

# Fetal tracheal occlusion in the rat model of nitrofen-induced congenital diaphragmatic hernia

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**Kitano, Yoshihiro, Paul Davies, Daniel Von Allmen, N. Scott Adzick, and Alan W. Flake.** Fetal tracheal occlusion in the rat model of nitrofen-induced congenital diaphragmatic hernia. *J. Appl. Physiol.* 87(2): 769–775, 1999.—Prenatal tracheal occlusion (TO) consistently accelerates lung growth in the sheep model of congenital diaphragmatic hernia (CDH). However, significant variability in lung growth has been observed in early clinical trials of TO. We hypothesized that lung hypoplasia created at relatively late stages of lung development may not be equivalent to human CDH-induced lung hypoplasia, which begins early in gestation. To test this hypothesis, we performed TO in the rat model of nitrofen-induced CDH. Left-sided CDH was induced by administering 100 mg of nitrofen to timed pregnant rats on *day 9* of gestation. On *day 19* of gestation, four to five fetuses per dam underwent surgical ligation of the trachea. At death (*day 21.5*), lungs from non-CDH (non-CDH group), left-CDH (CDH group), and trachea-occluded left-CDH fetuses (CDH-TO group) were harvested and compared by weight, DNA and protein content, and stereological morphometry. Wet and dry lung weight-to-body weight ratio, total lung DNA and protein contents, the volume of lung parenchyma, and the total saccular surface area of the CDH-TO group were significantly increased relative to the CDH group and were either greater than or comparable to the non-CDH controls. We conclude that TO accelerates lung growth and increases lung parenchyma in an early-onset model of CDH-induced lung hypoplasia.

fetal lung; lung development; lung hypoplasia; morphometry

CONGENITAL DIAPHRAGMATIC HERNIA (CDH) is a simple anatomic defect of the diaphragm that results in a devastating physiological consequence: pulmonary hypoplasia with abnormal pulmonary vascular development and secondary pulmonary hypertension. CDH is present in one in 2,000–5,000 live births, and defects are more common on the left side, with ~85–90% being left-sided (20). To date, the mortality of prenatally diagnosed isolated CDH remains ~60%, despite the availability of extracorporeal membrane oxygenation and other innovative postnatal treatment modalities (9). This persistent high mortality, in combination with recognition of the fetal pathophysiology, provides a compelling rationale for fetal intervention. The objective of prenatal treatment of CDH is to interrupt the

fetal pathophysiology and allow adequate lung growth before birth to improve survival after birth.

Initial experimental and clinical efforts to treat CDH before birth were directed toward mechanical reduction of herniated viscera and repair of the diaphragmatic defect in utero (12). Although technically feasible, this was abandoned with the realization that reduction of the herniated left lobe of the liver resulted in obstruction of umbilical venous flow to the fetus and secondary fetal demise (10, 23). More recently, the physiological observation that fetal tracheal occlusion (TO) accelerates lung growth has been applied to the problem of CDH in animal models and early clinical trials (8, 11, 13). However, in our initial clinical experience, the lung growth response after TO has been variable, with disappointing lung growth in some fetuses and impressive lung growth in others. This was unexpected because of the consistent lung growth observed in normal fetal animal models (3) as well as the sheep model of CDH (7, 14).

The variable lung growth observed in human CDH fetuses treated by TO suggests that there may be important differences between severe human CDH and the available animal models. There is indirect experimental and clinical evidence that the severity of pulmonary hypoplasia depends on the timing and the degree of compression of the developing lung (1, 2, 28). CDH in the sheep model is usually created during the pseudoglandular stage of lung development (75–85 days gestation), and the degree of lung hypoplasia may not be analogous to severe human CDH, which occurs during the embryonic stage of lung development. Furthermore, the sheep model of CDH does not mimic the severe liver herniation, which is always present in human cases selected for fetal intervention. Presence of the liver in the chest may limit reduction of the viscera and lung growth observed after TO. In contrast, in the rat model of nitrofen-induced CDH, abnormality of the diaphragm is detected as early as at 13–14 days of gestation, during the embryonic stage of lung development (4, 19), corresponding to 6-wk gestation in humans. In addition, a large amount of liver is herniated into the chest, analogous to the visceral herniation seen in severe human CDH.

