Video Article

A Murine Model of Experimental Necrotizing Enterocolitis Using Gavage Feeding, Lipopolysaccharide, and Systemic Hypoxia

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Abstract

This protocol describes a model of experimental necrotizing enterocolitis (NEC) using rats or mice. NEC is a gastrointestinal disease unique to premature infants. Nearly 10% of babies born <1.5 kg develop this disease, and the mortality rate approaches 50%. The pathogenesis remains incompletely understood, but involves feeding, ischemia, inflammation, and infection. Animal models are vital to advancing the collective understanding of NEC. Many laboratories study NEC using the murine model. Other models, including pigs and rabbits, have limitations, including cost, long gestation periods, and smaller litters. Many studies use known risk factors (enteral feeding, infection, inflammation, and ischemia) in NEC research.

One challenge in NEC research is enteral feeds. Pups, normally breastfed by their mother, must be fed by hand. Some methods include syringe or fine-tip applicator feeds. This requires animals to latch and swallow feeds without respiratory compromise. Risks include aspiration, regurgitation, and spilling of feeds. The complications often cause unintended mortality and inconsistent results. Gavage feedings avoid these complications. Feedings are gavaged using a silastic catheter, allowing for safe, efficient feedings. This reduces feeding-related complications and mortality. This method improves reproducibility, as the complete volume is appropriately administered.

The protocol utilizes three interventions associated with clinical NEC: diet, hypoxia, and inflammation. The diet is a high-calorie formula, which is associated with NEC. Pups receive enteral lipopolysaccharide (LPS). LPS, a Toll-Like Receptor 4 (TLR4) agonist, is associated with NEC in animals and humans. Following feeds, animals are subjected to hypoxia. Premature neonates are susceptible to hypoxemia, which, along with decreased intestinal perfusion following feedings, puts the infant at risk for post-prandial ischemia.

Introduction

The field of neonatology has evolved immensely over the last 50 years. Improvements in neonatal care have resulted in an increasing number of premature newborn who survive the first few days of life from respiratory insufficiency[1]. However, these infants face the risk of other complications of prematurity. One of these complications is Necrotizing Enterocolitis (NEC), a life threatening GI disease occurring almost exclusively in preterm neonates. The disease occurs in nearly 10% of all infants born less than 1.5 kg. The mortality rate approaches 50% in the most severely affected infants[2]. Despite decades of research, the collective understanding of the pathophysiology of NEC remains incomplete[3, 4].

NEC is a life-threatening gastrointestinal disease affecting neonates resulting in systemic inflammation, primarily affecting the small intestine. Breast milk has been shown to confer some protection[5]. Currently, treatment is largely supportive including bowel rest, antibiotics, the use of ventilators and inotropes to mitigate the effects of shock. Surgery is reserved for failures of medical management and includes resection of dead or perforated bowel. Therefore, the goal of NEC research has been to prevent NEC from occurring by concentrating on preventative factors such as breast milk feedings, growth factors, avoidance of stressful stimuli, and identification of molecular targets with therapeutic potential.

Performing clinical studies and interventions for NEC are difficult, given the uncertainty and complexity of its pathophysiology. Therefore, animal models are required to advance the field. Several models have been used throughout the years[6]. Some animal models, including pigs and rabbits, have limitations, including cost, time, and smaller litters[7, 8]. To maximize efficiency, many researchers use a murine (rat or mouse) model. Animals subjected to experimental NEC develop histological and biochemical changes similar to human neonates with the disease. However, there are several protocols used that will produce intestinal injury consistent with NEC, but may not resemble the clinical disease. The most significant risk factors for NEC are enteral feeding, ischemia, infection, and inflammation[9]. Many laboratories use some or all of these risk factors in their studies of NEC. The most important benefits of these models are that the findings could accurately represent the clinical disease.

One of the biggest challenges with those models is enteral feedings. The model uses animals that are only a few days old, and would normally be breastfed by their mother. Instead, animals must be fed by hand by the researchers. This is accomplished by using a syringe or a fine-tipped applicator. Animals must be able to adequate take feedings into their mouth, swallow the feed, and still be able to maintain adequate respiratory

efforts. However, this is often fraught with complications, including aspiration, regurgitation, and spilling of feeds. Consequently, studies are at risk for unintended mortality and inconsistent results.

