Effects of Tracheal Occlusion (TO)

on

Sonic-Hedgehog (Shh) expression

in

animal models

of

Congenital Diaphragmatic Hernia (CDH)

Dr. Nasir Khan, MD
Department of Surgery
McGill University, Montreal,
Quebec, Canada

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science in "Experimental Surgery".

Submitted January 2006

Your file Votre référence
ISBN: 978-0-494-24707-5
Our file Notre référence
ISBN: 978-0-494-24707-5

TABLE OF CONTENTS

Table of contents continued...

METHODS AND MATERIALS

A: **Rabbit experiments**

B: **Rat experiments**

RESULTS

A: **Rabbit results**

B: **Rat results**

Table of contents continued...

ABSTRACT

BACKGROUND: The mechanism by which tracheal occlusion (TO) induces lung growth is unknown. The sonic hedgehog (Shh) is a signaling protein essential for bronchial branching and lung maturation in rats. Shh expression is delayed in nitrofen-induced congenital diaphragmatic hernia (CDH). The aim of this study was to see if TO could up-regulate Shh expression in CDH.

METHODS: To create CDH in fetal rats, time-dated pregnant rats were gavaged 100 mg of nitrofen on day 9.5 post-conception. TO was performed on day 19 of gestation (term=22 days). Animals were sacrificed on day 20 and 21. Lung growth was measured by lung to body weight ratio (LW/BW) and histology. The expression of Shh was measured by immunohistochemistry (IHC) and by quantitative real time polymerase chain reaction (qRT-PCR).

RESULTS: TO induced growth in nitrofen-affected lungs in comparison to controls. Higher levels of Shh mRNA expression were observed in the nitrofen-affected lungs on day 21. TO in the nitrofen-affected animals lowered the mRNA expression levels and brought them to control levels.

CONCLUSIONS: The nitrofen-induced hypoplasia of CDH is reversible with TO. Shh-mRNA expression in the nitrofen-affected lungs is delayed and peaks later during gestation. TO decreases Shh-mRNA expression levels and brings them to control levels.

ABRÉGÉ

INTRODUCTION: L'occlusion trachéale (OT) incite la croissance de pulmonaire dans un modèle animal d'hypoplasie pulmonaire provoqué par une hernie diaphragmatique congénitale (HDC). Le mécanisme de cette croissance demeure inconnu. L'expression de la protéine *sonic hedgehog* (Shh), qui est essentielle pour le développement des bifurcations bronchiques et la maturation pulmonaire, est retardée dans la HDC. Le but de cette étude était de voir si l'OT pouvait corriger l'expression de Shh dans les poumons affectés par la HDC dans un modèle animal de rat.

MÉTHODES: Pour créer l'hypoplasie pulmonaire et la HDC dans les foetus, les rates enceintes ont reçu 100 mg de nitrofen dans l'huile d'olive le jour 9.5 après l'imprégnation. L'OT a été exécutée le jour 19 de grossesse (terme=22 jours). Les animaux étaient sacrifiés au jour 20 et 21. La croissance pulmonaire a été mesurée en obtenant le rapport entre poids pulmonaire et poids corporel et par l'histologie. L'expression de Shh a été mesurée par immunohistochimie (IHC) et par le *real time quantitative polymerase chain reaction* (qRT-PCR).

RÉSULTATS: l'OT a provoqué la croissance dans les poumons qui ont été affectés par le nitrofen. Nous avons remarqué que les niveaux d'expression de Shh-mRNA étaient hauts dans les poumons «nitrofen seul » . L'OT, dans les animaux affectés par le nitrofen, a baissé les niveaux d'expression de Shh-mRNA au niveau des animaux de contrôle.

CONCLUSIONS: l'hypoplasie pulmonaire de la HDC causée par le nitrofen est réversible avec l'OT. L'expresssion de Shh-mRNA dans les poumons « nitrofen seul » est retardée et culmine plus tard pendant la gestation. L'OT diminue les niveaux d'expression de Shh-mRNA.

ACKNOWLEDGEMENTS

The author wishes to acknowledge and express his appreciation to the many people who contributed to the completion of this work. Without their help the writing of this thesis would have been impossible.

I would like to thank Dr. Jean-Martin Laberge, my supervisor, for his high quality support, expert guidance and suggestions.

Thanks are due also to Dr. Helene Flageole, my co-supervisor and an encouraging teacher and surgeon.

Thank you, Dr. Pramod Puligandla, for your suggestions about writing and formatting this thesis. I always found you supporting and encouraging.

Thanks to Dr. Robert Baird, a colleague that kept me always at ease. Working with him was fun rather than a task.

I would like to thank Dr. Kaplan and Miss Katia Nadeau for their support in PCR analysis.

Thanks to Dr. Xinying He and Brian Meehan for their help on IHC and histology.

Thank are also due to Georgia Kalavritinos, the animal technician of MCH, for her valuable help in animal surgery and postoperative care.

I would like to thank Mrs. Vassiliki Kessaris, secretary to Dr. Laberge, for her support during the writing of this work.

ABREVIATIONS

BMP-4: Bone Morphogenic Protein 4.

CDH: Congenital Diaphragmatic Hernia.

DGA: Days of Gestational Age.

Dhh: Desert hedgehog.

DNA: DeoxyriboNucleic Acid.

ECMO: ExtraCorporeal Membrane Oxygenation.

E: Embryonic (days of gestation in mice and rats).

EDTA: EthylDiamine Tetetric Acid.

ELISA: Enzymed-Linked ImmunoSorbent Assay.

FGF: Fibroblast Growth Factor.

FGFR2: Fibroblast Growth Factor Receptor 2.

H&E: Hematoxylin and Eosin.

HFOV: High Frequency Oscillatory Ventilation.

IHC: ImmunoHistoChemistry.

Ihh: Indian hedgehog.

lgl1: late gestational lung protein1.

LW/BW: Lung Weight to Body Weight ratio.

mRNA: messenger Ribonucleic Acid.

MTBD: Mean Terminal Bronchial Density.

MyoD: Myogenic Determination factor genes for skeletal muscle formation.

Myo D- : Mice with MyoD knockout genes

P: Postnatal (days of rat life).

Ptch (1& 2): Patched (receptors 1 &2).

PHMP: Post Hepatic Mesenchymal Plate.

PPC: PleuroPeritoneal Canals.

qRT-PCR: quantitative Real Time Polymerase Chain Reaction.

RAC: Radial Alveolar Count.

Shh: Sonic hedgehog.

TGF: Transforming Growth Factor.

TO: Tracheal Occlusion.

TTF-1: Thyroid Transcription Factor 1.

VEGF: Vascular Endothelial Growth Factor

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a congenital anomaly that affects 1 in 3000 to 4000 live human births (Timothy et al, 2002). Despite the present day advances in neonatology, the mortality and morbidity associated with CDH remains high (Stege et al, 2003). This high mortality rate is mainly attributed to the consequences of pulmonary hypoplasia (Iritani et al, 1984) and persistence of pulmonary hypertension (Lagausie et al, 2005). The etiology of CDH-associated lung hypoplasia is complicated and not completely understood.

Although prenatal ultrasound has helped in early detection of the disease (Bahlmann et al, 1999), prenatal attempts at closure of the diaphragmatic defect does not solve the problem (Smith et al, 2002). Direct closure of the diaphragm has its own consequences associated with it such as a high operative mortality, surgical difficulty and high rate of preterm delivery and neonatal loss (Harrison et al, 1997). Prenatal tracheal occlusion (TO) has been tried in some centers with variable success rates possibly due to different selection criteria across centers (Smith et al, 2005). Although a prospective randomized trial did not show an advantage to fetal TO (Harrison et al, 2003), TO is still employed as a prenatal treatment option in Europe, for poor prognostic CDH (Smith et al, 2005). The exact mechanism by which TO induces growth in CDH-affected lungs is not known.

An array of secreted proteins such as fibroblast growth factor 10 or FGF-10 (Cardoso et al, 2001), Sonic hedgehog or Shh (Pepicelli et al, 1998), Bone morphogenic protein 4 or BMP-4 (Desai et al, 2002) and late gestational lung protein or lgl1 (Oyewumi et al, 2003) have been implicated in normal lung growth. Similarly, transcription factors such as GLi proteins (Pepicelli et al, 1998) and Foxa1& 2 (Besnard et al, 2004) play a role in the development of the lung. Although the exact role of these growth factors in the CDH-affected lungs is unknown, both FGF-10 and Shh have been found down-regulated in CDH (discussed below). It would be interesting to know if TO reverses the down-regulation of these proteins and normalizes them.

