gavage feeding. (**B**) Neonatal mouse being gavage fed with formula.

Pups were exposed to brief episodes of asphyxia (60 seconds in 100% $N_2$) followed by cold stress (4°C for 10 minutes) twice daily. Animals were observed closely for clinical signs of NEC such as severe abdominal distension, apnea, cyanosis, and lethargy. At signs of illness or at the end of the experimental period (72 hrs), animals were euthanized by decapitation and their small intestines collected and fixed in 10% buffered formalin for histological examination. Time of death was documented.

*DNA preparation*

Residual bacterial inoculates from five independent experiments were thawed and suspended in 1 mL of tris/EDTA (TE, pH 8.0 Ambion, Austin, TX), 150 µl tris saturated phenol and combined with 200 µl of 0.1 mm glass beads. Samples were vortexed for 15 minutes, and then placed at 0°C for five minutes. This was repeated twice and samples were centrifuged at 11,000 RPM for five minutes at room temperature. The bacterial DNA was extracted using phenol chloroform (PC). Two PC extractions were performed at room temperature, with centrifugation for five minutes at 11,000 RPM and the supernatant was combined with 100% ethanol and sodium acetate. The DNA was precipitated in an ice bath and centrifuged for five minutes at 11,000 RPM and room temperature. The pellet was washed with 70% ethanol, centrifuged again and then re-suspended in TE.

*PCR*

DNA samples underwent a polymerase chain reaction (PCR) to replicate 16s ribosomal DNA. A master mix of 4 µL/sample deoxyribonucleic acid nucleotides, 5 µL/sample 10X buffer, 0.25 µL/sample Hotstart Taq™ polymerase (Takara Bio, Otsu, Japan), 1 µL/sample of both forward and reverse primers, and sterile water 8.75 µL/sample was prepared. The forward primer used contained a GC clamp to allow greater stability during gel electrophoresis. The forward primer sequence was 5'CGCCCGCCGCGCGCGGCGGGC GGGGGGGGGCACGGGGGGGCCTACGGGAGGCAG-CAG 3' and the reverse primer sequence was 5'ATTACCGCGGCTGCTGG 3' (Invitrogen, Carlsbad, CA). 20 µL/sample of master mix was combined with 150 nanograms of DNA in water to a total reaction volume of 50 µL. PCR was performed utilizing a Perkins-Elmer Gene Amp PCR System 2400. The following conditions were used; an initial denaturation at 95° C for five minutes followed by: 20 cycles at 95°C for one minute, annealing for 45 seconds, and at 72°C for one minute. Initial annealing temperature was 65°C; this was ramped down 0.5°C per cycle over the 20 cycles. Following these initial 20 cycles, 10 additional cycles at 95° C for one minute, 55°C for 45 seconds, and 72°C for one minute were performed. A final step at 72°C for five minutes was performed followed by storage at 4° C. If the samples were not used immediately they were stored at -20°C.

*Denaturing gradient gel electrophoresis (DGGE)*

A Bio-Rad DCode™ system (Bio-Rad Laboratories, Hercules, CA) was used to perform DGGE. Solutions of 35% urea (20 mL 40% acrylamide, 2 ml of 50x tris/acetic acid/EDTA buffer [TAE,

Bio-Rad laboratories, Hercules, CA], 14.7 g urea, 14 mL of deionized formamide, and deionized water to make total volume of 100 mL) and 60% urea (20 mL 40% acrylamide, 2 mL of 50x TAE, 25.2 g urea, 24 mL of deionized formamide, and deionized water to make total volume of 100mL) were made. Sixteen μL of tetra-methyl-ethylenediamine (Temed, Bio-Rad laboratories, Hercules, CA) and 100 μL of 0.1 gram/ml ammonium persulfate (Bio-Rad laboratories, Hercules, CA) were combined with 16 mL of each stock solution. A Bio-Rad Gradient Delivery System (Model 475, Bio-Rad Laboratories, Hercules, CA) was used to make the gradient. The gel was allowed to polymerize for two hours, and then loaded with a combination of 25 μL of the PCR product and 25 μL of double strength loading buffer. Individual gels ran overnight in 1X TAE (Bio-Rad laboratories, Hercules, CA) at 58° C and 60 volts. The gel was stained with ethidium bromide (Bio-Rad Laboratories, Hercules, CA), visualized and imaged under ultraviolet light.

