Animal models of gastrointestinal and liver diseases. Animal models of necrotizing enterocolitis: pathophysiology, translational relevance, and challenges

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Animal models of intestinal and liver diseases. Animal models of NEC are the leading cause of death and challenges. The leading cause of morbidity and mortality from gastrointestinal disease in premature infants and is characterized by initial feeding intolerance and abdominal distention followed by the rapid progression to coagulation necrosis of the intestine and death in many cases. Although the risk factors for NEC development remain well accepted, namely premature birth and formula feeding, the underlying mechanisms remain incompletely understood. Current thinking indicates that NEC develops in response to an abnormal interaction between the mucosal immune system of the premature host and an abnormal indigenous microflora, leading to an exaggerated mucosal inflammatory response and impaired mesenteric perfusion. In seeking to understand the molecular and cellular events leading to NEC, various animal models have been developed. However, the large number and variability between the available animal models and the unique characteristics of each has raised important questions regarding the validity of particular models for NEC research. In an attempt to provide some guidance to the growing community of NEC researchers, we now seek to review the key features of the major NEC models that have been developed in mammalian and nonmammalian species and to assess the advantages, disadvantage, and major scientific discoveries yielded by each. A strategy for model validation is proposed, the principal models are compared, and future directions and challenges within the field of NEC research are explored.

Necrotizing enterocolitis; animal model; TLR4; innate immunity; microflora

NECROTIZING ENTEROCOLITIS (NEC) is the leading cause of death from gastrointestinal disease in premature infants and is now recognized as a major cause of morbidity in patients who are fortunate to survive the initial disease. NEC develops in ~1 in 1,000 live births (51), and since there were 3.95 million live births in 2012 according to the Centers for Disease Control and Prevention, ~4,000 infants were expected to develop this devastating disease in the past year alone. The typical infant with NEC is a premature child born at around 30 wk gestation, who was offered infant formula for nutrition, and who at ~2–3 wk of age develops feeding intolerance, abdominal distention, and bloody stools. In approximately half of cases, the child becomes progressively ill, with systemic sepsis and cardiovascular collapse requiring aggressive resuscitation. Clinical deterioration leads to abdominal exploration in nearly 30% of cases, where the hallmarks of NEC, i.e., death of parts or all of the small and/or large intestine, are encountered. Risk factors for NEC development include prematurity and the administration of formula feeds, and treatment is relatively nonspecific, which includes a combination of broad-spectrum antibiotics and removal of the diseased intestine (78). Although a full description of the clinical course and management of NEC are beyond the scope of the present review, the current consensus indicates that the incidence of NEC is either stable or increasing in frequency, that the overall survival for patients with this disease has not changed since the initial description 30 years ago (12, 72), and that the societal costs for patients who develop the disease, especially due to the long-term neurodevelopmental and intestinal effects, are enormous (33).

Insights into the pathophysiology of NEC have been gained through the study of clinical specimens and the careful selection and usage of animal models. Although the pathophysiology of NEC is generally agreed to be multifactorial (40, 78), our understanding of the cellular and physiological mechanisms that lead to NEC has changed significantly in the past decade or so. Initially, NEC was thought to develop as a result of an ischemic event leading to a disruption of an immature intestinal epithelium and mucosal barrier, which leads to bacterial translocation and the development of systemic sepsis (77). Although this explanation could account for the presence of intestinal epithelial disruption that is characteristic of NEC, it could not account for the presence of intestinal necrosis that is seen in patients with this disease, nor could this explanation readily reconcile the observation that most patients who develop NEC do not actually have an antecedent ischemic event (104). Previous authors have reviewed the major mechanistic factors thought to lead to the development of NEC (46, 78), and although a thorough evaluation is beyond the scope of the present review, current thinking suggests that formula feeding of the premature host that is colonized with a select microbial flora leads to an enhanced cellular and innate immune response in the context of host genetic factors and an impaired intestinal microvascular network, resulting in aggressive mucosal inflammation and reduced mesenteric perfusion. The ensuing tissue death leads to a cascade of bacterial translocation, systemic sepsis, and multisystem organ failure. There are many...
important pathogenetic questions that remain to be answered including how the process leading to mucosal inflammation is initiated, whether the abnormal bacterial colonization that is observed in the premature infant with NEC is a cause or a consequence of the disease, how the inflammatory response within the intestinal epithelium is coordinated, and the determinants of when a “tipping point” is reached, beyond which reversibility and restoration of the intestinal mucosal barrier are unachievable. To address these (among others) important gaps in our knowledge, careful study of clinical specimens from infants with and without NEC alongside carefully validated animal models of this disease is required. This review will now summarize the considerations in the selection of animal models for NEC research to address these important questions, will assess the key challenges associated with animal models in this field, and will then discuss the key animal models available to researchers with an assessment of the key contributions already gained from each, their strengths and their weaknesses. A summary of the major results from animal models that have provided a unifying approach to our understanding of the causes of NEC and the strategies that prevent this disease is shown in Fig. 1.

