

Review

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Animal models of necrotizing enterocolitis: review of the literature and state of the art

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Abstract: Necrotizing enterocolitis (NEC) remains the leading cause of gastrointestinal surgical emergency in preterm neonates. Over the last five decades, a variety of experimental models have been developed to study the pathophysiology of this disease and to test the effectiveness of novel therapeutic strategies. Experimental NEC is mainly modeled in neonatal rats, mice and piglets. In this review, we focus on these experimental models and discuss the major advantages and disadvantages of each. We also briefly discuss other models that are not as widely used but have contributed to our current knowledge of NEC.

Keywords: mice; mutant; NEC; pig; rat; transgenic.

Introduction

Necrotizing enterocolitis (NEC) remains the leading cause of gastrointestinal surgical emergency in preterm neonates [1, 2]. The incidence of NEC has increased over the years, partly due to the high number of preterm babies that are able to survive with the advancements in neonatal intensive care [3]. However, the morbidity and mortality of these babies with NEC remain high [4]. In an attempt to reduce the disease incidence and improve patient survival, many groups have advanced both clinical and laboratory research over the last five decades, leading to an exponential increase in the number of publications

associated with NEC. In particular, a variety of experimental models have been used to advance knowledge on the prevention of NEC and to study the effectiveness of novel therapeutic strategies.

Typically, investigators have reproduced experimental NEC using contributory factors similar to those that cause the disease in humans, such as bacteria and their byproducts. In 1970, Polotskiĭ and Vasser [5] induced NEC via the administration of enteropathogenic *Escherichia coli* in guinea pigs. In 1972, Touloukian et al. [6] reproduced what they termed “ischemic gastroenterocolitis” in neonatal piglets by inducing hypoxia followed by resuscitation. This experiment demonstrated that asphyxia increases the risk of neonatal intestinal necrosis and/or perforation caused by an initial reduction in perfusion followed by a rebound hyperperfusion in the intestinal blood flow.

Following these initial studies, other groups reproduced experimental NEC with different methods. In this review, we focus on the most popular experimental models of NEC that have been widely employed, and in particular we discuss experimental NEC reproduced in rats, mice and piglets. We also briefly discuss other models that are not as widely used but have contributed to our current knowledge of NEC.

The rat model of NEC

In a seminal study published in 1974, Barlow et al. [7] described a model of NEC based on factors that were recognized at the time to contribute to the development of human NEC: (1) intestinal immaturity, (2) hyperosmolar feeding, (3) hypoxic stress, and (4) bacteria. As NEC affects mainly premature and low-birth-weight neonates, Barlow et al. [7] opted to use neonatal rats, which have an immature intestine at term similar to that of human preterm babies. Moreover, as NEC mainly develops in neonates fed with formula, Barlow et al. [7] fed rats with a hyperosmolar formula. Their formula was made from a mix of human and canine artificial formulas, which was calculated to provide 163 calories/100 mL; this was close to rat maternal

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milk, which was calculated to provide 152 calories/100 mL. Formula was administered by a dropper 4 times a day, achieving approximately 300 kcal/kg/day. In this study, formula-fed rats grew similarly to mother milk-fed rats, but with 1 day of delay. Formula-fed rats were kept with a non-lactating foster mother. Additionally, to reproduce intestinal ischemic changes that are found in human neonates with NEC, rats were subjected to hypoxic stress. The latter was achieved by sealing a plastic bag around the head of the pup for 3–5 min, until cyanosis was observed. Finally, some rats were administered *Klebsiella* either orally or via transvaginal catheterization of the dam 24 h before delivery. With these experiments, Barlow et al. [7] concluded that to have a functioning model of NEC, hypoxia and formula feeding were essential.

Other authors have employed and modified the neonatal rat model of NEC. Caplan et al. [8] adjusted the NEC induction protocol by exposing pups to 100% nitrogen (N_2) for 60 s followed by cold stress at 4 °C for 10 min. The hypoxic insult was standardized by Nadler et al. [9] by placing the rat pups in an oxygen (O_2)-monitored chamber (5% O_2 with 95% N_2).

To decrease experimental variability and increase the severity of intestinal damage, some groups introduced another stress factor: lipopolysaccharide (LPS) [10–16]. LPS is an endotoxin and a major component of the outer membrane of Gram-negative bacteria, which are known to be involved in the pathogenesis of human NEC. In our laboratories, we administer 4 mg/kg/day of LPS from *E. coli* mixed with formula on days 1 and 2 of life [16].

