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Fetal Tracheal Occlusion in Mice
A Novel Transuterine Method

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Aim

Fetal tracheal occlusion (TO) is an emerging surgical therapy in congenital diaphragmatic hernia (CDH) that improves the fetal lung growth. Different animal models of CDH and TO present advantages and disadvantages regarding ethical issues, cost, surgical difficulty, size, survival rates and available genetic tools. We developed a minimally invasive murine transuterine TO model, which will be useful in defining how TO impacts lung molecular biology, cellular processes, and overall lung physiology.

Methods

Experimental Design
IACUC protocol #2016-0068 by the Cincinnati Children's Research Foundation Institutional Animal Care and Use Committee, age-matched wild-type (WT) C57BL/6 mice were mated and the date when the vaginal plug is seen was accepted as E0. At E16.5, pregnant dams underwent laparotomy and transuterine tracheal occlusion was performed upon 2 fetuses in each uterine horn with 2.5x magnification. At E18.5, all dams were harvested by cesarean section and fetuses were weighed by using a scale measuring accurately up to 0.001g (Mettler, Toledo). Lungs were dissected from mouse embryos and were weighed to calculate total lung to body weight ratio (LBWR). Normal fetuses of the litter served as non-manipulated controls.

TO Surgical Method
After performing a median laparotomy to the dams under general anesthesia, the uterine horn was positioned gently in a transverse fashion with the pups facing upward between two fingers of the surgeon. Using gentle pressure, pups' heads were extended allowing visualization of the neck. A 6.0 polypropylene suture (13mm 1/2c Taperpoint, Ethicon®) with an atraumatic needle was used for TO. The needle was inserted through the side of the uterus opposite to the placenta, through 1/3 anterior of the neck in a transverse fashion. (Figure 1A) The tip of the needle was gently advanced till it passes the midline of the pup where it was directed through the anterior of the neck and exits the neck between the trachea and opposite carotid sheath and uterus, respectively (Figure 1B). Lastly, it was knotted gently without tearing the membranes and uterine wall and avoiding the umbilical cord. The dam's abdominal wall and skin were then closed. Buprenorphine 0.1 mg/kg was administered intraperitoneally for analgesia and the pregnant mouse subsequently allowed to be recovered.

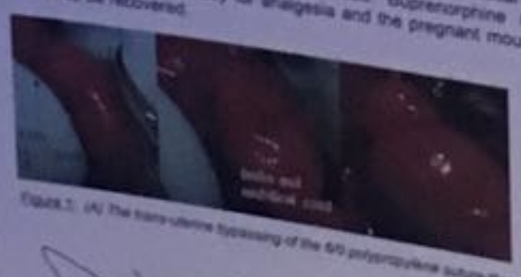
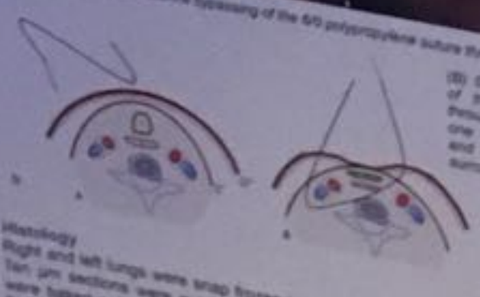


Figure 1: (A) The trans-uterine bypassing of the 6/0 polypropylene suture through neck



(B) Schematic representation of the trans-uterine suture through the neck, respecting one side vascular (carotid and jugular) elements and surrounding trachea.

Histology
Right and left lungs were snap frozen in OCT (Sakura) and snap frozen on dry ice. Ten µm sections were cut by cryostat and mounted on poly-lysine slides. Slides were baked a 60°C overnight, stained with hematoxylin and eosin, and mounted. Tile scanned 4X and individual 20X images were obtained using a Nikon 90i widefield microscope and Nikon Elements software.

Tissue Processing for Protein and DNA
Dissected fetal lungs were snap frozen and then homogenized in 300 mL of RIPA buffer (Sigma-Aldrich) and centrifuged. Trizol (Life Technologies) was used to extract DNA, RNA, and protein per manufacturer instructions. Protein was quantified using Bradford assay and DNA and RNA concentrations determined by Nanodrop (both Thermo Fisher) using previously published methods.[1]

Statistical Analysis
Data were normalized to fetal body weights, lung weights, and lung protein content and expressed as means ± standard deviations. Statistical analysis was performed using Students t-test with p-values of less than 0.05 considered significant. Data presented in bar chart form represents mean +/- standard error of the mean.

Results

Gross Results
There were 20 fetuses in the TO group and 17 fetuses in the control group. Four of the mice in TO group were excluded due to unsuccessful TO which were unable to be trachea occluded with the transuterine suture. The survival rate was comparable in both groups, 12 fetuses (75%) in the TO group and 15 fetuses (88%) in the control group survived the surgery. (p=0.334, OR 2.5 95% CI 0.39-16.05)

Morphometric Results
The mean body weight, lung weight, and LBWR were all higher in TO fetuses than the control group. (Table 1) When normalized to the mean LBWR of the control group, TO fetuses had significantly higher LBWR (Figure 2A). (p=0.006)

Table 1: Morphometrical results of groups

	TO	Control	p
Fetus weight (mg)	1100.52 ± 229.38	1087.15 ± 172.32	0.896
Lung weight (mg)	28.41 ± 5.87	23.38 ± 3.09	0.043
LBWR	0.0259 ± 0.0021	0.0217 ± 0.0028	0.006*

Values expressed as means ± standard deviations. LBWR refers to Lung to Fetal Body Weight Ratio. * 95% Confidence Interval 0.0222-0.0249

Learning the Model
The impetus to develop this technique derived from poor fetal survival using a previously published techniques that involve hysterotomy and deliver of the fetal head as was previously published. Our first three surgical attempts were unsuccessful with one mouse dying under anesthesia and two mice requiring euthanasia due to premature labor. The subsequent six surgeries were successful with >85% fetus survival noted at the time of collection. The average operation time was reduced from 90 to 45 minutes per dam over the course of this study, suggesting that reduced surgical times and reduced uterine manipulation are key factors in the success of this model.

