Dear Editor,

Thank you for your review of our manusctipt titled “Method of isolated ex-vivo lung perfusion in a Rat Model: Lessons Learned from Developing a Rat EVLP Program”. Below we have provided a point-by-point response to your comments and the reviewer comments. We have also made all associated revisions in the revised text. We look forward to publication of this method in the Journal of Visualized Experiments.

Samir Ghadiali & Bryan Whitson (co-corresponding authors)

**Editorial Comments:**

*1. All of your previous revisions have been incorporated into the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes.*

Thank you, we will use the most recent version of the manuscript for all subsequent changes.

*2. Please describe the data acquisition program setup in more detail. What is being recorded, how and to what are they connected?*

Protocol step 1.4 now includes the individual transducers and their purpose.  
  
*3. Please reduce/remove instances of the pronoun "our" throughout the manuscript.*

All instances of the pronoun “our” have been removed.  
  
*4. Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.*

We thank the Editor for the opportunity to proofread the manuscript.  
  
*5. Please disregard the comment below if all of your figures are original.  
If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."*

All figures are original.

**Reviewer #1 Comments:**

Major Concerns:

*1. Under representative results, page 12, it is unclear if the physiological data refers to control (low tidal volume/PEEP) or VILI (high tidal volume/PEEP) experiments.  
  
This needs to be clarified.*

Representative results are shown in Figures 10 and 11. The data in Figure 10 is from experiments run at low tidal volume/low PEEP (i.e. 4mL/kg tidal volume and 2cmH2O PEEP) while data in Figure 11 is from a high tidal volume/high PEEP of 10 mL/kg and 8 cm H2O PEEP. These conditions have been clarified in the text.  *If the graph refers to the control group and edema with increasing Pulmonary Artery pressures are observed during the first 60 minutes of perfusion this means that the proposed technique is not stable.*

First, while there may seem to be a trend of increasing lung weight with respect to time, statistical analysis (ANOVA) clearly demonstrates that there is no statistically significant increase in lung weight over 60 minutes (p=0.92). Furthermore, The lower pulmonary artery pressure (PAP) at the 0 min time point is a result of lower flow and ventilation settings used at the beginning of all experiments (3 mL/min) vs the larger flow rates (12 mL/min) used during the experiment. In addition, the PAP remains constant after the t=10 min time point with no statistically significant changes after t=10 min (ANOVA on ranks, p = 0.89). We therefore conclude that our technique is stable for 60 minutes and these point have been made clear in the manuscript.

*Several authors have described isolated lung perfusion with minimal edema formation during prolongued rat lung perfusion. Referring to previous published data with isolated/ex vivo rat lung perfusion and how these experiences help or not Nelson et al will be a great benefit to the reading audience.  
  
a- Noda K et. Al. Successful prolonged ex vivo lung perfusion for graft preservation in rats. Eur J Cardiothorac Surg. 2014 Mar;45(3):e54-60.  
  
b- Niemeier RW. The isolated perfused lung. Environ Health Perspect. Jun 1984; 56: 35-41.  
  
If the physiological data presented by Nelson et al in figure 8 refers to the VILI group, then data from the control (low tidal volume, low PEEP) should be added to the graph to demonstrate that the authors proposed method is stable when compared to an injurious method (VILI group).*

We thank the reviewer for the additional citations. They will be incorporated into the manuscript as suggested.

*2. Figure 9 is also difficult to interpret. If at time 0 rats were in a low tidal volume EVLP and at time 60 converted to a high tidal volume EVLP is the cytokine response related to the low tidal volume method or to the high tidal volume change. This should be clarified.  
  
My recommendation will be a set of experiments in which the rat lungs are evaluated ex vivo at low tidal volume for 60 minutes compared to a set of experiments in which the rat lungs are under VILI conditions for 60 minutes.  
  