One method to reduce these complications is to use gavage feedings. This technique allows for direct administration of feeds into the stomach, significantly reducing the risk of aspiration. In addition, the time required to feed the animals is drastically reduced, allowing for studying several litters simultaneously. The catheters are inexpensive, durable, and can be obtained from a neonatal intensive care unit (NICU). If a laboratory does not have access to these catheters, they can be easily ordered from a commercial vendor.

The induction of experimental NEC is accomplished by using several facets. Animals are fed a high-calorie formula, which is known to be a risk factor for NEC. Lipopolysaccharide (LPS) is also added to the feedings. LPS promotes overwhelming inflammation and is an agonist of the Toll-Like Receptor 4 (TLR4) pathway[10]. TLR4 activation is strongly associated with NEC pathogenesis in both animal and clinical studies. After feedings, pups are subjected to systemic hypoxia. Clinically, premature infants are at risk for significant hypoxia. This puts the infant at risk for intestinal ischemia, exacerbated by an increased metabolic demand in the post-prandial state.

The protocol is appropriate for both neonatal rats and mice. Rats are the preferred species, as they can be taken away from their mothers immediately after they are born, and can even be delivered prematurely by cesarean section of the mother. Taking the rats away from their mother immediately after they are born allows for the experimental protocol to occur prior to any effect of the pups receiving breast milk from their mother. However, there are few genetically modified rat strains, so knockout mice are needed. Neonatal mice are much smaller than rats, and must remain with their mother for seven days prior to any experiments.

This model of experimental NEC allows researchers to study the disease with known clinical risk factors. In addition to understanding pathogenesis, it allows for the opportunity to observe how diet modifications, supplements, and other interventions affect the disease.

Protocol

Ethical Statement: All protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The Medical College of Wisconsin. All procedures are non-survival surgery. No eye ointment was required. All syringes, utensils and tools were sterile. All chemicals were sterile. Euthanization of adult animals occurred by an overdose of carbon dioxide followed by a thoracotomy/pneumothorax. Euthanization of neonatal pups occurred by a lethal dose of ketamine and xylazine, followed by a thoracotomy/pneumothorax.

1. Catheter Selection and Procurement

1. Use 1.9 Fr (0.6 mm) single lumen catheters. Order commercially available catheters or obtain discarded catheters from a neonatal intensive care unit (NICU).
   NOTE: The 1.9 Fr is the standard size used, and can be readily obtained.

2. Catheter Disinfection

1. Disinfect catheters by flushing with 75% ethanol and 10% bleach. Submerge catheters in this solution for one week. Flush catheters with sterile water and allow to dry for several days prior to use.
2. Examine all catheters for patency.
   1. Flush sterile water through the catheter to ensure that the catheter is patent and that there are no punctures or leaks within the catheter. Store catheters in a clean plastic bag in a dry area until use.

3. Animal Selection, Breeding and Birthing

NOTE: Use rats if no genetically altered strains are needed, or if an established rat strain exists. Otherwise, use mice. The timing and method of birth depends on both the species used and the goal of the experiment.

1. If using rats, order pregnant female Sprague Dawley rats from an animal vendor with a known date of delivery so that they arrive within one week of expected delivery. Allow pups that will serve as control animals to delivery spontaneously and stay with their mother.
   1. Cesarean Delivery
      NOTE: Pups that will be subjected to NEC are prematurely delivered one day prior to the estimated date of delivery, or 20 days after conception.
      1. Euthanize the pregnant female by an overdose of carbon dioxide and thoracotomy.
      2. Immediately, place the animal in supine position. Make a midline vertical incision with scissors along the abdomen, starting from the pelvis and proceeding to the sternum. The pups are visible in the uterine horns.
         NOTE: The incision should be deep enough to penetrate the muscle and fascia, but not to puncture the intestines or uterine horns.
      3. Make a small incision with fine scissors into one horn. Remove pups by squeezing behind the pup through the uterine incision.
      4. Remove the placentas by pinching between the placenta and the animal to separate them.
         NOTE: Each pup has its own placenta.
      5. Dry the pup with paper towel. Observe that pups are adequately breathing. Pups may require gentile tactile stimulation to breathe.
      6. Place pups into an empty 1.25 ml pipette tip box or an equivalent container with paper towel at the bottom (to absorb urine and feces) and place under a heat lamp. Place the container into an egg incubator, set at 37 °C and 70% humidity, for three hours before any feeding is performed.