In our project, we attempted to study the effects of nitrofen-induced CDH on the expression of sonic hedgehog protein (Shh) in rat lung. Previously it has been shown that in the nitrofen-CDH animal model, Shh expression is delayed and peaks at a later

time compared to normal controls (Unger et al, 2003). Our goal was to go one step further and see if tracheal occlusion would reverse CDH-associated down regulation of Shh in this model. The importance of these studies cannot be overemphasized keeping in view the high mortality rate of CDH and the limited treatment options available for this deadly congenital anomaly (Sydorak et al, 2003).

The usefulness of these observations could be twofold. First, it would help us understand the mechanism of TO-induced lung growth. Second, though preliminary and not the focus of our present study, it could help us to develop growth factor-based therapeutic models in the future.

REVIEW OF LITERATURE

EMBRYOLOGY OF THE LUNG GROWTH

Lung growth in humans

Primordial lung buds originate as out-pouching of the primitive foregut endoderm (Jeffery et al, 1998). Airway branching is a prenatal event and almost all airways are present by mid-gestation. Formation of the alveoli, on the other hand, spans pre and postnatal life (Thurlbeck et al, 1992). One-third to one-half of the adult number of alveoli develops during the last trimester of pregnancy (Langston et al, 1984). Five stages of lung growth have been described in the literature, the embryonic, the pseudo-glandular, the canalicular, the saccular and the alveolar stage (McDonald et al, 1997). See Table 1.

During the embryonic stage (3-7 weeks) epithelial cells from the foregut endoderm invade the surrounding mesoderm to form the proximal respiratory tract consisting of trachea and the main bronchi. Further growth at this stage culminates in the formation of 18 major lobules (Bucher et al, 1964). Studies on lung morphogenesis suggest that the surrounding mesoderm regulates the branching of the tracheobronchial tree (Demayo et al, 2002).

The pseudo-glandular stage (7-16 weeks) is characterized by the formation of conducting airways, terminal bronchioles, and primitive acini. During this stage, the pseudo-stratified columnar epithelium is progressively replaced by tall columnar cells in the proximal airways and cuboidal cells in the distal acinar structures (Kitaoka et al, 1996).

During the canalicular stage (16-26 weeks), development of the distal airways results in the formation of respiratory bronchioles, alveolar ducts, and rudimentary alveoli (Sparrow et al, 2003).

In the saccular stage (26-36 weeks) of lung development, acinar tubules dilate while their surrounding walls become gradually thinner. As a result of this, the surface area for diffusion of gases (O_2 and CO_2) across the epithelial-endothelial barrier increases (Burri et al, 1984). Maturation of type-I cells into surfactant secreting type-II cells

occur during this stage of lung growth (Mercurio et al, 1976).

The alveolar stage does not start until very late in the gestation (36 weeks) and continues in postnatal life for up to 2 years (Dietert et al, 2000). This stage is characterized by the formation of secondary alveolar septae consisting of connective tissue projections with a double loop of capillaries (Cerna et al, 2002). Characteristics of this stage include further thinning of the alveolar walls, remodeling of the double capillary loops into a single capillary loop and proliferation of pneumocytes (Prodhan et al, 2002). This maturation is an essential part of the development and renders the lung compatible with extra-uterine environment (Zeltner et al, 1987).

Lung development does not stop in the early years of life and continues until the age of puberty or even adulthood (Tschanz et al, 1997). The estimated number of alveoli present in an adult lung is about 300 million (Kotecha et al, 2000). It has been reported that approximately one-third to one-half of the total number of alveoli are present at birth (De Jong et al, 2003).

Development of the Diaphragm in humans

Normal development of the diaphragm is essential for the growth and maturation of the lung (Inanlou et al, 2003). The diaphragm develops in the embryo during the 3rd week of gestation from four structures that are duly represented in the definitive (developed) diaphragm. These four structures are the septum transversum, the pleuroperitoneal membranes, the dorsal mesentery of the esophagus and the cervical myotomes (Rottier et al, 2005).

The septum transversum is that part of the embryonic mesoderm which separates the ventrally located pericardial cavity from the dorsally located gut. It eventually forms the central tendon of the diaphragm, an aponeurotic structure that fuses with the pericardium (Larsen: *Human Embryology*, 2001). A defect in this part of the diaphragm leads to a rare but important type of hernia called septum transversum diaphragmatic hernia (Paci et al, 2005).

During early embryonic life, the peritoneal cavity can hardly be demarcated from the thoracic cavity, both communicating through pleuroperitoneal canals (PPC) (Thebaud et al, 1998). The PPC usually close around 6-8 weeks of gestation by growth of the

pleuroperitoneal membranes (Kluth et al, 1996). In the fetal life, the pleuroperitoneal membranes represent a large portion of the diaphragm; however, they represent a small part of the definitive diaphragm. Failure of closure of the PPC by the pleuroperitoneal membranes is thought to be the major cause of the diaphragmatic defect in the posterolateral or Bochdalek type of CDH (Skandalakis: Embryology for surgeons, 1994), which is the topic of this thesis.

The dorsal mesentery of the esophagus, a double layer of peritoneum, forms the median portion of the diaphragm (Moore et al, 1988). Two strips of muscles called the right and left crura arise from the lumbar vertebrae to grow into the dorsal mesentery around the ninth to twelfth week of intrauterine life. Cervical myotomes contribute to the most peripheral part of the diaphragm. During the fifth week of development, the muscle cells from these somites migrate into the developing diaphragm, taking their nerves (phrenic nerve, C3, 4,5) with them from the cervical region (Larsen: Human Embryology, 2001).

Lung growth in animals (see table 1)

While all mammalian species undergo the same stages of lung development, the duration and timing of these stages vary in relation to their gestational age (Wu et al, 2000).

Pulmonary development of the rabbit mimics that of the human lung (Kikkawa et al, 1968). The embryonic stage lasts from the 5^{th} to the 19^{th} day of gestational age (DGA), followed by the pseudoglandular stage (19-24 DGA), the canalicular stage (24-26 DGA), the saccular stage (26-29 DGA) and the alveolar stage, which lasts from 29 DGA to term (Pringle et al, 1986). The gestational period (term) in does is 31 days. Unlike rats and mice, alveolization of the rabbit lung begins before birth (Wu et al, 2002) and is completed before birth (De Paepe et al, 1998).

Most of the current theories and knowledge about lung development is based on experiments conducted in rodents (Zoetis et al, 2003). The very short gestations of rats and mice, transgenic techniques and the availability of lung biomarkers (discussed below) have rendered the rat the most widely used model for studies on lung development. The gestational period in mice and rats is about 22 days. Lung growth

starts as an out-pouching from the ventral foregut around 9.5 days post-conception (Kaufman et al, 1992). The lung bud then proceeds though stages similar to human and rabbit lung morphogenesis.

The duration of these stages, according to the rat gestation expressed in days of embryonic (E) and postnatal (P) life can be summarized as: the embryonic stage (E9.5 to E12.5), the pseudoglandular stage (E12.5 to E16.5), the canalicular stage (E16.5 to E17.5), the saccular stage (E17.5 to P4) and the alveolar stage (P4-P14). The initial branching process that started at E9.5 reaches its peak in the pseudoglandular stage and continues until the end of the canalicular stage (Roth-Kleiner et al, 2005). At birth, rats are in the saccular stage of lung development. In rats, the alveolar phase of the lung growth, in contrast to humans and rabbits, occurs after birth and the lungs are not mature until postnatal day 14 (Morishige et al, 1982).

Growth factors in normal lung development

Lung morphogenesis has been widely studied in animal models such as mice and rats. A number of growth modulating proteins and genes regulating their expression have been identified to play a role in lung morphogenesis (Levy et al 2005). Some of the most common and widely studied factors are the FGF-7 (Ulich et al, 1997), FGF-10 (Sekine et al, 1999), BMP-4 (Bellusci et al, 1996), Shh (Litingtung et al, 1998), retinoic acid (Cardoso et al, 1995), vascular endothelial growth factor or VEGF (Lassus et al, 2001) and lgl1 (Oyewumi et al, 2003). Studies on knockout mice conclude that deficiencies in the expression of these factors may lead to defects in the branching process and maturation of the lung (Cardoso, 2001). There is increasing evidence suggesting that formation of the tracheo-bronchial tree and alveoli results from heterogeneity of the epithelial-mesenchymal interaction along the developing respiratory tract (Cardoso, 2001). Recent genetic data support this idea and show that this heterogeneity is likely the result of activation of distinct networks of signaling molecules along the proximal-distal axis (Perl et al 1999).

