Animal models of necrotizing enterocolitis

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ABSTRACT

Background Necrotizing enterocolitis (NEC) is one of the leading causes of death in premature infants. To determine the factors present in the disease that lead to increased morbidity and mortality, manipulation of variables that are shown to have a positive response has been tested using various animal models. Testing and manipulation of these variables are unwarranted in humans due to regulatory health standards.

Methods The purpose of this review is to provide an update to previous summaries that determine the significance of animal models in studying the mechanisms of NEC. A large variety of animal models including rats, mice, rabbits, piglets, nonhuman primates, and quails have been described in literature. We reviewed the reported animal models of NEC and examined the pros and cons of the various models as well as the scientific question addressed.

Results The animals used in these experiments were subject to gavage feeding, hypoxia, hypothermia, oxygen perfusion, and other methods to induce the disease state. Each of these models has been utilized to show the effects of NEC on the premature, undeveloped gut in animals to find a correlation to the disease state present in humans. We found specific advantages and disadvantages for each model.

Conclusions Recent advances in our understanding of NEC and the ongoing therapeutic strategy developments underscore the importance of animal models for this disease.

INTRODUCTION

Necrotizing enterocolitis (NEC) is a devastating disease affecting premature infants. Approximately 10% of infants born weighing less than 1500 g will develop the disease, with mortality rates for affected infants being as high as 20%–30%.1 Mechanisms leading to the development of NEC are not completely understood, which makes the study of this disease difficult.2 Although the exact pathophysiology of NEC is not fully understood, most experts agree that its etiology is multifactorial. It is felt that impaired gastrointestinal (GI) motility, intestinal barrier dysfunction, decreased digestive ability, poor circulatory regulation, intestinal microbial overgrowth, and immature immune defenses predispose premature newborns to intestinal injury leading to NEC.3,4

Naturally, development of robust animal models to aid in the study of this disease process is of critical importance. Traditionally, gavage formula fed/hypoxia/hypothermia models have been used for induction of NEC in mice, rats, and piglets. Asphyxia has been shown to hold a crucial role in the development of NEC because formula and bacterial exposure without asphyxia typically do not result in the phenotypical injury pattern seen in NEC.6 Another significant variable that has been manipulated in various models is the removal of breast milk. It is well known that breast milk contains large amounts of Immunoglobulin A (IgA), smaller amounts of Immunoglobulin G (IgG), active lymphocytes and macrophages, and specific antibodies against many types of microorganisms.5 Barlow et al6 theorized that without the passive immunity and intestinal flora control of breast milk, the enteric mucous barrier will be destroyed, leading to a pathologic cascade characterizing NEC.

To induce intestinal damage and microbial dysbiosis, pathogenic bacteria can be administered to disrupt the intestinal epithelial barrier leading to an inflammatory response and translocation of pathogens.3 Although this explains the presence of intestinal epithelial disruption that characterizes NEC, it does not explain the presence of intestinal necrosis that is seen in patients with this disease, nor can this explanation readily reconcile the observation that most patients who develop NEC do not actually have an antecedent ischemic event.6

The clinical NEC seen in different animal models resembles NEC presented in human patients. Robust animal models closely resembling human disease are critical to test biochemical pathways, cell receptors, enteral nourishment, mechanisms of direct bowel injury, and studies that cannot ethically be conducted in humans. Animal models of NEC utilizing rats, mice, nonhuman...
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primates, and piglets have been used to study the pathogenesis of NEC.

The advantages and disadvantages to use animal models are to be discussed in further detail. Because of the differences in size, genetic manipulation, cost of use, and preterm viability, each animal model can be examined to highlight the beneficial aspects of how NEC can be modeled.