General Considerations and Challenges in the Selection of Animal Models for NEC Research

There are some unique challenges associated with the selection of appropriate animal models for use in NEC research. Many of these challenges pertain to the unique features of NEC that distinguish this disease from other inflammatory or septic processes, and which together reduce the number of reliable options that are available for animal-based NEC research. For instance, NEC is known to primarily affect premature infants who receive formula as opposed to breast milk. Thus, if one wishes to reliably reproduce the clinical disease in animals, a model in an animal at an age that shows features of immaturity needs to be selected. Furthermore, although models in small animals such as mice and rats offer the advantages of being of relatively low cost and readily available in large numbers, they lack the clinical robustness that is seen in larger animal models such as piglets, in which the comparison to a premature human is more readily obvious (a premature human infant that develops NEC can be of a similar size and weight as a premature piglet). More important considerations for the selection of a particular animal model relate to the specific question that is being asked, and a subsequent determination of how “reductionist” the particular animal model needs to be. For instance, if the intent is to test a particular molecular pathway that is hypothesized to play a role in the pathogenesis of NEC, or perhaps a new therapeutic agent for the prevention or treatment of NEC, then an animal model should be selected in which this pathway is affected under conditions that mimic the key features of NEC as seen in humans. By contrast, if the investigator wishes to test a reagent that may be capable of inhibiting or enhancing the signaling through a specific pathway that is already known to be important in NEC pathogenesis, then a reductionist approach can be selected in which this particular pathway is manipulated and subsequent effects on remote tissues including the gut can be assessed. Furthermore, as knowledge of the underpinnings of NEC have evolved, the animal models that researchers employ to study the disease must necessarily evolve. For instance, with a greater understanding that an abnormal community of enteric bacteria plays an important role in the pathogenesis of NEC in humans, comes the need to actively incorporate enteric bacteria associated with NEC development in animal models of this disease. Additional considerations for the selection of a particular model include the cost, ease of implementation of the strategy for induction of NEC, need for gene deletion, and availability of the required reagents. However, the overriding determination of a particular model should be its relevance to the disease in question, and for this reason it is important to understand how a particular model of NEC should be validated, as will be discussed below. An overview of each of the commonly used animal models used in NEC research is provided in Tables 1–3, and a comparative analysis is summarized in Table 4.

How Should Animal Models of NEC Be Validated?

The relatively large number of animal models that investigators have used to study NEC suggests that an approach is required to clearly define what constitutes a model of NEC in the first place and to then apply a validation scheme so that the results of studies can be compared within an appropriate context. At a minimum, animal models should replicate most of the cardinal histopathological features that are seen in human NEC, which include the presence of mucosal edema, pneumatosis intestinalis (i.e., the presence of gas within the wall of the intestine due to microbial activity), epithelial sloughing/villous atrophy, evidence of enterocyte apoptosis, vascular thrombosis, and discontinuous necrotic segments of intestine (so called “skip lesions”; Ref. 78). In addition, as a

Fig. 1. Pathogenesis of necrotizing enterocolitis (NEC). Graphical representation of the mechanisms that lead to the development of NEC, as well as protection from NEC, based on findings from experiments performed in animal models as discussed in this review.
validation strategy, it should be possible to attenuate the severity of NEC in a particular animal model through the use of experimental strategies that have been shown to attenuate NEC in humans, such as the administration of broad-spectrum antibiotics (57) or the substitution of formula with breast milk (44). Furthermore, the mechanisms by which the models are induced should parallel the clinical condition in human NEC to the extent that is possible. In this regard, models that include the simple occlusion of the blood supply to the bowel (90) or mechanical obstruction of the distal bowel (27) are likely to have a reduced overlap with models that meet all of the above criteria. By contrast, models that include factors associated with NEC development, including prematurity, formula gavage, manipulation of the microbiome, and external stressors such as hypothermia or hypoxia, are more likely to result in a phenotype that resembles the clinical condition.