We hypothesized in this study that the difference in lung growth response to TO in animals and humans is due to differing severity of hypoplasia related to the early onset of human CDH or to differences in the reducibility of the herniated viscera related to the liver herniation. This hypothesis was studied by using the

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rat model of CDH. Only animals with left-sided CDH were included because it is more clinically relevant and because the degree of lung hypoplasia may differ between the right-sided and left-sided CDH.

## METHODS

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia and followed guidelines set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* [DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20892].

### CDH Model

Time-dated pregnant Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were housed in breeding cages and allowed food and water ad libitum. To create CDH in the fetuses, 100 mg of 2,4-dichlorophenyl-4-nitrophenyl ether (nitrofen; Wako Bioproducts, Richmond, VA) dissolved in 2 ml of olive oil were tube fed to a dam on *day 9* of gestation (vaginal smear positive = *day 0*, term = *day 22*).

### Surgical Procedure

Four to five fetuses per dam underwent surgical ligation of the trachea on *day 19* of gestation, and the littermates served as either the CDH or non-CDH controls. After intraperitoneal injection of pentobarbital sodium (15 mg/kg), one of the uterine horns was exposed through a maternal midline laparotomy. The head and neck of a fetus were exteriorized through a small hysterotomy, and the neck of the fetus was maintained in hyperextension by wet gauze. Drops of ritodrine solution (5 mg/ml) were applied directly to the uterine wall as necessary to minimize uterine contraction during the procedure. Under a stereo dissection microscope, the fetal trachea was exposed through a midline cervical incision and ligated with 9-0 nylon suture. The cervical incision was not approximated. The fetal head and neck were returned to the amniotic space, and the hysterotomy was closed with a 6-0 Prolene purse-string suture. The maternal abdominal wall was closed with 5-0 Vicryl running suture.

On 21.5 days gestation, the dam underwent a second laparotomy under deep anesthesia, with intramuscular injection of pentobarbital sodium (30 mg/kg). Methoxyflurane inhalation was added as needed. Fetuses were delivered by cesarean section one at a time and euthanized by intraperitoneal injection of pentobarbital sodium (5 mg). Each fetus was blotted dry, and body weight was measured. The fetal abdomen was opened through a transverse abdominal incision, the anterior chest wall was removed, and the presence and laterality of diaphragmatic defect were inspected. The lung and heart were dissected out below the ligation, and the complete tracheal obstruction was confirmed by dye injection as previously described (18). The fetuses with no leakage were designated ligated fetuses. The dam was euthanized by aortic transection under deep anesthesia after all the fetuses were harvested.

### Weight Measurements and DNA/Protein Analysis

Lung growth was evaluated by weight measurement and DNA and protein analysis among the three groups; the non-CDH group (12 fetuses from 6 litters), the CDH group (14 fetuses from 6 litters), and the CDH-TO group (9 fetuses from 3 litters). Only the pups with left-sided CDH were included in the CDH and CDH-TO groups. The right and left lung were

dissected free from the heart, trachea, and major bronchi and weighed separately. The entire left lung was kept in ice until lyophilization, which required at least 24 h. After the dry weight of the left lung was measured, it was sonicated in 500 ml of phosphate-buffered saline to make a crude suspension for the measurement of DNA and protein. DNA and protein concentration of the suspension were measured with a commercially available kit (QIAGEN tissue kit, QIAGEN, Santa Clarita, CA) and by the modified Lowry method (Bio-Rad DC Protein, Bio-Rad, Hercules, CA), respectively. Total lung DNA and protein were calculated assuming that the right lung contains the same amount of DNA and protein per milligram wet tissue as does the left lung.

### Lung Morphometry

For morphometrical analysis, five lungs from three to four litters were collected for each group. Again, only the fetuses with left-sided CDH were included. The trachea was cannulated with a 24-gauge Angiocath catheter (Beckton-Dickinson Vascular Access, Sandy, UT), the thorax was opened, and the lungs were inflated *in situ* at a constant pressure of 10 cmH<sub>2</sub>O with 1% glutaraldehyde and 2% formaldehyde in phosphate-buffered saline for 10 min. Each trachea was then ligated to maintain lung inflation during fixation, and the lungs were harvested from the thoracic cavity. Tissue was stored overnight in the same fixative, and fixed lung volumes (V<sub>L</sub>) were determined by the water-displacement method (26). After paraffin embedding, 5-mm-thick coronal sections were cut at five levels, 200–500 mm apart from each other, and stained with hematoxylin and eosin.