Interestingly, some authors observed that a single stress factor could be sufficient to reproduce severe bowel damage in pups. Nadler et al. [9] showed that formula milk alone could cause significant intestinal damage. Likewise, other groups have reported that severe hypoxic

insult alone (either 0% O_2 for 2 min or 5–10% O_2 for 30 min) is sufficient to induce bowel damage [17, 18].

To assess the degree of NEC severity, Nadler et al. [9] described the architectural changes of the small intestine at histology, which were then quantified by Dvorak et al. [19] using the following scoring system: 0, no damage; 1, mild changes, slight separation of submucosa and/or lamina propria; 2, moderate separation of submucosa and/or lamina propria and/or submucosal edema and muscular layers; 3, severe separation of submucosa and/or lamina propria and severe edema of the submucosal and muscular layers and sloughing of regional villi; 4, necrosis and loss of villi structure.

Our group has described a scoring system of the macroscopic appearance of the gut that could be helpful in assessing the severity of intestinal damage [16]. This scoring system, validated by histology, was based on the assessment of gut consistency, color, and dilatation. In the same study, we also described a clinical sickness score that we used to evaluate rat clinical status and to identify suffering pups to be euthanized. We used a modified version of an established scoring system [20] using parameters such as general appearance, response to touch, natural activity, and body color.

The rat model of NEC is most commonly used due to the low costs and ease of breeding (Table 1). Moreover, in this model, dams are removed from the pups soon after birth to avoid breastfeeding, which is known to be protective against NEC. This reproduces what occurs in human babies with NEC who are managed in the neonatal intensive care units (NICU) and whose mothers are likely not producing breast milk. However, the inability to provide intensive care to these rat pups limits the model to only the first few days of life after NEC induction. In fact, 100% of the animals that have undergone the NEC induction

Table 1: Advantages and disadvantages of the most widely used models of NEC.

Model	Advantages	Disadvantages
Rat	<ul style="list-style-type: none"> – Low costs – Easy breeding – Exclusive formula feeding since birth 	<ul style="list-style-type: none"> – Only few transgenic models available – Short-term model due to the inability to maintain pups alive for many days after NEC induction – Formula feeding does not provide sufficient calories and thus the pups are malnourished
Mouse	<ul style="list-style-type: none"> – Low costs – Easy breeding – Ability to genetically modify genes and create transgenic models 	<ul style="list-style-type: none"> – Gavage feeding technically challenging due to small animal size – Exposure to breast milk in the first hours/days of life – Frailty of transgenic animals
Piglet	<ul style="list-style-type: none"> – Anatomical and pathological similarities with preterm human intestine affected by NEC – Easily reproduced bowel injury even with a single stress factor (formula feeding) 	<ul style="list-style-type: none"> – High costs – Not available for genetic modification – Few commercially available antibodies – NEC induced on day 1 of life

protocol eventually die. Furthermore, the formula used to feed these pups does not provide the sufficient daily caloric intake and all pups are malnourished. This contrast with the current availability of parenteral nutrition for humans that ensures that babies with NEC receive adequate caloric intake.

The mouse model of NEC

The ability to genetically modify mice has created an enticing opportunity to better understand the genes and pathways that are involved in the pathogenesis of NEC. However, establishing NEC in mouse pups has been challenging due to the animal size and the frailty of transgenic pups. Compared to the rat model, there has been a bigger variability in modeling the disease, as reflected by the inconsistency in animal age at time of induction. Jilling et al. [14] were the first to create a transgenic model of NEC in neonatal mice, using the transgenic strain C3H/HeJ (TLR4 transgenic) delivered by cesarean section before term. In this study, NEC was induced in postnatal day 0 (P0) pups by gavage formula feeding, hypoxia (100% N₂ for 1 min) and hypothermia (4 °C for 10 min). Other groups used similar protocols to induce NEC but in P3 [21] or P5 mouse pups [22]. It is interesting to note the high degree of age variability of the mouse pups within the same laboratory: Dr Hackam's group has induced NEC in different transgenic mouse models at different ages, ranging from 7 to 21 days of life [23–28]. A study from another research group compared the incidence of NEC in mouse pups induced at different ages [29]. According to that study, there is no need to prevent breastfeeding immediately after birth as in the rat model, because initial breastfeeding does not influence the incidence of NEC. This would support studies that have induced NEC in mouse pups that are a few days old.

