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Transuterine Fetal Tracheal Occlusion Model in Mice

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TITLE:
Transuterine Fetal Tracheal Occlusion Model in Mice

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KEYWORDS:
Fetal tracheal occlusion; congenital diaphragmatic hernia; CHAOS; lung growth; fetal lung development; mice

SUMMARY:
Various animal models of congenital diaphragmatic hernia and fetal tracheal occlusion present advantages and disadvantages regarding ethical issues, cost, surgical difficulty, size, survival rates, and availability of genetic tools. This model provides a new tool to study the impact of both tracheal occlusion and increased luminal pressure on lung development.

ABSTRACT:
Fetal tracheal occlusion (TO), an established treatment modality, promotes fetal lung growth and survival in severe congenital diaphragmatic hernia (CDH). Following TO, retention of the secreted epithelial fluid increases luminal pressure and induces lung growth. Various animal models have been defined to understand the pathophysiology of CDH and TO. All have their own advantages and disadvantages such as the difficulty of the technique, the size of the animal, cost, high mortality rates, and the availability of genetic tools. Herein, a novel transuterine model of murine fetal TO is described. Pregnant mice were anesthetized, and the uterus exposed via a midline laparotomy. The trachea of selected fetuses were ligated with a single transuterine suture placed

behind the trachea, one carotid artery, and one jugular vein. The dam was closed and allowed to recover. Fetuses were collected just before parturition. Lung to body weight ratio in TO fetuses was higher than that in control fetuses. This model provides researchers with a new tool to study the impact of both TO and increased luminal pressure on lung development.

INTRODUCTION:

Congenital diaphragmatic hernia (CDH) occurs in 1:2500 pregnancies and results in pulmonary hypoplasia and neonatal pulmonary hypertension[1–6]. Fetal tracheal occlusion (TO) is an established prenatal therapy in severe CDH patients involving fetoscopy in the 26–30th gestational week in which a balloon is placed just above the carina and then removed in the 32nd gestational week. This temporary TO induces fetal lung growth and improves survival. Congenital High Airway Obstruction Syndrome is a lethal condition associated with lung hyperplasia, which inspired surgeons to perform artificial occlusion of the trachea to promote retention of the secreted epithelial fluid. This occlusion increased luminal pressure and induced lung growth[7]. However, the occlusion should be reversed to enable epithelial cell maturation.

Various animal models of CDH and TO—ovine, rabbit, rat, and mouse—have been developed to understand the pathophysiology of CDH and TO. All have their own advantages and disadvantages such as the difficulty of the technique, the size of the animal, cost, high mortality rates, and the availability of genetic tools. Although the surgical technique used for the ovine model is very similar to that used in humans and could be reversed, the major drawbacks of this model are the expense of the animal, the long gestational period, and the limited number of surgeries possible. The rabbit model has a shorter gestational period and is less expensive than the sheep model. However, the rabbit model is irreversible[8,9]. The murine model has the lowest cost, the highest number of fetuses per pregnancy, the best-characterized genome, and widely available tools for cellular and molecular analyses. However, a key drawback is the lack of reversibility of the TO, preventing full understanding of the impact of TO. Herein, a method is presented that combines all the advantages of the previously mentioned models and creates an easy, potentially reversible, and minimally invasive rodent TO model.

PROTOCOL:

All experiments have complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80023, revised 1978). The procedure was approved with IACUC protocol #2016-0068 by the Cincinnati Children's Research Foundation Institutional Animal Care and Use Committee.

1. Preparation

1.1. To mate age-matched wild-type (WT) C57BL/6 mice, place them in the same cage at 6:00 p.m. and separate them at 9:00 a.m. the next day.

1.2. To determine embryonic day 0 (E0), look at the vaginal plug, which has a homogeneous outer zone attached to the vaginal wall and an inner zone that is fibrous and includes some spermatozoa that form entangled masses mixed with the fibers of the plug material.

1.3. Record the weight of the mice at the time of mating.

1.4. Re-weigh the mice on E10 to ensure ongoing pregnancy.

1.5. Perform the surgery on E16.5 (early canalicular stage).

1.6. Sterilize the instruments that are going to be used during surgery: scissors, needle holder, forceps, clamps, and surgical knives and handles.

1.7. Pre-heat the surgery platform to 24 °C and prepare warm saline (24 °C) prior to surgery.

1.8. Create a warm environment for recovery, and leave wet food inside the cage for the early feeding.

1.9. Stay with the operated animals until they can feed themselves.

1.10. Keep the operated mice alone in their individual cages after the surgery.

2. Anesthesia

2.1. Apply subcutaneous 0.1 mg/kg of buprenorphine to the pregnant dams 1 h before the procedure.

2.2. Use inhaled 5 mL/h of isoflurane for induction and 2 mL/h continuously during the procedure for anesthesia.

2.3. Monitor the movements of the chins of the pregnant mice.

3. Laparotomy

3.1. Clean the abdominal surface with alcohol and povidone-iodine. Maintain sterile conditions throughout the operation.

3.2. Perform a vertical incision for the laparotomy of pregnant dams. Cut all layers separately.

3.3. Identify uterine horns on each side.

3.4. Determine the candidate fetuses for the surgery.

NOTE: Do not operate on the fetuses that are the nearest to the vagina.

3.5. Operate on two fetuses in each uterine horn if there are an even number of fetuses on each side (4 most of the time), and on 1 fetus in each uterine horn if there are an odd number of the uterus(3 most of the time).

4. Tracheal occlusion

4.1. Use 2.5x magnification glasses for visualization.

4.2. Position the uterine horn in a transverse fashion.

4.3. Take the pups, facing upward, between two fingers using the eyes of the pups and the tail as a guide to position the fetus.

