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Dynamic tracheal occlusion improves lung morphometrics and function in the fetal lamb model of congenital diaphragmatic hernia

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Abstract

Background—Congenital diaphragmatic hernia (CDH) is associated with significant neonatal morbidity and mortality. Although prenatal complete tracheal occlusion (cTO) causes hypoplastic CDH lungs to enlarge, improved lung function has not been demonstrated. Furthermore, cTO interferes with the dynamic pressure change and fluid flow associated with fetal breathing.

Purpose—To assess a novel dynamic tracheal occlusion (dTO) device that preserves pressure changes and fluid flow.

Methods—In this pilot study, CDH was created in fetal lambs at 65 days gestational age (GA). At 110 days GA, a cTO device (n=3) or a dTO device (n=4) was placed in the fetal trachea. At 135 days GA, lambs were delivered and resuscitated. Unoperated lamb co-twins (n=5), sham thoracotomy lambs (n=2), and untreated CDH lambs (n=3) served as controls.

Results—Tracheal opening pressure, lung volume, lung fluid total protein, and phospholipid were significantly higher in the cTO group than in the dTO and unoperated control groups. Maximal oxygenation and lung compliance were significantly lower in the cTO group when compared to the unoperated control and dTO groups.

Conclusion—Preliminary results suggest that in the fetal lamb CDH model, dTO restores normal lung morphometrics and function, whereas cTO leads to enlarged but less functional lungs.

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Keywords

Congenital Diaphragmatic Hernia; Tracheal Occlusion; Lung; Pulmonary Hypertension

Introduction

Congenital diaphragmatic hernia (CDH) is a common birth defect that afflicts approximately 1 in 2000 live births. Newborns with CDH face the morbidity and mortality of lung hypoplasia and pulmonary hypertension. Despite improvements in postnatal care, pooled results from over 50 centers worldwide indicate an overall survival of only 68% (CDH Study Group, <http://cdhsg.net>, February 2008). Those that survive immediate respiratory insufficiency are vulnerable to neurodevelopmental, nutritional, hearing, and pulmonary function deficiencies (1).

The high morbidity and mortality rates associated with CDH have led many investigators to pursue the technique of fetal tracheal occlusion (TO) to promote lung growth in this setting. Despite progression from animal experiments to ongoing human trials, TO has yet to be demonstrated to be superior to standard, postnatal therapy, and is currently reserved for use in only the most severely affected fetuses (2). In addition, TO may be detrimental, resulting in enlarged but functionally suboptimal lungs (3, 4).

The barriers to further progress in fetal TO for CDH may reside in our understanding of fetal breathing. Fetal breathing, lung fluid secretion, and efflux of fluid from the lung into the amniotic cavity are crucial for normal lung development (5). A positive pressure gradient exists at the level of the glottis and oropharynx, causing lung fluid to build up and distend the lungs (6). Fetal breathing movements have been defined as both the phasic changes in upper airway resistance as well as intermittent diaphragmatic contractions which together result in a net efflux of fluid from the lungs and are necessary for the expression of growth factors (7). Complete TO (cTO) eliminates the dynamic pressure changes and fluid flow caused by fetal breathing. We hypothesized that an intermediate between no treatment and cTO—dynamic tracheal occlusion (dTO)—would promote more normal lung growth and development in the fetal lamb CDH model.

To test this hypothesis, we constructed a novel tracheal occlusion device, incorporating a pressure-sensitive valve to allow for lung fluid efflux. The key parameter in this design is the opening pressure of the valve. The normal lamb oropharynx and glottis create a 20 mm H₂O pressure gradient between the lungs and amniotic fluid (8). Studies with cTO have demonstrated pressure gradients of ~160 mm H₂O (9). We chose a pressure of 80 mm H₂O in an attempt to mitigate the deleterious effects of high pressures while preserving the stimulus for growth. In this pilot study, we implanted this device or a cTO device into fetal lambs after they had undergone CDH creation. After lambs were delivered near term gestation, we measured respiratory function as well as lung morphometrics, histology, and fluid characteristics as indicators of the effects of the different TO devices on overall lung development.

Methods

Experimental Design

The Institutional Animal Care and Use Committee at the University of California, San Francisco and the University of California, Davis oversaw and approved the fetal lamb study design. We studied 5 groups of lambs: (1) the dTO group (n=4) that underwent CDH creation at 65 days gestational age (GA) and dTO device placement at 110 days GA; (2) the

cTO group (n=3) that underwent CDH creation at 65 days and cTO device placement at 110 days GA; (3) the untreated CDH group (n=3) that underwent CDH creation at 65 days GA but did not undergo device placement; (4) the sham thoracotomy group (n=2) that underwent fetal thoracotomy at 65 days GA without CDH creation or device placement; and lastly (5) the control group (n=5) of unoperated fetuses that were derived from co-twins of the intervention groups.