*16 s ribosomal sequence analysis*

After performing the DGGE, the individual bands were excised and stored in small tubes at -20° C. The bands were crushed and soaked to extract the DNA using 50 ml of DNA-free water and frozen at -20°C overnight. The samples were centrifuged at 10,000 RPM for five minutes, and the amount of DNA was quantified.

After determining the DNA concentration, the sample once again underwent PCR with the same primer as mentioned above but had the M13 vector sequences added to the end of the primer to permit sequences. Following PCR, the product was diluted to 200 ng/μL and sent to the Heflin Center for Human Genetics for sequencing. After obtaining the sequences, they were uploaded to the Ribosomal Database Project for identification (http://rdp.cme.msu.edu).

*Histological analysis*

The entire small intestine was embedded in paraffin, sectioned, and stained with hematoxylin-eosin. With the NEC protocol described above, pups developed intestinal histological injury within 72 hours with various degrees of severity (**Figure 2**). The following scoring system (from grade 0 to 4) was developed to describe
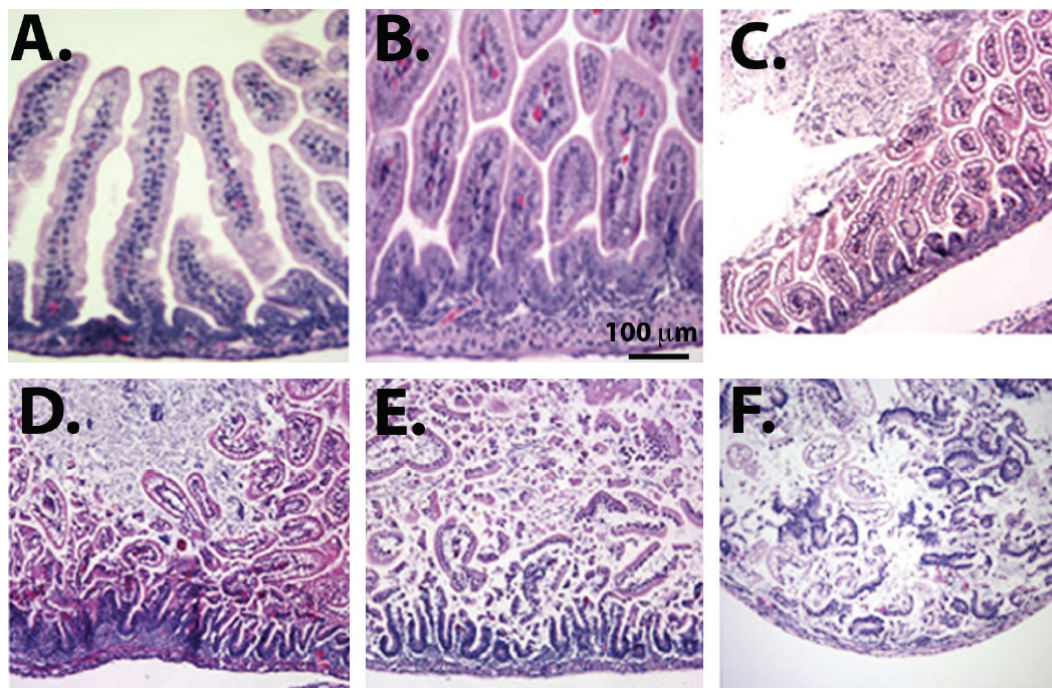


**Figure 2.** Histological grading of NEC severity in the neonatal mouse NEC model. Neonatal mice were submitted to the NEC protocol and euthanized when presenting signs of distress. Tissues were fixed overnight in 10% formalin and stained with hematoxylin-eosin. (**A**) Dam-fed control; (**B**) grade 0: intact villi; (**C**) grade 1 NEC: sloughing of the epithelial cells at the tip of the villi; (**D**) grade 2 NEC: mid-villous necrosis; (**E**) grade 3 NEC: complete villous necrosis; (**F**) grade 4 NEC: transmural necrosis. Mild NEC is limited to the tip of the villi while severe NEC involves whole villi.

the severity of NEC: Grade 0: intact villi; 1: superficial epithelial cell sloughing; 2: mid-villous necrosis; 3: complete villous necrosis; 4: transmural necrosis (**Figure 2**). A histological score was assigned to each specimen by an investigator blinded to the groups based on the area of worst injury (**Figure 2**). Severe NEC was defined as histological change of grade 2 or more.