FGF-10 is expressed in the mesenchyme and interacts with its receptor FGFR2, which is expressed throughout respiratory epithelium (Arman et al, 1999). Expression of FGF-10 is dynamic. It is expressed in higher concentrations preceding distal bud

formation. Its expression then decreases at the tip of the formed bud while new foci of higher expression appear adjacent to newly forming buds (Bellusci et al, 1997). FGF-10 acts as a paracrine hormone and its activity range is limited to the budding epithelium in developing lung. FGF-10, together with BMP-4 (which acts opposite to FGF-10) controls the proliferation and formation of lung branching (Hyatt et al, 2004). Fibroblast growth factors other than FGF-10, such as FGF-1 and FGF-7 appear to be comparatively less important in lung branching (Lebeche et al, 1999). There is however some evidence that FGF-7 plays a role in surfactant secreting type II cell maturation (Portnoy et al, 2004).

Sonic hedgehog (Shh), an important signaling glycoprotein, belongs to the family of the "Hedgehog proteins" including Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Indian hedgehog is implicated in bone growth and limb development (Chung et al, 2001), while Desert hedgehog plays a role in the development of germ cell lines and central nervous system (Wijgerde et al, 2005). Ihh and Dhh are named after the two different genes found in the drosophila (fruit flies), while Shh takes its name from a video game "sonic the hedgehog" (Ingham et al, 2001). Sonic hedgehog is found in various structures and organs since early embryonic life such as neural tube, primitive gut, kidneys, limbs and lungs where it regulates pattern formation (Gilbert: developmental biology, 7th edition, 2003).

Shh is expressed in the epithelium of the embryonic lung and acts through its receptors, called Patched (Ptch) receptors (Ptch1 and Ptch 2), situated in the mesenchyme (Walterhouse, 2003). Thus the expression and ligand-receptor interaction site for Shh is opposite to that of FGF-10, which is expressed in the mesenchyme while its receptors are situated in the epithelium. Shh, upon binding with its receptors (Ptch1&2), initiates activation of the Gli protein complex, especially gli-3 which then enters the cell nucleus and acts as a transcriptional activator for the target genes (Altaba et al, 2002). The correct regulation of the Hedgehog-Gli signaling pathway is essential for normal development of the lung (Stecca et al, 2002). Although Shh is present very early in embryonic lung, there is no evidence to show that Shh is required for the initiation of primary budding. Once primary buds are initiated by FGF-10, Shh becomes essential for subsequent lung branching (Pepicelli et al, 1998).

Studies on knockout animal models have strengthened the idea that Shh regulates the secretion of FGF-10 by the lung mesenchyme. If FGF-10 signals are diffuse rather than localized, directional clues are lost and branching is disrupted (Cardoso et al, 2001). Impaired signaling in the Shh pathway has been associated with a number of diseases in humans including lung diseases (Mullor et al, 2002) and currently there is a rising interest to know if modulators of this pathway have any therapeutic potential.

Bone morphogenic protein 4(BMP-4) plays important roles in regulating developmental processes of many organs, including the lung (Chen et al, 2005). A member of the transforming growth factor beta family (TGF-beta), BMP-4 is an important regulator of epithelial proliferation during lung morphogenesis (Cardoso, 2001). It is expressed at the distal lung epithelium and like Shh plays an opposite role to that of FGF-10 (Weaver et al, 2000). In vitro studies with lung explants have demonstrated that BMP-4 inhibits FGF-10 expression at the epithelial mesenchymal interface and therefore stops FGF-10 induced chemotaxis (Jeffery et al, 2005). This process seems to be important for lung morphogenesis. Although similar in their action, BMP-4 and Shh are expressed through pathways that are completely independent of each other (Bellusci et al, 1997).

Thus both BMP-4 and Shh stop the growing lung bud initiated by FGF-10 once the bud is sufficiently large. FGF-10 then appears in another lateral mesenchymal focus and initiates new budding. At the same time FGF-10 stimulates the production of BMP-4 at the distal epithelium, which along with Shh, inhibits the elongation of the lung bud. Similarly Retinoic acid, Sprouty and other transforming growth factors have been implicated in lung development but their roles are not as clear as the substances discussed above. Vascular endothelial growth factor (VEGF) and Platelet derived growth factor (PDGF) are associated with lung vascularization and maturation (Lassus et al, 2001). Recently lgl1, a product of late gestational gene 1(LGL-1) has been found associated with lung growth (Oyewumi et al, 2003). This list of growth factors and related substances is neither exhaustive nor complete but we chose to describe the most important ones studied at this time.

CONGENITAL DIAPHRAGMATIC HERNIA (CDH)

Embryology of CDH (in humans)

The two basic types of CDH frequently described in the literature are Bochdalek hernia and Morgagni hernia. The most common of them is the Bochdalek hernia. This posterolateral diaphragmatic defect, first described by Victor Alexander Bochdalek in 1848, develops due to patent pleuroperitoneal canals (PPC) beyond 10 weeks of human embryonic life (Timothy et al, 2002). PPC, a potential communication between the thoracic and abdominal cavities, usually close around 8 to 10 weeks of gestation (Kluth et al, 1996). Abnormal closure of the septum transversum causes defects in the anterior and central portion of the diaphragm. This may cause a wide retrosternal defect called Morgagni hernia (Robnett et al, 2003) or an isolated central defect called septum transversum diaphragmatic hernia (Goldstein et al, 2003). These are rarely symptomatic at birth. The abbreviation "CDH" used in this thesis should be considered as posterolateral (Bochdalek type) CDH.

Although chromosomal defects and other congenital anomalies such as Fryns syndrome, Simpson-Golabi-Behmel syndrome, tetrasomy 12p, Brachmann-de Lange syndrome and lethal multiple pterygium syndrome have been found associated with diaphragmatic defects, most cases of CDH occur as an isolated anomaly (Enns et al, 1998).

Pathophysiology of CDH

In CDH, the diaphragmatic defect provides a free channel for the abdominal contents to herniate through the defect into the thoracic cavity. Herniation of abdominal viscera such as intestinal loops and liver in the thorax, early during embryonic life, results in pulmonary hypoplasia presumably due to compression. Although the ipsilateral lung is more affected than the contralateral lung, both lungs are smaller and less mature when compared to their normal counterparts (Harrison et al, 1986).

Recently, some researchers have disputed the mechanism of hypoplasia secondary to mechanical factors. Their arguments are based on experiments conducted in rodents where CDH is produced by feeding nitrofen to these animals (described below).

According to these investigators:

1. Pleuroperitoneal canals (PPC) are too small to accommodate the intestinal loops early during development (Kluth et al, 1990).
2. PPCs are open as late as 8-10 weeks of gestation during normal embryogenesis. If abnormal herniation through a patent PPC caused the arrest in lung growth, this should not happen until after the 10^{th} week of intrauterine life (Kluth et al, 1996).
3. Abnormal closure of the post-hepatic mesenchymal plate (PHMP) rather than pleuroperitoneal canals may be responsible for the diaphragmatic defect in CDH. As the PHMP closes around 5^{th} week of gestation, failure of this process could explain the early hypoplasia of CDH (Iritani et al, 1984).
4. Hypoplasia of the lung has been proposed as the primary event, which leads to diaphragmatic defect (Iritani et al, 1984 and Allan et al, 1997).

Despite these new insights and developing concepts in the pathophysiology of CDH, mechanical factors are important in the causation of hypoplasia. Adequate intra-thoracic space, sufficient amount of amniotic fluid, normal volume of fluid in the potential respiratory airways and normal fetal movements have all been found to have some importance in normal lung growth (Joe et al, 1997). Disturbance in these physical factors due to any etiology leads to pulmonary hypoplasia (Thebaud et al, 1998). For example, impaired fetal respiratory movement caused by cervical cord section causes lung hypoplasia in the rabbit (Wigglesworth et al, 1979). As CDH may also affect diaphragmatic excursion, this supports the argument in the favor of mechanical (secondary) theory of hypoplasia. Further evidence has come from MyoD knockout mice in which the diaphragm is markedly thinned and nonfunctional, resulting in pulmonary hypoplasia due to lack of fetal breathing movements (Inanlou et al, 2003). At least two mechanical factors that influence lung growth are disturbed: abnormal herniation of the abdominal viscera into the thorax limiting the space available for lung growth and the diaphragmatic defect, which impairs fetal breathing movements. Lungs are also hypoplastic in the extensively studied lamb model of CDH (De Lorimer et al, 1967 and Lipsett et al, 2000). Similar results have been obtained in the rabbit model of CDH (Fauza et al, 1994 and Wu et al, 2000). In both of these animal models,

where the diaphragmatic defect is produced surgically in mid-to-late gestation, the presence of lungs hypoplasia at term suggests the importance of mechanical factors in lung morphogenesis. It may be possible that a dual-hit mechanism exists where a primary defect in lung morphogenesis and a secondary effect due compression, caused by herniation, contribute to the degree of hypoplasia associated with CDH (Keijzer et al, 2000).