4.4. Apply gentle pressure to the pup's head to allow extension of the head and therefore, visualization of the neck.

4.5. Use a 6.0 polypropylene suture with an atraumatic needle to perform TO (**Figure 1**). Keep the placenta on the side and far from the entrance and exit points of the needle.

4.6. Insert the needle transversely through the side of the uterus away from the placenta through the 1/3rd anterior part of the neck.

4.7. Move the needle gently until the midline of the neck and direct it to the anterior part, then exit the neck between the trachea and opposite the carotid sheath and uterus.

4.8. Knot the suture, taking care to maintain the integrity of the membranes and uterine wall, and keep the umbilical cord safe during knotting.

[Place Figure 1 here]

5. Abdominal wall closure

5.1. Replace the uterine horn in the abdomen.

5.2. Inject 2 mL of warm sterile saline into the peritoneal cavity before closure.

5.3. Put a running 5/0 polyglactin suture to close the abdominal wall, and close the skin with a non-running silk suture.

5.4. Apply 0.1 mg/kg of buprenorphine intraperitoneally for analgesia, and allow the recovery of the dam in a warm incubator.

6. Harvest

6.1. Apply anesthesia to the pregnant dam, and harvest all fetuses at E18.5 by cesarean section.

6.2. Check the viability of the fetuses by watching the movements of the fetuses.

6.3. Use at least two different techniques for the euthanasia: carbon dioxide insufflation and cervical dislocation.

6.4. Remove the bodies per the regulation of veterinary laboratory.

6.5. Weigh all fetuses.

6.6. Perform a vertical incision on the thorax for thoracotomy to remove the lungs.

6.7. Dissect the lungs of embryos, and weigh them to calculate total lung to body weight ratio (LBWR = (left lung weight + right lung weight)/body weight x100).

7. Histology

7.1. Snap-freeze the tissues in liquid nitrogen, optimal cutting temperature compound, and dry ice.

7.2. Cut the samples in 10 µm sections using a cryostat, and mount them on poly-lysine-coated slides.

7.3. Bake the slides at 60 °C overnight, and stain the baked slides with hematoxylin and eosin before mounting them for image acquisition at 10–20x magnification using a widefield microscope.

8. Tissue processing for protein and DNA analyses

8.1. Snap-freeze the dissected fetal lungs, and homogenize them in 300 µL of radioimmunoprecipitation assay buffer. Centrifuge at 4 °C for 5 min at 18,000 × *g*.

8.2. Extract and quantify protein, DNA, and RNA[10,12].

REPRESENTATIVE RESULTS:

This study examined 37 fetuses: 20 (54.1%) as TO vs. 17 (45.9%) as control. As the trachea could not be occluded in 4 fetuses in the TO group, they were excluded from the study. There was no significant difference in mortality in both groups: 4 fetuses (25%) in the TO group and 2 fetuses (12%) in the control group (p=0.334, odds ratio (OR) 2.5, 95% confidence interval (CI) 0.39–16.05). The mean body weight, lung weight, and lung to body weight ratio (LBWR) were higher

in the TO group than in the control group (**Table 1**). There was a significant difference in LBWR (p=0.006) between the TO and control groups.

DNA, RNA, and protein were quantified to determine the reason for the difference in LBWR (**Figure 2**). Lung DNA amounts and the DNA/protein ratio were higher in the TO group, no difference was observed in lung RNA, and protein amounts were lower in the TO group than in the control group, as previously observed in the rabbit TO model in which epithelial hyperplasia was noted[12]. The diameters of the airways in the TO group also demonstrated an increase.

Histological analyses of the E18.5 lungs showed the late canalicular/early saccular stage of lung development with developing airspaces and thickened interstitium between epithelial surfaces in the control samples while the lungs in the TO group had dilated central and distal airspaces with subjectively higher numbers of nuclei (**Figure 2**). This increased cellularity is consistent with the noted increase in the amount of lung DNA.

FIGURE AND TABLE LEGENDS:

Figure 1: Tracheal occlusion. (**A**) The transuterine suture passing through the neck. (**B**) Schematic representation of the structures after the suture passes through and before the knot. Abbreviations: C = Carotid artery; J = Jugular vein; T =Trachea; E = Esophagus; V = Vertebra.

Figure 2: **Features of the groups**. (**A**) Normalized lung to fetus weight ratio, (**B**) Lung DNA to protein ratio, (**C**) Lung DNA content normalized to lung weight, (**D**) Lung RNA content normalized to lung weight, and (**E**) Lung protein content normalized to lung weight. (**F**) Representative hematoxylin and eosin images of C57BL/6 E18.5 lungs without (scale bar = 50 μm) and (**G**) with fetal transuterine tracheal occlusion showing hyperplasia of conducting airways and increased size of distal airspaces; scale bar = 100 μm. Comparison of control (n=9) and tracheal occlusion (TO) (n=6) was performed using Student's *t*-test.

Table 1: Morphometrical results of groups

DISCUSSION:

This method describes a surgical procedure of fetal tracheal occlusion in mice and its impact on lung development. There are some critical steps in the protocol that should be carefully performed for successful TO. The warmth of the platform on which the surgery takes place and the saline introduced into the peritoneal cavity is crucial for the progression of the pregnancy. In addition, a slight pressure has to be applied to the head of the pups to ensure exposure of the neck.

A 6.0 polypropylene suture is the only suture that can be used for this technique. The needles of sutures larger than 6.0 are thicker and destroy the structures around the trachea in the neck, resulting in the loss of the fetus. The needles of the thinner sutures are very short and cannot pass through the neck of an E16.5 pup (early canalicular stage). Moreover, a cutting needle is not appropriate as it might destroy the adjacent structures.

