Journal of Visualized Experiments

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

--Manuscript Draft--

Manuscript Number:	JoVE54743R3
Full Title:	A Murine model of Necrotizing Enterocolitis Using Gavage Feeding, Lipopolysaccharide, and Systemic Hypoxia
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Necrotizing Enterocolitis, Gavage Feeding
Manuscript Classifications:	13.1.60.703.520.460.600: Infant, Very Low Birth Weight; 3.6.405.469: Intestinal Diseases
Corresponding Author:	Scott Welak Medical College of Wisconsin Milwaukee, WI UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	swelak@mcw.edu
Corresponding Author's Institution:	Medical College of Wisconsin
Corresponding Author's Secondary Institution:	
First Author:	Scott Welak
First Author Secondary Information:	
Other Authors:	Rebecca M Rentea, MD
	Shannon M Koehler
	David M Gourlay
Order of Authors Secondary Information:	
Abstract:	Necrotizing Enterocolitis (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. Given the uncertain pathophysiology, clinical studies of NEC are challenging. Animal models are vital to advancing our understanding. Although many laboratories study NEC, the murine model is commonly used. Other models, including pigs and rabbits, have limitations, including cost, time, and smaller litters. There are many methods to induce experimental NEC. Enteral feeding, infection, inflammation, and ischemia are hallmarks of the disease. Many researchers use these concepts 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 we ensure that the complete volume appropriately administered. 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, has been associated with NEC in animals and humans. Following feeds, animals are subjected to hypoxia. Premature neonates are susceptible to hypoxemia, which, along decreased intestinal perfusion following feedings, puts the infant at risk for post-prandial ischemia.

	This model is effective at inducing NEC in the laboratory using clinical risk factors. Findings from this have led to an increase in our understanding of the disease, and will hopefully yield fruitful results that lead to clinical interventions.
Author Comments:	N/A
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

Dear Editorial Board:

Thank you for the opportunity to submit our work to the Journal of Visual Experimentology (JoVE). Our laboratory has learned a great deal about experimental design and methodology from JoVE, and we are excited to present our study design to you. Our manuscript provides methodology details on a murine model of Necrotizing Enterocolitis (NEC). This disease is one of the most devastating diseases that occurs among premature infants. Unfortunately, the pathophysiology of the disease is complex and not well understood. Animal models are needed to improve our collective fund of knowledge.

Our journal submission describes our mouse and rat model of NEC. We have used this model for several years, and have produced several publications and funding opportunities with it. This model is unique in that it used the same risk factors to induce the disease that premature infants face—hypercaloric formula, systemic hypoxia, and endotoxin. The techniques we use are detailed, and would take a new researcher an extended period of time to master. We feel that by publishing our methodology in your journal, we will be able to provide a wealth of knowledge to many laboratories, and hopefully advance the care of premature newborns at risk of this disease.

Author Contributions:

Scott Welak: Corresponding author; wrote and edited manuscript; assisted with design and implementation of described protocol.

Rebecca Rentea: Wrote and edited manuscript; assisted with design and implementation of described protocol.

Shannon Koehler: Edited manuscript; has participated in use of described protocol.

David Gourlay: Edited manuscript; assisted with design and implementation of described protocol; mentor for the other authors

Peer Review Editors:

Misty Good, UPMC: goodml3@upmc.edu

Michael Caplan, North Shore University Health System: mcaplan@northshore.org

David Hackam, Johns Hopkins University: Dhackam1@jhmi.edu

Henri Ford, Keck Hospital of USC: hford@chla.osc.edu

Akhil Maheshwari, USF Health Morsani College of Medicine: akhilm@health.usf.edu

Josef Neu, University of Florida: neuj@peds.ufl.edu

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

AUTHORS:
Welak, Scott, R.
Pediatrics
Medical College of Wisconsin
Milwaukee WI, USA
swelak@mcw.edu

Rebecca, Rentea M.
Surgery
Children's Mercy Hospital
Kansas City, Missouri, USA
rrentea@cmh.edu

Koehler, Shannon M.
Pediatric Surgery
Medical College of Wisconsin
Milwaukee WI, USA
skoehler@mcw.edu

Gourlay, David M.
Pediatric Surgery
Medical College of Wisconsin
Milwaukee WI, USA
dgourlay@chw.org

CORRESPONDING AUTHOR:
Scott Welak

KEYWORDS:
Necrotizing Enterocolitis, Gavage Feeding, neonatal mice, Intestinal Alkaline Phosphatase, NADPH Oxidase, Premature Infants

SHORT ABSTRACT:
This protocol describes how to induce experimental necrotizing enterocolitis (NEC) in newborn rats and mice.

LONG 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

1.1 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

2.1 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.

2.2 Examine all catheters for patency.

2.2.1 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.

3.1 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.

3.1.1 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.

3.1.1.1 Euthanize the pregnant female by an overdose of carbon dioxide and thoracotomy.

3.1.1.2 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.

3.1.1.3 Make a small incision with fine scissors into one horn. Remove pups by squeezing behind the pup through the uterine incision.

3.1.1.4 Remove the placentas by pinching between the placenta and the animal to separate them.

Note: Each pup has its own placenta.

3.1.1.5 Dry the pup with paper towel. Observe that pups are adequately breathing. Pups may require gentile tactile stimulation to breathe.

3.1.1.6 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.

3.1.2 If a control litter of premature pups is needed, use a surrogate mother.

3.1.2.1 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.

3.1.2.2 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.

3.2 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.

4.1 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

5.1 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.

5.2 Add lipopolysaccharide (LPS) (0.1 mg LPS per 5 ml of fortified formula). Make enough LPS/formula for 24 h of feedings.

5.3 Take the pups from the incubator. With a moist soft towel, gently stimulate the perineal region to stimulate urination and defecation.

5.3.1 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.

5.4 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.

5.5 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.

5.6 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.

5.7 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).

5.8 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.

5.8.1 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.

5.9 Harvest the intestinal tissue using fine scissors.

5.9.1 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.