Beside the standard induction protocol for NEC, some investigators have employed other stress factors in mouse pups. Recently, Ginzl et al. [30] showed dextran sodium sulfate (DSS) is sufficient to induce NEC in formula fed neonatal mice. DSS, a chemical with anticoagulant properties, has been used for years to model inflammatory bowel disease [31]. In their mouse model, Ginzl et al. [30] noticed that DSS caused NEC-like lesions with humoral and cellular immune response in the small and large bowel. The authors state that this novel model of NEC has the advantage of avoiding physical stressors for the animals such as hypoxia and hypothermia and of inducing mucosal tissue changes in a relatively short time.

Another interesting model to induce NEC in mouse pups is based on Paneth cell elimination. Paneth cells are epithelial cells found in the villi of the small intestine and are responsible for produce antimicrobial peptides. With the increasing recognition of the importance of gut microbiota in the pathogenesis of NEC, Paneth cells have been identified as key players in these critical events [32]. Zhang et al. [33] reported that P14-P16 mice subjected to an intraperitoneal injection of dithizone to ablate Paneth cells and administered *Klebsiella* to promote the inflammatory response developed NEC within 10 h. Although this model addresses an important aspect of NEC pathogenesis, it cannot be reproduced in newborn mice, since Paneth cells in rodents are not present at birth unlike in humans.

The mouse model of NEC has become prevalent in basic research over the years. The main advantages are the low costs and ease of breeding the animals. As mouse models are more amenable to genetic manipulation, transgenic animals can be used to study specific pathways that are affected in NEC, which also allow investigators to genetically modify genes and create transgenic animals (Table 1). However, this model has limitations due to the small size of the pups that, as discussed, has led to variability in the animal protocols across centers. As the size of mouse pups at births makes it challenging to gavage feed them from birth, these pups are invariably left with their mothers and exposed to breast milk, which is protective against NEC.

The piglet model of NEC

In an attempt to create a model of NEC closer to the clinical representation of the human disease, some investigators have employed preterm piglets, whose intestine shares ontogenetic similarities to that of human neonates. As mentioned earlier, Touloukian et al. [6] were the first to model NEC in neonatal piglets by inducing asphyxia followed by resuscitation. This model displayed decreased gastrointestinal perfusion, intestinal hemorrhage and inflamed mucosal layers, all of which are histopathological hallmarks of necrosis. This study provided considerable understanding of the etiology of NEC, but it was criticized for the use of mature piglets (7–20 days old) and severe asphyxia insults. To avoid the latter, Cohen et al. [34] induced NEC via moderate asphyxia achieved with 50% reduction in partial pressure of oxygen for 30 min over a prolonged period of time in neonatal piglets aged 3–96 h. Other investigators have avoided the asphyxia stress factor to model NEC in piglets and have reproduced

similar bowel injury with a combination of ischemia and formula feeding [35] or even formula feeding alone [36]. In the latter case, Sangild's group has extensively investigated the pathogenesis of NEC and tested potential strategies for the prevention and treatment of this disease.

Compared with the rodent models, piglets offer unique advantages. Piglets share similarities in histopathological features and clinical signs of human NEC. Moreover, they reach gastrointestinal maturation only 2–3 weeks after birth. This has allowed the use of neonatal piglets to represent the immature gut of premature babies with NEC. Furthermore, as preterm piglets have similar body size and weight as those of human infants, some aspects of their management, such as surgical manipulation, replicate the human conditions and constitute an advantage for potential translation. In addition, Sangild et al. [36] have managed their animals in a “piglet intensive care unit”, which has an added advantage of offering similar quality of care to that of a hospital NICU and of exposing the piglets to similar risk factors associated with human NEC. In fact, piglets receive parenteral nutrition that on the one hand provides the adequate daily caloric intake, but on the other hand is associated with a morbidity risk related to central venous lines. Interestingly, the addition of parenteral nutrition in this model has shown to increase the incidence of NEC in piglets.

However, the piglet model has some recognized limitations, such as the difficulties of creating transgenic animals and the differences in the pathological intestinal changes caused by NEC. Specifically, piglets develop NEC-like damage between the stomach and the jejunum, whereas in human infants the ileum is the most affected area. These differences may impair the full understanding of the molecular mechanisms that are involved in NEC pathogenesis.

Other models of NEC

Beyond the models of NEC described above, some investigators have employed other techniques and/or animals to reproduce NEC-like bowel damage. At present, these techniques are not common, as they do not consider the multifactorial nature of NEC pathogenesis and do not reproduce the typical features of the human disease.