NOTE: Add distilled water to the incubator to provide humidity.

2. If a control litter of premature pups is needed, use a surrogate mother.
    1. Time a rat pregnancy so that the estimated date of delivery (EDD) occurs one day earlier than the mothers that are to be euthanized and delivered prematurely. After the surrogate mother delivers her pups, remove them from the cage. These pups may be used for other experiments or euthanized.
    2. After delivering the premature pups and allowing them to transition to extrauterine life, gently roll the premature pups in litter from the surrogate mother's cage. Then place the pups with the surrogate mother.

2. If using mice, place mice in harem breeding (one male, two females). Pups are born by spontaneous vaginal delivery. At one week of life, move pups to be subjected to NEC to an incubator (see step 3.1.1.6). Allow control pups to remain with the mother and feed on demand.
NOTE: Surrogate mothers are not needed for mouse experiments.

4. Catheter Preparation

NOTE: Use one catheter for each feeding condition. Some experiments require different feeding conditions, including the absence or presence of LPS, supplementation with intestinal alkaline phosphatase (IAP), or inhibitors of endogenous IAP.

1. Cut the catheter (any length greater than 4 cm) with scissors to ensure that the end is not jagged. Using a marker, draw a line at 4 cm from the end of the catheter tip. Fill a 1.0 ml syringe with the appropriate formula (see section 5). Flush the catheter with the formula to ensure that all contents are evacuated prior to feeding.

5. Diet and Feeding

1. Prepare hypercaloric formula consisting of powder milk replacer (1 part powder to 2 parts water). Fortify with premature infant powdered formula (1 g powdered formula per 5 ml milk replacer) to make a final concentration of 30 kcal/oz.
2. Add lipopolysaccharide (LPS) (0.1 mg LPS per 5 ml of fortified formula). Make enough LPS/formula for 24 h of feedings.
3. Take the pups from the incubator. With a moist soft towel, gently stimulate the perineal region to stimulate urination and defecation.
    1. Grasp the pup between the thumb and index finger near its neck. Apply gentle pressure to the lower jaw to open the mouth. Carefully insert the tip of the catheter, with a slight downward angle to ensure that it enters the esophagus, and insert to a depth of 4 cm. Briefly pause to ensure that the pup is still breathing adequately.

4. Administer the formula slowly (over 1-2 min) using a 1.0 ml syringe. On the first day, feed 0.1-0.2 ml of formula (per feed). Increase the amount by 0.1 ml/feed/day. If the animal does not tolerate a given feed, decrease the amount. If the animal displays signs of distress (discoloration, difficulty breathing, regurgitation, etc.), slow or discontinue the feeding.
NOTE: Using the above concentrations, a 5 g rat will receive 100 ml/kg/day, 107 kcal/kg/day, and 2.5 mg/kg/day of LPS. Pups will receive twice this on day 2 and three times this on day three of the feeding protocol. The volume of formula given depends on the day of life and tolerance. If the catheter is in the correct position, the stomach will fill with formula, which can be easily observed. The animal may become more active with the feeding, especially if the animal has intestinal injury consistent with NEC.
5. Once the desired volume is given, remove the catheter slowly. Monitor the pup for a few minutes, and then place it back into the incubator.
6. After all of the pups have been fed, place them into a hypoxia chamber. Use compressed nitrogen gas to bring the oxygen level to 5% through a regulator. Subject the pups to 5% oxygen for 10 min. Place them back into the incubator.
7. Feed the pups 5-6 times per day at even time intervals for a total of 72 h.
Note: If a pup appears to be morbidly ill and not survive the 72 h course, euthanize to prevent inappropriate suffering to the animal (see 5.8).
8. When the experiment is complete, euthanize the pups using a lethal dosing of ketamine (100 mg/kg) and xylazine (10 mg/kg), per IACUC guidelines. Dilute appropriately with sterile 0.9% normal saline to provide a pre-mixed solution containing both medications and draw up into sterile 31 gauge syringes.
    1. Remove the animal to be euthanized from the cage. Insert the needle containing the pre-mixed solution into the left lower quadrant of the abdomen. Once inserted, inject 0.01 ml of the solution into the peritoneum. Place the animal into a small box. When the animal has stopped spontaneous movement and breathing, stimulate the animal using light touch to the tail.
    NOTE: If the animal does not respond, it is considered expired. To ensure death, a small thoracotomy is placed in the left chest.