Experimental Necrotizing Enterocolitis in Rats

The earliest rat NEC model, which was developed by formula feeding and hypoxia in newborn rats, was described by Barlow and colleagues in 1974 (7), the results of which led to the conclusion that the gut flora and absence of breast milk were suggested to play important roles in NEC pathogenesis. One year later, this model was modified to include the combination of formula feeding, hypoxia, and hypothermia (6) to increase the severity of the histological changes that were seen. In this model, formula feeding and hypoxia-hypothermia (100% nitrogen for 50 s followed by exposure to 4°C for 10 min) are the two critical instigating factors, since all maternally fed animals or animals without exposure to hypoxia-hypothermia showed normal intestinal histology. To prevent any maternal milk exposure, researchers have studied premature neonatal rats delivered by the cesarean section 1 day before the scheduled birth (14) or rat pups collected immediately after birth (29). This rat NEC model includes the widely accepted major risk factors of NEC, i.e., intestinal immaturity, enteral feeding, and inappropriate bacterial colonization (78), and is therefore widely considered to be very applicable for the study of pathogenetic mechanisms associated with this disease.

Many laboratories have used this model to study various aspects of the pathogenesis of NEC. These include the expression of the cytokines interleukin 18 and interleukin 12 in NEC development (47); the roles of intestinal epithelial apoptosis (58), bacteria, and the lipopolysaccharide (LPS) receptor TLR4 (59); and the role of maternal milk (28). Additional studies using the rat model have evaluated the pathogenetic roles of platelet-activating factor (PAF, 15), epidermal growth factor (29), bile acids (48), and intestinal microcirculatory dysfunction (53) in NEC pathogenesis. Several laboratories have modified this formula-feeding/hypoxia/hypothermia rat NEC model by adding extra factors to increase the incidence of NEC, including the addition of commensal bacteria typically found in stool (13, 25), or by reducing various factors to simplify the protocol. This modified model has been used to determine the roles of platelet-activating factor receptor (14), nuclear factor-κB (25), heat shock protein 70 (HSP70, 66), probiotics (88), and nitric oxide dysregulation (37) in the pathogenesis of NEC. Feng et al. (32) utilized a modification in which formula-fed newborn or preterm born rats were exposed to intragastric LPS followed by hypoxia/hypothermia stress, and this formula-feeding/hypoxia/hypothermia/LPS rat NEC model was subsequently applied to demonstrate the pathogenic role of nitric oxide synthase dysregulation (38) and the protective role of heparin-binding epidermal growth factor-like growth factor (HB-EGF) in decreasing the NEC incidence (32), increasing intestinal microvascular blood flow (105), and protecting intestinal stem cells (21). Others used reoxygenation (100% O₂ for 5 min) to enhance the intestinal injury (62), whereas various laboratories have modified the hypoxia process using 5% oxygen for relatively long periods, i.e., 3 to 10 min, and omitted the addition of hypothermia, to simplify the induction of NEC (19). Because of its relative simplicity and reproducibility, the formula-feeding/hypoxia rat NEC model was widely accepted and used to determine the roles of endotoxin (19, 43), the glutathione antioxidant system (60), surface integrins (82), nitric oxide (20, 106), carbon monoxide (107), P-glycoprotein (44), human milk oligosaccharide (54), and the expression of TLRs and cytokines (67) in NEC. Some researchers have introduced Cronobacter sakazakii (CS), formerly known as Enterobacter sakazakii, which is a bacteria that was reported to be associated with NEC (99), to the formula-feeding/hypoxia rat NEC model (52). Okada and colleagues (79) have modified the particular feeding regimen, by utilizing formula containing casein as the protein source to exert a greater burden on the intestines than whey, and, strikingly, overfeeding caused NEC-like intestinal injury in half of the animals after 24 h.

Other Rat Models Used for the Study of NEC

Additional models in the rat have been developed to investigate the pathogenesis of NEC. For instance, the ischemia-reperfusion rat NEC model was developed based on the sensitivity of the intestine to oxidative stress. In this model, ischemia of the intestine is induced by interruption of blood flow in superior mesenteric artery or isolated ileal loop, and reperfusion generates reactive oxygen stress, which leads to intestinal injury (50). Although this model is clearly an oxidative stress model, the links to clinical NEC remain uncertain, since this model does not involve formula feeding, is often performed in older animals, and involves occlusion of the superior mesenteric artery, which is not seen in clinical NEC. The intestinal ischemia in this model can also be induced by hyoxia, and rats that underwent hypoxia and reoxygenation showed similar lesions as seen in neonatal NEC (80). Although several authors have used this model, the links to NEC remain speculative, since there is rarely a global ischemic injury associated with this disease clinically.