ANIMAL MODELS

Rat model

The rat model has been used most commonly for NEC analysis due to the similarity to human preterm infants. NEC mainly affects premature and low birthweight neonates and is characterized by ischemic necrosis of the bowel wall frequently leading to perforation and death. As such, neonatal rats can be useful models for comparison due to their level of bowel immaturity at day 21 of gestation. One of the earliest studies to use rats concluded that the gut flora and absence of breast milk were suggested to play important roles in NEC pathogenesis. In Barlow’s model, a formula was developed which simulated rat breast milk and could be mixed with a bacterial contaminant. In this model, the pups are removed immediately after Cesarian section and are placed in an incubator, with avoidance of breast feeding. The stressors used to induce a NEC like state in the animal models includes hypoxia, hyperthermia, addition of a lipopolysaccharide (LPS) and hyperosmolar formula during the course of feeding throughout a week-long experiment (figure 1). In this model, a formula is given four times per day with an increasing caloric intake each day of the week. At the end of the protocol, or when rat pups have clinical signs of NEC, intestines are harvested and histologically graded for intestinal injury 0–4 with lesions greater than 2 being considered as NEC (figure 2).

Several laboratories have modified this formula feeding/hypoxia/hypothermia rat NEC model using additional elements to help induce NEC. One model has administered commensal bacteria found in stool, whereas others have tried different types of bacteria. Some researchers introduced Cronobacter sakazakii (CS), formerly known as Enterobacter sakazakii, a bacteria that was reported to be associated with NEC, to the formula-feeding/hypoxia rat NEC model. Rat models are an attractive option when studying NEC due to their low cost, preterm viability post-Cesarian section, and resilience to common stressors used to induce the disease. One disadvantage seen is the lack of commercial antibodies produced to target selected receptors involved in NEC. Another disadvantage is that rats are highly tolerant to different types of bacteria and it can be difficult to achieve success when attempting to induce disease. Last but not least, another major drawback is the lack of genetic diversity found in rats compared with other species of rodents.

Using this model, many promising preventative and therapeutic interventions have been tested such as administration of probiotics, growth factors, stem cells, human milk oligosaccharides, tumor necrosis factor (TNF) blockers, and various antioxidants. To date, we found more than 400 papers that have used the rat model with or without modifications from the original description.

Mouse model

One of the major challenges and limitations of various animal models is that humans have an inherently different pattern of gene expression and therefore immune response to the development of NEC. In addition to this limitation, mice are also different due to their size compared with newborn rats or piglets. However, despite these limitation, mouse models have produced greater insights into the significance of prematurity in animals who develop NEC.

Humanized mouse models have initiated an area of interest for modeling NEC through genetic manipulation. Humanized mice can be defined as animals that are genetically manipulated to express human genes. This model also relies on the use of hyperosmolar formula gavage feeds, hypoxia, and hypothermia. The addition of LPS to increase the incidence of NEC is variable across the published literature.

The most useful advantage of the mouse model comes from the ability to monitor and manipulate genetic variability and the use of transgenic animals to compare knockout (KO) mice to wild-type.
humanized mouse model is the interrogation of human tissue while examining the progression of the disease. With this ability, the complex biological processes can be appreciated better in an attempt to model human neonates. For example, KO and transgenic (TG) models have been used to test the role of different growth factors in the prevention and treatment of NEC such as epidermal growth factor (EGF) and heparin-binding EGF-like growth factor (HB-EGF).

HB-EGF TG mice were designed to specifically over-express the human HB-EGF precursor (proHB-EGF) in the intestine. Using this model, the investigators were able to demonstrate that decreased endogenous HB-EGF expression predisposed the intestines to the development of NEC and the opposite being true for HB-EGF TG mice that overexpressed HB-EGF.

The advantages of this model lie in the ability to easily breed mice, the ability to genetically alter lines, and the cost–benefit of using a smaller animal model compared with the other animal models. On the other hand, the smaller size of the pups at birth predisposes them to a increased rate of complications. The mouse pups are more difficult to handle and gavage feed with formula through an orogastric catheter compared with rats.

**Piglet model**

Compared with rodents, piglets share a high degree of anatomical, developmental, nutritional, and physiological similarity to the GI tract of humans. The piglet is therefore a good model for studies looking at pathogenesis of NEC, evaluation of specific feeding regimens, preclinical drug studies for prevention and potential therapy, as well as for the development of radiological diagnostic methods. During the neonatal period, protein deposition is very rapid, and owing to similarities of postnatal nutrition and intestinal development to humans, the piglet can be viewed as an accelerated model of postnatal growth and development. In addition, the piglet model is also appropriate for modeling other biological systems that may have an impact on the development of NEC such as liver function and metabolism due to its similarity with human systems.