5.9.2 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.

5.9.3 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.

5.9.4 Using fine scissors, cut the intestine just proximal to the appendix. Make four 1-cm segments of the distal intestine.

5.9.5 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 Legends:

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).

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).

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).

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 (*).* $p < 0.05$.

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.

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.

ACKNOWLEDGMENTS:
The research for the representative publications was funded in part by the Clinical and Translational Science Institute (CTSI) at the Medical College of Wisconsin.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Stoll, B.J*., et al*. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics.* **126** (3), 443-456, doi:10.1542/peds.2009-2959 (2010).
2. Abdullah, F*., et al*. Necrotizing Enterocolitis in 20 822 Infants: Analysis of Medical and Surgical Treatments. *Clin Pediatr.* **49** (2), 166-171, doi:10.1177/0009922809349161 (2010).
3. Schnabl, K.L., Van Aerde, J.E., Thomson, A.B.R. & Clandinin, M.T. Necrotizing enterocolitis: A multifactorial disease with no cure. *World J Gastroentero.* **14** (14), 2142-2161, doi: 10.3748/wjg.14.2142 (2008).
4. Obladen, M. Necrotizing enterocolitis - 150 years of fruitless search for the cause. *Neonatology.* **96** (4), 203-210, doi: 10.1159/000215590 (2009).
5. Lucas, A. & Cole, T.J. Breast milk and neonatal necrotising enterocolitis. *Lancet.* **336** (8730–8731), 1519-1523, doi:http://dx.doi.org/10.1016/0140-6736(90)93304-8 (1990).
6. Lu, P*., et al*. Animal models of gastrointestinal and liver diseases. Animal models of necrotizing enterocolitis: Pathophysiology, translational relevance, and challenges. *Am J*

Physiol-Gastr L. **306** (11), G917-G928, doi: 10.1152/ajpgi.00422.2013 (2014).
7. Clark, D.A., Thompson, J.E., Weiner, L.B., McMillan, J.A., Schneider, A.J. & Rokahr, J.E. Necrotizing Enterocolitis: Intraluminal Biochemistry in Human Neonates and a Rabbit Model. *Pediatr.Res.* **19** (9), 919-921, doi:10.1203/00006450-198509000-00010 (1985).
8. Gonzalez, L.M., Moeser, A.J. & Blikslager, A.T. Porcine models of digestive disease: The future of large animal translational research. *Transl.Res.* **166** (1), 12-27, doi:10.1016/j.trsl.2015.01.004 (2015).
9. Kosloske, A.M. Epidemiology of necrotizing enterocolitis. *Acta Paediatr.Suppl.* **396**, 2-7, DOI: 10.1111/j.1651-2227.1994.tb13232.x (1994).
10. Leaphart, C.L.*, et al*. A Critical Role for TLR4 in the Pathogenesis of Necrotizing Enterocolitis by Modulating Intestinal Injury and Repair. *J Immunol.* **179** (7), 4808-4820, doi: 10.4049/ jimmunol.179.7.4808 (2007).
11. Welak, S.R.*, et al*. Intestinal NADPH Oxidase 2 Activity Increases in a Neonatal Rat Model of Necrotizing Enterocolitis. *PLoS ONE.* **9** (12), e115317, doi: 10.1371/journal.pone.0115317 (2014).
12. Lallès, J.-. Intestinal alkaline phosphatase: Multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. *Nutr.Rev.* **68** (6), 323-332, doi:10.1111/j.1753-4887.2010.00292.x (2010).
13. Bates, J.M., Akerlund, J., Mittge, E. & Guillemin, K. Intestinal Alkaline Phosphatase Detoxifies Lipopolysaccharide and Prevents Inflammation in Zebrafish in Response to the Gut Microbiota. *Cell Host and Microbe.* **2** (6), 371-382, doi:10.1016/j.chom.2007.10.010 (2007).
14. Heinzerling, N.P.*, et al*. Intestinal alkaline phosphatase is protective to the preterm rat pup intestine. *J.Pediatr.Surg.* **49** (6), 954-960, doi: 10.1016/j.jpedsurg.2014.01.031. (2014).
15. Rentea, R.M.*, et al*. Enteral intestinal alkaline phosphatase administration in newborns decreases iNOS expression in a neonatal necrotizing enterocolitis rat model. *J.Pediatr.Surg.* **48** (1), 124-128, doi:10.1016/j.jpedsurg.2012.10.026 (2013).
16. Rentea, R.M.*, et al*. Intestinal alkaline phosphatase administration in newborns decreases systemic inflammatory cytokine expression in a neonatal necrotizing enterocolitis rat model. *J.Surg.Res.* **177** (2), 228-234, doi: 10.1016/j.jss.2012.05.039 (2012).
17. Rentea, R.M.*, et al*. Intestinal alkaline phosphatase administration in newborns is protective of gut barrier function in a neonatal necrotizing enterocolitis rat model. *J.Pediatr.Surg.* **47** (6), 1135-1141, doi: 10.1016/j.jpedsurg.2012.03.018 (2012).
18. Riggle, K.M., Rentea, R.M., Welak, S.R., Pritchard Jr., K.A., Oldham, K.T. & Gourlay, D.M. Intestinal alkaline phosphatase prevents the systemic inflammatory response associated with necrotizing enterocolitis. *J.Surg.Res.* **180** (1):21-6*.,* doi: 10.1016/j.jss.2012.10.042. (2012).
19. Biesterveld, B.E.*, et al*. Intestinal alkaline phosphatase to treat necrotizing enterocolitis. *J.Surg.Res.* **196** (2), 235-240, doi:10.1016/j.jss.2015.02.030 (2015).
20. Neu, J., Walker, W.A. Necrotizing Enterocolitis. *N Engl J Med.* **364:** 255-264, doi: 10.1056/NEJMra1005408 (2011)
21. Desphande, G., Rao, S. Patole, S. Bulsara, M. Updated Meta-analysis of Probiotics for Preventing Necrotizing Enterocolitis in Preterm Infants. *Pediatrics*. **125 (5):** 921-930. doi: 10.1542/peds.2009-1301 (2010).
22. Jones, R.M. Symbiotic lactobacilli stimulate gut epithelial proliferation via NOX-mediated generation of reactive oxygen species. *EMBO J.* **32:** 3017-3028. doi: 10.1038/emboj.2013.224