*Statistical analysis*

Two-sided Student t-test was used for the comparison of two single groups and two-way analysis of variance (ANOVA) for multiple group comparisons. To evaluate the differences in the incidence and severity of NEC, and in 72-hour survival between two groups, $\chi^2$ analysis was used to compare results.

## Results

*Inoculation of adult commensal bacteria increases the incidence of severe NEC in neonatal mice*

When only hypoxia-cold stress-formula feedings were used in neonatal mice, we observed a low incidence of mild NEC (16 to 50% of grade I) (**Figures 3 A-B**). In an attempt to optimize the model, we examined whether adult commensal bacteria inoculated shortly after birth would increase the incidence of NEC. A standardized bacterial mixture was prepared from healthy adult mouse caecums (see methodology section) as previously described in a neonatal rat NEC model [16] and multiple aliquots were frozen at -80°C. In a pilot study, we found that 70% of pups inoculated with $1 \times 10^7$ bacteria prior to being placed into the NEC protocol developed severe NEC (grade 2 or more) (7/10), while none of the control pups did (0/9) ($\chi^2$=4.89, p<0.05). Pups inoculated with $1 \times 10^6$ bacteria had a 55% incidence of severe NEC (5/9) (**Figure 3A**). Therefore, we have included the inoculation of $1 \times 10^7$ adult commensal bacteria in our neonatal mouse NEC model.

To rule out inter-experimental variations in bacterial content, DGGE of residual bacterial preparations from five independent experiments done several months apart was performed to evaluate the microbial populations in different samples (**Figure 3C**). Lanes 1, 2, and 9 were 1 kilobase pair DNA ladder. Lane 3 was a water control containing no DNA. Lanes 4-8 showed the

PCR products of 16s ribosomal DNA amplification. While there are differences in intensity of some bands, the populations of each of the samples showed marked similarity, confirming that the bacterial inoculation of the mouse pups was uniform. Band identification and homology was performed using Gel Compar II software (Applied Maths, Austin, TX). There was a 92.5% homology between the five samples. Twelve distinct bands were observed following DGGE. Using sequence analysis, we identified 12 individual organisms based on previous sequences reported in the Ribosomal Database Project (**Table 1**). All five samples contained *E. fecalis.*

*Inoculation of E. fecalis ($10^5$ CFU) produces an incidence of NEC similar to that of adult commensal bacteria preparation ($10^7$ CFU)*

The bacterial preparation was studied with aerobic (5% sheep blood agar and McConkey agar plates) and anaerobic (selective and non-selective medium) cultures. Many colonies of *E. fecalis* grew under aerobic conditions. *E. fecalis* was isolated from the preparation and its purity was examined based on colony morphology and confirmed by DGGE analysis (single band) and PCR/sequencing as 15 independent colonies were sequenced for the 16S ribosomal RNA and revealed to be *E. fecalis.* In an attempt to simplify the model, we examined whether *E. fecalis* alone isolated from the preparation would be able to induce intestinal injury in the model. When pups were inoculated with $10^5$ CFU of *E. fecalis*, we observed an incidence of severe NEC of about 37% when exposed to hypoxia-cold stress-formula feedings vs 6% in the control group without bacteria inoculation ($c^2$=4.98; p<0.05) (**Figure 3B**).