9. Harvest the intestinal tissue using fine scissors.
    1. Make a midline incision along the abdomen, starting at the pelvis and proceeding superiorly to the sternum. Taking two fine forceps, gently push the intestines to the right side of the abdomen. Locate the descending colon left of the animal's midline. Dissect the most distal portion the colon.
    2. Grasping the distal colon segment, gently pull the intestines apart. The mesentery is firmly attached to the intestine, but should peel off easily from the intestine. If the mesentery remains attached, use two forceps and dissect the mesentery. Continue to pull the colon until the appendix is visible.
    NOTE: The appendix will be an outpouching from the colon, usually in the shape of a comma.
    3. After finding the appendix, continue to pull the intestine out. Continue to pull the intestine until 4-5 cm is visible and free of mesentery.
    NOTE: The terminal ileum is the portion of the intestine just proximal to the appendix.
    4. Using fine scissors, cut the intestine just proximal to the appendix. Make four 1-cm segments of the distal intestine.
    5. Place the small intestinal segments into appropriate containers. Place one segment into a histology cassette. Place segments into test tubes for mRNA quantification, protein quantification or enzymatic activity assays[11].

Representative Results

Using this protocol, several manuscripts have been published regarding the pathogenesis of NEC.

NADPH Oxidase in NEC

The NADPH Oxidase (NOX) family of enzymes generates reactive oxygen species. There are several isoforms, which generate either superoxide or hydrogen peroxide. NOX enzymes have physiologic functions. NOX2, the most thoroughly studied isoform, contributes to host defense by providing superoxide for the respiratory burst in killing bacteria. NOX1 also generates superoxide, but functions in intracellular communication. However, both isoforms can cause deleterious effects in inappropriately activated. Pathologic NOX2 activity has been observed in atherosclerosis, hypertension, and diabetes. Overexpression of NOX1 has been found in inflammatory bowel disease. NOX activity can be quantified using a well-studied activity assay. NOX-derived superoxide reacts with lucigenin, which produces light, and can be detected using chemiluminescence. Because there is some artefactual signaling that occurs with lucigenin, the free radical scavenger, disodium 4,5-dihydroxy-1,3-benzenedisulfonate is added to some samples, and the difference is expressed as disodium 4,5-dihydroxy-1,3-benzenedisulfonate-inhibitable chemiluminescence (TIC)[11]. TIC values are normalized to the amount of intestinal tissue added, with a final unit of TIC per milligram of protein (TIC/mg).

Inflammation and oxidative stress are overwhelming in NEC. NOX activity increases in this rat model of NEC (Figure 1)[11]. Over the course of four days, NOX activity increases in pups exposed to the NEC protocol. Inhibition of NOX2 activity reduced the overall NOX-derived superoxide, indicated that NOX2 is the main isoform responsible for the increases in NOX activity in the NEC model. When pups were exposed to only some components of the NEC model, NOX activity did not increase (Figure 2). Furthermore, NOX2 mRNA expression increased in NEC pups, while NOX1 expression was unchanged (Figure 3).