Pathology of CDH-affected lungs

Lung growth can be assessed using different methods of assessment such as lung weight to body weight ratio (LW/BW), radial alveolar count (RAC), mean terminal bronchial density (MTBD) and histology. Hypoplasia is said to be present when lung weight to body weight ratio (LW/BW) is less than 0.012(67 % of the normal) and radial alveolar count is less than 4.1(75 % of the normal) (Askanazi et al, 1979). During normal lung growth the number of alveoli increases with time while terminal bronchiole supplying them gradually decreases. Lung maturity can be assessed by counting the number of terminal bronchioles in a given histological slide. Thus MTBD is another indicator of lung hypoplasia (Bratu et al, 2001).

Irrespective of the underlying process, the lungs in CDH are smaller with a reduced lung weight to body weight ratio (Thibeault et al, 1998). Total alveolar surface area of human CDH-affected lungs is decreased (George et al, 1987). Histology of the lungs of the newborns with CDH shows that developmentally these lungs are at an earlier stage than their normal counterparts when compared for the date and time of gestation (George et al, 1987). Compared to normal lungs, CDH-affected lungs have fewer bronchi, less differentiated epithelium and thicker alveolar interstitial septae (Bratu, 2001). Collagen content of CDH lungs is increased, making them less distensible owing to increased intrinsic lung stiffness (Hassett et al, 1995). Lung growth in CDH is arrested at the pseudoglandular stage characterized by cuboidal epithelium lining the respiratory airways instead of squamous epithelium of the normal lung (Groenman et al, 2005). These changes are more marked on the side of the lung ipsilateral to the hernia but nevertheless are present to some extent in the contralateral lung as well (Brandsma et al, 1994).

The net effect is a significantly decreased surface area available for gas exchange.

There is evidence that a minimal lung volume of 45% of the predicted normal for the age and weight of the newborn is needed for survival of human babies (Thibeault, 1998). CDH-affected hypoplastic lungs have increased stiffness due to excessive collagen, increased distance between alveolus-capillary interface and thick cuboidal epithelium. All of these factors negatively affect the diffusion of gases across the epithelial-endothelial barrier of the CDH-affected lung.

Pulmonary hypertension is the second main contributor towards the mortality and morbidity associated with CDH (Skari et al, 2000). Just as for hypoplasia, the exact mechanism of pulmonary hypertension is unknown. Two factors considered to be important in the CDH induced pulmonary hypertension are the structural changes in pulmonary vasculature and hyperreactivity of the pulmonary vascular system (Thibeault et al, 1998). Structural changes in the pulmonary vasculature of the CDH include: increased muscularization of pulmonary arterioles extending beyond the pre-acinar level, increased medial wall thickness of the intra-alveolar vessels, reduction in pulmonary arterial cross-sectional area, reduced total pulmonary vascular bed and decreased number of vessels per volume unit of lung (Geggel et al, 1985 and Shehata et al, 1999).The pulmonary vascular bed of CDH seems to be more reactive than that of normal lungs (Stolar et al, 1985). A number of vasoactive substances are implicated in this altered state. The levels of nitric oxide, a potent and selective vasodilator of the pulmonary vessels, are decreased due to altered nitric oxide synthetase activity (Kinsella et al, 1997). Similarly the levels of the vasoactive peptide Endothelin-1 and its receptors are elevated in CDH leading to an exaggerated vasoconstrictor response (Kobayashi et al, 1994). Endothelin-1 is believed to be responsible for the structural changes as well as hyperreactivity of the pulmonary vessels (Okazaki et al, 1998). VEGF-A, an essential mediator of normal pulmonary vascular development, has altered expression in lungs affected with CDH (Kitterman et al, 2005).

The surfactant secreting ability of the type II cells is decreased in CDH. Similar to hypoplasia and pulmonary hypertension, the mechanism leading to immature surfactant system is unknown (Asabe et al, 2003). It has been suggested that decrease lung distension due to CDH is partly responsible for retarded type1 cell maturation in to type II cells (Chapin et al, 2005). Inadequate amount of surfactant at term and

increased lung stiffness of the hypoplastic lung decreases lung compliance. This leads to atelectasis in newborns with CDH. Atelectasis increases the work of breathing causing hypoxia and hypoxemia, further increasing pulmonary vascular resistance (Haugen et al, 1991).

CDH-affected heart

Apart from changes in the lung, CDH is associated with cardiac abnormalities, such as left ventricular (LV) hypoplasia (Siebert et al, 1984). The degree of LV-hypoplasia, evaluated by prenatal echocardiography, has been used in outcome predictions for fetuses affected with CDH (Crawford et al, 1989). Similar to lung hypoplasia, anatomical distortion by mechanical factors are thought to induce ventricular hypoplasia. In the context of the nitrofen model of CDH, researchers now propose non-mechanical, primary defects as possible etiology (Correia-Pinto et al, 2003). The presence of LV hypoplasia further increases CDH-associated mortality in humans (Sweed et al, 1993).

Clinical manifestations of CDH

CDH commonly occurs on left side, comprising 80 to 90% of cases (Greer et al, 2000). Right-sided CDH accounts for 8-20% of all cases of CDH (Harrison et al, 1994) and has a worse prognosis (Sakari et al, 2000). It is always associated with herniation of liver into thoracic cavity (Hedrick et al, 2004). Bilateral cases are rare and usually lethal. The clinical manifestations of lung hypoplasia and pulmonary hypertension include hypoxemia, hypercarbia and difficulty in breathing soon after birth (Ontario Congenital Anomalies Study Group, 2004). Because of the high resistance in the pulmonary vascular bed pulmonary blood flow is shunted away from the lungs, passing through the foramen ovale and ductus arteriosus, from the right side to the left side of the circulation (R-->L shunt), further reducing oxygen tension in the systemic blood (Muratore, 2000). Hypoxia is a strong vasoconstrictor stimulus for pulmonary vasculature, thereby increasing the pulmonary vascular resistance and worsening the pulmonary hypertension (Demiryurek et al, 1993). Thus a vicious cycle is established causing severe hypoxemia, hypercarbia and respiratory acidosis, culminating in acute

respiratory and cardiac failure. Death ensues soon after birth if the situation is not treated immediately and vigorously.

Despite all advances in the postnatal care, such as extracorporeal membrane oxygenation (ECMO), high frequency oscillatory ventilation (HFOV), approximation of delivery rooms to postnatal care facilities, better staff training and use of prenatal ultrasound for early detection, the mortality rate has not significantly changed over the years (Ontario Congenital anomalies group, 2004). Poor prognostic indicators associated with high mortality include herniation of liver into the chest, lung to head ratio less than 1 and associated chromosomal or congenital anomalies (Gosche et al, 2005). Babies, who may survive with aggressive postnatal therapy using ECMO, face a number of long-term complications (Stefanutti et al, 2004). Some of these complications include gastro-esophageal reflux and failure to thrive (Muratore et al, 2001)' right ventricular hypertrophy (Jillard et al, 2003)' sensori-neural hearing loss (Van Meurs et al, 1993) and chronic pulmonary diseases (Cortes et al, 2005).

Lung growth factors in CDH

Once it was determined that lung morphogenic proteins and transcription factors were important for normal lung morphogenesis, there was a growing interests to know if these substances have any role in CDH-induced hypoplasia and pulmonary hypertension. Two important modulators of the lung bronchogenesis, Shh and FGF-10, have been found down-regulated in CDH (discussed elsewhere).