*The intestinal injury mimics human NEC in a neonatal mouse model*

When neonatal mice were inoculated with $10^7$ CFU of a standardized mixture of adult commensal bacteria or with $10^5$ CFU of *E. fecalis* and submitted to hypoxia-cold stress-formula feedings, they consistently developed various degrees of histological intestinal injury (**Figure 2**). In mice with severe NEC, the histological lesions found (**Figure 4D**) were similar to the lesions found in surgical specimen of infants with NEC (**Figure 4B**). These were characterized by transmural coagulative necrosis with villous sloughing.
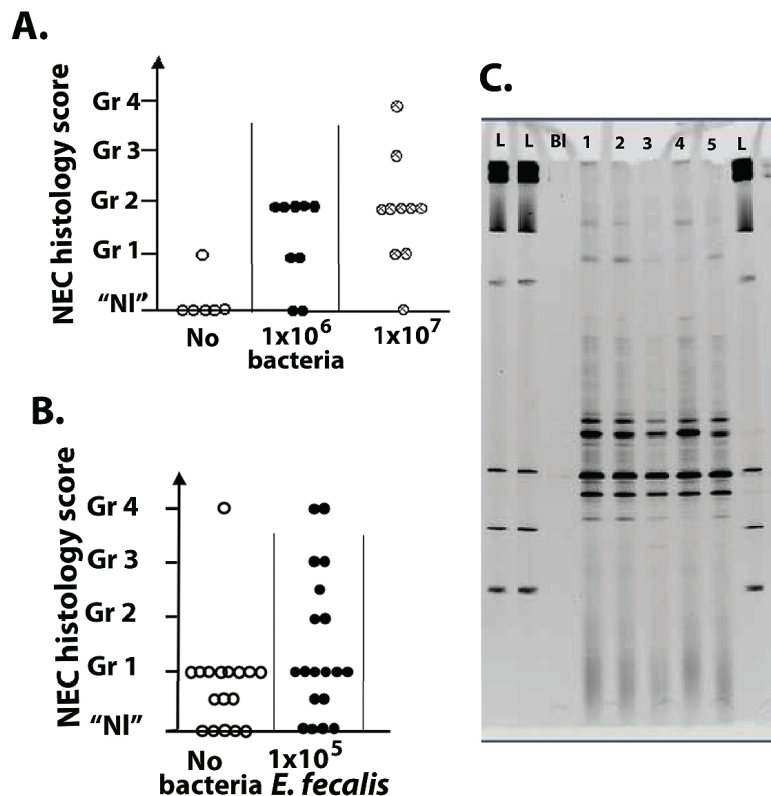
**Figure 3.** Inoculation of adult commensal bacteria or of *E. fecalis* increases the incidence of NEC: (**A**) Pups born naturally and dam fed for less than 12 hours were divided in 3 groups: Group 1 received no bacteria, group 2 was inoculated with 1x10⁶ bacteria and Group 3 with 1x 10⁷ bacteria. All groups were submitted to the NEC protocol. We found an increased incidence of NEC in animals inoculated with 1x 10⁷ bacteria compared to controls. (**B**) Pups born naturally and dam fed for less than 12 hrs were divided into two groups: Group 1 received no bacteria and group 2 was inoculated with 1x10⁵ CFU *E. fecalis*. All groups were submitted to the NEC protocol. We found an increased incidence of NEC in animals inoculated with 1x 10⁵ *E. fecalis* compared to controls. (**C**) When using adult commensal bacteria, similar bacteria were identified amongst bacterial inoculums of independent experiments. DNA was extracted from bacterial preparations (five independent experiments) and 16S ribosomal DNA amplified by PCR. PCR products were run on a denaturing gradient gel electrophoresis (DGGE). The 5 lanes were from the 16s ribosomal DNA amplification of our five bacterial preparations, showing five separate PCR products. Pearson similarity coefficients were calculated between the lanes. The similarity tree showed >92% similarity between individual lanes.

**Table 1.** Bacterial composition of bacteria preparation given to neonatal mice. The order of the organisms in the **Table** reflect the relative density of the bands (with *E. fecalis* being the dominant species)

*Enterococcus fecalis*
*Escherichia coli*
*Enterococcus casseliflavus*
*Escherichia vulneris*
*Enterobacter asburiae*
*Sporosarcina macmurdoensis*
*Streptococcus hyointestinalis*
*Enterococcus canintestini*
*Alicyclobacillus sendaiensis*
*Inquilinus limosus*
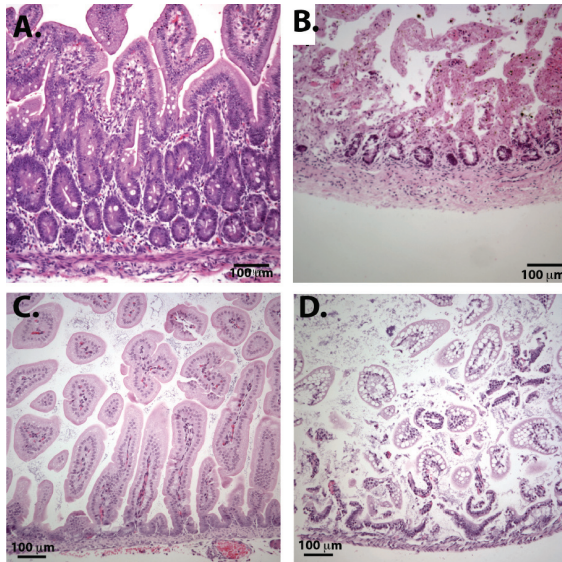*Mannheimia glucosida*
*Clostridium perfringens*