Device Fabrication

The dTO device consists of a 3.0-mm inner-diameter cuffed endotracheal tube (ET) (Covidien-Nellcor, Boulder, CO) attached to a ventriculoperitoneal shunt valve (Medtronic, Santa Barbara, CA) via luer fittings (Fig. 1A). The cTO device was identical to the dTO device, except for replacement of the valve-luer apparatus with a luer cap to prevent all flow through the ET tube.

Fetal Surgical Procedures and Neonatal Resuscitation

At 65 days GA, ewes underwent anesthesia with IV ketamine and diazepam. The ewes were then intubated and maintained under general anesthesia with isoflurane. A left-sided fetal CDH was created after maternal laparotomy and hysterotomy as previously described (10). Defect creation at this timepoint in gestation results in severe lung hypoplasia and replicates the severity of disease for which fetal tracheal occlusion is currently offered in ongoing human trials. Sham-operated lambs underwent a thoracotomy without CDH creation. At 110 days GA the ewes with lambs in the intervention groups underwent a second laparotomy and hysterotomy and a dTO or cTO device was placed in the fetal trachea via direct laryngoscopy. The ET tube balloon was inflated with two milliliters of saline for both devices. The devices were then secured to the fetus using 4-0 prolene suture.

At 135 days GA, ewes underwent a third laparotomy and hysterotomy. While still on placental support, fetal arterial and venous catheters were placed. Lambs were paralyzed with pancuronium. The balloons of all implanted devices were inspected and found to be intact. The opening pressure of the lung fluid was measured in tracheal-occluded lambs using a fluid-column manometer. Lung fluid was then collected by aspiration. The entire device was removed and the lambs were re-intubated with a 4.5-mm inner-diameter cuffed ET tube. Control unoperated lambs, sham-operated lambs, and untreated CDH lambs were at this stage intubated for the first time. The umbilical cord was then clamped and cut and the lambs were connected to a neonatal ventilator set to deliver 100% oxygen (V.I.P. Bird; Carefusion, San Diego, CA). The arterial catheter was transduced and arterial blood gases were drawn and vital statistics and ventilator settings were recorded every 10 minutes. Ventilator settings were tailored to each lamb with a target pH of 7.4 and arterial carbon dioxide partial pressure of 40 mmHg. Intravenous Lactated Ringer's boluses and sodium bicarbonate were used to treat metabolic acidoses. The primary endpoint of the resuscitation was maximal achievable arterial partial pressure of oxygen (pO_2) during the resuscitation. Compliance was calculated using the following equation: compliance = tidal volume / (peak inspiratory pressure – peak end expiratory pressure). After two hours the lambs were euthanized with sodium pentobarbital. The lungs were perfused with Microfil (Flow Tech, Inc., Windsor, CT) via the main pulmonary artery and then inflated via the trachea with 10% formalin at a constant pressure of 25cm H₂O until they were completely fixed. Lung volumes were determined by water displacement.

Lung Fluid Analysis

At the time of delivery, lung fluid in the unoperated lambs and intervention groups was collected for surfactant protein-B (SP-B), phospholipid (PL) and total protein (TP) analysis. Samples of lung fluid were centrifuged at 500 × g for 5 minutes to remove cells. Lung fluid

supernatant was centrifuged at $27,000 \times g$ for 60 minutes to isolate large aggregate surfactant pellet and supernatant fractions. The large aggregate pellet was re-suspended in surfactant buffer (10mM Tris, 154mM NaCl, 1.5mM CaCl_2 , pH 7.4) and an aliquot was extracted (11) and assayed for PL content (12). TP was measured on both fractions using QuantiPro BCA assay system (Sigma, St. Louis, MO). SP-B was assayed using serial dilutions (50%) of PL with the first well containing 1.2 μg of PL. SP-B was detected using a polyclonal anti-SP-B antibody (Millipore, Billerica, MA) as previously reported (13, 14).