Generally, with small variations, the model involves piglets that are delivered by C-section at 90% term. Following birth, preterm piglets go through a period of natural hypoxia/hypothermia, and then are formula fed to induce injury. A variation uses total parental nutrition at birth followed by a transition to enteral formula feeds on the second day of life. Many investigators have used variations of this model as outlined in table 1.

Cohen et al described a model in which newborn piglets were subjected to a hypoxic insult for 30 min and hypothermic stress (core temperature reduced to 35°C for 30 min). After 3–4 days, approximately half of the animals developed histological changes that resembled NEC. Other investigators induced NEC through a combination of cow-based formula and ischemia/reperfusion such as the model described by Crissinger et al or by means of administered isosmolar acidified casein solution into intestinal segments of 1day-old piglets.

All these reported variations of the piglet model serve to underline the importance of this model to study the pathogenesis of NEC.
Table 1  A summary of the studies using the piglet model

<table>
<thead>
<tr>
<th>Author</th>
<th>Title</th>
<th>NEC protocol</th>
<th>Gestational age (days)</th>
<th>Area of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Robinson et al</td>
<td>Prematurity reduces citrulline-arginine-nitric oxide production and precedes the onset of necrotizing enterocolitis in piglets</td>
<td>Piglets gavaged with formula, and tracer induced TPN for 42 hours, starting day 3 of life (DOL)</td>
<td>103 110 114</td>
<td>Effects of tracers including citrulline, arginine, and nitric oxide in lowering the incidence of necrotizing enterocolitis</td>
</tr>
<tr>
<td>Zamora et al</td>
<td>Low abdominal near-infrared spectroscopy values and elevated plasma intestinal fatty acid-binding protein in a premature piglet model of necrotizing enterocolitis</td>
<td>Piglets gavaged with 3–5 mL/kg/h of infant formula every 3 hours for 48 hours starting on day of life 3</td>
<td>103</td>
<td>Detecting necrotizing enterocolitis prior to the onset of clinical symptoms through continuous abdominal near-infrared spectroscopy measurements</td>
</tr>
<tr>
<td>Burrin et al</td>
<td>Delayed initiation but not gradual advancement of enteral formula feeding reduces the incidence of necrotizing enterocolitis in preterm pigs</td>
<td>Early abrupt group: gavaged with formula every 3 hours starting day of life 2, late abrupt: gavaged with formula every 3 hours starting day of life 5</td>
<td>103</td>
<td>Early versus late initiation and abrupt versus gradual advancement of enteral feeding of an intact versus hydrolyzed protein formula on incidence of necrotizing enterocolitis</td>
</tr>
<tr>
<td>Burrin et al</td>
<td>Near-infrared spectroscopy measurement of abdominal tissue oxygenation is a useful indicator of intestinal blood flow and necrotizing enterocolitis in premature piglets</td>
<td>Piglets gavaged with cow’s milk formula every 3 hours after 72 hours of life. Piglets attached to abdominal near-infrared spectroscopy probe for continuous monitoring</td>
<td>105–107</td>
<td>Developing a non-invasive method for early detection for necrotizing enterocolitis using near-infrared spectroscopy probes</td>
</tr>
<tr>
<td>Sun et al</td>
<td>Necrotizing enterocolitis is associated with acute brain responses in preterm pigs</td>
<td>Gavaged with increasing doses of human donor milk for 8 days, starting day of life 0</td>
<td>106</td>
<td>The effects of necrotizing enteroctilis lesions on the neuro development of the hippocampus</td>
</tr>
<tr>
<td>Jensen et al</td>
<td>Similar efficacy of human banked milk and bovine colostrum to decrease incidence of necrotizing enterocolitis in preterm piglets</td>
<td>Piglets gavaged with either human donor milk, bovine colostrum, and infant formula every 2 hours, starting day of life 3</td>
<td>105</td>
<td>Developing supplemental replacements for formula</td>
</tr>
<tr>
<td>Bjornvad et al</td>
<td>Enteral feeding induces diet-dependent mucosal dysfunction, bacterial proliferation, and necrotizing enterocolitis in preterm pigs</td>
<td>Piglets gavaged with either porcine colostrum, bovine colostrum, or infant formula every 3 hours, starting day of life 0 for 20–40 hours</td>
<td>105–108</td>
<td>Effects of formula on the incidence of necrotizing enterocolitis</td>
</tr>
<tr>
<td>Buddington et al</td>
<td>The risk of necrotizing enterocolitis differs among preterm pigs fed formulas with either lactose or maltodextrin</td>
<td>Piglets gavaged with colostrum, lactose, and maltodextrin</td>
<td>105</td>
<td>Removing maltodextrin in formula to lower incidence of necrotizing enterocolitis</td>
</tr>
<tr>
<td>Good et al</td>
<td>Lactobacillus rhamnoses HN001 decreases the severity of necrotizing enterocolitis in neonatal mice and preterm piglets: evidence in mice for a role of TLR9</td>
<td>Piglets were gavaged with 15 mL/kg of formula every 3 hours for 4 days containing Lactobacillus rhamnoses DNA or live Lactobacillus rhamnoses</td>
<td>105–108</td>
<td>Potential of Lactobacillus rhamnoses in lowering the incidence of necrotizing enterocolitis</td>
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</tbody>
</table>