Although Shh was implicated in normal lung morphogenesis several years ago (Pepicelli et al, 1998), it was not until recently that it was studied in the context of CDH and a direct relationship between the CDH and Shh was established. Shh expression is delayed in the nitrofen-induced murine model of CDH (Unger et al, 2003). The peak mRNA expression of Shh that normally occurs in the late pseudoglandular/early canalicular stage, was found delayed with a maximal Shh expression at term (22 days) instead of day 18 in control lungs. In the same study the authors compared rat and human fetuses with CDH and again observed a similar pattern between the two species. In CDH-lung samples from human fetuses, Shh was delayed with a maximal expression from 21 to 32 weeks of gestation, corresponding to the late canalicular/early

saccular period. The expression of Shh subsequently diminished quite rapidly in human pulmonary hypoplasia with advancing gestation (Unger et al, 2003).

A similar pattern of expression has been reported for FGF-10 in the context of CDH. Previously, Acosta et al, have shown than nitrofen-induced hypoplasia in mice is associated with decreased and temporospatially abnormal expression of FGF-10. In a parallel experiment, the investigators added FGF-10 to the *in vitro* culture of nitrofen-affected lungs. The lungs from this culture were less hypoplastic compared to controls lungs (nitrofen–affected but FGF-10 not added) (Acosta et al, 2001). This observation was repeated by Teramoto et al who reported that FGF-10 genes are down-regulated in nitrofen-induced CDH in rats (Teramoto et al, 2003).

Similarly, mediators of vasculogenesis and lung maturation such as VEGF, endothelin-1 and thyroid transcription factor (TTF-1) show altered expression in CDH. VEGF is a potent modulator of pulmonary vasculogenesis and plays an important role in the maturation of type II cells. It is expressed throughout normal lung development and peaks at the onset of canalicular stage (E16) in mice and rats (Hara et al, 2005). VEGF hyperactivity is detected in postmortem lung specimens from babies affected with CDH (Tibboel et al, 2004). In the nitrofen-induced murine model of CDH, down regulation of VEGF has been reported by some (Chang et al, 2004). In a fetal respiratory distress model in premature mice, VEGF administered during gestation and postnatally to premature pups, VEGF protected against respiratory failure and increased survival (Compernolle et al, 2002). Endothelin-1, a vasoactive peptide and a proposed modulator of pulmonary hypertension, is over-expressed in CDH (see above). TTF-1 which functions as a regulator of proximal-distal patterning in early embryonic development, as a transcriptional regulator of the Clara cell secretary protein and type I cell differentiation in late gestation, is down regulated in nitrofen-induced CDH (Losada et al, 2000). However, others have shown a late phase over-expression of TTF-1 in the distal epithelium associated with surfactant deficiency in CDH (Chapin et al, 2005). The increasing search for these proteins fulfills two objectives: (i) to understand the pathological process of CDH-associated changes in the lung and (ii) to find out if these molecules could permit antenatal lung rescue by in-utero growth factor therapy (Jesudason, 2002).

ANIMAL MODELS OF CDH

Most of the current knowledge bank regarding CDH-associated changes in the lung is based on the data gathered from animal models of CDH. Broadly these animals could be divided into two main groups depending on the method of CDH creation, the surgically created CDH models and the teratogenic CDH models. The surgical CDH model comprises lambs and rabbits, while the teratogen nitrofen is used to create CDH in mice and rats.

Surgical CDH models

The lamb is perhaps the model most widely studied in the context of CDH in general and tracheal occlusion (discussed below) in particular. The large size of the fetal lamb makes it suitable for intrauterine CDH creation, tracheal occlusion and postpartum ventilation/resuscitation trials (Bratu et al, 2004). In lambs the CDH is created at 65 to 80 days of gestation (term 145 days) by left lateral thoracotomy, to mimic left sided CDH. Use of sheep as an experimental model has been abandoned in many centers due to Q-fever risks (Bernard et al, 1982) and perhaps due to the high cost and logistics reasons.

The rabbit has provided an alternative for the lamb model. The history of experimental CDH in rabbits dates back to 1976 (Ohi et al, 1976). Fauza et al experimented two approaches for creating CDH in rabbit. With the abdominal approach a diaphragmatic defect was created after performing a small laparotomy. This approach was associated with a 100% loss. In the lateral thoracotomy approach, the CDH was created by a small nick in the lateral part of the left diaphragm. This approach led to 70% fetal loss (Fauza et al, 1994). This latter approach is now employed by investigators to create CDH in rabbits. CDH in fetal rabbits is created by an intrauterine surgical procedure at 23 days of gestation (term 33 days), in the canalicular phase of lung development (see above + table1). The possible advantages of rabbit over lamb as a model of CDH include lower costs, no need of special veterinary facilities, smaller body size, year-round availability, higher number of fetuses per pregnancy, and shorter gestational period (Fauza et al, 1994). The disadvantage includes a clearly high fetal loss rate.

Teratogenic CDH models

Chemical compounds that create pharmacological CDH in mice and rats include: 4-biphenyl carboxylic acid (BPCA), bisdiamine, benzofuranyl ureas (SB-210661) and 2, 4-dichloro-phenyl-*p*-nitrophenyl ether (nitrofen). The thromboxane A2-receptor blocker, BPCA produces defects in the diaphragm that are dose dependent. An oral dose of 150 mg/kg twice daily during days 7-16 of embryonic life induces a diaphragmatic hernia in up to 42% of pups (Sutherland et al, 1989).

Bisdiamine or Bis (dichloroacetyl) diamine is a spermatogenesis inhibiter, used as male contraceptive in domestic animals (Munson et al, 2004). The drug is capable of reliably producing congenital diaphragmatic hernia (100%) by a single treatment at gestation day 11(Taleporos et al, 1978). It is an effective oral abortifacient in rats and leads to other structural anomalies such as those of snout and heart making it less desirable for CDH creation.

The benzofuranyl ureas (SB-210661), a 5-lipoxygenase inhibitor, produce CDH and cardiac and thymus anomalies at an oral dose of 50 and 100 mg/kg/day from day 7-16 of gestation (Solomon et al, 2000).

The 2, 4-dichloro-phenyl-*p*-nitrophenyl ether (nitrofen) is the most commonly used agent for inducing CDH in rats and mice. Nitrofen, a potent herbicidal, was withdrawn from the market because of its carcinogenic effects in humans (National Toxicology Program, 1978). In rats and mice the drug is found to be teratogenic following a single oral dose (Francis, 1986). Given orally nitrofen is partially absorbed from the gastrointestinal tract. The lethal dose in adult rodents is about 2400 to 2600 mg/ kg body weight. Neonatal death due to induced malformations in the absence of maternal toxicity may occur at a much smaller dose. Pups exposed to nitrofen in-utero, show signs of respiratory distress associated with morphological alterations of the lungs, cardiac malformations and diaphragmatic hernias (Hurt et al, 1983).

Nitrofen, in an oral dose of 100 mg on day 9.5 of gestation, causes a diaphragmatic defect in 50-80 % of the pups (Guilbert et al, 2000). The diaphragmatic defect is frequently on the left side and more frequently unilateral than bilateral (Cilley et al, 1997). Dose and timing of administration of the drug is important for creation of the defect (Cilley et al, 1997). The lungs of pups from the nitrofen-fed dams are

hypoplastic with or without diaphragmatic hernia (Iritani et al, 1984).

The mechanism by which nitrofen induces CDH in animals is not known. Interference with thyroid hormone metabolism, due to competition between the hormone and nitrofen for thyroglobulin (Manson al, 1986) or thyroid hormone receptor (Brandsma et al, 1994), has been suggested by some as the possible mechanism of CDH induction. Vitamin A, administered during pregnancy decreases the incidence and severity of nitrofen- induced CDH in rats, suggesting that nitrofen could be causing CDH by blocking retinoic acid receptors (Thebaud et al, 1999 and Baptista et al, 2005). The pulmonary vascular effects (increased thickness of the media and adventitia) in the nitrofen- induced CDH are more pronounced than in the surgical model of CDH indicating a direct role of nitrofen on the vascular system (Taira et al, 1998).

Although no animal model studied so far can perfectly mimic human CDH, each one has its own merits and demerits. The teratogenic murine-CDH model has certain advantages over the surgical model despite the difficulties that arise when surgical intervention such as TO is considered. Firstly, lung morphogenesis has been extensively studied in rodents with the help of knockout mice. The previously available data provide a quick and reliable reference for comparisons to be drawn between CDH and normal lungs. Secondly, antibodies are readily available against mice and rat lung growth factors and could be used to detect these growth factors by techniques such as immunohistochemistry, Western blot, ELISA and other biomedical methods. Thirdly, nitrofen-induced hypoplasia of the lung closely resembles clinical CDH in humans (O'Toole et al, 1996). This point has been emphasized by investigators who believe that the hypoplasia of CDH is a primary event due to alterations in lung morphogenesis before the closure of diaphragm (Leinwand et al, 2002). Lastly, the short gestation period of rodents (22 days) render them particularly suitable for laboratory use.