Valve Characterization

The Medtronic CSF-Flow Control Valve is specified to have an opening pressure of 85mm H_2O at a flow rate of 5 mL/hour, with a linear increase to 105mm H_2O at 50 mL/hour (Fig. 2A, inset). This specification is defined within a range of $\pm 25\text{mm H}_2\text{O}$. Of note, opening pressures at flow rates less than 5 mL/hour are not specified by Medtronic. The valves were characterized via the following technique (pressure-flow curves detailed in Fig. 2A). Valves were detached from the explanted ET tubes and attached to a PX26 differential pressure sensor (Omega Engineering, Stamford, CT). An in-line syringe pump was set to various flow rates and the pressure was recorded 10 times for each rate using a multimeter (Agilent, San Jose, CA).

Histology

Serial uniform sampling techniques were used to randomize lung tissue from the right lower lobe of lambs for histologic analysis. Five micron sections were stained with hematoxylin and eosin. Lung histologic injury scores were calculated as previously described (15) by a single, blinded observer (SCS).

Statistical Analyses

Continuous variables are represented as means $\pm$ standard deviation. Comparison of two means was made using an unpaired Student's t-test. Comparison of three or more means was made using ANOVA. Compliance data over time was compared using repeated-measures ANOVA. The Student-Newman-Keuls method was used for direct post-hoc testing between groups. P values ≤ 0.05 were considered significant. Prism 5 software (Graphpad Inc., La Jolla, CA) was used for all calculations.

Results

There were no significant differences in body weight among the groups. In the lambs that underwent CDH creation, gross analysis revealed that no defects spontaneously healed. All defects were consistently large with abdominal viscera present in the chest (Fig. 1B) and hypoplastic left lungs in untreated CDH controls.

Tracheal pressure, lung fluid volume, and lung volume

Tracheal opening pressure at the time of delivery and resuscitation was significantly lower in the dTO group than in the cTO group (Fig. 3A, $p < 0.003$). Lung fluid volume was significantly higher in the cTO group when compared to the unoperated control group (Fig. 3B). There was a non-significant trend toward normalization of lung fluid in the dTO group. Total lung volume was significantly increased in the cTO group when compared to the unoperated control, dTO, and untreated CDH groups. As expected, the untreated CDH group had significantly smaller lungs than the unoperated control, dTO, and cTO groups (Fig. 3C).

Lung fluid analysis

Sufficient lung fluid was available for analysis in the unoperated control, dTO, and cTO groups (Fig. 3D-F). TP and PL content in the lung fluid were both significantly higher in the cTO group than the in dTO and unoperated control groups. Although not statistically significant, there was a trend towards decreasing SP-B/PL percentage with the highest amount in the unoperated controls, followed by the dTO group and then the cTO group.

Lung function

The maximum achievable pO_2 was significantly decreased in the cTO group when compared to the unoperated control group (Fig. 2B). Despite a trend towards an increased pO_2 in the dTO group when compared to the cTO group, the trend was not statistically significant. When analyzing these results we noticed a surprisingly wide range of values for maximum pO_2 in the dTO group. This variability led us to analyze the valves that we had used.

Post-hoc valve characterization and repeat analyses

The pressure-flow characteristics of all the valves in this study are summarized in Fig. 2A. At flow rates lower than 5ml/hr, devices V3 and V4 remain close to factory tolerance, whereas the opening pressures of V1 and V2 decline precipitously. At a physiologic lung fluid secretion rate of a ~2.5 mL/kg/hr at 110 days GA (16), V1 and V2 have a lower opening pressure than the 80 mm H₂O that we had targeted. Thus, we excluded these two lambs from the dTO group. Analysis without these incorrectly operating valves demonstrated that the maximum pO_2 was significantly higher in this dTO-V3V4 subgroup compared to the cTO group (Fig. 2C). Re-analysis of all of the data with the two incorrectly operating valves excluded did not change the relationships between groups or statistical significance for opening pressure, lung volumes, lung fluid volume or lung fluid content (PL, TP, SP-B).

Lung compliance and histology

Complete lung compliance data for all lambs was available for the first 80 minutes of the resuscitation (Fig. 4A). In general, during the resuscitation period, compliance improved slowly for all groups. Lung compliance was significantly higher in the dTO-V3V4 group when compared to the cTO group. Both intervention groups were significantly less compliant than unoperated controls but significantly more compliant than the untreated CDH group. Preliminary histologic analysis of the right lower lobe of the ventilated lambs in the unoperated control, untreated CDH, dTO-V3V4, and cTO groups was performed (Fig. 4C-F). This analysis demonstrated significantly more severe histologic lung injury in the cTO group when compared to the unoperated control group. The dTO-V3V4 group was statistically indistinguishable from the unoperated controls (Fig. 4B).

