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Models of Necrotizing Enterocolitis

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Abstract

Necrotizing enterocolitis (NEC) is the leading cause of death and disability from gastrointestinal disease in premature infants. The mortality of patients with NEC is approximately 30%, a figure that has not changed in many decades, reflecting the need for a greater understanding of its pathogenesis. Progress towards understanding the cellular and molecular mechanisms underlying NEC requires the study of highly translational animal models. Such animal models must mimic the biology and physiology of premature infants, while still allowing for safe experimental manipulation of environmental and microbial factors thought to be associated with the risk and severity of NEC. Findings from animal models have yielded insights into the interactions between the host, the colonizing microbes, and the innate immune receptor Toll-like Receptor 4 (TLR4) in driving disease development. This review discusses the relative strengths and weaknesses of available *in vivo*, *in vitro*, and *NEC-in-a-dish* models of this disease. We also highlight the unique contributions that each model has made to our understanding of the complex interactions between enterocytes, microbiota, and immune cells in the pathogenesis of NEC. The overall purpose of this review is to provide a menu of options regarding currently available animal models of NEC, while in parallel hopefully reducing the potential uncertainty and confusion regarding NEC models to assist those who wish to enter this field from other disciplines.

Keywords

NEC; Necrotizing Enterocolitis; Models of Necrotizing Enterocolitis

Introduction

It is widely recognized that the leading cause of death from gastrointestinal disease in premature infants is necrotizing enterocolitis (NEC), a statistic that has changed little since the disease was described some 40 years ago.[1,2] The fact that NEC remains a condition with such remarkable morbidity and mortality despite decades of experience in its management underscores the need for additional, detailed studies of its pathogenesis – studies that will involve, in part, highly relevant animal models. This review will highlight our understanding

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of the animal models that are available to study NEC, with an emphasis on their advantages and disadvantages. We will also review the recently described "NEC-in-a-dish" models, which use human intestinal cells exposed to NEC's microbial and environmental conditions to reproduce the relevant biochemical pathways in the actual disease. The overall purpose of this review is to provide a menu of options regarding currently available animal models of NEC, while in parallel hopefully reducing the potential uncertainty and confusion regarding NEC models to assist those who wish to enter this field from other disciplines.

Risk factors and clinical manifestations for NEC in the design of animal models

The ability to recapitulate NEC in animal models is predicated upon a clear understanding of the relevant risk factors for disease development and an appreciation of the various forms of presentation of this complex disease. Risk factors for NEC and its clinical manifestations are reviewed below.

Risk factors for NEC

The typical NEC patient is a premature infant, typically several weeks of age, who has recently received formula feeds.[3,4] The contribution of formula feeding to the induction of NEC has been shown in several large series.[5,6] It is highlighted by the consistent finding that over 90% of infants who develop NEC are enterally fed, and those infants who receive infant formula develop NEC more frequently than those who are fed breast milk.[7,8] Additional risk factors for NEC include the recent administration of blood transfusions,[9] use of H2 blockers,[10–12] broad-spectrum antibiotics,[13–15] chorioamnionitis,[16] bacterial colonization of the GI tract with dysbiotic bacteria,[17] congenital heart disease,[18,19] remote infection,[20,21] or sibling with NEC.[21] By contrast, protective factors for NEC include the administration of breast milk,[5,6] the use of probiotics,[22–24] and strategies that minimize exposure to the key risk factors described above. An understanding of these risk factors is critical in the development of clinically relevant animal models. For instance, the combined administration of infant formula and intestinal microbes can induce NEC in rodents and piglets. In immature mice, transfusion of red blood cells can cause NEC-like lesions[25].