This model has some limitations. First, there is a difference in the correlation of the lung developmental stages and gestational period between mice and humans. Second, it is difficult to develop CDH in mice and finally, hemodynamic studies are difficult to conduct in mouse models. However, the short learning curve in this study resulted in a dramatic decrease in the fetal mortality rate. As stated earlier, the cost of the animals as well as their maintenance, the number of fetuses per pregnancy, the length of the pregnancy period, and limited availability of genetic tools are the main limiting factors in rabbit and sheep models.

The first advantage of this mouse model is that it eliminates the need for hysterotomy for TO and has the potential to be reversed in utero[11], which accounts for low mortality rates observed in this study. Second, the reduction in the cost of the animals and their maintenance and a shorter pregnancy period facilitates a greater range of experiments. Third, non-technical causes of complications, such as hypothermia and anesthesia, are prevented by the short duration of surgery. Finally, the wide variety of genetic tools available in mice will lead to more studies to understand the pathophysiology of CDH. The removal of the transuterine suture with the live birth of the fetus in the nitrofen and knockout models of CDH will be the future applications of this technique.

ACKNOWLEDGMENTS:
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. All authors have made substantial contributions to the conception and design of the study, acquisition, analysis, and interpretation of data, drafting the article, and revising it for important intellectual content and final approval of the version to be submitted. The authors thank Can Sabuncuoğlu for his kind efforts on the production of the artwork of the surgical technique.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Wright, N. J. Global PaedSurg Research Collaboration. Management and outcomes of gastrointestinal congenital anomalies in low, middle and high income countries: protocol for a multicentre, international, prospective cohort study. *BMJ Open*. **9**, e030452 (2019).
2. Aydin, E. Current approach for prenatally diagnosed congenital anomalies that requires surgery. *Turkish Clinics Journal of Gynecology and Obstetrics.* **27**, 193–199 (2016).
3. Nolan, H. et al. Hemorrhage after on-ECMO repair of CDH is equivalent for muscle flap and prosthetic patch. *Journal of Pediatric Surgery*. **54** (10), 2044–2047 (2019).
4. Aydin, E. et al. Congenital diaphragmatic hernia: the good, the bad, and the tough. *Pediatric Surgery International*. **35** (3), 303–313 (2019).
5. Aydın, E., Özler, O., Burns, P., Lim, F. Y., Peiró, J. L. Left congenital diaphragmatic hernia-associated musculoskeletal deformities. *Pediatric Surgery International*. **35** (11), 1265–1270, (2019).
6. Aydın, E. et al. When primary repair is not enough: a comparison of synthetic patch and

muscle flap closure in congenital diaphragmatic hernia? *Pediatric Surgery International*. **36** (4), 485–491, (2020).
7. Wilson, M., Difiore, J. W., Peters, C. A. Experimental fetal tracheal ligation prevents the pulmonary hypoplasia associated with fetal nephrectomy: Possible application for congenital diaphragmatic hernia. *Journal of Pediatric Surgery*. **28** (11), 1433–1440 (1993).
8. Mudri, M. et al. The effects of tracheal occlusion on Wnt signaling in a rabbit model of congenital diaphragmatic hernia. *Journal of Pediatric Surgery*. **54** (5), 937–944 (2019).
9. Khan, P. A., Cloutier, M., Piedboeuf, B. Tracheal occlusion: a review of obstructing fetal lungs to make them grow and mature. *American Journal of Medical Genetics. Part C, Seminars in Medical Genetics.* **145 C** (2), 125–138 (2007).
10. Chomczynski, P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques*. **15** (3), 532–534, 536–537 (1993).
11. Beurskens, N., Klaassens, M., Rottier, R., De Klein, A., Tibboel, D. Linking animal models to human congenital diaphragmatic hernia. *Birth Defects Research Part A: Clinical and Molecular Teratology*. **79** (8), 565–572 (2007).
12. Varisco, B. M. et al. Excessive reversal of epidermal growth factor receptor and ephrin signaling following tracheal occlusion in rabbit model of congenital diaphragmatic hernia. *Molecular Medicine*. **22**, 398–411 (2016).