Taken together, these studies performed in rats have been used to generate important observations regarding the pathogenesis of NEC. The low cost, high litter size, and ready availability of rats makes them an attractive species for performing these studies. The major drawbacks of the use of rats include the lack of readily available transgenic strains and the increased tolerance for bacteria/endotoxin between rats and other animals including humans (9). These drawbacks have led investigators to pursue models of NEC in other species, as reviewed below. A summary of the experimental details, strengths, and limitations of NEC models in rats is shown in Table 1.

AJP-Gastrointest Liver Physiol • doi:10.1152/ajpgi.00422.2013 • www.ajpgi.org
Table 1. Experimental details, strengths, and limitations of models of NEC in rats

<table>
<thead>
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<th>Experimental Details</th>
<th>Strengths</th>
<th>Limitations</th>
<th>References</th>
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<tbody>
<tr>
<td>Formula feeding and hypoxia (newborn rats).</td>
<td>Utilizes known clinical risk factors.</td>
<td>Rats may be exposed to maternal breast milk before induction of the model.</td>
<td>7</td>
</tr>
<tr>
<td>Formula feeding, hypoxia and hypothermia (newborn rats).</td>
<td>Utilizes known clinical risk factors.</td>
<td>Rats may be exposed to maternal breast milk before induction of the model.</td>
<td>6</td>
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<tr>
<td>Formula feeding, hypoxia and hypothermia (premature rats).</td>
<td>No maternal milk exposure.</td>
<td>Cesarean section is required.</td>
<td>14, 15, 28, 29, 47, 48, 53, 58, 59</td>
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<td></td>
<td>Intestinal barrier is immature.</td>
<td>In patients, NEC does not develop immediately after birth questioning the clinical relevance.</td>
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<tr>
<td>Formula-feeding, hypoxia and hypothermia with the addition of commensal bacteria from adult rats (premature rats).</td>
<td>Includes risk factors associated with bacterial colonization.</td>
<td>The bacterial components that are added may not be representative of the clinical situation.</td>
<td>13, 14, 25, 37, 66, 88</td>
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<tr>
<td>Formula-feeding, hypoxia and hypothermia with the addition of intragastric LPS.</td>
<td>High disease severity.</td>
<td>LPS is not representative of all bacterial components relevant to NEC.</td>
<td>21, 32, 38</td>
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<tr>
<td>Formula-feeding, hypoxia, hypothermia reoxygenation (100% O₂ for 5 min).</td>
<td>The introduction of bacterial component (i.e., LPS).</td>
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<tr>
<td>Formula-feeding and hypoxia, reoxygenation (5% oxygen for 3 to 10 min), no hypothermia.</td>
<td>Increased intestinal injury.</td>
<td>The delivery of 100% oxygen to premature infants is avoided given known toxicity.</td>
<td>62</td>
</tr>
<tr>
<td>Formula-feeding and hypoxia, addition of Cronobacter sakazakii.</td>
<td>Avoidance of hypothermia simplifies the model.</td>
<td>Clinical relevance is uncertain given the prolonged reoxygenation.</td>
<td>19</td>
</tr>
<tr>
<td>Ischemia and reperfusion of the intestine in older rats.</td>
<td>Allows for the direct investigation of the role of Cronobacter sakazakii, which is associated with NEC.</td>
<td>Cronobacter sakazakii causes a minority of NEC cases in patients, and is more likely to be associated with other infections (i.e., meningitis).</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>More accurately is an oxidative stress model.</td>
<td>Performed in older animals when the intestine is more mature; relevance to clinical NEC is uncertain.</td>
<td>50, 80</td>
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NEC, necrotizing enterocolitis.

Experimental Necrotizing Enterocolitis in Mice

Compared with rat models, the induction of experimental NEC in mice offers many advantages including high genetic similarity of inbred strains and the opportunity to utilize sophisticated transgenic approaches. However, the biggest challenge to establish the mouse NEC models is the need to feed the relatively small and fragile mouse pups. Similar to the rat NEC models, the most popular mouse NEC models are based on intestinal immaturity and enteral feeding. In 2006, Jilling and colleagues (59) developed a NEC model using mice delivered by cesarean section between embryonic day (E)20 and E21, and NEC was induced by combined formula feeding, hypoxia, and hypothermia, resulting in 66% NEC incidence with gross and microscopic evidence of intestinal necrosis. This formula-feeding/hypoxia/hypothermia mouse NEC model was subsequently validated in preterm and term mice delivered by cesarean section (30, 44) and was used to assess the role of various genes in NEC pathogenesis, including TLR4 (59), interleukin 18 (49), MDR1a (44), and MUC2 (71). Various investigators have modified this mouse model of NEC by colonizing neonatal mice with adult commensal bacteria in addition to formula feeding, hypoxia, and hypothermia, resulting in increased NEC incidence (10), and the roles of bacteria (16), endogenous PAF-acetylhydrolase (68), and probiotics (10) were investigated with this modified model.