Rabbit model

Gurien et al evaluated the protective role of Lactococcus lactis against NEC using a rabbit model of NEC. In their description, 2-day-old preterm New Zealand white rabbit pups were gavage fed and given CS in addition to oral ranitidine and indomethacin after performing an anal blockage using adhesive tape. The anal blockage was intended to simulate the poor intestinal function and dysmotility of preterm neonates. Bozeman et al provided evidence that in this model the incidence of NEC increased with the duration and completeness of the anal blockage. Their model is similar to that in research of Gurien et al and utilized premature Csection delivered rabbits.

To examine the clinical applicability of Doppler sonography in a rabbit model of NEC, Choi et al. induced experimental NEC using a combination of endotoxin, hypoxia, and cold stress. Reduced oxygen to the small intestine induces mucosal injury and may contribute to neonatal NEC. Since little is known about the relationship between fetal hypoxia and GI motility, Sase et al. assessed the potential effects using a rabbit model. These experiments gave insight into the GI motility, proving that it was significantly decreased by maternal hypoxia during the last third of gestation. It also highlighted that hypoxia-induced reduction in GI motility might contribute to neonatal NEC.

Miller et al. reproduced NEC in rabbits by means of transmural injection of intestinal loops with an acidified solution of casein and calcium gluconate, mimicking the luminal milieu of afflicted neonates. Using this model, they demonstrated that NEC could be established by a luminal insult that caused local generation of free radicals and exaggerated release of prostaglandins and leukotrienes.

Predominant enterobacteria from infants with NEC was examined for detecting unusual ability of fermenting lactose by Carbonaro et al. One such isolate, a Klebsiella pneumoniae strain, partially induced lactose operon expression in tryptone containing media, and was pathogenic in a rabbit ileal loop model for NEC. The results of these studies on the intraluminal biochemistry of infants with NEC support the hypothesis that an increased ability for lactose fermentation may be a bacterial pathogenic trait with respect to NEC. Many investigators have used variations of this model as outlined in Table 2.

### Quail model

The quail model has been used in studies evaluating the role of bacteria in NEC. Development of cecal NEC-like lesions in quails requires the combination of two major factors that are present in preterm human infants:

<table>
<thead>
<tr>
<th>Author</th>
<th>Title</th>
<th>NEC protocol</th>
<th>Gestational age (days)</th>
<th>Area of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gupta et al.</td>
<td>Occurrence of necrotizing enterocolitis may be dependent on patterns of bacterial adherence and intestinal colonization: studies in caco-2 tissue culture and weanling rabbit model</td>
<td>Adherent E. coli isolates, from necrotizing enterocolitis cases, were able to cause pathologic changes typical of necrotizing enterocolitis in a weanling rabbit ileal loop model</td>
<td>No age specification</td>
<td>The role of intestinal microbial ecology in the pathophysiology of necrotizing enterocolitis</td>
</tr>
<tr>
<td>Garston et al.</td>
<td>The role of intraluminal tension and pH in the development of necrotizing enterocolitis: an animal model</td>
<td>A closed intestinal loop, 20 cm distal to the cecum was created. A solution containing fatty acids at pH 3 or 5 were infused into the loop at a pressure of 10 or 40 mm Hg</td>
<td>6- to 8-weeks old</td>
<td>Relative effects of pH and intraluminal tension on colonic mucosa</td>
</tr>
<tr>
<td>Bozeman et al.</td>
<td>An animal model of necrotizing enterocolitis in preterm rabbits</td>
<td>Pups were intestinally blocked to stimulate abdominal distention. Pups were then bolus fed 120 kcal/kg/day with kitten milk replacer mixed with Enterobacter cloacae</td>
<td>2 days premature</td>
<td>Developing a non-invasive, premature animal model that closely mimics clinical conditions</td>
</tr>
<tr>
<td>Mark et al.</td>
<td>Super oxide dismutase prevents damage and attenuates eicosanoid release in a rabbit model of necrotizing enterocolitis</td>
<td>Transmural injection of intestinal loops with an acidified solution of casein and calcium gluconate, mimicking the luminal milieu of afflicted neonates</td>
<td>No age specification</td>
<td>Localizing eicosanoids released from necrotic and healthy intestine in response to proinflammatory agents</td>
</tr>
<tr>
<td>Hwa-Young et al.</td>
<td>Bowel sonography in sepsis with pathological correlation: an experimental study</td>
<td>Animals received 1 mg/kg of E. coli O55-B5 lipopolysaccharide to induce sepsis. Bowel wall thickness and injury were evaluated.</td>
<td>1-week old</td>
<td>Facilitating early detection of intestinal injury in septic infants with necrotizing enterocolitis</td>
</tr>
<tr>
<td>Erdener et al.</td>
<td>Pentoxifylline does not prevent hypoxia/ reoxygenation-induced necrotizing enterocolitis</td>
<td>Pups were pretreated with pentoxifylline 15 min prior to being exposed to 5 min hypoxic/ reoxygenation episodes 3 times a day for 3 days</td>
<td>1-day old</td>
<td>Investigating the protective effects of pentoxifylline in necrotizing enterocolitis</td>
</tr>
</tbody>
</table>
lactose in diet and colonization by lactose-fermenting bacteria. Experimental infection of germ-free quails with either *Clostridium butyricum* (CB) strains (monobiotic quails) or fecal specimens (polybiotic quails), both originating from preterm infants with NEC, reproduced many aspects of the pathology of NEC, such as thickening of the cecal wall with gas cysts, hemorrhagic ulcerations, necrotic areas, and intestinal pneumatosis. This model allowed the study of the role of bacterial strains involved in NEC in gnotobiotic quails as the experimental model. This model looked at Clostridia species, and demonstrated that this species were strongly implicated in NEC through excessive production of butyric acid as a result of colonic lactose fermentation. This model also served to understand the protective role of bifidobacteria through a decrease in clostridial populations and in butyric acid. Oligofructose dietary supplementation was shown to enhance the effect with an increase in the bifidobacterial level and consequently a greater decrease in clostridia, as described in table 3.

In addition, inoculation of germ-free quails with bacteria associated with NEC have improved our understanding of the inducible nitric oxide synthase (iNOS) pathway activation prior to the development of macroscopic lesions in NEC.

### Nonhuman primate model

Though not as common, nonhuman primates have been utilized as models to examine the disease progression of NEC. Nonhuman primate models most closely resemble human disease states because of their homology in several organ systems, specifically seen in regard to their gastrointestinal development.