Clinical presentation of NEC

The development of accurate and valuable models for the study of NEC must account for the recognition that the presentation of this disease can be quite variable. For instance, its presentation in a very low birth weight premature infant (where NEC typically takes several weeks to develop) may be quite different from its presentation in a neonate born closer to term, who may have, where the disease onset is usually much sooner. To clarify our understanding of how NEC can present clinically, we have recently described five clinical presentations of the disease, as follows:[26]

1. *"Textbook" NEC* – this is the presentation of NEC that perhaps first comes to mind when the diagnosis of NEC is considered. "Textbook NEC" refers to the presentation in which a premature infant who is predominantly formula-fed develops abdominal distention and bloody stools, and which is associated with the presence of a characteristic finding on abdominal plain films termed *pneumatosis intestinalis,* which refers to the presence of gas within the wall of

the bowel. After an initial period of treatment consisting of cessation of all feeds, nasogastric decompression, resuscitation, and administration of broad-spectrum antibiotics, patients may either recover (in 50% of cases) or progress towards the development of intestinal necrosis and intestinal perforation[27], as revealed by the presence of peritonitis and the finding of pneumoperitoneum on abdominal films. Abdominal exploration then reveals the presence of patchy necrosis involving regions of the small and large intestine[28], and overall survival is determined by a host of factors, including the reversibility of the accompanying septic process and presence of comorbidities.

2. *Persistent NEC without free air* – this presentation refers to the infant who develops NEC as above (presence of *pneumatosis intestinalis*) and fails to improve clinically but does not demonstrate obvious intestinal perforation. In the absence of clear improvement, exploratory laparotomy may reveal patchy necrosis and evidence of acute or indolent intestinal perforations.

3. *Portal venous gas and abdominal tenderness* – This presentation refers to the child with abdominal findings of air within the portal system, which generally suggests significant intestinal necrosis in the setting of abdominal tenderness.

4. *Staccato NEC* – this presentation refers to the child with NEC who is initially relatively stable yet rapidly develops deterioration characterized by overwhelming sepsis accompanied by clinical and radiographic evidence of NEC that evolves over hours.

5. *NEC totalis* – the child with *NEC totalis* exhibits extensive necrosis that involves nearly all the small and large intestines. While *NEC totalis* may be suspected on abdominal X-rays based upon the extent of *pneumatosis intestinalis,* the diagnosis of *NEC totalis* is often only made at laparotomy.

It is obvious that no single animal model can reliably mimic all these five presentations of NEC. Indeed, no human presents with all 5, although there is significant overlap between presentations (i.e., presentations 1 and 2 overlap to a degree, as do 3 and 4). Given the clinical complexity of NEC, it may be helpful to recognize that there is not one “best” NEC model. Rather, the choice of animal model should be based upon the specific question to be answered (for example, the investigation of a particular pathway that may be implicated in disease pathogenesis versus the assessment of a potential treatment), the desired phenotype (patchy versus diffuse intestinal necrosis) and the resources available (see Table 1).

Mouse models of NEC

Mouse models are some of the earliest[29,30] and most frequently utilized models to study NEC. In general terms, as with other diseases, mouse models offer the opportunity for genetic modification to test disease pathogenesis and have the advantage of being relatively inexpensive to purchase and house compared with other animals.[31] While individual models vary somewhat, the typical mouse models are performed on mice within the first week or so of life and utilize a combination of gavage with infant formula, brief exposure to hypoxia and/or hypothermia, and the addition of either bacterial lipopolysaccharide or bacteria obtained from human infants with clinical NEC.[32] The complexity of the mouse

model also gives rise to its inherent disadvantages: mouse models of NEC require regular feeding and a team of investigators trained to administer formula by gavage to tiny mice. The model typically lasts 4 or 5 days, upon which a vast majority of mice will display the patchy intestinal edema, *pneumatosis intestinalis,* and necrosis that characterizes human NEC.[33]

The mouse NEC model performed in our laboratory occurs as follows:[34–40] Pups are separated from their mother on day seven after delivery and housed in a temperature-controlled (37ºC) neonatal incubator to maintain homeostasis. The pups are fed by gavage formula five times daily using a 22ga feeding catheter and colonized with enteric bacteria isolated from stool obtained from infants with NEC. Mice are subjected to brief periods of hypoxia twice daily (10 min at 95% N_2, 5% O_2). On day 5 of the model, mouse pups are euthanized by decapitation with surgical scissors. We typically sample the intestine at a fixed point in all cases to avoid sampling bias, mainly 1cm from the terminal ileum. Tissues are obtained for PCR analysis, histology, and western blot. A typical study will involve fifteen to twenty p7 mice per experimental group, and expected mortality is approximately 20%, with a disease penetrance of over 80%.