TRACHEAL OCCLUSION (TO)

Tracheal occlusion induces growth in normal lungs as well as CDH-affected hypoplastic lungs (Keller et al, 2004). The idea of tracheal occlusion as a prenatal therapeutic intervention came from the clinical observation that babies suffering from laryngeal atresia have larger lungs, possibly due to fluid retention in their respiratory airways (Silver at al, 1988). Subsequent studies in animal models of CDH substantiated this idea and suggested that intrauterine tracheal occlusion may reverse the hypoplastic lung changes associated with CDH (Wilson et al, 1993).

Clinical trials in babies have reported variable outcome across centers, possibly due to difference in the selection criteria (Smith et al, 2005). Experiments in the lamb model of CDH have shown that tracheal occlusion accelerates lung growth, which gradually reduces abdominal viscera into the abdominal cavity (DiFiore et al, 1994). The beneficial effects of TO on the pulmonary hypoplasia are reflected by improvement in morphometric indices of the lung. With TO, lung to body weight ratio (LBWR) is normalized to non-CDH levels, total alveolar surface area increases and alveolar septal thickness decreases (Wilson et al, 1993).

Effects of TO on the pulmonary vascular system include, less muscularized arteries compared to CDH, number of vessels per unit area of lung similar to non CDH controls, normal capillary wall structure and normal thickness of the capillary-alveolar interface (DiFiore et al, 1995 and Bratu et al, 2001). CDH-affected lungs have increased pulsatility index (PI), an indicator of pulmonary hypoplasia and hypertension calculated from Doppler blood flow waveform of pulmonary arteries. Fetal tracheal occlusion reverses this finding, and results in a normal fetal physiological response to changes in oxygen tension at term (Sylvester et al, 1998). Thus TO-induced remodeling of pulmonary arterial system may decrease CDH-associated pulmonary hypertension (Bratu et al, 2001).

Similar pulmonary responses were observed in the rabbit model of CDH with tracheal occlusion. De Paepe et al showed that TO in this animal model, performed on day 24 of gestation, show better lung growth compared to control. They observed that immediately after tracheal occlusion lung growth stops for about 3 days (the so called lag- phase) followed by distension of airspaces, increased cell proliferation, and

accelerated architectural and cellular maturation by post-occlusion days 4 and 5 (De Paepe et al, 1998). Subsequently Wu et al found that TO (28 GA), performed in fetal rabbits that had undergone CDH at an earlier date (23 GA), reversed the CDH-induced hypoplasia of the lung (Wu et al, 2002). Roubliova et al has shown that TO, carried out at day 28 of gestation, leads to a lesser degree of muscularization of smaller, intra-acinar pulmonary arterioles, with a greater effect (75% reduction in muscularization) on vessels of less than 30 μm size (Roubliova et al, 2004).

TO in the rat model has shown that nitrofen-induced changes in rat lungs are reversible (Kanai et al, 2001). Tracheal occlusion in the rat model is performed on day 19 of gestation (E-19) when rat lungs are in the canalicular stage of development corresponding to 24-28 weeks of human prenatal life (Kitano et al, 1999). TO in this animal model reverses lung hypoplasia induced by nitrofen and leads to better lung maturation if performed late (E 20) rather than early (E 19)) during gestation (Kitano et al, 2001).

There is at least one major disadvantage associated with tracheal occlusion: it leads to an important decrease in the number of type II cells. Increased lung proliferation caused by TO may be responsible for this deleterious effect on type II pneumocytes (Flecknoe et al, 2000). Other mechanisms that may be involved are increased de-differentiation of type II cells into type1 cells (Yoshizawa et al, 2003), TO-associated increased apoptosis (De Paepe et al, 2004) and change in lung fluid composition (Luks et al, 2001). Since type II cells are the only source of surfactant, a deficiency would theoretically render the lung less compliant, thereby off shooting the beneficial effects of tracheal occlusion. This may explain the lack of significant improvement in postnatal lung function and respiratory gas exchange with tracheal occlusion, as observed in some animal studies (O'Toole et al, 1997; Davey et al, 2003). However a more recent study showed improved gas exchange and lung compliance with TO, despite the decrease in surfactant proteins (Bratu et al, 2004).

The deleterious effects on type II cell density seems to be related to timing and duration of tracheal occlusion (Wu et al, 2002). The earlier approaches that "plug the lung until it grows" may increase the lung mass but not necessarily the function (Hedrick et al, 1994). Others have shown that the so called plug-unplug approach of a temporary

15-day occlusion, initiated at 95 days' gestation in lambs, is associated with better maturation of type II cell mass (Flageole et al, 1998). Similarly, tracheal occlusion experiments in the fetal rabbits with or without CDH have shown that type II cell density is greater when occlusion is performed at 27 or 28 days instead of 24 days of gestation. Late TO (Liao et al, 2000) or temporary tracheal occlusion (Luks et al, 2000), while restoring type II cell number, is comparatively less effective than early and prolonged TO for lung growth induction (Wild et al, 2000).

The exact mechanism by which tracheal occlusion enhances lung growth is yet to be discovered. While TO has already been used experimentally in human fetuses with CDH, understanding these mechanisms may lead to more effective and less invasive therapies. Studies in animal models have indicated that a sustained increase in intratracheal pressure somehow stimulates the lungs to grow (Nardo et al, 1998). Others have argued that increased in the lung fluid volume is more important than a mere increase in the intra tracheal pressure caused by TO (Kitano et al, 2000 and Kitano et al, 2001). Alternatively, some investigators have claimed that it is the retention of growth factors in the lung fluid due to stagnation caused by TO that enhances lung growth (Sylvester et al, 1998).

As pointed out earlier, lung growth factors and signaling proteins are responsible for normal lung growth. Furthermore the expression of these substances was found down-regulated or disorganized in CDH. Investigators therefore started to look for these growth factors and signaling proteins as the possible mechanism of TO-induced lung growth. Earlier Quinn et al have reported that increased TGF-beta2 expression may contribute to accelerated lung growth and decreased surfactant production observed after tracheal occlusion (Quinn et al, 1999). TTF-1 is believed to have a role in branching morphogenesis of the lung, proximal-distal patterning and lung maturation (Ramirez et al, 1997). Recently it has been reported that in CDH-affected lungs TTF-1 is over-expressed in distal epithelium, TO reverses this pattern of over-expression to normal levels (Chapin et al, 2005). Hara et al reported that CDH is associated with decreased VEGF-A, a mediator of vasculogenesis in the lung; fetal lung distension by tracheal occlusion (TO) increased VEGF-A protein (Hara et al, 2005).

Although the current interest in finding the mediators of tracheal occlusion has lead to

some very interesting studies in vasculogenesis and maturation process of the lung, their role in lung branching has not been investigated so far. In this context, we aimed to study the effects of tracheal occlusion on Sonic Hedgehog (Shh), which is a mediator of branching morphogenesis, and previously has been shown to be down-regulated in CDH-affected lungs.

----------x----------

THE PROJECT

Our project and its rationale

Our project was divided in two parts. The first part consisted of establishing, in our laboratory, the rabbit as an alternative model to the sheep for surgically created CDH. Most of the lung markers believed to play a role in CDH have been investigated either in normal, transgenic mice or in CDH-affected rodent lungs. Conversely, the effects of tracheal occlusion in rabbits as well as sheep models have been studied using morphological (naked eye and microscopic) methods of detection. Very few studies have attempted to study the molecular effects of tracheal occlusion in CDH lungs. We therefore aimed in the second part of our project to study the expression pattern of sonic hedgehog (Shh) in CDH and to see how this marker of branching morphogenesis is affected by tracheal occlusion, comparing the teratogenic (rat-nitrofen) with surgical (rabbit) model of CDH.

Hypothesis

There is decreased bronchial branching in CDH lungs and Shh expression is delayed in CDH-affected lungs.

We propose:

1- TO-induced lung growth is associated with increased lung branching.

2- TO up-regulates Shh.

METHODS AND MATERIALS

A: **RABBIT EXPERIMENTS**

A: 1 **Animal protocol**

(McGill university animal care committee, protocol number 4663). Time-dated pregnant white New Zealand rabbits were purchased from Charles River, Quebec, Canada. Animals were delivered to the animal care facility one week before the proposed procedure time to get them acclimatized to the facility. They were housed in standard laboratory conditions, providing them free access to food, water and toys. Animals were arbitrarily divided in 3 groups:

The CDH Group, where we attempted to create CDH at 23 DGA (days of gestation age) in one ovarian end of the bicornuate uterus.