(2013)

Figure 1

Click here to download Figure Fig 1.tif

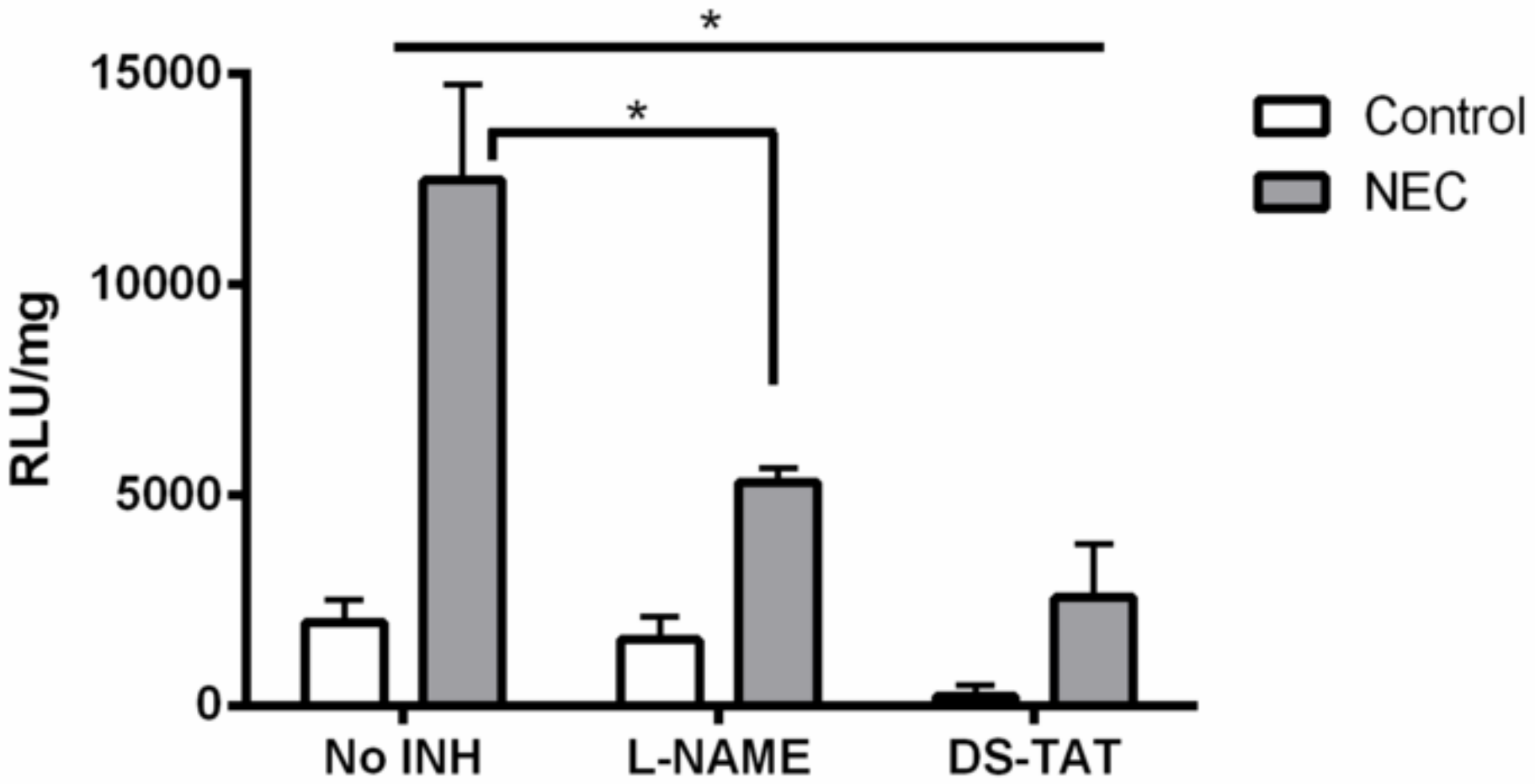


Figure 2