**Figure 4.** Intestinal microscopic changes of mice submitted to the NEC protocol and of human NEC. H&E staining of (A) human control small intestine; (B) human NEC (10 day old 28 week premature infant); (C) 2-day-old dam-fed mouse; (D) 2-day-old mouse with experimental NEC. The histological lesions in the neonatal mouse NEC model resemble human NEC. Note the transmural coagulative (ischemic) necrosis with complete loss of villi. The inflammatory cell infiltration is minimal. Human tissue collection was approved by the hospital internal review board committee.

*More than 12 hours exposure to breastfeeding decreases the incidence of NEC*

To assess the role of breast milk on the incidence of NEC, mice were delivered by cesarean section (group 1) (n=50), allowed to deliver naturally and to be dam fed for <12 hours (group 2) (n=41) or for 12 to 24 hours (group 3) (n=28) before being separated from the dams (**Figure 5A**). These three groups were then placed on the NEC protocol (adult commensal bacteria inoculation/hypoxia and cold stress/formula feeding).

We observed an early mortality (<24 hours) of 19/50 (38%), 10/41 (24%) and 2/28 (7%) in groups 1, 2 and 3 respectively. Since NEC-like histology does not develop typically before 24 hrs of age, we excluded these pups in our analysis on the incidence of histological NEC, as others have previously published [17]. We found that breastfeeding for more than 12 hours protected mice against NEC, as we observed a 32(±12)% increase in survival (p<0.05)(**Table 2**) and a 43(±12)% (χ²=4.88, p<0.05) decreased incidence of NEC in these animals (**Figure 5B**) when compared to breastfeeding for less than 12 hours. We did not find a statistically significant difference in survival and in the incidence of severe NEC in the naturally born pups allowed to breastfeed for less than 12 hrs compared to the ones delivered by ce-
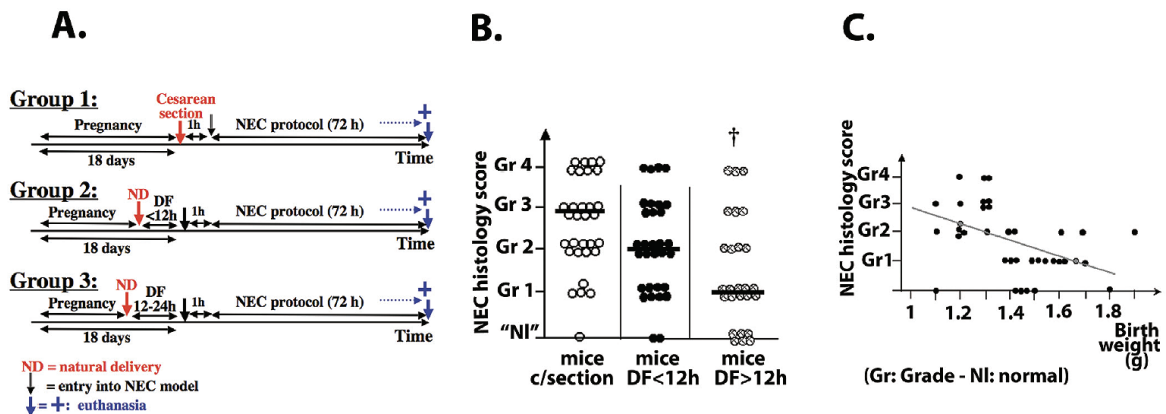


**Figure 5.** (A) More than 12 hours exposure to breastfeeding decreases the incidence of NEC. To assess the role of breastfeeding on the incidence of NEC, mice were delivered by cesarean section (group 1), allowed to deliver naturally and to be dam fed for <12 hours (group 2) or for 12 to 24 hours (group 3) before being separated from the dams. (B) Mice delivered naturally and dam fed (DF) for > 12 hours have a decreased incidence of severe NEC (grade 2 or more) compared to those dam fed for <12 hours or those delivered by cesarean (c/s) section. Histological grades of NEC. (C) Severe NEC was associated with low birth weight in a neonatal mouse model: Mouse pups were delivered naturally and dam fed for <12 hrs. They were then submitted to the NEC protocol and sacrificed when presenting signs of distress. Histological grading of NEC was assigned by an investigator blinded to the group, and correlated to birth weight.