Intestinal Alkaline Phosphatase in NEC

Intestinal Alkaline Phosphatase (IAP), a member of the Alkaline Phosphatase enzymes, is an intestinal brush border enzyme normally found in the intestine. Four important functions of IAP are 1) regulation of bicarbonate secretion and duodenal surface pH; 2) modulation of intestinal long chain fatty acid absorption; 3) detoxification of LPS, resulting in amelioration of intestinal and systemic inflammation; and 4) regulation of gut microbial communities and their translocation across the gut barrier[12]. IAP thus has several mechanisms of actions that may be beneficial in ameliorating NEC. It contributes to maintaining commensal bacterial colonization, as well as inactivation of lipopolysaccharide (LPS), a highly immunogenic constituent of Gram-negative bacteria outer membranes[13]. Both LPS and its cell surface receptor, Toll-Like Receptor-4 (TLR4), are strongly associated with the development of NEC[10]. The interaction of LPS and TLR4 are known to lead to inflammation, cell death and loss of gut barrier function in the intestine, which are important events in the pathogenesis of NEC.

Using this hypoxia and LPS-induced NEC rat model the effects of IAP in NEC were examined. Early administration of IAP decreases intestinal permeability and inflammatory cytokine expression in pups subjected to the NEC protocol[14]. Total alkaline phosphatase (AP) activity was significantly decreased in NEC, but in was increased after exogenous enteral administration of IAP (Figure 4A). NEC scores were significantly decreased with IAP administration (Figure 4B). In addition, intestinal permeability, as measured by translocation of the large molecule FITC-Dextran, was increased in NEC, but was improved to near baseline levels with IAP administration (Figure 4C).

Importantly, enteral administration of IAP attenuates the systemic inflammatory response, and has improved efficacy in reducing NEC related histology injury (Figure 5), systemic and local tissue inflammation, and intestinal permeability compared with intraperitoneal injection[15-17]. There were no differences in intestinal IAP activity or NEC-related injury despite attenuation of the serum pro-inflammatory response with intraperitoneal administration of IAP[18]. This effect may have been due to local interaction with higher levels of IAP delivered directly to the intestines with enteric administration compared with an intraperitoneal route. Finally, IAP is best used preventatively as NEC histologic injury decreases and native IAP increases when NEC stressors are removed from the animal model[19].

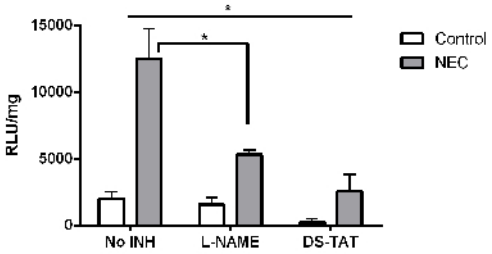

Figure 1: NOX2 activity increases in experimental NEC. Rat pups treated with the NEC protocol had significantly increased small intestinal NOX activity compared to same-day control animals that remained with their mother and were breastfed. NOX activity in samples treated with a NOX2 inhibitor (GP91-ds-*tat*) had significantly reduced NOX activity. The addition of an inhibitor of nitric oxide synthase (L-N^G-Nitroarginine methyl ester, L-NAME) did not affect NOX activity. * $p < 0.05$. Error bars indicate SEM. Y-axis is the relative light units detected that were inhibited by Disodium 4,5-dihydroxy-1,3-benzenedisulfonate normalized to milligrams of protein per well (RLU/mg). Please click here to view a larger version of this figure.

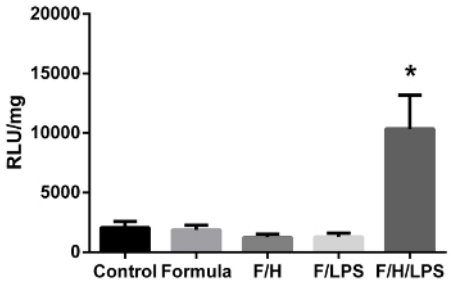

Figure 2: Maximum NOX activity requires the complete NEC model. Rat pups were subjected to either the complete NEC protocol (formula feeding, LPS, and hypoxia [F/H/LPS]), or just some of the components (formula feeding, formula and hypoxia [F/H], or formula and LPS [F/LPS]. NOX activity of pups in the partial NEC protocol was not significantly increased compared to untreated controls. * $p < 0.05$. Error bars indicate SEM. Y-axis is the relative light units detected that were inhibited by Disodium 4,5-dihydroxy-1,3-benzenedisulfonate normalized to milligrams of protein per well (RLU/mg). Please click here to view a larger version of this figure.