In 2007, Hackam and colleagues (64) developed a NEC model in 10-day-old mice using a combination of gavage formula feeding and intermittent hypoxia, resulting in the gross appearance of edema and necrosis in the small intestine that resembles human NEC. Representative histomicrographs revealing the typical histology of the terminal ileum from mice that were either untreated or were induced to develop NEC by using this model are shown in Fig. 2, in which the comparison with human NEC can be observed. This formula-feeding/hypoxia mouse NEC model was validated in several studies and was utilized to induce NEC in mice with modifications in various genes including IFN-γ (64), global TLR4 (63), intestinal TLR4 (95), endothelial TLR4 (103), TLR9 (42), inducible nitric oxide synthase (iNOS; Ref. 20), HSP70 (2), and autophagy-related protein 7 epithelium conditional knockout mice (75). The protective functions of TLR9 (42), NOD2 (83), amniotic fluid (39), and small molecule inhibitors of TLR4 (74) were also investigated by this model. This model was slightly modified by other laboratories and was used to determine the roles of phosphatidylinositol 3-kinase (3), TNF-α (4), oxidative stress (5), and heme oxygenase-1 (86) in NEC pathogenesis.

Studies performed in mice have shed light on the reasons for which prematurity contributes to the pathogenesis of NEC through effects, in part, on the regulation of the innate immune system of the developing gut (69). Specifically, we have shown that the TLR4 expression is significantly increased in the premature intestine compared with the full-term intestine, which reflects the recently recognized role of TLR4 in regulating normal intestinal differentiation and development (95). We and others have shown that, in the postnatal period, TLR4 activation drives intestinal injury and reduces intestinal perfusion, as reviewed above, implying that the signaling properties of TLR4 in utero are distinct from its signature role in host defense. Given that the expression of TLR4 in the developing mouse intestinal lining rises during embryonic development and then falls precipitously at birth (42, 102), it can be
speculated that the premature infant, in which the expression of intestinal TLR4 remains persistently elevated, the developmental role for TLR4 switches to a proinflammatory role upon its interaction with colonizing microbes, leading to mucosal inflammation and NEC development. Additional studies are needed to further identify the molecules that are involved in the switch of TLR4 from a predominantly developmental to a defensive role. Moreover, strategies that can attenuate the extent of TLR4 signaling in the infant born prematurely may offer effective therapeutic approaches to the development of NEC.

Other Mouse Models Used in NEC Research

Researchers have developed additional mouse models for NEC research, each having varying degrees of relevance to the clinical disease. The ischemia-reperfusion model involves surgical occlusion of both superior mesenteric vessels, resulting in intestinal lesions (61), although the direct relevance to clinical NEC is uncertain. A very novel approach to model human NEC includes the “humanized mouse model,” or the “xenograft transplant model.” This model, as first described by Walker and colleagues (34, 73), involves the implantation of 2-cm sections of human fetal ileum from 12 to 20 wk gestation subcutaneously into mice with severe combined immunodeficiency. Xenografts are then harvested 20 and 30 wk posttransplantation to allow for examination of the intestine, as well as expression of cytokines and innate immune inflammatory genes. This model allows for the use of a highly reductionist approach to study NEC in mice, while allowing for the interrogation of human tissue.

Clear advantages of mouse work include the low cost and ready availability of these animals, as well as the ability to harness the power of transgenics, which is of particular importance given the homology of the mouse and human genome.