**Table 2.** Survival of mice without or with severe NEC ($\geq$ grade 2) in the three groups

| Survival | Cesarean section (n=50) | DF < 12 hours (n=41) | DF >12 hours (n=28) |
|---|---|---|---|
| Mean (±SEM) | 33.6 h (±2.17) | 34 h (±2.399) | 45 h (±4) |
| Median (95% CI) | 32.2 h (29-38) | 32.7 h (29-39) | 41.2 h* (37-54) |

NOTE. Mice were delivered by cesarean section (group 1), allowed to deliver naturally and be dam fed for <12 hours (group 2) or for 12 to 24 hours (group 3) before being separated from the dams. These three groups were then submitted to the NEC protocol (commensal bacteria inoculation-hypoxia-cold stress-formula feeding).
DF: Dam fed. *:$p<0.05$. Survival of mice delivered naturally and dam fed for 12 to 24 hours (>12 h) is increased compared to those dam fed for <12 hours or those delivered by cesarean section.

sarean section.

*The incidence of severe NEC is higher in low birth weight mice*

In an experiment where naturally-delivered pups were allowed to breastfeed for less than 12 hours and submitted to the NEC model, we found that mice with a birth weight of less than 1.4 g had a 93% incidence of severe NEC (15/16) while those with a birth weight equal or superior to 1.4 g had a 24% incidence of severe NEC (5/21) ($\chi^2=17.6$, $p<0.0001$) (**Figure 5C**).

## Discussion

Until recently, there was no suitable neonatal mouse model of NEC available and investigators were limited to using young mouse models of acute intestinal injury, such as the ones induced by ischemia/reperfusion [18] or by PAF [6]. However, these models do not take into account the developmental differences characteristic of the premature neonate at risk for NEC. Recently, a neonatal mouse NEC model has been developed [17] in which neonatal pups are delivered by cesarean section and exposed to formula feeding, cold stress and hypoxia. This model helped identify TLR4 as playing a role in NEC [17]. In our lab, we observed a variable incidence of NEC in the model and a high mortality rate associated with cesarean section birth in mouse pups. Therefore, we have modified the model in our laboratory. In this study, we characterized a neonatal mouse model of NEC and determined whether bacteria, breast milk and low birth weight, factors associated with human NEC [19-21] modulate the incidence of NEC in our model. We found that: 1) the administration of adult commensal bacteria

increased the incidence of NEC and made the model more reproducible; 2) when a strictly standardized procedure was used to prepare bacteria inoculums, there was minimal inter-experimental variation in bacterial content from inoculums used in experiments performed several months apart; 3) naturally-delivered pups dam fed for 12 hours or more had a decreased incidence of severe NEC compared to those breastfed for less than 12 hours and to those born by cesarean section and never dam fed; and 4) the rate of severe NEC was higher in mice with low birth weight. We did not find a protective effect of vaginal delivery compared to cesarean section on the incidence of severe NEC in our neonatal NEC model.

While dispensable for the induction of NEC [22,23], bacteria inoculation has been shown to increase the incidence of NEC in a neonatal rat model [12,17] and therefore, the inoculation of single species of specific bacteria such as *E. coli* [12,24,25] and *Klebsiella* [11,17] has been commonly used in NEC models. Other investigators have used enteral administration of lipopolysaccharide to optimize the model [14]. Here, we show that a preparation of adult commensal bacteria maximizes the incidence of NEC in a neonatal mouse NEC model, and the bacterial preparation remains consistent from experiment to experiment, as the same strains could be identified in independent inoculums. As opposed to the inoculation of single species of specific bacteria, which may lead to single species bacterial overgrowth, this model might better mimic what may happen during intestinal colonization at birth as many species of bacteria are present in the environment. However, the use of a mixed bacterial preparation could jeopardize the reproducibility of the model in the

future. We now show that the model can be simplified by using *E. fecalis* $10^5$ CFU, which also produces a significant incidence of NEC. In a small study, *E. fecalis* has been found to be detected in the stools of infants who developed NEC, but in none of the controls (Pisharody U., personal communication). In a recent study, an increased frequency of *Enterococcus*-like sequences has been detected in the stools of premature infants with NEC compared to controls using 16S ribosomal RNA sequence analysis [26].