A coherent multipurpose test lattice, consisting of 42 test points and a discontinuous series of line probes (KR-821, Klarmann Rulings, Litchfield, NH), was fitted in the eyepiece of a light microscope (Leica DMRBE, Leica, Allendale, The Netherlands) and used for point counting. Parameters measured were as follows.

- 1) Volume density (V<sub>v</sub>) of parenchyma in lung (V<sub>v<sub>p</sub></sub>).
- 2) V<sub>v</sub> of saccular air space in parenchyma (V<sub>v<sub>sa</sub></sub>).
- 3) V<sub>v</sub> of septal tissue in parenchyma (V<sub>v<sub>ss</sub></sub>).
- 4) Surface density (S<sub>v</sub>) of the saccular epithelium in parenchyma (S<sub>v<sub>s</sub></sub>).
- 5) Numerical density (N<sub>v</sub>) of saccules in parenchyma (N<sub>v<sub>s</sub></sub>).
- 6) Radial count (RC) of saccules.

V<sub>v</sub>. V<sub>v</sub> is the volume of a tissue compartment per unit volume of reference compartment. The lung is divided into two compartments, parenchyma and nonparenchyma. By definition, parenchyma consists of the gas-exchanging compartment of the lung that contains the air spaces (saccule ducts and saccules) and the intervening septa. Nonparenchyma is the lung compartment containing structures in which gas exchange does not occur and consists of conducting airways to the level of terminal bronchioles, large blood vessels, and the connective tissue associated with these structures. Four randomly chosen fields in each slide (20 fields/lung) were point counted at a magnification of ×50 for V<sub>v<sub>p</sub></sub> and ×200 for V<sub>v<sub>sa</sub></sub> and V<sub>v<sub>ss</sub></sub>.

S<sub>v<sub>s</sub></sub>. S<sub>v<sub>s</sub></sub> was estimated by counting the number of points on parenchyma and the number of intersects of the test line with saccular air space-epithelial interface at a magnification of ×200 in the same field that was used to measure V<sub>v<sub>sa</sub></sub> and V<sub>v<sub>ss</sub></sub>.

S<sub>v<sub>s</sub></sub> was calculated as

$$S_{v_s} = 2/d (I_s/P_p)$$

where *d* is the length of one line probe of the test lattice at the magnification of ×200 (*d* = 85 mm).

$Nv_s$ . The potential gas-exchanging air spaces in fetal lungs differ morphologically from adult alveoli and are referred to as saccules. Saccules were defined as the air spaces distal to terminal bronchioles either wholly enclosed by respiratory epithelium or partially enclosed, with the remaining boundary formed by an imaginary line that connected the distal ends of two septa. The number of saccules present within the test area ( $0.0625 \text{ mm}^2$ ) was counted at  $\times 400$  magnification and included those partially overlaying two selected borders (top and left).  $Nv_s$  was calculated by using the formula

$$Nv_s = (N_A)^{3/2}/b(Vv_{sa})^{1/2}$$

where  $N_A$  is the number of saccules per unit area and  $b$  is the shape factor taken to be 1.55 (32).

$RC$ .  $RC$  was defined as the number of saccules transected by a line drawn from a terminal bronchiole perpendicular to the closest pleura or lobular septum. Whenever possible, four  $RC$ s per slide (20 counts/lung) were counted at a magnification of  $\times 100$ .

Absolute values for all features were calculated by using the volume of lung parenchyma ( $V_L \times Vv_p$ ). Because there was no apparent histological difference between right and left lungs in any of the specimens, we did not count them separately.

#### Statistical Analysis

Data are means  $\pm$  SD. Statistical analysis was performed by using one-way ANOVA with Scheffé's test using Statview software (Abacus, Berkeley, CA). Statistical significance was confirmed at  $P < 0.05$ .