Click here to download Figure Fig 2.tif

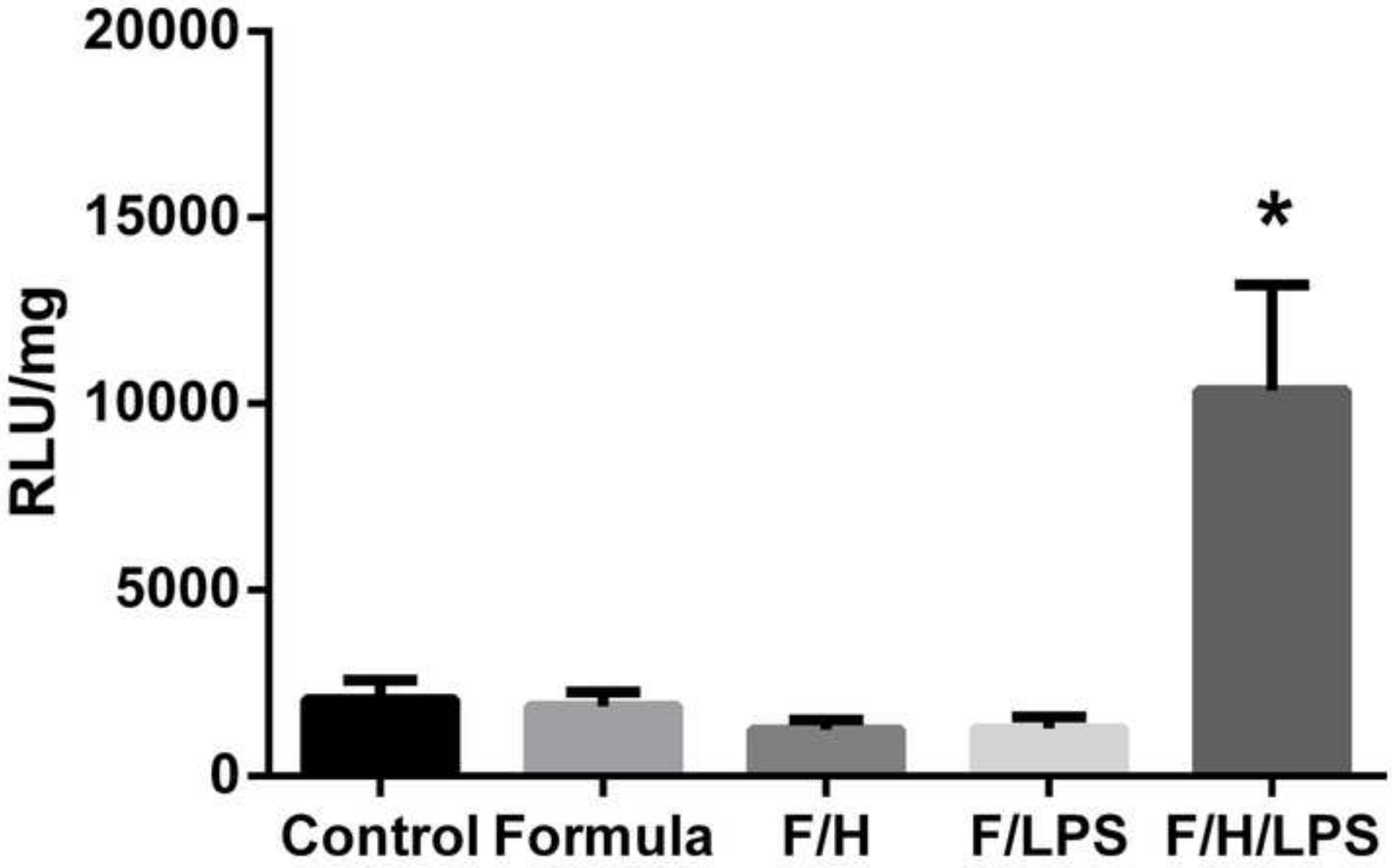


Figure 3

Click here to download Figure Fig 3.tif