In the traditional model [17], dams need to be sacrificed during cesarean section. Therefore, dam-fed control pups used in biochemical studies have to be obtained from different litters and dams cannot be used more than once to produce litters, which is inconvenient when working with expensive genetically manipulated mice. In addition, we observed an increase in immediate neonatal mortality when neonatal mice were delivered by cesarean section that could confound the interpretation of our data. The early mortality noted within 24 h of entry into the NEC model could be due to other causes of perinatal morbidities than NEC, such as respiratory distress syndrome, apnea and infection. In this study, we found an incidence of severe NEC of about 70% in naturally-born pups allowed to breastfeed for less than 12 hrs that was similar to these delivered by cesarean section. Therefore, we do not believe that cesarean section is important in the neonatal mouse NEC model. This is consistent with what we know about human NEC, for which no studies have shown a clear association with mode of delivery [27]. As on the other hand we found a low incidence of NEC in pups allowed to be dam fed for more than 12 hrs, we have optimized our NEC model by using mice delivered naturally and dam fed less than 12 hrs. Our model has the advantage to allow natural delivery to take place and therefore minimize potentially confounding effects from differences in the cesarean section surgical procedure. Because it does not require cesarean section, it allows part of the litter to be kept with the dams, serving as dam fed controls while the other part is placed on the NEC protocol. This permits better control for genetic background in subsequent molecular studies.

Low birth weight has been found to be strongly associated with human NEC [28]. In this study,

we found that the rate of severe NEC is increased in mice with low birth weight. Therefore, when testing the effect of pharmacological interventions in the NEC model, special attention should be taken to divide litters between groups so that each experimental group not only contains pups of equivalent genetic background, but also of similar birth weight.

In conclusion, our laboratory has developed a neonatal mouse NEC model that shares several risk factors with human disease. This model uses naturally-delivered mouse pups, which are dam fed for less than 12 hours. The model consists of inoculating the pups with adult commercial bacteria or *E. fecalis* once at the entry into the NEC model, gavaging them with formula every three hours and exposing them to hypoxia-stress twice a day. Since transgenic mice are now available, we believe that our model will be a useful tool to better define and characterize the pathway leading to NEC, so novel therapies could be developed, thus improving the outcome of this deadly disease.

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### References

[1] Flidel-Rimon O, Friedman S, Lev E, Juster-Reicher A, Amitay M, and Shinwell ES: Early enteral feeding and nosocomial sepsis in very low birthweight infants. Arch Dis Child Fetal Neonatal Ed 2004; 89: 289-292.

[2] Fanaroff AA, Stoll BJ, Wright LL, Carlo WA, Ehrenkranz RA, Stark AR, Bauer CR, Donovan EF, Korones SB, Laptook AR, Lemons JA, Oh W, Papile LA, Shankaran S, Stevenson DK, Tyson JE, and Poole WK: Trends in neonatal morbidity and mortality for very low birthweight infants. Am J Obstet Gynecol 2007; 196: 147-148.

[3] Lin PW, Stoll BJ: Necrotising enterocolitis. Lancet 2006; 368: 1271-1283.

[4] Stoll BJ: Epidemiology of necrotizing enterocolitis. Clin Perinatol 1994; 21: 205-218.

[5] Gonzalez-Crussi F, Hsueh W: Experimental model

of ischemic bowel necrosis. The role of platelet-activating factor and endotoxin. Am J Pathol 1983; 112: 127-135.

[6] Sun X, Caplan MS, Liu Y, Hsueh W: Endotoxin-resistant mice are protected from PAF-induced bowel injury and death. Role of TNF, complement activation, and endogenous PAF production. Dig Dis Sci 1995; 40: 495-502.

[7] Garstin WI, Kenny BD, McAneaney D, Patterson CC, Boston VE: The role of intraluminal tension and pH in the development of necrotizing enterocolitis: an animal model. J Pediatr Surg 1987; 22: 205-207.

[8] Cohen IT, Nelson SD, Moxley RA, Hirsh MP, Counihan TC, Martin RF: Necrotizing enterocolitis in a neonatal piglet model. J Pediatr Surg 1991; 26: 598-601.

[9] Sibbons PD, Spitz L, van Velzen D: Necrotizing enterocolitis induced by local circulatory interruption in the ileum of neonatal piglets. Pediatr Pathol 1992; 12: 1-14.