This model has been utilized with modification by several investigators, including Besner et al.,[41] who added hypothermia, and Pierro et al.,[42] who combined hypoxia with LPS administration. Maheshwari et al., on the other hand, employed asphyxia (100% nitrogen gas) and cold stress (4°C for 10 min) twice daily[43] and have also utilized a blood transfusion model to induce NEC in a mouse correlate of this important risk factor in pathogenesis[25]. Genetic modifications used include the innate immune receptor TLR4,[44] IL-18,[45] eNOS[46] and others. Further, Ginzel et al. have utilized the mucosal irritant DSS to induce NEC-like lesions in the small and large bowel in the absence of hypoxia and hypothermia.[47] McElroy et al. have used both chemical and genetic ablation of Paneth cells in mice at P14-P16 days of age, resulting in NEC-like intestinal injury and a shift toward Enterobacteriaceae species.[48,49] These studies reveal the utility of mouse models for NEC and the various modifications that can be used to achieve a reliable platform for understanding pathogenesis.

Several approaches to validating the mouse and human NEC models have been performed. First, the newborn mouse shares features consistent with a 28-week infant, i.e., minimal subcutaneous fat, eyes closed most of the time, poor thermoregulation, and immature gut motility.[50] Second, the newborn mouse with NEC exhibits a pattern of gene expression in component immune cells within the small intestine that resembles the human infant with NEC.[51] Further, the intestinal microbiome seen in mice with NEC parallels that in humans.[46,52] Most importantly, biochemical and genetic pathways that play causative roles in mouse NEC are also observed in the human disease. Among these are pathways involving TLR4, IgA, EGF, and HMGB1.[38,53–56] To this end, agents that prevent or ameliorate NEC in mouse models have shown promise against human disease in clinical studies[46] or *ex vivo* studies with human tissue.[33] Taken together, despite the potential drawbacks of the mouse model based on its small size and incomplete overlap between mouse and human immune cells, mouse NEC models have emerged as essential tools in studying NEC pathogenesis.

Rat models—Rats were the first animal used to create an experimental model of NEC. In 1974 and 1975, Barlow and Santulli reported that a NEC-like histopathological phenotype could be achieved in prematurely delivered pups by gavage feeding formula and treating them with intermittent episodes of cold or hypoxia[57,58]. Several researchers have successfully adapted this model to study specific variables thought to play a role in the risk and severity of NEC. In 1994, Caplan et al. reported that the introduction of bacteria into the formula led to NEC, thus revealing a role for bacterial colonization in NEC development[59]. Additional laboratories have made further adaptations to the rat NEC model to study the effects of colonization with different species of bacteria found in the stool of infants with necrotizing enterocolitis, including Cronobacter sakazakii[60], Klebiella[61,62], and Clostridia[63–67]. LPS has also been used to increase the extent of intestinal injury in the rat model[68]. In one iteration of the rat model, Zani *et al.* induced NEC by administering 4 mg/kg/day of LPS obtained from *E. coli* mixed with formula to e21.5 pups beginning on the fifth day of life.[69] Similar to other animal models of NEC, rat models have been shown to exhibit morphological changes in the intestinal epithelium similar to those seen in patients with acute NEC, and iNOS mRNA upregulation, enterocyte apoptosis, and decreased IL-12 production in the intestinal epithelium have been implicated at important aspects of NEC pathophysiology[70].