Fig. 2. Histological appearance of necrotizing enterocolitis in the human, piglet, and mouse. Representative micrographs showing hematoxylin and eosin-stained sections of the terminal ileum from a premature human infant (A), piglet (B), and mouse (C) without (left) or with (right) NEC as described in this review. Size bar = 50 μm.
Implantation of human fetal ileum
Ischemia and reperfusion by surgical
Formula feeding and hypoxia in 10-day-old piglet
Hypoxia and hypothermia in neonatal term piglets
Administration of total parenteral nutrition

Experimental details, strengths, and limitations of models of necrotizing enterocolitis in mice

Table 2. Experimental details, strengths, and limitations of models of necrotizing enterocolitis in mice

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<tr>
<th>Experimental Details</th>
<th>Strengths</th>
<th>Limitations</th>
<th>References</th>
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<tbody>
<tr>
<td>Formula feeding, hypoxia and hypothermia in preterm or term mice delivered by cesarean section.</td>
<td>Preterm pups.</td>
<td>Technical challenges of handling premature pups. Clinical NEC does not occur immediately after birth so the clinical relevance is somewhat in question.</td>
<td>30, 44, 49, 59, 71</td>
</tr>
<tr>
<td>Formula feeding, hypoxia and hypothermia in mice delivered by cesarean section, with the addition of commensal bacteria obtained from adult mice.</td>
<td>Preterm pups. High NEC incidence. Addition of commensal bacteria increases clinical relevance. Technically easier to handle the 10-day-old mice. NEC is induced at a time in which it is observed clinically within the postnatal period.</td>
<td>Difficulty in handling preterm pups. Clinical NEC does not occur immediately after birth. The model does not use preterm pups.</td>
<td>10, 16, 68</td>
</tr>
<tr>
<td>Ischemia and reperfusion by surgical occlusion of both superior mesenteric vessels.</td>
<td>More accurately is an oxidative stress model.</td>
<td>Clinical NEC does not involve occlusion of the mesenteric vessels, so the relevance of this model is uncertain.</td>
<td>61</td>
</tr>
<tr>
<td>Implantation of human fetal ileum subcutaneously into SCID mice.</td>
<td>Allows for interrogation of human tissue.</td>
<td>Requirement for human fetal tissue. Technically challenging.</td>
<td>73, 34</td>
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especially for inflammatory and metabolic genes (101). However, important drawbacks of mice include their small size and associated difficulty with feeding and handling small pups. A summary of the experimental details, strengths, and limitations of NEC models in mice is shown in Table 2. These disadvantages may be addressed through the use of the piglet model, as described below.

Experimental Necrotizing Enterocolitis in Piglets

Compared with rodents, piglets share a high degree of anatomical, developmental, nutritional, and physiological similarity of the gastrointestinal tract with human infants (81). However, unlike primates, which are viable from 70% gestation. Because piglets are not viable until after 90% gestation (84), and models of NEC are typically induced in piglets under conditions that do not require significant resuscitative cardiac or ventilator support. Therefore, the first piglet models of NEC were developed in neonatal term piglets based on the fact that preterm infants often undergo ischemic injury that can be induced by hypoxia (89). Subsequently, hypothermia was introduced in piglet models of NEC, which yielded high NEC severity (18, 23). Given the acceptance that enteral feeding is one of the most important risk factors associated with NEC development in humans, infant formula or casein was introduced in neonatal piglets to develop NEC. In 1994, Crissinger and colleagues (24) produced a piglet NEC model by luminal perfusion of 1-day-old piglet jejunum with predigested and bile acid-solubilized preterm cow milk-based infant formula coupled with ischemia and reperfusion, and the importance of the lipid fraction of the formula in NEC pathogenesis was suggested. Di Lorenzo and colleagues (26) injected acidified casein solution into the intestinal loop of neonatal piglets and successfully induced intestinal damage. In 2006, Sangild and colleagues (85) induced NEC in preterm piglets, which were delivered by cesarean section at 92% gestation, by administering total parenteral nutrition, then administering infant formula without exposure to hypoxia or hypothermia. This preterm-birth/formula-feeding piglet NEC model was further validated.

Table 3. Experimental details, strengths, and limitations of models of necrotizing enterocolitis in piglets