[10] Crissinger KD, Burney DL, Velasquez OR, Gonzalez E: An animal model of necrotizing enterocolitis induced by infant formula and ischemia in developing piglets. Gastroenterology 1994; 106: 1215-1222.

[11] Barlow B, Santulli TV: Importance of multiple episodes of hypoxia or cold stress on the development of enterocolitis in an animal model. Surgery 1975; 77: 687-690.

[12] 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: 1017-1028.

[13] Dvorak B, Khailova L, Clark JA, Hosseini DM, Arganbright KM, Reynolds CA, Halpern MD: Comparison of epidermal growth factor and heparin-binding epidermal growth factor-like growth factor for prevention of experimental necrotizing enterocolitis. J Pediatr Gastroenterol Nutr 2008; 47: 11-18.

[14] F 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: 144-149.

[15] Zuckerbraun BS, Otterbein LE, Boyle P, Jaffe R, Upperman J, Zamora R, Ford HR: Carbon monoxide protects against the development of experimental necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 2005; 289: 607-613.

[16] De Plaen IG, Liu SX, Tian R, Neequaye I, May MJ, Han XB, Hsueh W, Jilling T, Lu J, Caplan MS: Inhibition of nuclear factor-kappaB ameliorates bowel injury and prolongs survival in a neonatal rat model of necrotizing enterocolitis. Pediatr Res 2007; 61: 716-721.

[17] Jilling T, Simon D, Lu J, Meng FJ, Li D, Schy R, Thomson RB, Soliman A, Arditi M, Caplan MS: The roles of bacteria and TLR4 in rat and murine models of necrotizing enterocolitis. J Immunol 2006; 177: 3273-3282.

[18] Souza DG, Pinho V, Soares AC, Shimizu T, Ishii S, Teixeira MM: Role of PAF receptors during intestinal ischemia and reperfusion injury. A comparative study between PAF receptor-deficient mice and PAF receptor antagonist treatment. Br J Pharmacol 2003; 139: 733-740.

[19] Krediet TG, van Lelyveld N, Vijlbrief DC, Brouwers HA, Kramer WL, Fleer A, Gerards LJ: Microbiological factors associated with neonatal necrotizing enterocolitis: protective effect of early antibiotic treatment. Acta Paediatr 2003; 92: 1180-1182.

[20] Sisk PM, Lovelady CA, Dillard RG, Gruber KJ, O'Shea TM: Early human milk feeding is associated with a lower risk of necrotizing enterocolitis in very low birth weight infants. J Perinatol 2007; 27: 428-433.

[21] Zaw W, Gagnon R, da Silva O: The risks of adverse neonatal outcome among preterm small for gestational age infants according to neonatal versus fetal growth standards. Pediatrics 2003; 111: 1273-1277.

[22] Israel EJ, Schiffrin EJ, Carter EA, Frieberg E, Walker WA: Cortisone strengthens the intestinal mucosal barrier in a rodent necrotizing enterocolitis model. Adv Exp Med Biol 1991; 310: 375-380.

[23] 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: 587-595.

[24] Caplan MS, Hedlund E, Adler L, Lickerman M, Hsueh W: The platelet-activating factor receptor antagonist WEB 2170 prevents neonatal necrotizing enterocolitis in rats. J Pediatr Gastroenterol Nutr 1997; 24: 296-301.

[25] Caplan MS, Miller-Catchpole R, Kaup S, Russell T, Lickerman M, Amer M, Xiao Y, Thomson R, Jr.: Bifidobacterial supplementation reduces the incidence of necrotizing enterocolitis in a neonatal rat model. Gastroenterology 1999; 117: 577-583.

[26] Mshvildadze M, Neu J, Shuster J, Theriaque D, Li N, Mai V: Intestinal microbial ecology in premature infants assessed with non-culture-based techniques. J Pediatr 2010; 156:20-25.

[27] Craig A, Ling LN, Beardsley DJ, Wingate-Pearse N, Walker DW, Hohimer AR, Back SA: Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. Exp Neurol 2003; 181: 231-240.

[28] Guthrie SO, Gordon PV, Thomas V, Thorp JA, Peabody J, Clark RH: Necrotizing enterocolitis among neonates in the United States. J Perinatol 2003; 23: 278-285.