The TO group, where we performed TO at 27 DGA in one ovarian end of the bicornuate uterus. The littermate at the other end of the uterus was subjected to a sham operation.

The CDH+TO group, where we attempted to create CDH at 23 DGA in one fetal rabbit in each horn. This group had to undergo a second operation on day 27 DGA for tracheal occlusion.

The Control group, the non-operated fetuses in each of the above 3 groups, served as controls.

A: 2 **Creation of CDH**

Time dated pregnant rabbits were operated on gestational day 23 (term = 31 days). Each doe was given preoperative analgesia using synthetic morphine in therapeutic dose, followed by intramuscular Ketamine (50 mg per kg body weight) and Xylazine (5mg per kg body weight) as induction anesthesia. Endotracheal intubation was done using a 3mm uncuffed endotracheal tube according to McGill university's animal committee guidelines. General anesthesia was maintained with halothane (2%-3%) in oxygen. Cefazoline (100mg/kg IM) was given to protect against infection and Medroxyprogesterone acetate (5 mg IM) was administered as tocolytic agent.

The doe was placed in a supine position and the operative site was shaved. Under aseptic conditions a midline abdominal incision was made and one horn of the gravid uterus was delivered. Fetal parts were identified. A small (1.5 cm) transverse incision was made in the uterine wall on anti-mesometrial border, close to the cephalic end of fetus. Three tension sutures of 6-0 prolene were placed on the uterine incision to prevent membrane dislodgment. The fetus was partially delivered through the uterine incision. Fetal parts were identified. With the help of a sharp pair of scissors a left diaphragmatic defect was produced through a lateral thoracic approach. During the procedure the uterus was kept moist by intermittent spray of warm normal saline. The chest wall was closed with 6-0 prolene. After reducing the fetus to the uterus, warm normal saline was injected into the uterine cavity to compensate for the lost amniotic fluid. The hysterotomy incision was closed with 6-0 prolene. The uterus was reduced to the abdominal cavity and the abdominal incision was closed with 4-0 Vicryl. Skin suturing was done with 4-0 prolene. Adequate postoperative analgesia was provided.

A:3 **Tracheal Occlusion**

On day 27 of gestation pregnant dams were pre-medicated and anesthetized as described above. Thorough a midline laparotomy incision one uterine horn was delivered; a purse string suture applied and a small hysterotomy performed using techniques discussed above. The fetal head and neck were exposed and a midline cervical incision was made to expose the trachea. The trachea was identified by its rings and after further dissection a 3-0 Ethibon ligature was passed around it and tied to occlude the trachea completely. The uterine purse string was released, the fetus returned to the uterus and warm saline injected to compensate for the fluid loss during procedure. The hysterotomy wound was then closed, the uterus returned to the abdomen and the abdominal wound was closed, as described above.

A: 4 **Animal Sacrifice and Tissue Processing**

At 30 days gestation, animals were euthanized by a lethal dose of intravenous Euthanosol (125 mg/kg pentobarbital). The abdomen was opened through the previous scar. The operated horn was identified and the operated fetuses were harvested. Where

appropriate the presence of a tracheal ligature was confirmed using a surgical microscope. The total weight of each fetus in the study was measured. Also under the microscope, the chest of the operated fetus was opened; heart and lungs were dissected free along with the trachea, followed by careful removal of the heart and great vessels. The wet lung weight was measured after the excess tracheal fluid was allowed to escape particularly in TO group, as TO has been shown to increase retained lung fluid. A 20-gauge cannula was placed in trachea and advanced until the tip was just short of carina (tracheal bifurcation). Both lungs were perfused with 10% formaldehyde by slow injection to avoid lung damage. The lungs were then transferred to a perfusion system for continuous perfusion, at pressure of 25cm of H_2O for 24 hours. For light microscopy and morphometric measurements the lung tissue was embedded in paraffin.

A: 5 **Airspace Morphometry**

4-5 micron thick sections from each lung specimen were stained with hematoxylin and eosin (H&E staining). Mean terminal bronchial density (MTBD) in each slide was calculated. For each lung, 20 sections were assessed by two separate investigators blinded to the study group and a total of 40 sections per animal were examined (both lungs).

A: 6 **Statistical Analysis**

Statistical analysis was done using Microsoft excel and employing student t-test. A P-value < 0.05 was considered significant. Values (variables) were expressed as Means $\pm$ standard deviation.

B: RAT EXPERIMENTS

B.1: **Animals protocol**

(McGill University animal care committee protocol # 4463, revised). Time- dated pregnant rats (identified by vaginal semen plug = day 0) were purchased from Charles River, Quebec, Canada. Animals were shifted to the animal care facility of the Montreal Children's Hospital five days before the start of any experiment. Animals were housed in groups of two per cage. They were allowed free access to food (standard rat chow) and water and toys were provided in each cage. Animals were housed in standard constant temperature (21 degree Celsius) with 12 hours day and night cycles.

B.2: **Creation of CDH by Nitrofen**

In our experiment, we gavaged nitrofen (Chem Service Inc, West Chester, PA, USA) to animals on day 9.5 of gestation, according to published protocols. Immediately prior to administration, we dissolved nitrofen (100mg) in olive oil (2.5 ml). A gavage needle made up of stainless steel was passed per orum and the drug was administered. Control dams were gavaged with olive oil only. Dams were returned to their cages and the animal care continued as prior to gavage. Animals at this stage were divided into nitrofen and control group.

B.3: **Animal sacrifice and tissue preservation for CDH group**

In nitrofen-affected rat lungs Shh peaks later during gestation (day 20-21) compared to the control lungs (day 18-19) (discussed elsewhere). We therefore chose to sacrifice nitrofen-fed animals on day 20 and 21 to study the expression pattern of Shh. Each animal was anesthetized according to established protocol (discussed below) and a midline laparotomy incision made. The uterus was delivered, hysterotomy performed and fetuses delivered out one by one, after ligating their umbilical cords. Each fetus was weighed. The presence of a diaphragmatic defect (CDH) was confirmed and lungs were dissected out using microsurgical techniques. Wet lung weight of each fetus was measured. The dam was given a lethal dose of Pentothal and her laparotomy wound closed in a single layer using vicryl 4-0.

B.4: **Tracheal occlusion**

Tracheal occlusion was performed on Day 19 in selected animals from the nitrofen as well as the control group. Preoperatively, animals were anesthetized with a cocktail of drugs consisting of 5 ml Ketamine; 2.5 ml Xylazine; 1 ml Acepromazine; 1.5 ml sterile saline given at the dose of 0.1ml per 100 grams body weight. Antibiotic (Ancef 125 mg IM) was given to decrease the risk of infection. A midline laparotomy was performed under aseptic conditions. One uterine horn was delivered and a fetus near to ovarian end selected for operation. The rest of the uterus was replaced back in to abdominal cavity to avoid cooling and drying of the uterus. A purse string suture was applied in a less vascular part of the fetal sac using 5-0 silk. A small hysterotomy was performed and the head and neck of the fetus were delivered. The purse string was tightened to avoid expulsion of the fetus. The neck of the fetus was extended by placing a string (silk) in the mouth of the fetus and pulling back. Using a surgical microscope, gentle neck dissection was performed avoiding trauma to jugular vessels and thyroid gland. The trachea was identified as whitish midline structure and a surgical clip was applied. The purse string was released and fetus returned to the uterus. Warm normal saline (1-2 ml) was injected slowly into to uterus through the hysterotomy hole to compensate for the fluid loss. The uterus was closed by tying the purse string suture. A maximum of three TO were performed per dam, two on one horn and one on the other. Buprenorphine 0.01 to 0.05 mg/kg was given intramuscularly every 8-12 hours for the first 1-2 days, as required, for postoperative analgesia.

B.5: **Animal sacrifice and Tissue preservation for TO group**

Dams, whose fetuses underwent TO on day 19, were sacrificed on day 21 (term 22 days). The abdomen was opened through the previous incision. TO fetuses were identified, delivered through a hysterotomy and their total body weight measured. Through an abdominal approach the presence or absence of the CDH was confirmed in the nitrofen-fed animals. The lungs were then dissected out under a surgical microscope. TO was confirmed by examining the clip around trachea. Wet lung weights were measured. Tissues were preserved in formalin (for histology), 10 % formaldehyde (for IHC) and snap-frozen in liquid nitrogen (for IHC & PCR).