## RESULTS

### Survival and Success Rate of TO

A total of 24 pregnant rats were used in this study. Five rats did not undergo autopsy because of anesthesia-related deaths in four and premature delivery in one. In the 19 surviving dams, 61 of 85 operated fetuses were found alive (mortality rate of TO: 28.2%). Of 61 live fetuses, complete TO was confirmed by dye injection in 45 fetuses (success rate of TO: 73.8%). Sixteen fetuses were found to have either transection of the trachea or a loose ligature.

### Gross Appearance

The lungs of the fetuses with CDH appeared hypoplastic. The abdominal organs herniated into the chest in fetuses with left-sided CDH included the two left lobes of the liver, the stomach, and the spleen. Figure 1, A and B, shows the gross appearance of the enlarged lungs in the CDH-TO group. The CDH-TO lungs were much larger and filled the entire thoracic cavity, obscuring visualization of the heart. Although lung size was markedly increased by TO, the herniated liver consistently remained above the rim of the diaphragm (Fig. 1B), suggesting that the liver may impede complete reduction of herniated viscera by lung expansion.

### Weight Analysis

The results are summarized in Table 1. Fetal body weight and the right-to-left lung weight ratio were not significantly different among the three groups. In the

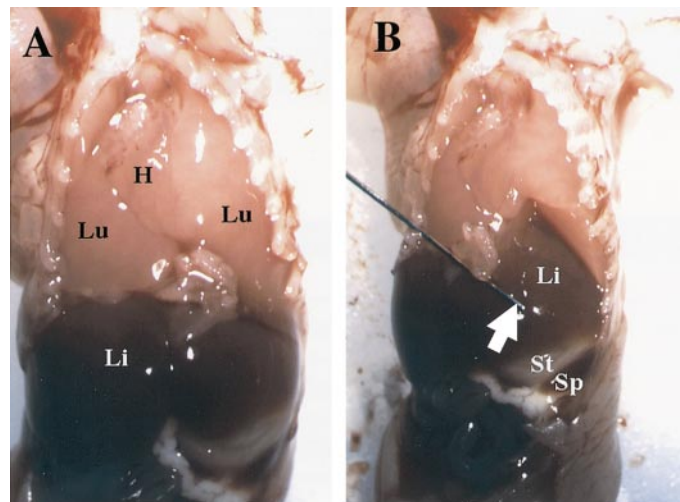


Fig. 1. A: gross appearance of enlarged lungs after tracheal occlusion (TO) in a rat fetus with left-sided congenital diaphragmatic hernia (CDH). Note hyperplastic lung filling chest cavity. Li, liver; H, heart; Lu, lung. B: herniated left liver is still seen in chest, behind enlarged left lung. Stomach (St) and spleen (Sp) have been reduced into the abdomen. Arrow, level of diaphragm.

CDH group, the wet lung weight (LW;  $P < 0.05$ ), wet lung weight-to-body weight ratio (LW/BW;  $P < 0.01$ ), and dry lung weight-to-body weight ratio (dLW/BW;  $P < 0.05$ ) were significantly decreased compared with the non-CDH group. Dry-to-wet weight ratio was significantly increased in the CDH group compared with the non-CDH group ( $P < 0.01$ ). The dLW tended to be smaller in the CDH group, but this did not reach statistical significance.

When the CDH-TO group was compared with the CDH group, LW, LW/BW, dLW, and dLW/BW were all significantly increased in the CDH-TO fetuses ( $P < 0.01$ ). When the CDH-TO group was compared with the non-CDH group, LW ( $P < 0.01$ ), LW/BW ( $P < 0.01$ ), dLW ( $P < 0.05$ ), and dLW/BW ( $P < 0.05$ ) were all significantly increased in the CDH-TO fetuses, indicating that TO can induce lung growth in hypoplastic lungs and that the resultant lung size can exceed that of the controls. The dry-to-wet weight ratio of the CDH-TO group was significantly lower ( $P < 0.01$ ) compared with either the CDH or non-CDH group.