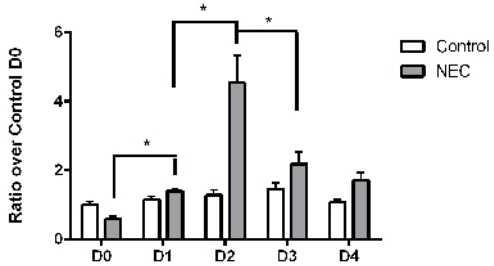

Figure 3: NOX2 expression increases in experimental NEC. Newborn rat pups were either allowed deliver at term and stay with their mother (control) or were delivered one day prematurely and subjected to the NEC protocol (NEC). Pups were euthanized on Day of Life 0-4 (D0-D4), and intestinal NOX2 expression was quantified by RT-PCR and normalized to the housekeeping gene GAPDH. Rat pups subjected to the NEC protocol had significantly increased small intestinal NOX2 expression compared to controls on D2 and D4. * $p < 0.05$. Error bars indicate SEM. Y-axis is the ratio of the expression of the condition to the ratio of control animals on Day of Life 0. X-axis is the Day of Life of each animal (D = Day of Life). Please click here to view a larger version of this figure.

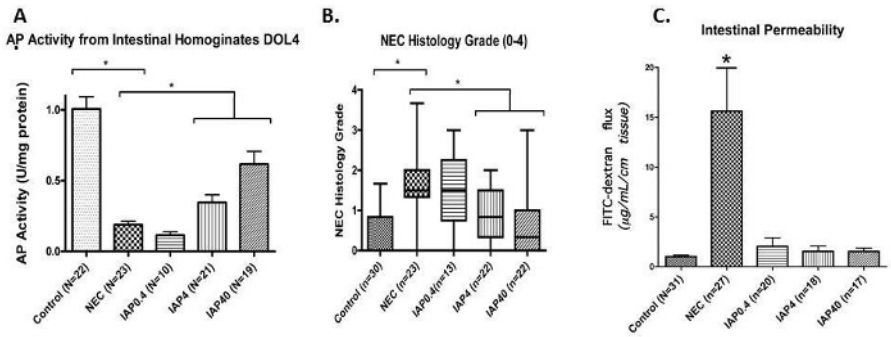

Figure 4: IAP affects histological injury grade, Alkaline Phosphatase (AP) activity and intestinal permeability. A. AP activity (y-axis) is reported as Units (U)/mg protein. DOL4 = Day of Life 4. B. Histological injury grading mean score of terminal ileum sections grade 0 (no injury) to grade 4 (full thickness necrosis). C. Permeability of ileal loops of day 3 pups was measured using 10kDa Fluorescein isothiocyanate-dextran (FITC-dextran). Data are means of five experiments; error bars represent SEM and P values ≤0.001 are indicated with an asterisk (*).* $p < 0.05$. Please click here to view a larger version of this figure.