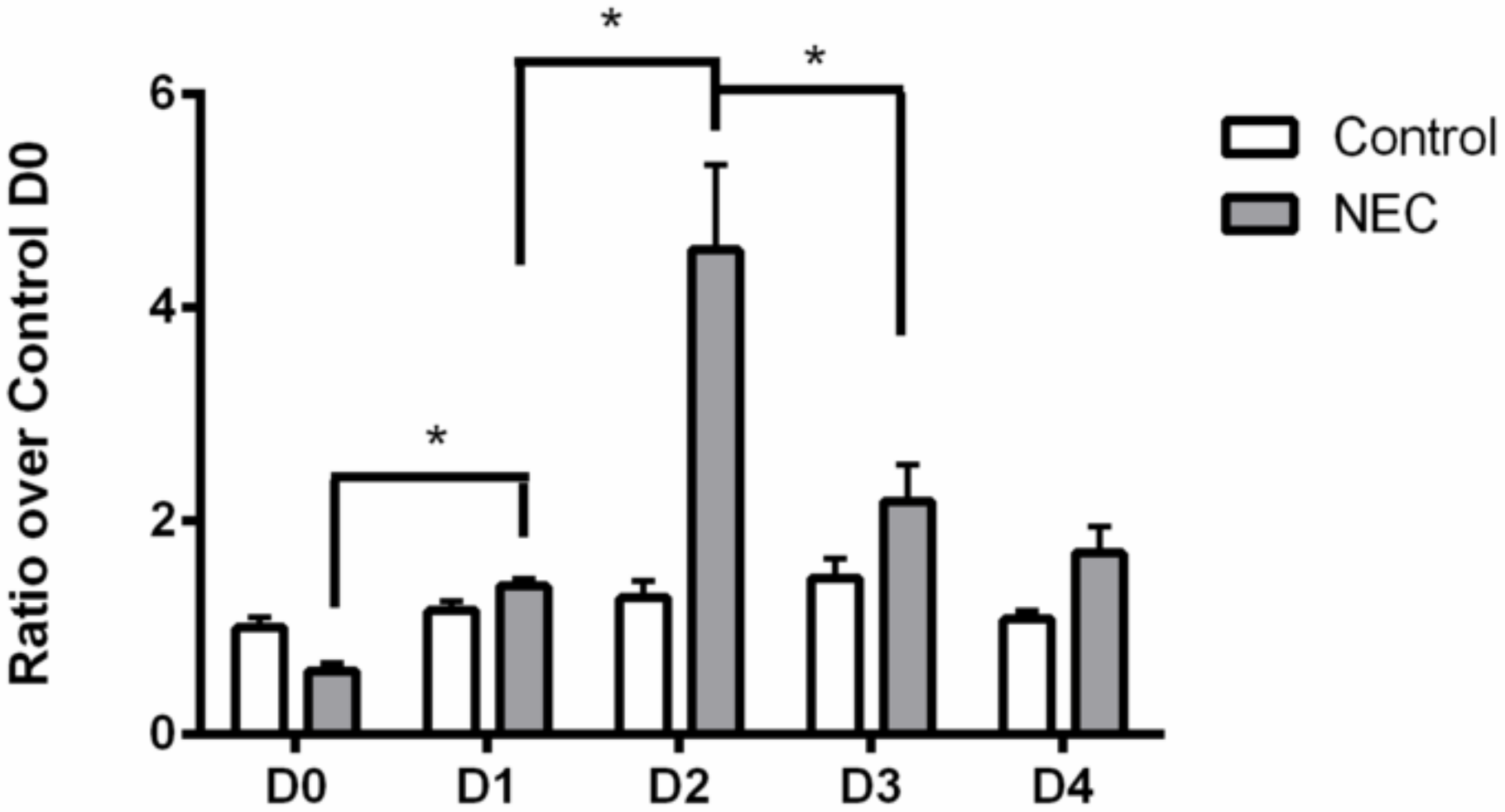


Figure 4

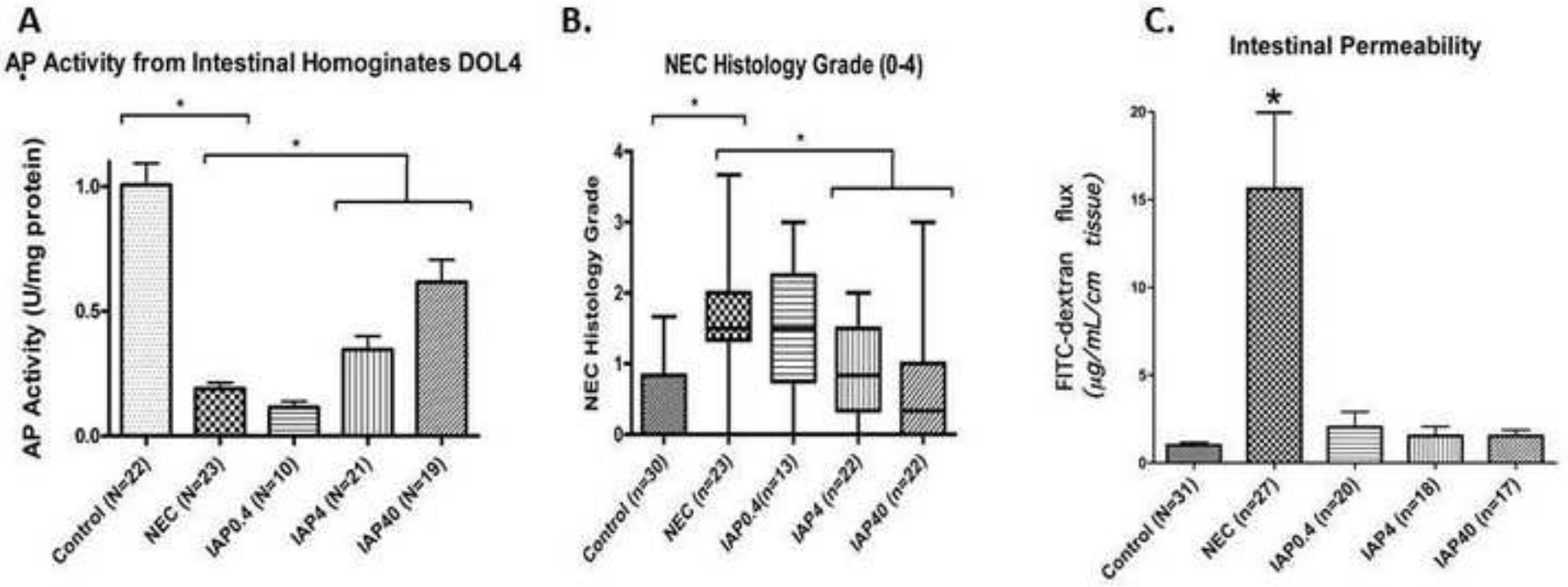
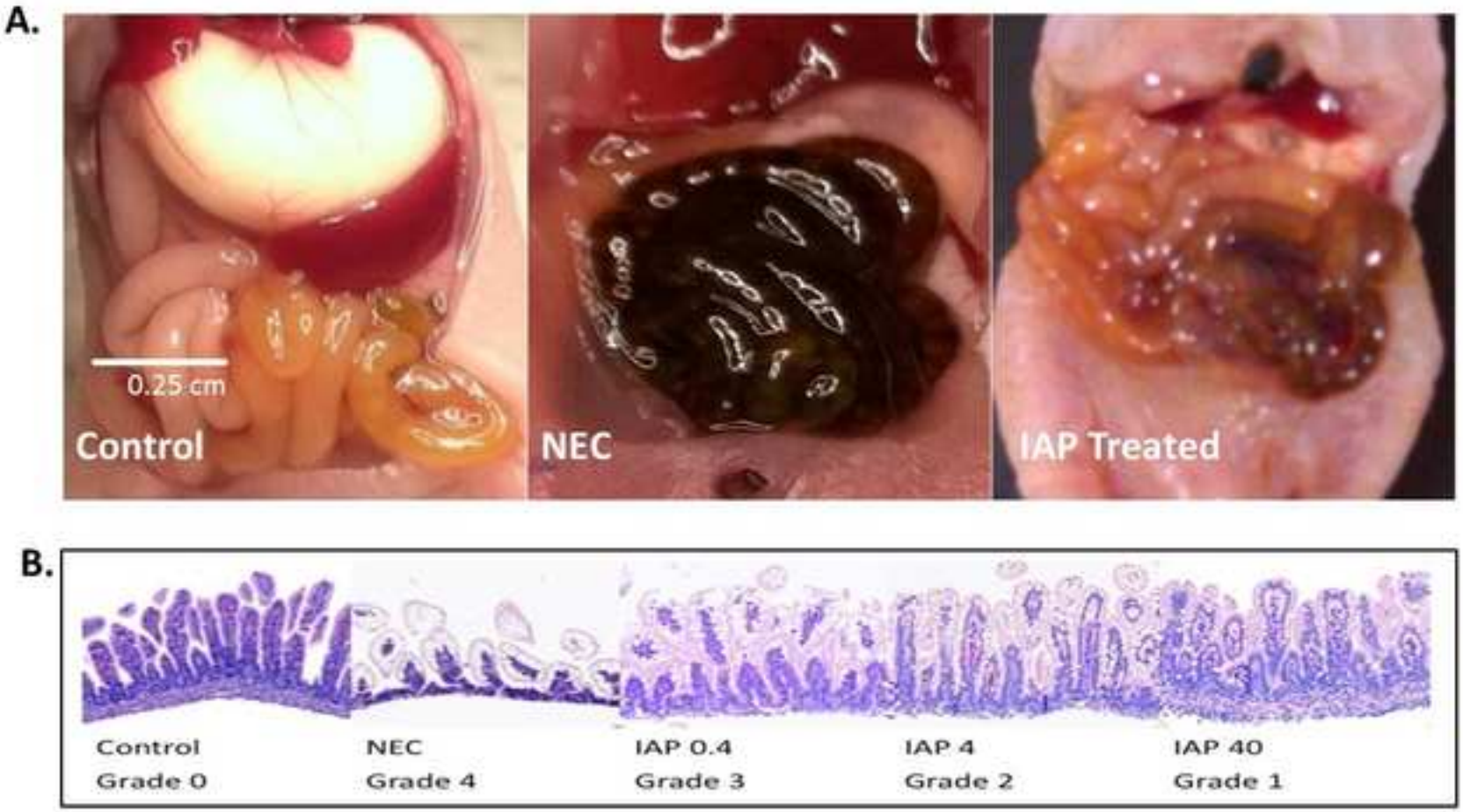