Discussion

The aim of this study was to analyze the effect of dTO on lung growth and function in a fetal lamb model of severe CDH. This model is well described and mimics the lung hypoplasia, pulmonary hypertension, decreased airway generation, and abnormal vascular muscularization that characterizes human CDH (10, 17). Complete TO has been shown to promote lung growth but at the expense of surfactant-producing type II pneumocytes (18). Furthermore, cTO interferes with the cyclical mechanical strain that is necessary for proper lung development and cellular differentiation (19). We hypothesized that a dTO device that maintained a set intra-tracheal pressure, while allowing for some dynamic exchange of fluid, would promote more normal lung development and function. We found that CDH lambs

with correctly operating dTO valves had superior physiologic outcomes compared to cTO-treated CDH lambs.

Normal lung development occurs in the setting of active lung fluid secretion that distends the lungs and is intermittently released by glottic, pharyngeal, and diaphragmatic movements (5). CDH leads to lung hypoplasia and malfunction. Mechanical stretch alone—as induced by cTO—to stimulate lung growth in this setting not only ignores the dynamics of intermittent stretch, but likely overstretches the lungs, causing *ineffective* lung growth. The optimal pressure characteristics to stimulate lung growth in the setting of CDH are unknown. In this study, we targeted a baseline pressure of 80 mm H₂O and relied upon fetal breathing movements to create pressure fluctuations.

Consistent with previous work, the lungs of the cTO group were significantly larger than those of the control groups and had significantly lower mean maximum pO₂. This mean pO₂ was nonetheless much higher than historical non-treated CDH lambs (20) as well as untreated CDH lambs in this study. In contrast, lungs that were treated with dTO with properly functioning valves had a mean maximum pO₂ and lung volume that mimicked unoperated controls. Most importantly, the mean maximum pO₂ in the dTO-V3V4 group was significantly higher than that of the cTO-treated lambs. In addition, lung compliance and overall lung histology (likely a reflection of ventilator trauma) was improved in the dTO-V3V4 group. Thus, dTO resulted in significantly improved pulmonary function.

The tracheal opening pressure in all dTO lambs was well below the pressure setting of the valves (Fig. 3A). As the lung approached a normal size and as the lambs neared term gestation, the lung fluid secretion rate may have decreased, leading to a decrease in tracheal pressure (considering the valve characteristics shown in Fig. 2A). Alternatively, changes in lung compliance *in utero* could also explain these differences in opening pressure. The increased TP content of the cTO lung fluid is consistent with previous reports (21), but the increased PL content has not been described before. One simple explanation for the increased TP and PL in the cTO lung fluid, which may reflect another critical benefit of dTO, is an accumulation of cellular debris that cannot be eliminated and may have deleterious effects on cTO-treated lungs. The trend towards an increase in SP-B when comparing dTO to cTO suggests that surfactant may contribute to the dTO-V3V4 group's superior function, but also implies that surfactant-independent mechanisms may be at work. Overall, dTO may provide a more physiologic cellular milieu in which the developing lung epithelial cells are bathed.

Admittedly, our selection of a target tracheal pressure of 80 mm H₂O was a best-educated guess. Some studies suggest that even lower, less traumatic tracheal pressures may be sufficient to stimulate sufficient lung growth and development (22, 23), however, these studies were performed on lambs without CDH. Difiore et al, working in the fetal lamb model with the diaphragmatic defect created at 90 days GA and tracheal ligation performed at 110 days GA, discovered an opening pressure similar to our target pressure (6 mm Hg or 83 mm H₂O) (24). In this study, the underlying histology and lung function of the tracheal ligation lambs was basically normal. Differences in the duration of occlusion and in the model severity may explain the differences in opening pressure and lung function in the completely-occluded lambs in the Difiore study versus our study. However, considering their data alongside the correlation of our validated valve opening pressures with lung function may indicate that the optimal distending pressure rests at approximately 80 mm H₂O. Future studies will determine the actual changes in airway pressure in the setting of both untreated and dynamically-occluded CDH over time during gestation. These studies will improve our understanding of the contributions of these physical forces, both to the

underlying pathophysiology of lung hypoplasia and to the mechanism of tracheal pressure modulation in the treatment of CDH.