The advantages of using rats in NEC research include their low cost and relative tolerance of the model, while disadvantages center primarily on the inability to manipulate the genome to interrogate specific pathways involved in disease development. Rat models of NEC may have essential roles in pharmacokinetics studies and safety and efficacy studies of potential NEC therapies, as reported by several groups[71–75].

Piglet models—Piglets offer an attractive model for studying NEC because of their anatomical, physiological, and developmental similarities to the gastrointestinal tract of humans.[76] The premature piglet model, in particular, offers specific advantages in that the model is performed on animals weighing between 1000–1300g, which approximates the size of the human infant with NEC. The model's technical details, as described by Sangild et al., include delivery at 90% term, which results in a natural period of hypoxia and hypothermia[77], and subsequently formula fed to induce intestinal injury.[77] Sangild et al. also used TPN to induce the intestinal changes seen in the human disease.[78]

The piglet model as performed in our lab makes use of timed-pregnant White Yorkshire sows whose piglets are delivered prematurely via cesarean section at 95% gestation. NEC is induced by feeding 20 mL/kg of formula supplemented with enteric bacteria obtained from infants with surgical NEC five times daily. Piglets are housed in an incubator for the duration of the model and euthanized with intracardiac potassium chloride injection following anesthesia with ketamine. The piglet NEC model has been used to understand early NEC detection methods, potential therapies, and preventative strategies. For instance, Zamora, Burrin, and Chen employed this model to develop non-invasive methods for early detection of NEC using near-infrared spectroscopy.[79] Other researchers, including Burrin *et al.* and Buddington *et al.,* used the piglet model to systematically evaluate the impact of various feeding protocols on the incidence of NEC-like pathology and the effects of lactose versus maltodextrin content in formula.[80,81] Utilizing this model, Puiman *et al.*

found that formula feeding, as opposed to feeding with bovine colostrum, was associated with decreased gut protein synthesis and lower levels of MUC2, the predominant secretory mucin in the human intestinal tract.[82] The model has elucidated findings including reactive oxygen species[83], platelet-activating factor,[84] intramural intestinal pH,[85] and the role of T-cell mediated mucosal immunity.[86] Our lab has used the piglet model to demonstrate the effects of human milk oligosaccharides[87] and bacterial DNA[1] as potential NEC therapy.

While the piglet model closely mimics human NEC since the piglets used are premature and are close in size to newborn infants, there are significant drawbacks. These include the high cost associated with model development, the large team needed to care for a litter of piglets around the clock, and the facilities required to care for a large sow and her offspring. Moreover, the piglet genome is difficult to manipulate, thus limiting the applicability for pathogenesis studies. As with the rat models, the piglet models may be most effective in pharmacologic and drug safety studies and pre-clinical evaluation of specific nutrient components relevant to premature newborn health.

NEC-in-a-DISH models—Organoid models are three-dimensional cultures of cells that recapitulate some functional aspects of whole organs.[88] These *in vitro* systems can be derived from progenitor and stem cells from both mouse and human tissue.[88] Intestinal organoids can be applied to NEC research by representing a functional intestine-in-a-dish.[89] To accomplish this, organoids may be obtained from the differentiation of iPSCs, or enteroids may be cultured *ex vivo* from the intestinal crypts of experimental animals or patients. While in culture, these organ units are exposed to NEC bacteria and hypoxia, critical components of the NEC model. Importantly, organoids exposed to hypoxia or NEC bacteria alone show no overt changes in structure or gross cell morphology. However, those exposed to a combination of these conditions display distorted architecture, an increase in inflammatory cytokine up-regulation, and reduced proliferation,[56] consistent with the structural and functional changes observed in human NEC. This model, termed *"NEC-in-a-dish"*, has been used to assess biological features relevant to NEC, including barrier function, immune response, and transcriptional profiles in intestinal organoids.[56,90] Furthermore, they have been used to confirm the importance of TLR4 activation in human tissue, elucidate the role of milk oligosaccharides, and discover novel cellular pathways involved in NEC, such as necroptosis.[44,56]