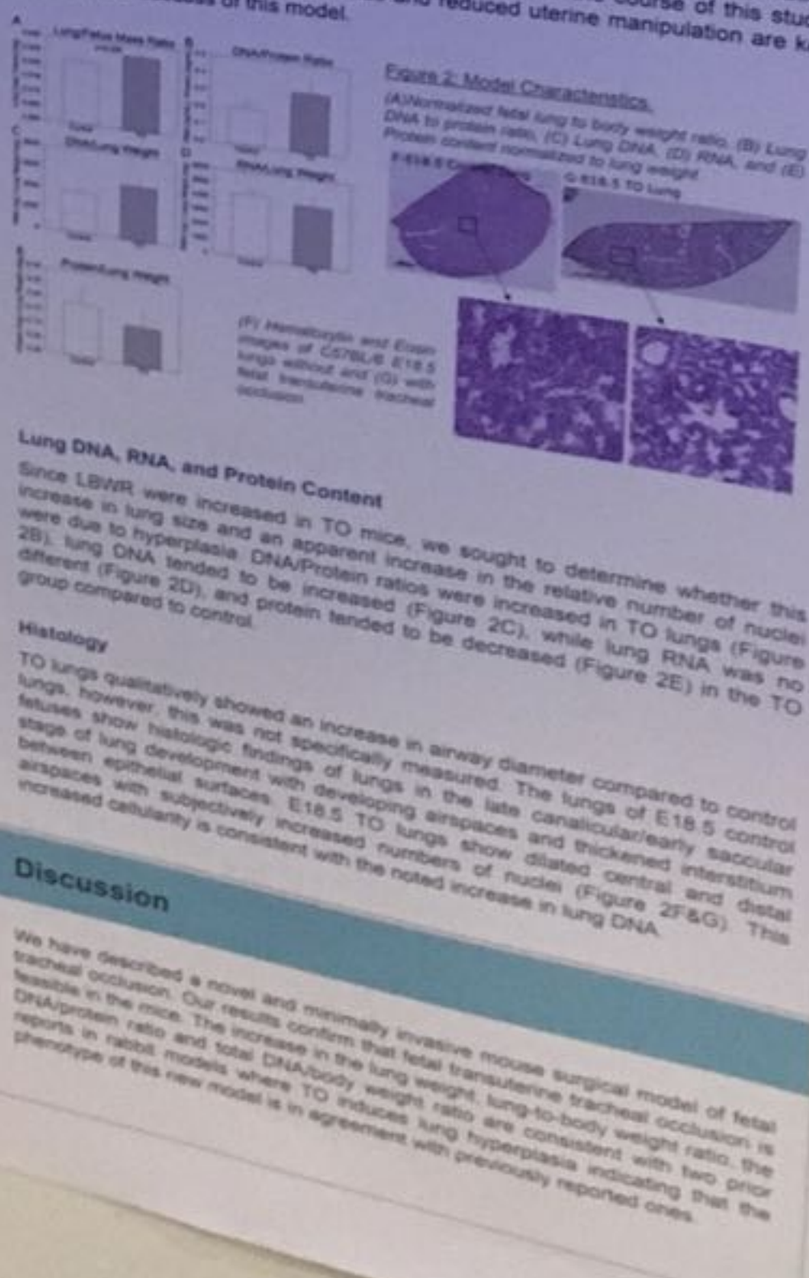


Figure 2: Model Characteristics.
(A)Normalized fetal lung to body weight ratio, (B) Lung DNA to protein ratio, (C) Lung DNA, (D) RNA, and (E) Protein content normalized to lung weight. (F) Hematoxylin and Eosin images of C57BL/6 E18.5 lungs without and (G) with fetal transuterine tracheal occlusion.

Lung DNA, RNA, and Protein Content
Since LBWR were increased in TO mice, we sought to determine whether this increase in lung size and an apparent increase in the relative number of nuclei were due to hyperplasia. DNA/Protein ratios were increased in TO lungs (Figure 2B), lung DNA tended to be increased (Figure 2C), while lung RNA was no different (Figure 2D), and protein tended to be decreased (Figure 2E) in the TO group compared to control.

Histology
TO lungs qualitatively showed an increase in airway diameter compared to control lungs, however, this was not specifically measured. The lungs of E18.5 control fetuses show histologic findings of lungs in the late canalicular/early saccular stage of lung development with developing airspaces and thickened interstitium between epithelial surfaces. E18.5 TO lungs show dilated central and distal airspaces with subjectively increased numbers of nuclei (Figure 2F&G). This increased cellularity is consistent with the noted increase in lung DNA.

Discussion

We have described a novel and minimally invasive mouse surgical model of fetal tracheal occlusion. Our results confirm that fetal transuterine tracheal occlusion is feasible in the mice. The increase in the lung weight, lung-to-body weight ratio, the DNA/protein ratio and total DNA/body weight ratio are consistent with two prior reports in rabbit models where TO induces lung hyperplasia indicating that the phenotype of this new model is in agreement with previously reported ones.