Figure 1

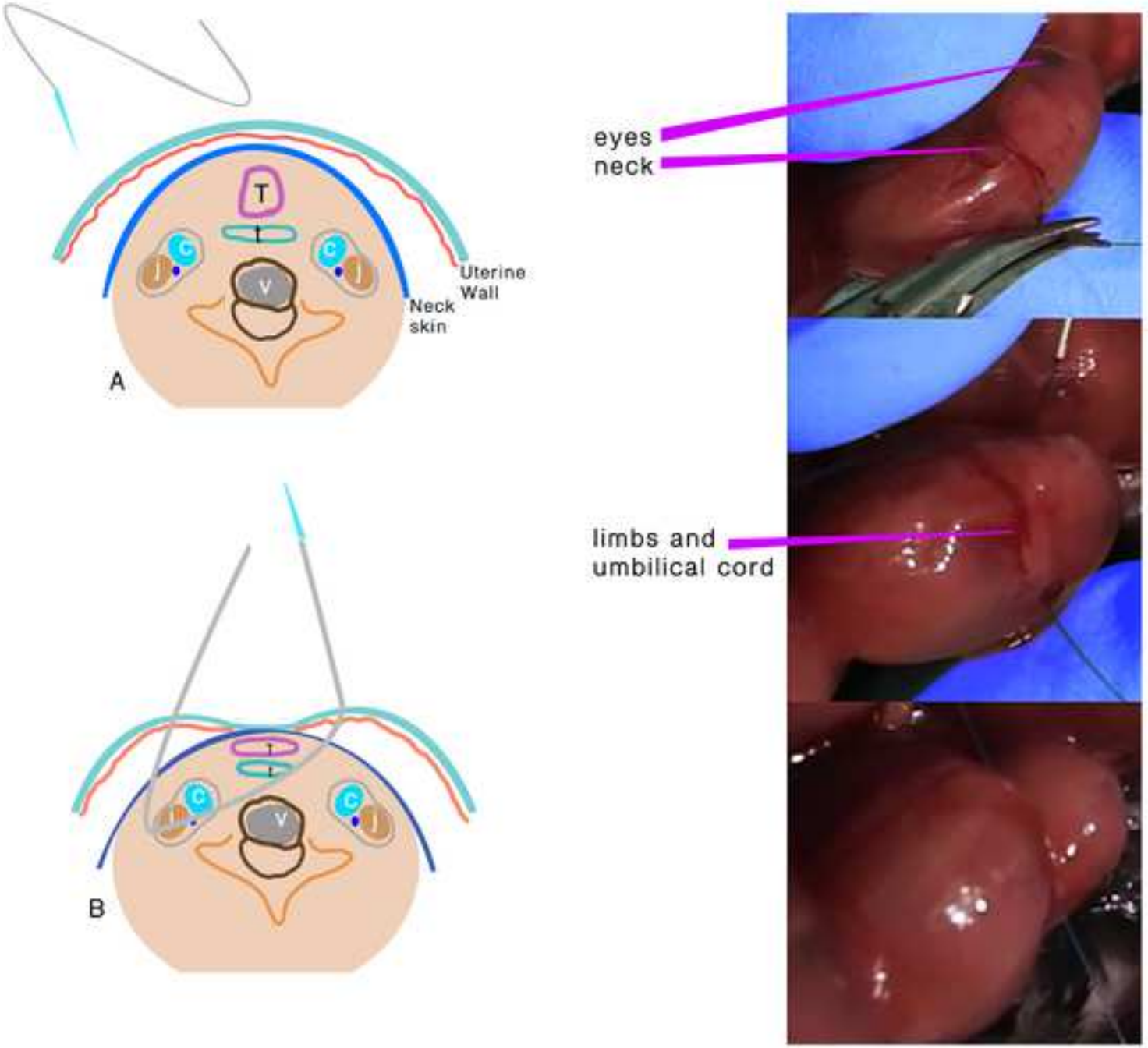

Figure 2

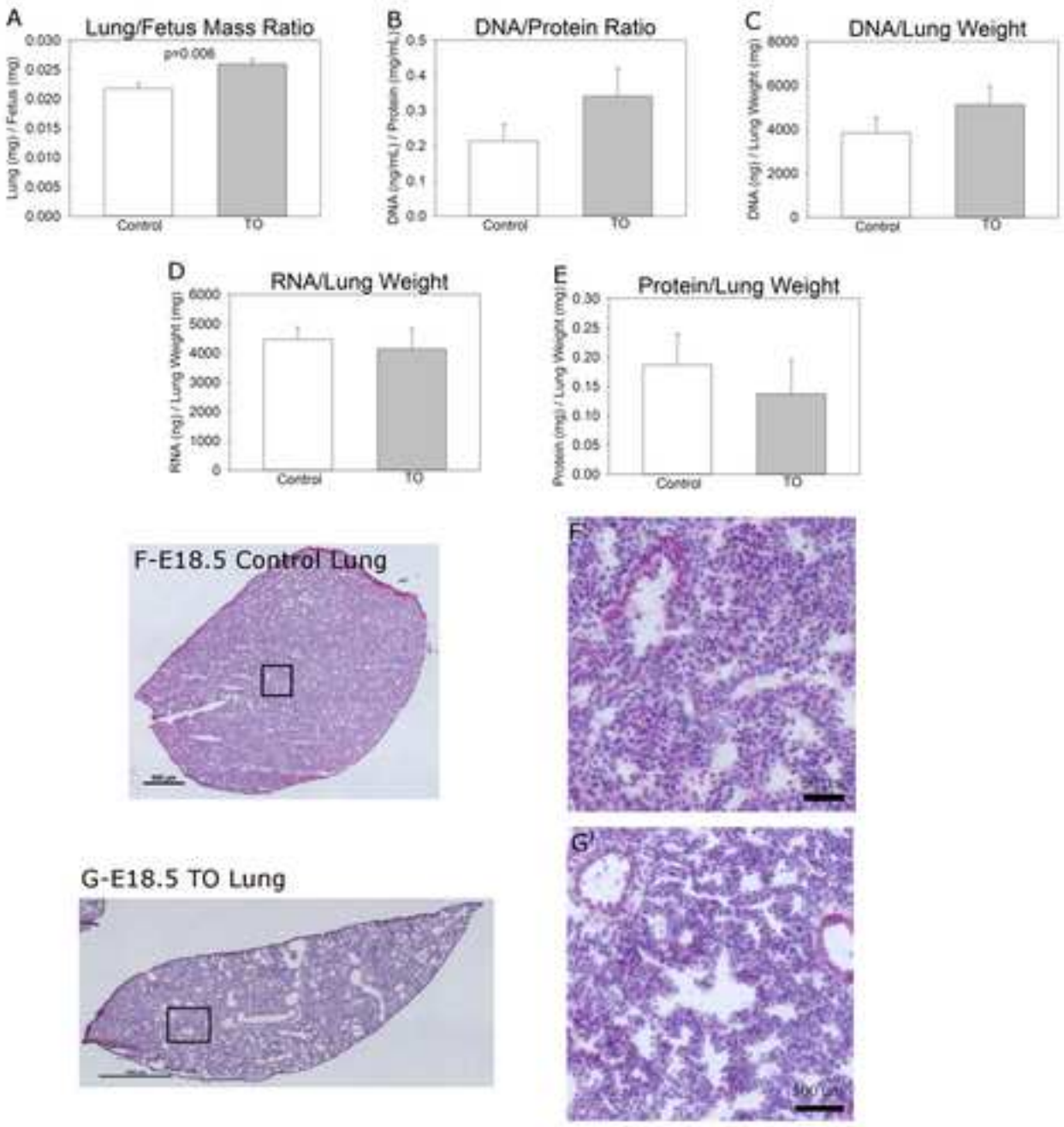

Table 1: Morphometrical results of groups

Fetus weight (mg)			
Lung weight (mg)			
LBWR			

Values expressed as means ± standard deviations. Abbreviations: LBWR = Lung to Fetal Body W

*95% Confidence Interval 0.0222–0.0249. Groups compared by Student's *t* -test.