In summary, although the “n” number in this study is small and the valves did not perform uniformly, preliminary results suggest that in the fetal lamb model of CDH, dTO restores normal lung morphometrics and function, whereas cTO leads to enlarged but less functional lungs. Despite the severity of this particular model, with the defect creation at gestational day 65, dTO-V3V4 lambs exhibited oxygenation levels that were normal and compliance levels that were near-normal. It is clear that a dynamic form of tracheal occlusion is a viable area of inquiry in the pursuit of *in utero* therapy for CDH. This study suggests that promotion of lung growth in CDH may be improved with dynamic devices that more accurately replicate a normal fetal pulmonary developmental environment. *In utero* “gentle distension” may benefit the *fetus* with CDH in the same way that postnatal “gentle ventilation” benefits the *neonate* with CDH.

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Figure 1.

A. The dynamic tracheal occlusion device. The proximal end, with the Button CSF-Flow Control Valve, is on the right. B. Representative image of CDH defect at necropsy with anterior chest wall removed. Viscera are visible in the left hemithorax and the hypoplastic left lung is hidden behind the heart.

Figure 2.

A. Pressure-flow characteristics for dynamic tracheal occlusion (dTO) devices labeled V1-V4. At physiologic flow rates of about 4 cc/hr at 110 days GA, V1 and V2 had an opening pressure well below the 80 mm of H₂O that was targeted. Inset; factory specification of valves is shown for the range defined by the manufacturer. B. Scatter-plot of maximum pO₂ (mmHg) including all of the valves. There was a wide degree of variability in the dTO group. C. Mean maximum pO₂ after exclusion of inappropriately operating valves. The dTO-V3V4 (488.0 ± 26.9) and unoperated control groups (531.3 ± 52.5) had significantly higher mean maximum pO₂ than the complete tracheal occlusion (cTO) (192.7 ± 88.7), sham (255 ± 31.1), untreated CDH (89 ± 39.6) and dTO-V1V2 (145.5 ± 61.5) groups. * = $p < 0.05$.

Figure 3.

Lung fluid and volume. A. Mean tracheal opening pressure in mm H₂O transduced through the complete tracheal occlusion (cTO) or dynamic tracheal occlusion (dTO) device. The dTO mean was significantly lower than the cTO mean (23 ± 8.5 vs. 130 ± 19). B. Mean volume of lung fluid in mL/kg aspirated from lungs of all groups. The mean volume of lung fluid was significantly higher in the cTO (55.4 ± 15.3) than in the unoperated control (12.4 ± 3.1), sham-operated (7.6 ± 2.5) and untreated CDH (3.3 ± 0.9) groups. There was a non-significant trend towards normalization of dTO lung fluid volume. C. Total lung volume in mL/kg was significantly higher in the cTO (149.1 ± 30.2) vs. the unoperated control (94.9 ± 9.8) and dTO (91.4 ± 28.6) groups. Untreated CDH lambs (47.7 ± 7.6) had significantly less lung volume than the unoperated control, dTO, and cTO groups. D. Mean total protein (TP) content (mg) in lung fluid obtained from lambs in the control, cTO, and dTO groups. Mean TP was significantly higher in the cTO group when compared to the dTO and control group (5287.4 ± 1134.7 vs. 635.0 ± 658.8 and 217.9 ± 175.5). E. Mean phospholipid (PL) content (mg) in lung fluid obtained from lambs in the unoperated control, cTO, and dTO groups. Mean PL was significantly higher (mg) in the cTO group when compared to the dTO and unoperated control group (2.3 ± 1.5 vs. 0.27 ± 0.37 and 0.15 ± 0.2). F. Mean percentage of surfactant protein B/phospholipid (SP-B/PL) in lung fluid obtained from lambs in the unoperated control, cTO and dTO groups. The trend was unoperated control > dTO > cTO but was not statistically significant. * = $p < 0.05$.

Figure 4.

Lung histology and compliance. A. Compliance curves for all groups. The dynamic tracheal occlusion (dTO) group had significantly higher compliance than the complete tracheal occlusion (cTO) group. Both intervention groups were significantly less compliant than unoperated controls but significantly more compliant than the untreated CDH group (repeated-measures ANOVA, $p < 0.05$ for all comparisons described). B. Lung injury histology scores. The cTO group had significantly higher lung injury histology scores when compared to unoperated controls, which were statistically indistinguishable from the dTO group (2.4 ± 0.4 vs. 1.2 ± 0.5 and 1.5 ± 0.3). C-F. Representative sections from the right lower lobe of the unoperated control, untreated CDH, dTO and cTO groups. A = alveolus, Art = artery, T = terminal bronchiole, Hem = hemorrhage. More alveolar hemorrhage and parenchymal damage was visible in the untreated CDH group and cTO group when compared to the unoperated control group and dTO group. * = $p < 0.05$.