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<th>Strengths</th>
<th>Limitations</th>
<th>References</th>
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<tbody>
<tr>
<td>Ischemia induced by hypoxia in neonatal term piglets.</td>
<td>Mimics the ischemic injury that may be seen in NEC.</td>
<td>Term piglets are used raising questions regarding the clinical significance. Lack of enteral feeding.</td>
<td>89</td>
</tr>
<tr>
<td>Hypoxia and hypothermia in neonatal term piglets.</td>
<td>Mimic the ischemic injury. High NEC severity. Short duration of the model.</td>
<td>Term piglets are used. No enteral feeding. Uncertain clinical significance. Term piglets were used. Uncertain clinical significance.</td>
<td>18, 23</td>
</tr>
<tr>
<td>Luminal perfusion of 1-day-old piglet jejunum with predigested and bile acid-solubilized preterm cow milk-based infant formula coupled with ischemia and reperfusion.</td>
<td>Introduction of intestinal dysmotility, which is a risk factor for NEC.</td>
<td>Term piglets are used and the experimental model is very short, raising questions regarding the clinical significance. Need for a well-equipped veterinary surgical facility.</td>
<td>24</td>
</tr>
<tr>
<td>Injection of acidified casein solution into the intestinal loop of neonatal piglets.</td>
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<td>26</td>
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<tr>
<td>Administration of total parenteral nutrition followed by the administration of infant formula in preterm piglets which are delivered by cesarean section at 92% gestation.</td>
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<td></td>
<td>8, 55, 56, 85, 91, 92, 93, 98, 100</td>
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via demonstration of an increase in the proinflammatory cytokine IL-6, one of the signature cytokines found in NEC infants (93). This model was used to determine the effects of colos-
trum (85), human milk (55), amniotic fluid (91), carbohydrate mal digestion (98), and glucagon-like peptide-2 (8) and was also used to study the changes in the microvasculature (100). Furthermore, a recent report on the role of antibiotics in this model, and an initial report demonstrating the role of gut colonization, provide additional important evidence for the validity of the model (56, 92). We have recently modified this model by delivering piglets at 92% estimated gestation and administered infant formula without exposure to hypoxia or hypothermia or the provision of total parenteral nutrition and observe mucosal inflammation similar to mouse and human NEC (Fig. 2).

The utilization of piglet models for the study of NEC offer the advantages of using a large animal model for this disease that has anatomic and functional properties of the intestine that are comparable to the human infant. These factors provide a suitable clinical platform for the investigation of drugs for potential use in humans, as well as for an assessment of whether biochemical pathways that were identified in rodent models may have relevance across species. However, the large cost of performing piglet NEC work, as well as the relative difficulty of maintaining and feeding a group of premature piglets, offer unique disadvantages of this approach (Table 3).

Experimental Necrotizing Enterocolitis in Hamsters and Rabbits

Besides the most commonly used mouse, rat, and piglet NEC models, other animals, such as hamster and rabbit, have been used in the past to induce experimental NEC. In 1989, hypothermia was applied to weanling hamsters, and 50% of cold-stressed animals showed colonic damage that resembled NEC (18). This hypothermia hamster NEC model was used to determine the role of reactive oxygen species in NEC, and cold-stressed hamsters showed a significant decrease in xanthine dehydrogenase/xanthine oxidase activity ratio (18). In 2004, Erdener and colleagues (31) developed a hypoxia/reoxygenation rabbit NEC model, in which newborn rabbits received 5-min hypoxia followed by 5-min reoxygenation, which

| Table 4. A comparative overview of the advantages, disadvantages, and applications of experimental models for the study of NEC |
|-----------------|-----------------|-----------------|-----------------|
| **Model Organism** | **Advantages** | **Disadvantages** | **Applications** |
| Hamsters and rabbits | Relatively large size. Ease of handling. | High cost relative to rats and mice. Variability of genetic background. Need for a well-equipped surgical facility to perform cesarean section and safely manage a litter of piglets. | |
| Invertebrate (Drosophila) | Low cost. Easy genetic manipulation. | Relevance to disease in mammalian systems is uncertain. | Study of underlying mechanisms. |
yielded intestinal lesions similar to human NEC, and which was applied to determine the effect of pentoxifylline on NEC. In a modification of this rabbit NEC model, Choi et al. (22) used a combination of LPS, hypoxia, and hypothermia in term New Zealand White rabbits before weaning (i.e., 0–4 wk) to assess intestinal perfusion by noninvasive, Doppler ultrasound-based methodology. Although hamster and rabbit models of NEC may offer advantages with respect to their relatively large size and ease of handling, their costs are much higher than mice or rats, and they lack the physiological and anatomic advantages of piglets, making their overall role in NEC research somewhat unclear. A comparison of the advantages, disadvantages, and applicability of models of NEC in each of these species is shown in Table 4.