### DNA and Protein Contents

The results are summarized in Table 2. The total lung protein content was smaller in the CDH group compared with the non-CDH group, but this was significant ( $P < 0.05$ ) only after it was corrected for body weight. It was significantly increased in the CDH-TO group ( $P < 0.01$ ) compared with the CDH group. When it was compared between the CDH-TO group and the non-CDH group, the former had higher protein content, although the statistical difference was confirmed ( $P < 0.05$ ) only after it was corrected for body weight. The same trend was observed in the total lung DNA content, but the difference between CDH-TO and the non-CDH group was not statistically significant. Protein-to-DNA ratio remained constant among the three

Table 1. *Body and lung weight measurements*

Group	BW, g	LW, mg	LW/BW, %	Right-to-Left LW Ratio	dLW, mg	dLW/BW, mg/g	Dry-to-Wet Weight Ratio, %
Non-CDH ( $n=12$ )	$3.99 \pm 0.54$	$86.2 \pm 16.2$	$2.16 \pm 0.28$	$1.82 \pm 0.15$	$9.44 \pm 2.11$	$2.36 \pm 0.36$	$10.9 \pm 0.86$
CDH ( $n=14$ )	$3.80 \pm 0.53$	$50.7 \pm 10.2$	$1.33 \pm 0.14$	$2.01 \pm 0.26$	$7.33 \pm 1.89$	$1.92 \pm 0.35$	$14.4 \pm 2.35$
CDH+TO ( $n=9$ )	$4.11 \pm 0.51$	$199 \pm 56.5$	$4.81 \pm 1.11$	$2.17 \pm 0.54$	$12.0 \pm 2.72$	$2.92 \pm 0.52$	$6.28 \pm 1.31$

Values are means  $\pm$  SD;  $n$  = no. of rats/group. BW, body weight; LW, lung weight; dLW, dry lung weight. Non-CDH fetuses without congenital diaphragmatic hernia on either side; CDH, fetuses with left-sided CDH; CDH + TO, fetuses with left-sided CDH and tracheal ligation [tracheal occlusion (TO)] at 19 days gestation. \*  $P < 0.01$ , †  $P < 0.05$ , compared by one-way ANOVA with Scheffé's test.

groups, suggesting that the increase in protein content after TO was the result of cell proliferation rather than mere inflation of existing structures or cell hypertrophy.

### Lung Morphometry

The histology of lung specimens after inflation fixation is shown in Fig. 2. The CDH-TO lungs have a less cellular appearance with larger saccules and thinner septa, compared with either the CDH lungs or the non-CDH control lungs. Lungs from all groups appear to be in the saccular stage of lung development.

Morphometric values per unit parenchymal volume are presented in Table 3.  $V_{V_p}$  and  $V_{V_{sa}}$  were decreased in the CDH lungs compared with the controls ( $P < 0.05$ ). They were increased after TO compared with the CDH lungs ( $P < 0.01$ ) and with the non-CDH control lungs ( $P < 0.05$  for  $V_{V_p}$  and  $P < 0.01$  for  $V_{V_{sa}}$ ). Accordingly,  $V_{V_{ss}}$  was decreased in the CDH-TO lungs ( $P < 0.01$ ), supporting the visual impression of thinner septa in this group. There was no difference in the  $S_{V_s}$  among the three groups.  $N_{V_s}$  was larger in the CDH lungs compared with the control ( $P < 0.05$ ) and was smaller in the CDH-TO lungs compared with the CDH lungs ( $P < 0.01$ ). There was no statistical difference between the CDH-TO and the non-CDH groups.

Absolute values calculated from the morphometric data are summarized in Table 4.  $V_L$  and the saccular surface area of the CDH-TO lungs were increased three- to fourfold compared with the CDH lungs ( $P < 0.01$ ). They were even greater than those of the non-CDH controls ( $P < 0.05$ ). Total saccular number was increased in the CDH-TO group compared with the CDH group ( $P < 0.01$ ), suggesting that TO induces an increase in saccular number. RCs of the CDH-TO lungs

were significantly higher compared with both the CDH lungs and the non-CDH controls ( $P < 0.01$ ).