One of the most popular models in the past was based on the concept of bowel ischemia/reperfusion injury. Recognizing that the intestine of human babies with NEC suffers from profound ischemia, some investigators have occluded the superior mesenteric vessels to induce NEC-like bowel damage in rodents [37–41]. Given the technical

challenge to ligate very small vessels in newborn rodents, these studies were conducted mainly in adult or weanling animals.

Another technique to create a NEC-like bowel damage was described by Clark et al. [42] in weanling rabbits. Using an already established model for the study of cholera toxins [43], the authors ligated the rabbit intestine to form 10-cm long loops while preserving their blood flow. The tested ligated loops were injected with acidified casein and protein, which resulted in NEC-like bowel damage at histopathology. With the same concept of a close-loop obstruction, Bozeman et al. [44] induced NEC in preterm rabbits by blocking the anal canal using cyanoacrylate tissue adhesive while feeding with formula mixed with *Enterobacter cloacae*. This resulted in a picture of abdominal distention and intestinal dysmotility, which promoted bacterial translocation.

In the past, goats were also employed to study experimental NEC, as described by Sweeny et al. [45]. In this study, the authors administered hypertonic formula to induce damage to the intestinal mucosa.

To study specific bacterial strains in their capabilities to induce NEC, a French group employed gnotobiotic quails, which are raised in a germ-free or known bacterial environment [46, 47]. In these studies, the authors investigated the role of *Clostridium* in the pathogenesis of NEC and the protective role of bifidobacteria. They also induced bowel damage by inoculating germ-free quails with known flora. As quails are not milk consumers, they were fed a diet that contained lactose and was sterilized by irradiation.

Conclusion

Over the years, a variety of experimental models of NEC have been described. Typically, models that employ stress factors similar to those that contribute to the development of human NEC have been the most widely used. For this reason, the neonatal rat model developed by Barlow et al. [7] in 1974 and most recently adapted to neonatal mice remains the most commonly used of all. However, an exact model of human NEC is yet to be described. To date, investigators would have chosen one model over another depending on their research question. If the study is directed toward translation into clinical practice, the piglet model is the most suitable. Conversely, if the study is aimed to investigate molecular mechanisms of NEC pathogenesis or therapeutic effectiveness of a novel drug, the transgenic mouse model is a good option. Overall, the animal models of NEC have increased our understanding

of this devastating disease, particularly in contributing factors, prevention and therapeutic options.

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Informed consent: Informed consent is not applicable.
Ethical approval: The conducted research is not related to either human or animals use.

Author Contributions

Adrienne T Sulistyo: writing – original draft; Abidur Rahman: writing – original draft; George Biouss: writing – original draft; Lina Antounians: writing – review and editing; Augusto Zani: conceptualization; supervision; writing – review and editing.

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Reviewer Assessment

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Reviewers' Comments to Original Submission

Reviewer 1: anonymous

Jan 09, 2018

Reviewer Recommendation Term: Accept with Minor Revision
Overall Reviewer Manuscript Rating: N/A

Custom Review Questions	Response
Is the subject area appropriate for you?	4
Does the title clearly reflect the paper's content?	4
Does the abstract clearly reflect the paper's content?	4
Do the keywords clearly reflect the paper's content?	4
Does the introduction present the problem clearly?	4
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	4
How adequate is the data presentation?	4
Are units and terminology used correctly?	4
Is the number of cases adequate?	4
Are the experimental methods/clinical studies adequate?	4
Is the length appropriate in relation to the content?	4
Does the reader get new insights from the article?	4
Please rate the practical significance.	4
Please rate the accuracy of methods.	4
Please rate the statistical evaluation and quality control.	4
Please rate the appropriateness of the figures and tables.	4
Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	4
Please judge the overall scientific quality of the manuscript.	4
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

In this study different animal models of NEC are introduced. Their advantages and disadvantages are discussed. Overall, this review gives a good overview of methods and models to induce NEC.

Minor revision:

Page 3: "...Dvorak et al proposed the following histology based scoring system...". The cited publication is from 2002, but this scoring system has been introduced earlier.

Page 6: The authors state, that Paneth cells were identified as key players in NEC pathogenesis. In contrast to rats and mice, which develop Paneth cells after around two weeks after birth, humans develop these cells during the first trimester of gestation. So in humans NEC occurs in the presence of Paneth cells. This difference should be addressed.