**Highly Reductionist Experimental Approaches to NEC Research: Nematode and Invertebrate Systems**

Although the models of NEC described above have clear translational significance to clinical NEC, they are by definition limited by the complex nature of mammalian in vivo systems. Important questions in the field, such as the role that individual cell types may play in governing the extent of intestinal injury in NEC, may not be readily addressed by mammalian models, given the complex and interrelated systems that are activated during the experimental conditions. To address this potential pitfall, various authors have utilized highly reductionist approaches that allow for the study of single cells and cell-microbial interactions, to gain insights into the pathogenesis of NEC. For instance, the nematode *Caenorhabditis elegans* is characterized by a remarkably well-characterized genome and has an epithelialized gastrointestinal tract with a predictable number and arrangement of cells, that can be fed a diet of bacteria of various species. By using this model, mechanisms leading to gut epithelial necrosis in response to bacterial products can be readily assessed without needing to control for other cell types found in mammalian systems. The genome of *C. elegans* is completely sequenced and is known to share homology with that of mammals (94). Work in *C. elegans* has been used to identify the role of proteases in mediating necrotic gut epithelial death, which approximates that seen in clinical NEC (11, 70). Similarly, investigators have performed important studies using flies, which have shed light on the processes that regulate epithelial homeostasis within the intestine (96), and have identified critical molecular determinants required for intestinal epithelial apoptosis (87) and proliferation (45). Although it would be inaccurate to indicate that these models in flies and worms are a surrogate for complex studies that can be performed in mammalian systems, it is fair to indicate that studies in these systems offer advantages of easy genetic manipulation while assessing conditions known to be important in NEC research (Table 4).

**An Approach to Model Selection for NEC Research**

Having reviewed the various animal models available for NEC research, we now propose an approach to model selection. For the majority of NEC studies, the development of models in rodents will be most appropriate, given their advantages of low cost, ready availability, and relative ease of disease induction. Full-term rats and mice are relatively immature compared with full-term infants, so that delivery of premature pups by cesarean section may not be necessary. Experimental rats and mice have relatively uniform genetic backgrounds and a short gestational period and can mimic the macroscopic, microscopic, and biochemical features of NEC; they are therefore appropriate models for identification of biomarkers, pathogenetic mechanisms, and drug discovery. Compared with rodents, neonatal piglets share greater anatomical, physiological, and metabolic similarities with the human neonate, and the preterm-delivered piglet approximates the preterm human infant in terms of size. The piglet is therefore useful in studies of pathogenesis, and also in the evaluation of specific feeding regimens and in preclinical drug studies for prevention and potential therapy, as well as for the development of early radiological diagnostic methods (35). However, the relatively high cost, the variability of genetic background, and the need for a well-equipped surgical facility to perform a cesarean section limits the application and widespread use of piglet NEC models. Although no particular animal model can fully incorporate all the characteristics in human NEC, significant progress has already been achieved in advancing our understanding of this disease by use of these animal models,
and the major advantages, disadvantages, and applicability of these models are shown in Table 4. Through an understanding of more than one particular model, and collaboration between investigators who work across models, greater insights into this devastating disease can be gained.

Future Directions in NEC Model Development

As we learn more about the pathogenesis of NEC, in part through the studies described above, as well as in part from the careful study of clinical tissues obtained from patients with and without NEC at similar gestational ages, it is imperative that our modeling evolves to more readily count for nuances in the understanding of the disease. For instance, as a greater understanding of the role of the microbiome in NEC becomes apparent, it will be important to factor in various strains of bacteria to induce NEC and to take into account the relative roles of varying microbial communities in animal experiments in which NEC is observed to varying degrees. Similarly, as the role of the host genome on NEC development becomes more apparent, specific experiments in mice, or in lower organisms as described above, could be performed to better understand the role of host genetics in disease pathogenesis, which until recently was considered a passive bystander in the process (41). In addition, owing in part to the recently emerging gene editing technologies using zinc-finger nucleases (36), transactivation-activator-like effector nucleases (97, 17) and CRISPR-Cas systems (65), genetically manipulated rats, pigs, and other mammals may be available for NEC study in the near future. Finally, the recent identification of techniques for the culture and growth of intestinal stem cells from mice and humans with and without NEC has opened doors for the possibility of generating cultures of the intact intestinal epithelium for mechanistic studies, as well as to provide a platform for drug discovery (Fig. 3) (1, 76). The immediate future holds great promise for the field of NEC research, in part due to the powerful and rapid advances that have been made recently in fundamental mechanisms that lead to NEC development, but also in the identification of specific preventative or treatment approaches.

ACKNOWLEDGMENTS

The authors acknowledge the large number of talented researchers in the field and apologize to those dedicated scientists whose important work was inadvertently not referenced.

GRANTS

D. J. Hackam is supported by The Hartwell Foundation, Memphis, TN and by R01GM078238, R01DK083752, and P50GM053789 from the National Institutes of Health.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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AJP-Gastrointest Liver Physiol • doi:10.1152/ajpgi.00422.2013 • www.ajpgi.org


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