### DISCUSSION

This study documents the induction of proliferative lung growth by TO in the nitrofen-induced rat CDH model. Compared with the hypoplastic lungs of CDH animals, the lungs of CDH-TO animals are strikingly larger and more mature in structure, with an increased volume of lung parenchyma and surface area available for gas exchange. TO induced consistent lung growth in the hypoplastic lungs of this model, resulting in lungs that were larger than, or at least comparable with, the non-CDH controls. We also observed that TO did not result in reduction of the herniated liver segments back to the peritoneal cavity, unlike other mobile abdominal organs. This finding is clinically important, since TO is currently only indicated for human cases with liver herniation and is expected to gradually reduce the abdominal organs from the chest cavity.

Because of the small size and the fragility of fetal tissue prior to 18 days of gestation, the timing of TO in the rat model was limited to a relatively late period of gestation. Fortunately, 19-day-gestation rat lung is in the canalicular stage of lung development. This corresponds to the stage of human lung development at which TO has been performed for the prenatal treatment of CDH (24–28 wk), making this study clinically relevant.

LW/BW, dLW/BW, and total lung DNA and protein content per body weight were all significantly decreased in the CDH animals, which confirms that the lungs in the CDH animals were actually hypoplastic. Despite a relatively short period of TO, the growth of hypoplastic lung was impressive by all the parameters

Table 2. *DNA and protein content*

Group	Total Lung Protein, mg	Total Lung Protein/BW, mg/g	Total Lung DNA, $\mu$ g	Total Lung DNA/BW, $\mu$ g/g	Protein-to-DNA Ratio
Non-CDH ( $n=11$ )	$5.83 \pm 0.84$	$1.42 \pm 0.14$	$459 \pm 44.2$	$112 \pm 7.95$	$12.7 \pm 1.38$
CDH ( $n=11$ )	$4.83 \pm 0.87$	$1.20 \pm 0.15$	$371 \pm 80.4$	$92.3 \pm 18.0$	$13.3 \pm 2.22$
CDH+TO ( $n=7$ )	$7.10 \pm 1.48$	$1.67 \pm 0.28$	$537 \pm 102$	$126 \pm 14.6$	$13.2 \pm 1.31$

Values are means  $\pm$  SD;  $n$  = no. of rats/group. \*  $P < 0.01$ , †  $P < 0.05$ , compared by one-way ANOVA with Scheffé's test.