TO	Control	p
1100.52 ± 229.38	1087.15 ± 172.32	0.896
28.41 ± 5.87	23.38 ± 3.09	0.043
0.0259 ± 0.0021	0.0217 ± 0.0028	0.006*

eight Ratio; TO = tracheal occlusion.

Table of materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_Aydin.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Buprenorphine	Par Pharmaceutical	NDC 42023-179-05	For regional anesthesia
Isoflurane	Halocarbon Life Sciences	NDC 66794-017-25	For general anesthesia
Magnification glasses	USA Medical-Surgical	SLR-250LBLK	At least 2.5x
Nikon 90i microscope	Nikon	3417	Motorized Fluorescence
Nucleospin Tissue Kit	Macherey-Nagel, Düren, Germany	740952.5	DNA isolation
Pierce BCA Protein Assay Kit	Thermo Fisher, IL, USA	23225	Protein quantification
Polyglactin suture	Ethicon	VCP451H	4-0, 24 mm, cutting
Polylysine slides	VWR	48382-117	Microscope adhesion slides
Polypropylene suture	Ethicon	Y432H	6-0, 13 mm 1/2c Taperpoint
RIPA buffer	Sigma-Aldrich, Missouri, USA	R0278-50ml	Protein isolation
Silk suture	Ethicon	VCP682G	4-0, 24 mm, cutting
Trizol	Invitrogen	15596026	RNA isolation

November 9th, 2020

The Jove Methods Collections

Dear Jove Methods Collections Editors:

Please find enclosed the revised manuscript entitled *Transuterine Fetal Tracheal Occlusion Model in Mice*. We appreciate your consideration of our manuscript for publication as an article in the *Transuterine Fetal Tracheal Occlusion Model in Mice*.

All necessary revisions were done per your comments.

We appreciate your consideration of our work for publication and look forward to hearing from you soon!

Dr. Emrah Aydın, MD, MBA

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling and grammar. Please define all abbreviations at first use.

The authors have proofread the manuscript and defined abbreviations as requested.

2. In the summary, please use the full forms of CDH and TO.

It was revised accordingly.

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), before punctuation (but after a closed parenthesis).

It was revised accordingly.

4. In the abstract (line 40), you stated that TO increases lung volume, but does not promote lung maturation. In the introduction, line 72 where you state that this method has all the advantages of previously mentioned models, does this model promote maturation?

The authors thank to editor for the kind comments. Our group previously showed that TO induced lung growth but that the gene expression of this lung was more like earlier time point mouse lung (Varisco Mol Med 2016). The proposed model has similarities with previous models however whether TO has the same effect on lung maturation is unknown. In an ongoing study that the occlusion is reversed, the lung appears more mature.

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

It was revised accordingly.

6. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

It was revised accordingly.

7. Step 1.1: Do you mean "To mate age-matched …"?

Sorry for the typing mistake. It was revised.

8. 1.2: Please specify characteristics to determine E0.

The definition was included.

9. 1.6: What is the temperature of the platform?

It was revised accordingly.

10. Please specify surgical instruments to be sterilized and used during 3-6.

It was revised accordingly.

11. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) Please specify what happens to the dams and the fetuses after the study. Please specify the euthanasia method.

It was revised accordingly.

b) Please mention how proper anesthetization is confirmed.

It was revised accordingly.

c) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

The authors stated that vet ointment was not used during the surgery.

d) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

It was revised accordingly.

e) Discuss maintenance of sterile conditions during survival surgery.

It was revised accordingly.

f) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

It was revised accordingly.

g) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.
It was revised accordingly.
h) Please do not highlight any steps describing euthanasia.
12. 4.6: To which needle are you referring?
It is the needle of the suture.
13. How do you prepare the polylysine slides (as you don't have them in the Table of Materials)?
The polylysine slides come pre-coated.
14. 7.3: Do you use a normal oven or do you need to adjust the humidity?
The oven is not humidified.
15. 7.5: Please specify magnification etc for light microscopy.
These are 20X tile scanned images.
16. For 8, please provide more details (especially if this is to be filmed) or cite them (if they will not be filmed): how much RIPA buffer, what are the centrifugation conditions, what is the extraction procedure—how much of lung tissue do you take?
The lungs were homogenized in 300 microliters and centrifuged at 4^{o}C for 5 minutes at 18,000 g.
17. Please address the earlier question in representative results, do the histology results indicate more lung maturation?
We modified the manuscript to indicate that the representative results are indicative increased distal airspace size and epithelial cell hyperplasia.
18. Please add table legend/caption for Table 1 after Representative results. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.
It was revised accordingly.
19. Line 180: please replace "destructs" with "destroys".
It was revised accordingly.
20. Lines 182-183: what do you mean by "distract the adjacent structures"?
It was revised accordingly.
21. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please write the journal names fully.
It was revised accordingly.
22. Please add scale bars in the micrographs in Fig. 2.
The scale bar was added, and it represents 50 and 100 microns respectively. Since these were tile scanned images upon which we zoomed in, the magnification is 20X for both although the scale bars are different.
23. Please sort the materials alphabetically in the Table of Materials.
It was revised accordingly.