Figure 5



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.9 Fr Argyle Catheter	Coviden-Medrtonic	43309	**Can contain human** Obtained discarded cathete
Ethanol	Sigma Aldrich	459844	
Bleach	Up and UP/Target	003-07-0058	**TOXIC**
Incubator	Georgia Quail Farm (Savannah, GA)	1588 Hova-Bator	
Puppy Formula	Pet Ag (Hampshire IL)	Esbilac Powder Milk Replacer FG99501	
Infant Formula	Mead Johnson	Enfacare Infant Formula	
Lipopolysaccharide (LPS)	Sigma Aldrich	L2630	**TOXIC.** Dissolved in ddH2O, 2 mg/ml
Hypoxia Chamber	Biospherix (New York)	A-Chamber	
Nitrogen Gas	Praxair (Danbury CT)	Compressed Nitrogen Gas	**Potentially Harmful**
Regulator	Biospherix (New York)	Proox Model 110	
Ketamine (Ketaject)	Clipper Distributing Co	2010012	**Potentially Harmful**
Xylazine (Anased)	Lloyd Laboratories		**Potentially Harmful**
Rats	Jackson Laboratories	Timed-Pregnant Sprague Dawley Pregnant Female Rats	
Mice	Jackson Laboratories		
L-NAME (N5751 SIGMA Nω-Nitro-L-arginine methyl ester hydrochloride)	Sigma Aldrich	N5751	
Lucigenin (N,N'-Dimethyl-9,9'-biacridinium dinitrate)	Sigma Aldrich	M8010	Toxic. Dissolved in ddH2O, Stock concentration

GP91-ds-*tat*	Blood Center of Wisconsin	Made from previously described publication (Circ Res. 2001 Aug 31;89(5):408-14.)
FITC-Dextran, 10 kDa	Sigma Aldrich	FD10S
Intestinal Alkaline Phosphatase (IAP)	donated by AM-Pharma (Netherlands)	

ers from NICU at Children's Hospital of Wisconsin

of 5 mg/ml

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: A Murine Model of Necrotizing Enterocolits Using Gavage Feeding, Lipopolysaccharide, and Systemic Hypoxia

Author(s): Scott R. Welak, Rebecca M. Rentea, Shannon M. Koehler, David M. Gourlay

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

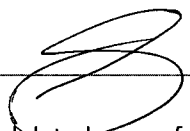
CORRESPONDING AUTHOR:

Name: Scott Welak

Department: Pediatrics

Institution: Medical College of Wisconsin

Article Title: A Murine Model of Necrotizing Enterocolitis Using Gavage Feeds Lipopolysaccharide and Systemic Hypoxia

Signature: 

Date: 3/15/16

Please submit a signed and ~~dated~~ copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Dear Editors and Reviewers:

Thank you for taking the time to review our submission to JoVE for the article entitled, "A Murine Model of Experimental Necrotizing Enterocolitis Using Gavage Feeding, Lipopolysaccharide, and Systemic Hypoxia." We appreciate the insightful feedback, and have addressed the suggestions and concerns raised. We will address each comment from each editor/reviewer below. We kept the original comments from each editor/reviewer in the text and underlined it, followed by our responses to each individual comment.

Editorial comments:

•Grammar: "Ligate" appears to be misused. Possibly dissect should be used instead, especially in 5.8.2:

Changed as suggested

•Additional detail is required:
-3.1.1.1 – How much prior to delivery does this occur?
-3.1.1.6 – How many hours is several?

Details added.

•Branding should be removed from Results and Figure Legends (Tiron)

Changed brand name to common name.

•Results:
-Figure 5B – Please include a scale bar.

Added a magnification specification.

•Discussion: The discussion is repetitive, starting with line 421 at least, maybe earlier. Please condense the discussion while maintaining all five required discussion items: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The discussion was appropriately edited to remove unnecessary redundancy. We tried to keep the discussion succinct and organized as recommended.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
superb description of a robust model of nec

Major Concerns:
no concerns

Minor Concerns:
information regarding limitations and comparison with the human disease would be helpful

Additional Comments to Authors:
N/A

Appreciate review of article.

Reviewer #2:
Manuscript Summary:
This manuscript by Welak et al describes the preparation of a murine model of necrotizing enterocolitis (NEC), which includes gavage formula feeding, LPS and intermittent hypoxia. The paper highlights the importance of animal models to study the pathogenesis of NEC and would be an addition to the JOVE literature, however several issues should be addressed.

Major Concerns:
-Overall: This is an important addition to the JOVE publications, but the writing style consists of abruptly short sentences and needs some editing to this regard so that the manuscript flows with ease. One example of a paragraph that needs reworked is lines 59-66, as the paragraph seems disjointed and doesn't flow. However, the authors should ensure that the overall writing style is edited and each sentence leads into the next.

We attempted to use the writing style recommended by the JoVE editors during the initial revisions. We reviewed the article and attempted to make the introduction, representative results, and discussion sections easier to read, including the section specifically mentioned by this reviewer.