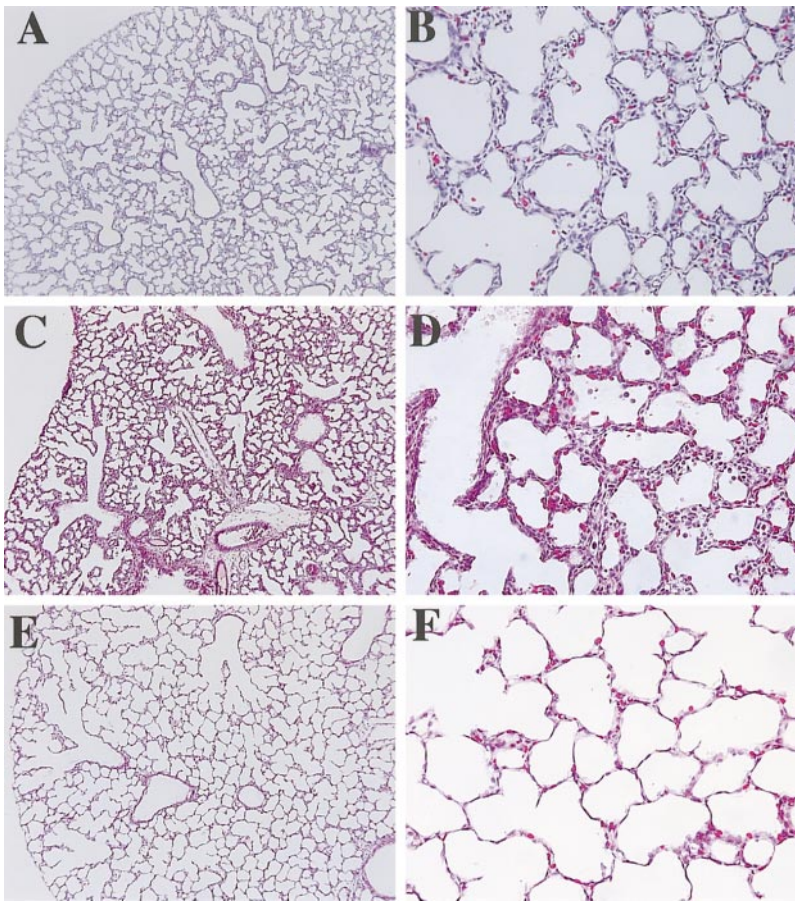


Fig. 2. Histology of inflation-fixed lungs from rat fetuses without CDH (non-CDH group; *A* and *B*), fetuses with left CDH (CDH group; *C* and *D*), and fetuses with left CDH and TO (CDH-TO group; *E* and *F*). All lungs were inflation fixed under a pressure of 10 cmH<sub>2</sub>O. Note the larger saccules and thinner saccular septa in lungs after TO. Difference can also be appreciated between the CDH-TO lungs and the non-CDH lungs. Original magnifications for *A*, *C*, and *E*:  $\times 50$ ; for *B*, *D*, and *F*:  $\times 200$ .

examined. These lungs were even hyperplastic compared with the non-CDH controls. LW/BW, dLW/BW, and total protein content/BW ratios were significantly increased. Lung DNA content/BW was also increased, but this did not reach statistical significance. Protein-to-DNA ratio was unchanged among the three groups, confirming the proliferative nature of TO-induced lung growth. Right-to-left LW ratio tended to be increased in the CDH animals compared with the non-CDH group and further increased in the CDH-TO lungs, but the difference was statistically insignificant when compared by ANOVA among the three groups.

Our morphometric data confirm that TO increases lung volume and surface area in this model. These changes reflect an increase in the volume of parenchyma relative to that of nonrespiratory conducting

airways and blood vessels. Within the parenchyma, the volume of saccular spaces was increased relative to that of intervening septal tissue, but the fact that the surface density of saccular epithelium was not reduced suggests that the changes were achieved by a more complex process than simple enlargement of existing saccules. The numerical density determination is potentially less reliable than the other morphometric parameters because it depends on assumptions of the shape and size distribution of saccules. The results suggest, however, that the increased volume and surface area of air spaces in the ligated lungs were due to an increase in the total number of saccules, i.e., an almost twofold increase in the CDH-TO lungs compared with the CDH lungs. This result was reinforced by an independently determined index of saccular number, the RC, which

Table 3. Morphometric results: relative values

Group	Vv of Parenchyma, %	Vv of Saccular Air Space, %	Vv of Saccular Septa, %	Sv of the Saccular Epithelium, saccules/mm	Nv of Saccules $\times 1,000/\text{mm}^3$
Non-CDH ( $n=5$ )	$79.0 \pm 2.9$	$57.0 \pm 3.3$	$43.0 \pm 3.3$	$24.4 \pm 3.4$	$5.23 \pm 0.74$
CDH ( $n=5$ )	$70.1 \pm 2.2$	$47.9 \pm 4.1$	$52.1 \pm 4.1$	$25.4 \pm 2.3$	$7.12 \pm 1.39$
CDH+TO ( $n=5$ )	$86.5 \pm 4.2$	$69.7 \pm 5.8$	$30.3 \pm 5.8$	$24.8 \pm 1.7$	$3.66 \pm 0.69$

Values are means  $\pm$  SD;  $n$  = no. of rats/group. Vv, volume density; Sv, surface density; Nv, numerical density. \* $P < 0.01$ , † $P < 0.05$ , compared by one-way ANOVA with Scheffé's test.

Table 4. Morphometric results: absolute values

Group	Lung Volume, μl	Total Saccular Surface Area, ×1,000 mm <sup>2</sup>	Total No. of Saccules, ×10 <sup>5</sup>	Radial Count
Non-CDH (n=5)	180 ± 42	3.44 ± 0.79	7.3 ± 1.3	1.6 ± 0.1
CDH (n=5)	91 ± 26	1.64 ± 0.58	4.6 ± 1.9	1.1 ± 0.1
CDH + TO (n=5)	343 ± 143	7.31 ± 2.87	10.1 ± 1.9	1.8 ± 0.1

Values are means ± SD; n = no. of rats/group. \*  $P < 0.01$ , †  $P < 0.05$ , compared by one-way ANOVA with Scheffé's test.

showed a 1.6-fold increase. Because this count is made within the acinus, the results suggest an increase in the number of saccules per acinus. However, the final total number of saccules is not necessarily different from that in the non-CDH control lung.