Reviewers' comments:

Reviewer #1:

The paper by Aydin et al. presents an mice model for fetal tracheal occlusion that has certain benefits compared to already established models. In my opinion the main advantages are indeed the well characterized genome and availability of a broad cellular and molecular toolbox. The model is well described but I have some comments that probably should be addressed prior to publication of this paper.

Main comments:
- The authors have not really discussed an essential limitation of the mice model that is important to consider when translating the results to humans. The alveolar stage of lung development in mice only occurs after birth (P4-P21) and thus making them immature at birth. This means that surfactant production is limited. In addition, the tracheal occlusion is done in the pseudoglandular stage whereas in humans it is performed in the saccular stage. This should be added to introduction/discussion
It was revised accordingly. The tracheal occlusion was done on 16.5 which is the early canalicular stage.

- Abstract/Introduction: the authors describe fetal tracheal occlusion as 'a well-established' treatment method. As long as the results of the TOTAL trials have not been published, I think the authors should be more nuanced in their description. The same applies to the statement in line 57, 'improves survival'.
The authors thanks to reviewer's kind comments. The treatment strategy has already been described and the majority of the clinics worldwide do the surgery in this timeframe. However, the authors also agree that there is still certain need of improvement in the treatment and management of the disease, hence the current study was proposed. Despite these facts, tracheal occlusion has been proven to improve the survival of the CDH patients.
- Abstract, line 48-50: the fact that lung mass increase is not a correct reflection as fetal size also increases, please change to lung-to-body weight ratio. Also in TO it was 28.41mg and in controls 23.38mg that is not really twice as much?
The authors are sorry for the typing error. It was revised accordingly.
- Results, line 155-160: there is no description of how the lungs were evaluated, did the authors perform a formal morphometric analysis? If so, this should be added to methods, which outcome measures, how many lungs, how many fields/lung etc. If not, then I don't think any conclusion about histology can be made based on just a subjective evaluation.
We agree with the reviewers' statement. mRNA-seq experiments are currently being analyzed. We have revised the manuscript to indicate that hyperplasia was present.
- Discussion, line 173: see previous comment, without a formal evaluation one can only comment about the increase in size.
The discussion was revised to reflect increased size of distal airspaces and the redundancy of the epithelium in conducting airways.
Minor comments:
- Introduction, line 55-56: the balloon is usually placed between 27-30w in severe cases (some series 26-30w) and later in moderate cases, the balloon is removed between 34-35wks.
It was revised accordingly.
- Introduction, line 60: 'This temporary occlusion', I would suggest rewording this as the fact that it is temporary is not really clear from previous sentence.
It was revised accordingly.
- Introduction, line 66: I am not sure what the authors mean by limited number of surgeries?
When compared to other animal models, there is 1 or 2 fetuses per pregnant animal in the ovine model. The limited number of surgeries refers to this fact.
- Methods, line 101 (3.7): this explanation is not entirely clear to me, for instance what happened in case of 2 fetuses and what if there were 5?
In case of two fetuses, only one fetus can be operated if both fetuses are located on the same side. If not, this mom should not be included in the study. However, the authors did not meet with this case. In case of 5 fetuses, then the options depend on the where fetuses are located.
R:0-1-0 L:1-0
R/L: 0-1-0-1-0
R: 1-0-1-0 L: 0
1: operate, 0: do not operate. The ranking is from proksimal to distal.
- Methods, line 106 (4.2): transverse is not entirely clear, transverse to what?
The transverse refers to the long axis as seen in Figure 1.
- Methods, line 117 (4.10): what do the authors mean by 'to spare the membranes and uterine wall'? I would suggest rewording.
It was revised accordingly.
- Critical steps, line 175-176: this statement suggests that prior to the current study there was a pilot study or was this changed/observed after the first 1-2 animals?
This is an observation gained from many different studies with mice fetuses including this one. Moreover, there was a pilot study before starting the current study.
- Significance, line 192: can the authors add mean/median surgery times to results to fund this claim? (anesthesia time, not just skin-to-skin).
The time for the surgery is highly dependent on the number fetuses that the mice have. The median operation time for a single fetus was 1.5 minutes while the median operation time on a mice with 4 fetuses was 13 minutes from skin to skin and 14 minutes for the anesthesia.

- Significance, line 192-194: just out of curiosity, in future experiments do the authors have any thoughts on how to determine that the fetuses that are candidates for TO (point 3.7 methods) also have a diaphragmatic hernia?
The authors thank to the reviewer for the interest in the future applications. The authors have some tools to define CDH in utero prior to operation one of which is applying artificial intelligence tools to the radiological imaging techniques. The first part of it was published while the second part is currently under review. The authors are also developing an oligohydramnios model that may overcome the variability of the nitrofen model.
- Future applications, line 198: not entirely correct, if a balloon is used in lambs it can also be reversed in utero.
The reversibility is a major advantage over the small animal models which none has the potential to be reversed. When compared to the ovine model, the time, the cost, the number of fetuses, the ease of the operation and housing of the animal are the major advantages.
- Figure 1: I would suggest adding a description to the legend for the last photo (I suspect it is at the end of surgery?)
It has already been described in the figure and table legends in line 170.
- I would suggest some grammatical changes:
1. Line 45: trachea instead of tracheas, the fetus only has 1
It was revised accordingly.
2. Line 81: mate instead of meet?
It was revised accordingly.
3. Line 82: separate instead of separated?
It was revised accordingly.
4. Line 100 (3.6): that instead of those?
It was revised accordingly.
5. Line 108 (4.4), line 109 (4.5): I would suggest using singular for tails and heads (see point 1)
It was revised accordingly.
6. Line 144: Per the trachea?
It was revised accordingly.
7. Line 148: found higher, add other verb?
It was revised accordingly.
8. Line 182: per it might distract? Please reword
It was revised accordingly.