### Table 3 A summary of the studies using the quail model

<table>
<thead>
<tr>
<th>Author</th>
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<th>NEC protocol</th>
<th>Gestational age (days)</th>
<th>Area of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dupriet et al</td>
<td>Evidence for clostridial implication in necrotizing enterocolitis through bacterial fermentation in a gnotobiotic quail model</td>
<td>2-week old, germ-free quail were orally inoculated with bacterial strains: <em>K. pneumoniae</em>, <em>C. perfringens</em>, <em>C. difficile</em>, <em>C. paraputrificum</em>, <em>C. butyricum</em> Quails were sacrificed after 3 weeks</td>
<td>2-week old</td>
<td>To analyze the role of <em>K. pneumoniae</em>, <em>C. perfringens</em>, <em>C. difficile</em>, <em>C. paraputrificum</em> in the development of necrotizing enterocolitis</td>
</tr>
<tr>
<td>Dupriet et al</td>
<td>Short-chain fatty acids (SCFAs) and polyamines in the pathogenesis of necrotizing enterocolitis: Kinetics aspects in gnotobiotic Quails</td>
<td>3-day old quail were orally inoculated with <em>C. butyricum</em>. Quails were sacrificed at day 7, 12,18, and 24</td>
<td>3-day old</td>
<td>Investigating if short-chain fatty acids and polyamines are primary elements in the pathogenesis of necrotizing enterocolitis like lesions</td>
</tr>
<tr>
<td>Butel et al</td>
<td>Oligofructose and experimental model of neonatal necrotizing enterocolitis</td>
<td>Quails were fed a 6% lactose diet that included lactose-fermenting bacteria such as <em>C. butyricum</em> or fecal flora specimens</td>
<td>No age specification</td>
<td>Assessing how low endogenous lactase activity, lactose in diet and lactose-fermenting bacteria onset intestinal lesions</td>
</tr>
<tr>
<td>Butel et al</td>
<td>Clostridial pathogenicity in experimental necrotizing enterocolitis in gnotobiotic quail and protect role of bifidobacteria</td>
<td>Quails were fed a lactose diet and orally inoculated either <em>C. butyricum</em> or whole necrotizing enterocolitis flora (including three clostralid species <em>C. butyricum</em>, <em>C. perfringens</em>, <em>C. difficile</em>—each from premature infants suffering from necrotizing enterocolitis) or Bifidobacterium strain</td>
<td>2-week old</td>
<td>Determining the involvement of clostridia in the development of necrotizing enterocolitis. As well as determining the protective role of bifidobacterial in necrotizing enterocolitis</td>
</tr>
<tr>
<td>Bouseboua et al</td>
<td>Experimental cecitis in gnotobiotic quails monoassociated with clostridium butyricum strains isolated from patients with neonatal necrotizing enterocolitis and from healthy newborns</td>
<td>Animals were initially fed a commercial diet ad libitum then transitioned into a lactose-based diet after day 3 of life. At day 13, quails were split and given <em>C. butyricum</em> bacterial strains were obtained from sick premature babies</td>
<td>13-day old</td>
<td>Determining the involvement of clostridia strains in the development of necrotizing enterocolitis</td>
</tr>
</tbody>
</table>
Animal models of NEC have been around for a long time and they are of significant value to help us better understand the pathogenesis of NEC. Without them, our current understanding of pathways that lead to NEC would be very limited. These models have helped identify a number of promising and novel strategies such as probiotics, growth factors, stem cells, and breast milk to reduce the burden of NEC. There are significant variabilities to each and every model whether it is related to the animal size, genetic variability, or the protocol used to generate the model. However, they each have distinct advantages and drawbacks. As there is no “gold standard” animal model for the study of NEC, our paper highlights the importance of using animal models that are appropriate to answer the specific scientific question. We found that the most commonly used model remained the rat pup one, mainly due to its low cost, larger size of the pups and ease to learn and reproduce. As many authors have emphasized, there is a significant need for research and further development and funding in the field of NEC.

**CONCLUSION**

Animal models of NEC have been around for a long time and they are of significant value to help us better understand the pathogenesis of NEC. Without them, our current understanding of pathways that lead to NEC would be very limited. These models have helped identify a number of promising and novel strategies such as probiotics, growth factors, stem cells, and breast milk to reduce the burden of NEC. There are significant variabilities to each and every model whether it is related to the animal size, genetic variability, or the protocol used to generate the model. However, they each have distinct advantages and drawbacks. As there is no “gold standard” animal model for the study of NEC, our paper highlights the importance of using animal models that are appropriate to answer the specific scientific question. We found that the most commonly used model remained the rat pup one, mainly due to its low cost, larger size of the pups and ease to learn and reproduce. As many authors have emphasized, there is a significant need for research and further development and funding in the field of NEC.

**REFERENCES**