Because TO is performed after the completion of airway development during the pseudoglandular stage of lung development, the components of the lung that grow in response to TO should be distal to the terminal bronchiole, resulting in a "polyalveolar lung." Polyalveolar lung is a term first applied by Hislop and Reid (15) to a newborn with an enlarged lobe in which several segments of the lung had around five times the normal alveolar number and demonstrated the clinical features of lobar emphysema. Our morphometric results confirm that the lung growth following TO occurred in parenchyma, rather than in nonparenchyma, and in saccular air space, rather than in saccular septa. In this study, TO resulted in an increased number of saccules and an increased RC. The long-term function of polyalveolar lung is not known. However, an increase in alveolar number without an increase in airway branching is known to occur in compensatory lung growth after pneumonectomy (16) and in patients with CDH (5) and unilateral pulmonary aplasia (25). Emphysema does not necessarily accompany the polyalveolar lung in these circumstances. We speculate that fetal TO may accelerate the same process and we are optimistic about the long-term function of the enlarged lungs. Davey et al. (6) reported that in a fetal sheep model of pulmonary hypoplasia induced by lung fluid drainage subsequent fetal TO resulted in the actual survival of the neonatal lamb with almost normal pulmonary function after birth. Harrison et al. (13) reported that the oxygen requirement after birth was low in human CDH survivors after TO. As a result, seven of eight survivors did not require extracorporeal membrane oxygenation. These results indirectly suggest that the enlarged lungs do function after birth.

Although we did not perform physiological or vascular morphometric evaluations, several studies have documented the improvement of arterial blood pH,  $PCO_2$ ,  $PO_2$ , and lung compliance after TO in the sheep model of CDH (7, 14, 24). We have previously reported indirect evidence that TO could reverse the increased pulmonary resistance that accompanies CDH in the sheep model (30). Morphometric studies suggest that the diminution of total capillary surface area in CDH-induced lung hypoplasia is due to a global deficit in lung volume and alveolar surface area, rather than any

difference in composition at the level of the acinus (31), and that the diminution could be reversed by TO (21). Taken together, we speculate that the CDH-TO lungs created in this study would have better gas-exchanging capacity and lower pulmonary vascular resistance compared with CDH lungs and might approximate the values seen in non-CDH lungs. This supports the clinical potential of prenatal TO as a treatment for severe CDH, because survival is primarily limited by pulmonary hypoplasia and pulmonary hypertension.

Recently, antenatal glucocorticoid therapy has been shown to improve not only biochemical maturation and lung compliance but also lung morphology in experimental CDH animals (17, 22, 27, 29). However, parameters of lung growth such as LW, LW/BW, total number of air spaces, and the gas-exchanging surface area were not increased by this therapy in either the rat or the sheep model of CDH. This is a striking difference between antenatal glucocorticoid therapy and TO. We believe that "increasing lung parenchyma" is an indispensable requirement for successful prenatal treatment of severely hypoplastic lungs. Despite significant risk for both the fetus and mother, we feel that TO remains the most promising approach for the prenatal treatment of severe pulmonary hypoplasia.

Thus our hypothesis that lung growth in human CDH is variable because of the early onset of CDH or the presence of herniated liver in the chest was not supported by our results. In humans, we have found that the growth response of fetal lungs to TO is gestation dependent; i.e., fetuses late in gestation (28–30 wk) do not respond to TO as consistently as those which undergo occlusion at 25–27 wk. The mechanisms to explain this phenomenon may include variable ability of hypoplastic lungs to produce lung fluid and variable susceptibility to betamimetic tocolytic agents, which are not used in animals but frequently used in humans and are known to reduce net lung fluid production in late-gestation fetuses. In addition, presumably high intrathoracic pressure due to the defect in the diaphragm may also compromise the results of TO. Further studies are necessary to develop strategies that consistently induce lung growth after TO.

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