-Protocol: Cesarean delivery - Why are the controls not also delivered early and fostered to another timed pregnant Dam? This means that the controls aren't age-matched with the animal that undergo experimental NEC. The authors should comment on this in the manuscript.

We have added a surrogacy section to our protocol.

-Protocol: Diet: It is very unclear to me how someone would make the experimental NEC formula from this description. Per manufacturer's protocol is not adequate especially when you

are adding in another formula to it. Please provide exact details of formula preparation including number of scoops, whether you make it up fresh every feed, every day, how it is stored, is LPS added once per day or to the formula that is made up for each day?

We removed every mention of "per manufacturer recommendations" and have replaced it with more clear directions, including the points that the reviewer has raised above.

-Protocol: Volume of Feeding: How does one decide the amount of 0.1-0.2ml per feed? Is it based on weight? If so, please add those details. When the amount is increased each day, is there a maximum that the animals receive? Also do the mice and the rats get the same amount and advance the same rate? If you have to decrease the amount, how much do you decrease it if the animals aren't tolerating it. (and how do you monitor for animal feeding tolerance)? Also how many times are the mice and rats fed per day and how often?

We have clarified each of the questions and concerns raised by the reviewer for this point.

-Protocol: Time Course: Expand more on goals of the experiment and list examples of time points that you typically perform.

We have tried to clarify the goals of the experiment, but have left this somewhat open-ended. Our goal is to provide information on the model, and allow readers to use this protocol to develop their own goals. We believe that the information provided in the representative results section is sufficient in providing examples.

-Representative Results: The results section appears like a background section and does not reference any of the figures that the authors provide.

We have not changed the results section. Because these results are representative findings in already published manuscripts, we did not go into great detail. The goal of this section was to provide examples of how this protocol can be used.

Also some of the methodologies included in the figures are not included in the protocol such as NEC severity determination and measurement of intestinal permeability.

The goal of this paper was to focus on the induction of experimental NEC. The scope of the manuscript was not intended to focus on different assessments of how experimental NEC can be quantified or evaluated.

Minor Concerns:
-Line 63-64: "This model is effective at inducing NEC in the laboratory…using clinical risk factors". Change to This model is effective in inducing experimental NEC in rats and mice and includes many of the accepted risk factors for NEC in human infants.

-Line 81: Change septic shock to refractory shock because all of the patients will not have sepsis, they have a shock-like phenotype.
-Line 83: Change concentration to concentrating.
-Lines 93: Change "intestinal injury consistent with…" to "intestinal injury that histologically appears similar to NEC, although the direct relation to clinical NEC is not clear. "
-Line 99: Change sentence to: One of the biggest challenges with this experimental murine model of NEC is enteral feedings.
-Line 114: Add citation for high calorie formula being a risk factor for NEC.
-Paragraph 113-119: Needs reordered for flow. After risk factor for NEC on line 114, add sentence on TLR4 activation (line 116), then add LPS promotes overwhelming… (line 115), then add that you are adding LPS to the feedings (Line 114-115).
-Line 153: Change increases to increased
-Line 154: Add citation about incidence and severity by delivering the animals prematurely.
-Line 157: Delete "a" before scissors.
-Lines 174-175 Delete these two sentences as they are duplicates.
-Line 181: Change "different" to "each"
-Line 185: Delete "a" before scissors.
-Line 294: change "are" to "include"
-Line 301: advantages over other methods…please list these advantages here.
-Line 314: change "should" to "can"
-Line 315: delete "some"

The grammar and word choices mentioned by this reviewer have been addressed and altered as needed.

-Figure 1: Add more detail to the Y axis. Such as, NOX2 activity RLU/mg tissue

Provided more detail in the figure legend.

-Figure 2: Change the name of the Y axis to NOX2 mRNA expression. Also is this fold change relative to a housekeeping gene? If so, please describe in the legend.

The graph represents an activity assay and not RT-PCR results. The RT-PCR results are for Figure 3. We changed the figure legend for Figure 3 to be more clear, including the description of the housekeeping gene.

-Figure 3: The IAP Treated picture is somewhat blurry. For 3B, Please create a table and list the histological changes that dictate whether a slide is scored as Grade 1, 2, 3, or 4 and add this to the Protocol section for Determining NEC severity in the model.

The histology scoring is in Figure 4. Because the determination of NEC severity was not within the scope of the paper, we did not change the figure legened.

-Figure 4: There is no discussion about the methods for measuring intestinal permeability. The authors should describe this in detail under Protocol.

This should be for Figure 5. The quantification of intestinal permeability is not within the scope of this paper. Readers can find citations that will address this in the bibliography.

-Table of Materials: Add incubator to list.

The incubator is already in the list of materials.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
The authors have described a hypoxia/gavage model for Necrotizing Enterocolitis. The authors conclude that the model is effective at inducing NEC in the laboratory using clinical risk factors. Findings from this have led to an increase in our understanding of the disease, and will hopefully yield fruitful results that lead to clinical interventions.

Major Concerns:
The major concern is that this model has been well studied and extensively characterized in the literature. The current submission does not add additional knowledge to what is already known. The data shown about the use and manipulation of IAP is intriguing.

This submission was requested by the editors of JoVE, as they felt the topic has not been covered in their journal. While it may not add novel findings, this article is useful in that it will provide a thorough description and visual demonstration of the experimental NEC protocol.

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #4:
Manuscript Summary:
The manuscript "A Murine model of Necrotizing Enterocolitis Using Gavage Feeding, Lipopolysaccharide and Systemic Hypoxia" aims to provide a clear and effective protocol to induce NEC-like intestinal injury, which accurately represents the clinical disease. Although this protocol in principle may lead to the induction of NEC-like intestinal injury in neonatal rats or

mice, reproducibility of this protocol for the reader will be challenging. Critical steps of the model are not described detailed enough or explained clearly. Moreover, in the result section, clear demonstration that this model induces severe enough intestinal damage in a significant number of animals is missing or not convincing enough.