Then I will plot the physiological and cytokine data from both EVLP conditions (Low vs High tidal volume).  
By doing this the readers will understand a couple things:  
  
a- that the proposed method (low tidal volume/PEEP) of EVLP leads to a very stable and non-injurious preparation providing a platform for research studies. Low edema, low inflammatory response.  
  
b- that high tidal volume/PEEP during 60 minutes of EVLP is associated to high inflammatory response and edema formation.*

We believe the reviewer is referring to Figure 11. The goal of this figure is to demonstrate that a high tidal volume/high PEEP ventilation protocol generates significant inflammation and that our technique is able to reproduce this component of ventilation induced lung injury. We have clarified this point and note that the tidal volume and PEEP was set at time 0 and was maintained throughout the 60 minute perfusion. This data clearly demonstrates that 60 min of high tidal volume/PEEP ventilation induces a large increase in pro-inflammatory cytokines (IL1β, TNFα) and no statistically significant change in the anti-inflammatory cytokine (IL-4). The reviewer’s suggestions for further investigation in the effect of VILI is intriguing and we will work toward pursuing answers to that question in our upcoming work. The intent of the current work was to describe and detail this reproducible approach to rodent EVLP, and to provide the granularity that the literature is lacking so that other researchers can benefit from the steep learning curve involved in this procedure.

Minor Concerns:

*1. Referring to background in EVLP the authors comment "Typically, the lungs are ventilated at 50% of total lung capacity or 20 cmH20 of peak airway pressure with a fraction of inspired oxygen (FiO2) of 30 to 50% [4]. Preservation solution is perfused at 40-60ml/kg (approximately 40% of the predicted cardiac output of 100mL/kg) [5, 6] at a temperature 4 to 8°C with an ischemic time less than 8 hours, but possibly up to 12 hours [7]."  
  
This paragraph is not clear. Are the authors referring to cold lung preservation or ex vivo lung perfusion? In addition is not clear if the authors refer to human and large animal ex vivo lung perfusion protocols or small animal protocols. Reference 6 is from a large animal model of EVLP and 4 is from a rat model of isolated lung perfusion.  
  
I recommend reviewing this paragraph for consistency. EVLP methods for humans and large animals present different challenges to small animals as the authors comment.*

The paragraph has been modified to improve the clarity and to note the differences between human/large animal and small animal EVLP protocols.

*2- Under the Perfusate section, the authors briefly analyze what characteristics the ideal perfusate should have. In addition they comment on a commercially available solution "Steen Solution" for EVLP, which contains human albumin, and reflect on the potential effects of human albumin across species and how this could potentially affect results.  
  
One aspect that the authors overlook is that there are also potential detrimental effects of dextran across species. Especially in rats, with some strains being allergic to it, which favors pulmonary edema.  
  
I recommend the authors take into account the potential detrimental effects of dextran in a rat model of ex vivo lung perfusion and add this to their discussion/comments.  
  
Harris J.M. Differences in responses between rat strains and colonies. Food and cosmetics Toxicology Volume 3, 1965, Pages 199-202.*

Thank you, this citation has been added.

*3- Some pictures reveal that no PPE other than gloves are being used when handling the animals. I recommend the use of pictures were a lab coat or similar PPE protection is being used to comply with international animal handling techniques.*

Noted.

Additional Comments to Authors:  
*1. I would like to thank the authors for the opportunity to review their work.*

We thank the Reviewer for their insightful review of our manuscript.

**Reviewer #2 Comments:**

Major Concerns:

*1. Lacked additional data about perfusate used in infusion:  
- Association anti-inflammatory drugs?*

No anti-inflammatory drugs or agents were evaluated. These data are for variable ventilation strategies alone and as a description/model manuscript.

*2. About stabilization time of the system:  
The ventilatory parameters and flow were changed. Was there a time limit?*

Step 4.3 in the procedure states the parameters are to be changed in the initial 15 minutes of the experiment.

*3. Recently a very important work in the area was published and would be interesting to include in the discusson by highlighting important points in common with this article:  
Noda K, Shigemura N, Tanaka Y, et al.  
Successful prolonged ex vivo lung perfusion for graft preservation in rats.Eur J Cardiothorac Surg (Germany), Mar 2014, 45(3) pe54-60.*

This citation has been added.

Minor Concerns:

*1. Line 125, 127 and 137 What figures are referred?*

Lines 125 and 127 refer to Figure 1, and line 137 refers to Figure 2.