Reviewer 2: anonymous

Jan 02, 2018

Reviewer Recommendation Term:	Accept
Overall Reviewer Manuscript Rating:	N/A
Custom Review Questions	Response
Is the subject area appropriate for you?	5 - High/Yes
Does the title clearly reflect the paper's content?	5 - High/Yes
Does the abstract clearly reflect the paper's content?	5 - High/Yes
Do the keywords clearly reflect the paper's content?	5 - High/Yes
Does the introduction present the problem clearly?	5 - High/Yes
Are the results/conclusions justified?	5 - High/Yes
How comprehensive and up-to-date is the subject matter presented?	5 - High/Yes
How adequate is the data presentation?	N/A
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	N/A
Are the experimental methods/clinical studies adequate?	N/A
Is the length appropriate in relation to the content?	5 - High/Yes
Does the reader get new insights from the article?	4
Please rate the practical significance.	4
Please rate the accuracy of methods.	N/A
Please rate the statistical evaluation and quality control.	N/A
Please rate the appropriateness of the figures and tables.	5 - High/Yes
Please rate the appropriateness of the references.	5 - High/Yes
Please evaluate the writing style and use of language.	5 - High/Yes
Please judge the overall scientific quality of the manuscript.	5 - High/Yes
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

Animal models of Necrotizing Enterocolitis: Review of the literature and State of the Art for Innovative Surgical Sciences.
The author described all established animal models for Necrotizing Enterocolitis (NEC). He discussed specific details of each model and their implication for NEC in humans. Particularly, the summarizing table makes clear the advantages and disadvantages of the models of different animals including their impact for specific scientific problems.

Authors' Response to Reviewer Comments

Feb 15, 2018

Dear Editor,

Re: Ms. No. ISS-2017-0050

Many thanks for giving us the opportunity to revise our manuscript entitled: "Animal Models of Necrotizing Enterocolitis: Review of the Literature and State of the Art". We have considered the Reviewers' comments, implemented the suggested changes (highlighted in yellow in the text), and provided a point-by-point response as indicated below.

Thank you in advance for further consideration,

Reviewer #1: Minor revision:

Page 3: "...Dvorak et al proposed the following histology based scoring system...". The cited publication is from 2002, but this scoring system has been introduced earlier.

We have added a reference of an earlier publication from the same research group (Nadler et al 2000), where the authors had qualitatively described the histological differences that were then quantified by Dvorak et al in 2002.

Page 6: The authors state, that Paneth cells were identified as key players in NEC pathogenesis. In contrast to rats and mice, which develop Paneth cells after around two weeks after birth, humans develop these cells during the first trimester of gestation. So in humans NEC occurs in the presence of Paneth cells. This difference should be addressed.

We are grateful to the Reviewer's comment as it gave us the opportunity to expand on this topic. A sentence explaining the late development of Paneth cells in rodents has been added to the text (page 6).

Reviewer #2: The author described all established animal models for Necrotizing Enterocolitis (NEC). He discussed specific details of each model and their implication for NEC in humans. Particularly, the summarizing table makes clear the advantages and disadvantages of the models of different animals including their impact for specific scientific problems. I have no further comments and suggest accepting the manuscript for publication.

We are thankful for the comments made.

Reviewers' Comments to Revision

Reviewer 1: anonymous

Feb 17, 2018

Reviewer Recommendation Term:	Accept
Overall Reviewer Manuscript Rating:	N/A
Custom Review Questions	Response
Is the subject area appropriate for you?	5 - High/Yes
Does the title clearly reflect the paper's content?	5 - High/Yes
Does the abstract clearly reflect the paper's content?	5 - High/Yes
Do the keywords clearly reflect the paper's content?	5 - High/Yes
Does the introduction present the problem clearly?	5 - High/Yes
Are the results/conclusions justified?	5 - High/Yes
How comprehensive and up-to-date is the subject matter presented?	5 - High/Yes
How adequate is the data presentation?	5 - High/Yes
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	5 - High/Yes
Are the experimental methods/clinical studies adequate?	5 - High/Yes
Is the length appropriate in relation to the content?	5 - High/Yes
Does the reader get new insights from the article?	5 - High/Yes
Please rate the practical significance.	5 - High/Yes
Please rate the accuracy of methods.	5 - High/Yes
Please rate the statistical evaluation and quality control.	5 - High/Yes
Please rate the appropriateness of the figures and tables.	5 - High/Yes
Please rate the appropriateness of the references.	5 - High/Yes
Please evaluate the writing style and use of language.	5 - High/Yes
Please judge the overall scientific quality of the manuscript.	5 - High/Yes
Are you willing to review the revision of this manuscript?	No: done

Comments to Authors:

It's ok