Organoid *NEC-in-a-DISH* models offer significant advantages compared to *in vivo* models for future research. These systems are now developed with standardized protocols and are scalable for large-scale approaches such as drug and therapy screening. *NEC-in-a-dish* models can also be derived from fresh or frozen tissue and be grown from human tissue, leading to conclusions more relevant to human disease.[48,90] Organoid models derived from patient samples have the same genetic background as the patient, allowing for the assessment of precision treatments specific to each patient.[48] Finally, organoid NEC-in-a-dish models are low cost, allowing for widespread adoption in various workflows. However, *NEC-in-a-dish* models also have limitations. Notably, current organoid approaches lack the immune component of the intestine, which is implicated in NEC development.[91] As an isolated system, there is also a lack of circulating factors that contribute to NEC.[48] Further developments to *NEC-in-a-dish* models, including the use of co-culture systems with

isolated primary circulating and intestinal epithelial lymphocytes, may remedy these issues and lead to further understanding of NEC pathogenesis.

Conclusion

NEC remains a leading cause of morbidity and mortality among premature infants.[92] Over the last several decades, many experimental models have been developed to enhance our understanding of this disease. Because multiple risk factors are involved in the pathophysiology of NEC, modeling this disease has often proven to be beyond the scope of studies in traditional single-cell cultures. To this end, animal models, and "*NEC-in-a-dish*" models all play a crucial role in modeling and deciphering the biological pathways and potential therapeutic avenues in NEC. Recent discoveries utilizing various models in parallel underscore the importance of understanding each model's distinct advantages, drawbacks, and applicability.

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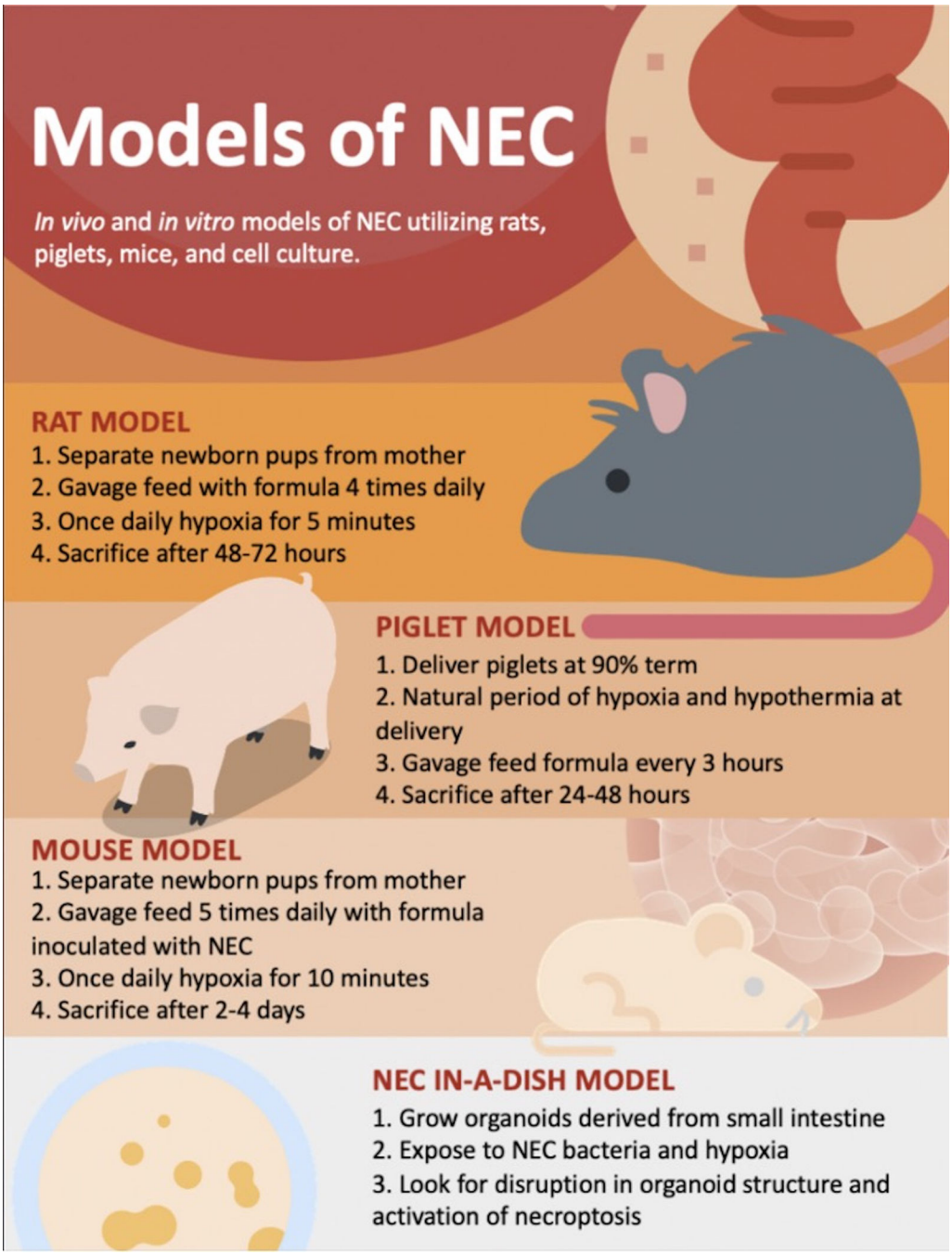