Reviewer #2:
Manuscript Summary:
This is a report on a modified model of tracheal occlusion (TO) in rat fetuses. The TO is obtained with a suture passing behind the trachea, Jugular vein and Carotid artery on one side. The suture is passed without opening the uterus. The Authors applied this method of TO to 20 fetuses, and was successful in 16. 4 fetuses died in the TO group vs 2/17 in the control group. According to the Authors, this model allows a simpler, quicker and less costly model of tracheal occlusion.
The methodology is well explained in a point to point description.
This is an interesting manuscript introducing a modification of the TO methodology in the rat model, that seems to simplify it, thus allowing to spread it further.

Major Concerns:
None

Minor Concerns:
- The Authors suggest that present model combines all the advantages of previous models, including reversibility that is usually not possible in existing rat models. Did the authors attempt to reverse TO? If yes, which were the results?
The authors thank to kind comments of the reviewer. The authors state that the reversibility of the suture is successful. However, there are certain molecular analysis needed to demonstrate the efficacy of the technique.

- TO was unsuccessful in 4 fetuses. What was the cause for failure? Could the Authors suggest some technical tips to reduce the risk of failure? This relates also to the issue of how to be sure that the stitch is in correct position.
All the tips are mentioned in the manuscript. The reason for the unsuccessful occlusion was due to improper occlusion. The trachea was either disrupted or couldn't be occluded.
- 4 fetuses in the TO group died. Was this out of 20 fetuses that had TO attempted (20%) or was it out of the 16 that had TO successful (25%)? This is an important information. What was the cause of death? Could it be related to the ligation of neck vessels on one side? If yes, did the Authors develop some technique to tighten the stitch to the right tension?
They were out of them, ie 25%. This was mentioned in the results section in line 155. The main cause was the occlusion of the neck vessels on "both" sides. Although all care was given to prevent this, it could not be succeeded in 4.

Reviewer #3:
General comments:
This manuscript describes a method to perform tracheal occlusion (TO) in mice. As compared to previous models, the major advantage of this model is the limitation of fetal losses as hysterotomy is avoided. As compared to untouched controls, this mouse model induces gross anatomy changes and increased DNA content compatible with lung growth.
The title and the abstract are appropriate (see little remark below). The protocol per se should be more detailed to allow the replication by other researchers. I would also suggest adding some experimental steps (see below). The use of bold font to highlight crucial actions may be useful for the experimenter. The representative results may be more depicted. The discussion section lacks a paragraph on the limitations of the model and discrepancies with other existing models. Regarding references, I understand that the authors would like to refer to their own previous works. However, other pivotal publications can be cited with respect to TO in humans and in animal models.

Specific comments:
1) Abstract
The authors state that tracheal occlusion promotes lung growth but not maturation (line 40). However, several studies have demonstrated that tracheal occlusion can modulate alveolar epithelial cell maturation, myofibroblast differentiation, and elastogenesis among others. Therefore, I would simply suppress the above sentence from the abstract, as this is not the place to discuss the numerous consequences of tracheal occlusion on lung development.
It was revised accordingly.

2) Introduction

a) Line 53; please add the abbreviation 'CDH' after Congenital Diaphragmatic Hernia.
It was revised accordingly.

b) The authors mention the reversibility of TO. However, they did not mention why temporary TO is important for alveolar epithelial cell maturation, justifying the removal of the plug in human fetuses, more often around the 34th week of gestation (and not 32). Also, they state that the rabbit model is irreversible. This sentence should be modulated by the fact that temporary TO has already been tested using gel plugs instead of tracheal ligation in fetal rabbits (Muensterer et al. JPS 2012).
The importance of the reversibility was included in the manuscript. For the rabbit model, although it has been realized by the authors, it has not gained a wide acceptance throughout the researchers in the field.

c) Rabbits have also large litters up to 8 pups per rabbit doe (3-4 per uterine horn).
Having a larger fetus is not an advantage in this technique. The rabbit fetuses are larger than mouse fetuses but the suture needle that would be required is very large and the need for a deeper bite increases the risk of malposition. Furthermore, the mouse offers genetic tools not readily available in the rabbit.

3) Protocol

a) Before the first step 'preparation', I would add a first paragraph detailing how the dams were housed before mating as well as before and after surgery. Please specify the temperature, type of light (daylight, 12:12?), and access to food and water (ad libitum?).
It was revised accordingly.
b) Preparation
- Step 1.1 Why did the authors choose the C57BL/6 mice? Are there differences with other strains in terms of litter size?
It is easy in B6 mice fetus to locate them in utero. The eyes are black in B6 in utero which is recognizable via membranes and uterus. In the other models such as CD1 the eyes are colorless which makes it difficult to locate the body.
- Step 1.5. Specify to which lung stage E16.5 does correspond (i.e. beginning of the canalicular stage).
It is early canalicular stage.
- Step 1.6. Specify to which temperature the platform should be heated.
24°C.
- Step 1.7. Specify to which temperature saline should be heated.
24°C.
c) Anesthesia
- Step 2.1. Specify the concentration of isoflurane. Was there oxygen provided (this is regularly done), and if so, what was the flow?
2ml/min O_2 was provided during surgery.

