**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.