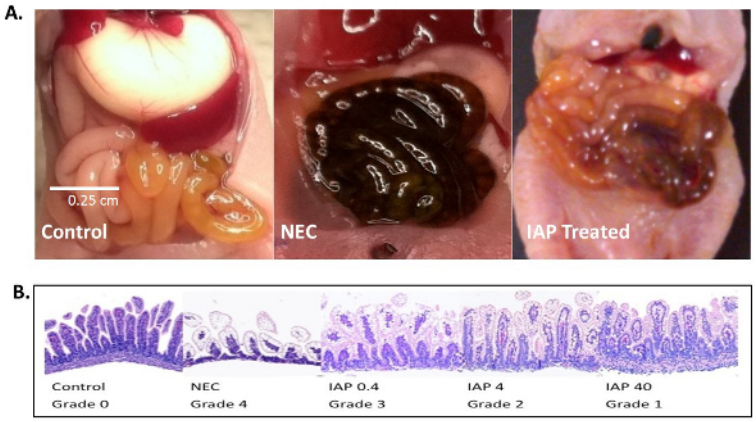

Figure 5: IAP treatment effects morphological characteristics of the intestine and histological injury grade. A. Intestine of rat pups on day of life 4- control (normal intestines), NEC (dusky intestines with full thickness necrosis) and NEC with IAP treated pups (variable patchy injury of the intestine occurring most near the terminal ileum). Bar = 0.25 cm. B. Histological injury of the terminal ileum grade 0 (no injury) to grade 4 (full thickness necrosis) with increased doses of enteral IAP treatment NEC injury grades decrease. Magnification 200 X. Please click here to view a larger version of this figure.

Discussion

NEC is a devastating disease among premature infants that desperately requires research to better understand the disease. This murine model of NEC allows researchers to potentially uncover vital knowledge that may benefit these infants. The main advantages of this model include using interventions that are known risk factors for NEC, efficient administration of feedings that reduce mortality, and improved consistency and reproducibility. Additionally, there is limited cost associated with these studies. The catheters can often be obtained from the hospitals for free, and only require minimal disinfection. The other materials required, including formula, incubators, and surgical tools, are often already in laboratories or can be purchased at a reasonable cost.

While the murine model has many advantages, there are some limitations. It is impossible to deliver pups as premature as the human infants that are at risk for NEC. While there are similarities, the newborn, full-term mouse and rat intestinal physiology is not a perfect replicate of the extremely premature human infant. The piglet model would be better to validate studies prior to clinical interventions, especially those studies that focus on dietary modifications. Given the extreme costs of both money and time, investigators are best served to explore initial hypotheses with the murine model.

One significant advantage of this NEC model is the ability to modify the protocol. Using transgenic mice or rats provides the ability to quantify differences that occur in the absence or presence of a single gene or protein. A wide variety of agonists, antagonists, or other chemicals targeting specific pathways can be added to the formula. In addition, each animal can provide several data points for one experiment, including mRNA and protein expression, histological changes, and even systemic involvement by analyzing serum or plasma.

There are several different animal models that are used in studying NEC, including rat, mouse, hamster, piglet, *Drosophila*, and baboon[6]. The murine model is one of the most commonly used, and has some advantages over other models. Relatively large litters and a short gestation period allow researchers to efficiently generate data. Investigators are able to obtain a great deal of data in a short time span. In addition, there are many different genetically modified mice strains, allowing researchers to study the effects of a single gene alteration.

Because the collective understanding of NEC is significantly incomplete, many more studies are required. The disease is considered multifactorial, and it is likely that many different factors play a role in the development and progression of NEC. Investigators can use this murine model to obtain data on novel hypotheses. For example, the use of probiotics has been shown to reduce the incidence of NEC in premature infants[20]. The pathophysiology regarding this reduction is not clear. Researchers have shown that probiotics reduce TLR4 signaling and subsequent inflammation[21]. Others have shown that probiotics stimulate pathways that may promote intestinal epithelial wound healing[22]. The murine model would allow researchers to examine specific pathways that are affected by the enteral administration of probiotics.

There are several aspects of the protocol that require the research team to learn and gain experience before achieving success. Learning how to handle and feed the pups requires time and practice. The pups are quite fragile, and any mishandling of the animals during the learning phase often leads to death. Successful insertion of the catheter into the esophagus can be challenging, especially when learning the protocol. Complications include placing the catheter into the trachea (which results in complete aspiration) or injury to the oropharynx, both of which usually cause death to the animal. The feedings must be accurately timed. If the animals are fed too soon, they often will regurgitate and aspirate. If there is a delay in feeding, the animals may become hypoglycemic or dehydrated. One must also be careful of feeding volumes. The litters are not uniform, and smaller animals may not tolerate the same volume as larger pups.

Disclosures

The authors have nothing to disclose.

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