d) Laparotomy
- As a first step before surgery, how many operators are necessary? Does one operator position the fetus, and another perform tracheal occlusion?
One operator is enough to perform whole procedure.
- Step 3.3. Precise which layers are to be cut (e.g. peritoneum, inner and outer muscle layer).
A formal laparotomy was performed including incision to the peritoneum.
- Step 3.4. When identifying uterine horns, how should the surgeon proceed (e.g. one horn at a time or both horns together)?
Both horns were taken out of the body to see the actual number of the fetuses and distribution among them. The authors preferred to operate while both horns were out of the body, however, one can prefer to operate one horn at a time.
- Step 3.5. It is not detailed how the surgeon should determine candidate fetuses. From step 4, it seems that the optimal fetus is positioned with the nose pointing upwards. Maybe it could be useful to precise that this is not difficult to identify fetuses by transparency through the uterine wall.
The fetus is determined by the location (3.8) not the position since the position of the fetus could be changed in utero.

e) Tracheal occlusion
- Step 4.9. Could the authors precise the approximate length between the entrance and the exit points?
The authors state that there is no precise length, but the landmarks as described in the manuscript help the researcher to locate the needle.
f) Abdominal wall closure
- Before step 5.1., the operator should replace the uterine horn in the abdomen.
It was revised accordingly.
- Step 5.1. Are the peritoneum and muscle layers closed using a single suture?
Yes
- Step 5.5. Please detail how the dams are allowed to recover (e.g. in their cage, on the heating platform, and for how many time).
It was revised accordingly in 1.9-1.12.
- Could the authors precise whether or not antibiotics and/or tocolytics were administered?
None of the antibiotics or tocolytics were administered.
g) Harvest

This step requires additional details.
Although it was revised, per the editorial comments, the euthanasia was not detailed.
- Step 6.1. The method to harvest dams is not detailed. Euthanasia is an important step with respect to the ARRIVE guidelines. Please specify whether cervical dislocation or pentobarbital (and dose if applicable) was used.
Although it was revised, per the editorial comments, the euthanasia was not detailed.
- Step 6.2. Before weighing fetuses, the authors should detail how they were harvested (e.g. cesarian section) and how viable fetuses (operated and controls) were discriminated (clinical signs). This is important to be mentioned as survival rates are calculated in the results section.
It was revised accordingly. The differentiation of the viability of the fetuses was easy. It can easily be differentiated by the movements if they are alive. Otherwise, there was a clear destructive process if they were expired.
- Step 6.3. The method to open the chest and dissect the lungs is not detailed. To be very complete, please add the formula : LBWR = (left lung weight + right lung weight)/body weight x100.
It was revised accordingly.

h) Histology
- Step 7.5. Specify how images were obtained (e.g. type of microscope, magnification used, etc.).
I don't see the step by step protocol. Three lungs per group were snap frozen, embedded in OCT, stained for H&E, and tile scanned at 10X20X magnification using a Nikon 90i widefield microscope.

i) Tissue processing
- This step is described a bit too straight forward. This can be detailed to my opinion, unless reference 7 describes the whole procedure.
It was revised to provide more detail and also referenced the rabbit paper in which an identical method was used.
4) Representative results
- What is the average time for surgery? And how did it improve overtime? The authors mentioned their learning curve in the discussion but did not explain how they assessed this outcome.
The time for the surgery is highly dependent on the number fetuses that the mice have. The median operation time for a single fetus was 1.5 minutes while the median operation time on mice with 4 fetuses was 13 minutes from skin to skin and 14 minutes for the anesthesia.
- The authors mentioned that the mean body weight is higher in operated pups (line 147), but this does not appear in Table 1. Please correct the error in the body text.
The authors rechecked the table and the body text but could not find any discrepancy.
- Histological results are described but were not supported by morphometry. It would be more convincing for the reader to provide such data to confirm the success of the protocol, which can be easily obtained on H&E sections.
The authors stated that they did not perform formalize morphometry as this was a method paper and have changed the manuscript to reflect the presence of hyperplasia rather than maturation since that seems to imply distal lung maturation which is not what we intended.

5) Discussion

a) While the mouse model displays several advantages, the authors did not discuss the limitations of this model. Of note, the mouse model allows limited access to functional studies. While respiratory function may be evaluated using rodent ventilators, it precludes from hemodynamic measurements (indirectly by ultrasound or directly by catheters). Moreover, because alveolarization in rodents starts postnatally, it would be worth mentioning that lung evaluation at birth prevents from alveolarization studies, unless the pups are left alive.
It was revised accordingly in means of limitations. On the other hand, the model enables the pups to be delivered via vaginal route and kept alive.

b) The authors found a statistical difference in terms of LBWR between controls and operated fetuses.

However, this effect seems somewhat attenuated (20% increase). Indeed, TO can increase LBWR values up to 200% in other models. Could the authors explain this difference?
The authors thank to reviewer for the kind comments. While there is no certain explanation of this difference, it might be due to the limited time frame between TO and harvest. As a future application, the time frame might be extended to see if there will be more increase.

c) Another difference as compared with other models is that protein amount did not increase, and even decrease, after TO. Usually, both total DNA and protein contents increase after TO, which indicates lung growth and not hypertrophy (Nardo et al. Pediatr Res 1998). Could the authors explain this difference?
We believe that this is an indication of hyperplasia, and the same pattern was observed in the rabbit model. The rabbit model indicates that this may be EGFR related.

d) Do the authors think that this model, applied to an earlier gestational age, could reproduce cystic dilation of the lungs, such as seen in congenital pulmonary malformations?
The application should be tried before making any comments. Theoretically it is possible. However, the dimension of the fetus is the main factor that will determine the applicability.

6) Figures
- Scale bar is missing in Figure 2, panels F and G.
It was revised accordingly.
- Specify the statistical test use.
It was revised accordingly.