We feel that the results section is sufficient. We are able to demonstrate that this model of experimental NEC does induce sufficient injury based on histology and other factors, including changes in inflammatory cytokines and oxidative stress. The reviewers provided excellent feedback on ways to make the protocol more clear, and have been incorporated into the most recent revision.

Major Concerns:
Title:
The title seems appropriate except that a clear NEC pathology is not demonstrated in this manuscript. Therefore, I recommend using NEC-like intestinal injury or similar terminology.

We agree with the point, and have changed the title to explain that this is an experimental model of NEC, and not the same as the disease seen in humans.

Abstract:
The abstract is not written in a clear structured way. It would benefit from a clear division into "Background; Aims, Methods, Results, and Conclusion." It can also be shortened.

The format used is per JoVE guidelines.
Introduction:
The introduction is OK but would benefit from a clear but very brief overview which models are used on rats/mice (see minor concerns).

Will address with minor comments.

Protocol:
The protocol is lacking a lot of detailed information, which are crucial to run this model accurately.
"Diet and Feeding" What is the caloric intake/day/g BW? What is the LPS concentration given per mouse/g BW? The feeding volume is indicated with 0.1 - 0.2 ml per feed and needs to be increased 0.1 ml /feed/day." This is imprecise. Indicating the volume/g BW is advantageous. Why are animals treated with hypoxia after the feeding and not before?

The calculations have been added to the new revision to provide a more specific protocol. We have always performed hypoxia after feeding, and have achieved success in causing appropriate injury to the animals.

5% hypoxia for 10 min seems long, especially for mice. Is that feasible?

Exposing the animals to this hypoxia protocol is sufficient. To date, there have been no mortalities from the hypoxia in either the rat or mouse model.

4 Feedings per day are not enough, unless the caloric intake is really high. Feedings should be continued over 24h with maybe one feeding rest per night.

We amended this to make it 5-6 feedings. The number of feedings also depends on the amount of formula and calories provided. We feel that this is sufficient, especially for labs that do not have enough technical support to allow for 24 hours of feedings.

The animals need to be stimulated for urination and defecation at least twice daily, better with each feeding. This information is completely lacking in the protocol.

This has been added to the protocol.

Results (Figures):
It would be beneficial to show more histology. The main purpose of this protocol is to induce NEC-like intestinal damage. The achievement should be demonstrated properly. The histology images provided are not fully convincing.

The images provided are representative results that we feel accurately demonstrate that this model can provide sufficient intestinal injury. Readers are provided with references to review from our previous publications. However, if the reviewers prefer additional images, we will provide them.

Comparison between hypoxia treatment and oral feeding plus additional stressors like bacterial load or hypothermia has been made in the past. However this protocol does not show data if the LPS treatment is needed to increase intestinal damage.

We have not performed experiments looking at the individual components of the protocol and examined histology. Unpublished data from our lab indicates that formula, LPS, and hypoxia are all needed. In Figure 2, we show that all three components are needed to induce NOX2 activity, and that using only parts of the protocol do not change NOX2 activity.

Discussion:
Seems mainly appropriate but limitation of this model could be explained in more depth and comparison to other models is lacking.

We have made the discussion more concise, and did not compare to other NEC models. There is evidence that a number of different protocols will induce experimental NEC, but this is outside the scope of our manuscript.

Minor Concerns:

Page 2 line 46 - 47 "Although many laboratories study NEC, the murine model is commonly used". The context of this sentence is unclear.

Clarified

Page 2 line 48 - 49 "There are many methods to induce experimental NEC. Actually there are not that many methods. The main methodology uses hypoxia+enteral feeding and often additionally either hypothermia or bacterial injection or both. Those models then vary in terms of species (mouse or rat) starting age of pups, bacteria strain, hypoxia concentration and length. Other models use Paneth cell depletion and Klebsiella treatment or PAF and LPS.

Clarified.

Page 3 line 101: Sentence should probably be " one of the biggest challenge with those models…" instead of "…. with this model…".

Clarified.

Page3 line 109: "One method to reduce this complications is to use gavage feeding". There are a number of laboratory groups using gavage feeding in their model. Adequate references are missing (e.g. Jilling T et al. J Immunol 2006; Leaphart C et al. J Immunol 2007; Schulz S et al. 2015 Pediatric Research).

I'm not exactly sure what this means. The citations could be added but do not add anything to the article.

Page 3 line 112 - 113 and Page 4 line 142 - 145 : "The catheters are inexpensive, durable, and can be obtained from a neonatal intensive care unit (NICU)." Not every laboratory has access to a NICU. There are very good disposable plastic feeding tubes for neonatal rats and mice available from Instech (FTP-22-25). They are soft and flexible.

We added instructions for laboratories that do not have access to the PICC lines.

Page 5 line 184 - 191 "Catheter Preparation" by using the commercially available feeding tubes (see above) this can be simplified.

The details provided were requested during a previous review by JoVE editors.

Page5 line 181 - 182: The usage of an incubator for 1week old mice is not clear to me. Why is this necessary? In our hands, mice suffer from overheating at this age at a temperature of 37C. A small local source of heat, where animals can move towards or away from might make more sense.

Our experience is that the mice become cold very quickly and require constant heat at 37 degrees. Both rat and mouse pups at their respective ages are usually huddled their mother to maintain heat.

Page6 line 198 - 199: The pups will swallow the gavage tube; this is the best sign that the tube is placed correctly. The gavage tube should not be forced.

We have corrected the protocol to make this clear.

Page 7 line 249 - 254: Harvesting proximal colon additionally to terminal ileum is beneficial since NEC can affect both areas. Harvesting the tissue as a "jelly role" provides most information regarding the pathophysiological changes.

We do not typically harvest the colon. Our analyses have shown that the terminal ileum is the most affected area. In addition, the terminal ileum is the intestinal segment most affected in clinical NEC.

References seem not to be in a unifying format.

We have reviewed the format, and believe that the references are per JoVE recommendations. If not, we will correct them.