Figure 1.

Experimental models have served a critical role in the study of necrotizing enterocolitis pathogenesis. *In-vivo* models involve exposing young rats, piglets or mice to hypoxia and bacteria to recapitulate conditions involved in NEC. More novel *in-vitro* models have made use of organoids growing in culture under similar conditions.

Table 1.

Models of NEC and associated phenotypes. Most significant resource requirements and some significant aspects of NEC pathogenesis learned from these models are listed.

Animal Model	Phenotype	Resources	Examples of specific questions answered with each model
Rat	• Sloughing of epithelial cells at tips of villi • Attenuation of normal developmental increase in heart rate variability • Increased serum levels of proinflammatory cytokines (e.g., IL-1B, IL-6)	• Low cost • Easy breeding • Limited availability of transgenic models	• Role of probiotics in NEC prevention[93] • Therapeutic applications of anti-TNF-alpha antibody[94] • Protective effect of antioxidants[95] • Correlation of intestinal fatty acid binding protein on timing of NEC onset[96] • Role of protein kinase A inhibitor as a potential therapy in experimental NEC [97]
Mouse	• Ileum is scattered with patchy lesions of injury • Tissue destruction ranging from mild destruction to the tips of villi to transmural necrosis • Upregulation of inflammatory factors and antimicrobial peptides (e.g., IL1-B, Cxcl2, Reg3g)	• Low cost • Easy breeding • Able to create transgenic models	• The role of TLR4 in the pathogenesis of NEC[30] • Therapeutic potential of short-chain fatty acids in alleviating intestinal inflammation in NEC[98] • Impact of human milk oligosaccharides on experimental NEC [99]
Piglet	• Severe abdominal distension • Hemorrhagic discoloration and sloughing throughout small and large bowel • Necrosis and sloughing of epithelium	• High cost • Limited availability of transgenic models	• Role of nitric oxide production in the pathogenesis of NEC[100] • Diagnostic applications of transabdominal near infra-red spectroscopy[101] • Impact of NEC on hippocampal development [98] • Impact of abrupt versus gradual advancement of enteral feeds on the incidence of NEC[81] • Potential of probiotics in lowering the incidence of NEC [102]