B.6: **Immunohistochemistry (IHC)**

For Shh protein analysis by IHC, Shh-19D antibody kit was purchased from Santa Cruz, California U.S.A. Prior to IHC, formaldehyde preserved lungs from animals at gestational day 20 and 21, were passed through increasing concentrations of alcohol solutions for the dehydration and antigen preservation purposes. These processed lungs as well as fresh frozen lungs were cut into thin sections using microtome and were mounted on glass slides. For IHC, sections were air dried for 5 minutes and were transferred to buffer solution-PBS. Next, they were transferred to hydrogen peroxide solution for 20 min and then incubated with blocking serum for 20 min. Then the primary antibody was added to the slides and after they were incubated for 2 hours the secondary antibody was added and incubated again for 30 min at room temperature. After washing in PBS, the Avidin-Biotin Complex (ABC) reagent was added and kept for 30 min. Slides were counterstained with H&E. The slides were mounted and examined by light microscopy. Staining was evaluated on a scale 0-3, comparing proximal vs. distal airway vs. mesenchyme. About 10 high power fields (HPF) were examined per slide and at least 4 slides per animal. Intensity of staining was examined in each location (proximal vs distal airway vs mesenchyme) and was averaged for each animal and then within each group, before comparisons were made.

B.7 Measurement of Shh-mRNA by quantitative Real Time Polymerase chain reaction (qRT-PCR)

B.7.1 **Isolation of RNA**

The snap-frozen (frozen in liquid nitrogen) specimens, from day 21 animals were used for PCR analysis. Total RNA was isolated by homogenizing the lung tissue in TRIZOL Reagent (Invitrogen, USA). TRIZOL Reagent is a mixture of phenol and guanidine isothiocyanate that is used for cell lysis and RNA extraction. After addition of chloroform followed by centrifugation, the solution was separated into an aqueous phase and an organic phase. The RNA (in the aqueous phase) was precipitated with isopropyl alcohol and was re-suspended in DEPC (diethylpyrocarbonate) treated RNAse-free water (Ambion Inc, USA). To remove DNA contaminants, samples were treated with Turbo DNase (Ambion, USA). The Turbo DNase is an engineered enzyme

that exhibits a much greater binding efficiency than wild type DNase 1 and which effectively removes DNA contaminants from the RNA samples prior to PCR. The integrity of RNA was confirmed by fractionation of the RNA samples on 0.7% (weight/volume) agarose gels stained with ethidium bromide.

B.7.2 **RNA Quantification and PCR**

Quantification of RNA was performed by using light spectrophotometry (260/280 wave-length ratio). Total RNA content was diluted in TE (Tris + EDTA) solution to a uniform concentration of 10ng/ml. This solution was run on a quantitative one-step PCR machine, using the QuantiTect Probe Master Mix system (Qiagen, USA). QuantiTect Probe Master Mix is a premixed solution containing HotStarTaq DNA Polymerase, QuantiTect Probe PCR buffer, the deoxynucleotide triphosphates (dNTPs) and ROX (a passive reference dye that normalizes fluorescent reporter signal in real-time quantitative PCR)

Shh probe sets were designed using the QuantiTect custom assay (Qiagen, Netherlands). The Sequence of the probe was AGGAAA ACACTG GAGC. The oligonucleotide sequences for the primers were as follow:

Forward = CCAATTACAACCCCG ACAT

Backward = AGTCTCCACGTT TCTGTTCA

These probes were added to the QuantiTect Probe Master Mix, after the addition of reverse transcription enzyme. For quantification, 18s RNA was used as an internal control and normal fetal rat lung was used to generate a standard curve.

The relative number of molecules of each transcript was determined by interpolating the values of the unknown samples to each standard curve and the obtained values were normalized.

B.8: **Statistical analysis**

Statistical analysis was carried out using Microsoft excel and *statpro add-in* software. Data was grouped as mean plus/minus standard deviation. The different groups were compared using the student t-test for paired and unpaired analysis. A p-value of less than 0.05 was considered significant. Confidence intervals were calculated where appropriate.

RESULTS

A: **RABBIT RESULTS**

A.1: **Animal outcome**

Six rabbits had creation of CDH (CDH group) in one fetus at 23 days of gestational age (DGA). Another group of six rabbits underwent tracheal occlusion (TO group) in one fetus at 27 DGA. One dam died during induction of anesthesia. In one dam the procedure had to be abandoned due to technical reasons. Survival in the CDH group was zero. Of the 6 fetuses in the TO group, 5 survived (harvested at 30 DGA) while one dam aborted on the morning of the planned operation. The overall animal survival was therefore 83.33 % in the TO group. The third group (CDH +TO), which was the main focus of our study, where we contemplated to create TO after successful CDH creation, could not be completed due to failure to create CDH successfully.

A: 2 **Lung weight to body weight ratio (LW/BW)**

Grossly TO lungs were bigger compared to the controls (see figure 1). The fetuses in the TO group showed a significantly higher LW/BW ratio when compared with the control lungs. The mean LW/BW in the TO group was 0.032 ± 0.003; while in the control lungs we observed a mean value of 0.021 ± 0.001. The p-value for the difference in the mean values of these two groups was 0.006.

These results are shown in table 2.

A.3: **Histology and MTBD**

The histology of the lungs in the TO group was quite different from that of control lungs (See figure 2 and 3). Lungs in the TO group had thinner inter-alveolar septae than control lungs. There were more alveoli in the TO group than in control lungs. MTBD in control lungs was 10.31± 0.089. In contrast, the TO group had lower values with mean MTBD of 7.725± 0.49. This difference was significant (p-value =0.006) indicating that lungs in TO group were hyperplastic when compared with the controls.

Values for MTBD are shown in table 2.

B: **RAT RESULTS**

B: 1 **Animals outcome**

The total number of animals in the study was 32. Nitrofen was given to 16 animals, the remaining animals were used as controls with or without TO. Several fetuses from each pregnant dam underwent surgery. Based on our study animals were divided into five potential groups:

1. CDH+, where all the animals had CDH due to nitrofen.
2. CDH- (negative), where pups had no CDH although dams were given nitrofen.

Animals from these two groups were sacrificed at 3 different time points, day 19, day 20 and day 21 of gestation to observe changes in the Shh staining with time.

3. The Control group comprised of animals where pups were examined without any intervention (no nitrofen but olive oil, no TO).
4. Control +TO, where we performed TO on animals that were not given nitrofen.
5. CDH+TO, consisted of nitrofen-fed animals that underwent tracheal occlusion and were found CDH positive on dissection.

We examined 120 pups from nitrofen-fed dams (n= 16) and found 75% of them having a diaphragmatic defect. We achieved an overall survival of 40 % for TO, with better outcome in the control group (53%) than in nitrofen-fed animals (27%). This could be due to the fact that exposure to nitrofen leads to systemic damage and thus these already feeble fetuses were unable to withstand TO. On naked eye examination lungs from control animals were bigger than CDH lungs. CDH+TO lungs were the same size as controls.

B: 2 **Lung weight to body weight ratio**

Lung to body weight ratio (LW/BW) was used to assess the effects of nitrofen and tracheal occlusion on lung growth. Values for Mean ± standard deviations of LW/BW were 0.038 ± 0.003 and 0.031± 0.005 for the control group and the CDH+ group respectively. The difference between the two groups was statistically significant (p-value =0.001). Thus nitrofen-induced CDH was associated with smaller ratio (and therefore smaller lungs) than controls. Values for CDH- (negative) group were 0.033± 0.006. A p-value of 0.003 indicated a statistically significant difference between this

group of animals and controls. We compared LW/BW of the CDH+ lungs (mean = 0.0307 ± 0.0052) with LW/BW of the CDH- lungs (mean = 0.0327 ± 0.0062) and found that there is no significant difference (p-value 0.307) between these two groups. Thus nitrofen-affected lungs were smaller than controls irrespective of the presence or absence of the CDH. These results are shown in table 3.

Tracheal occlusion in our study induced lung growth in the control as well as in the CDH lungs. In the Control +TO group mean LW/BW was 0.0544 ± 0.0077 and significantly different (p-value = 0.001) from the control group values of 0.0379 ± 0.0033. These results are shown in table 4. Conversely, lungs in CDH+ TO group had a mean LW/BW of 0.0361 ± 0.0022. Thus mean LW/BW in this group was larger than values in CDH+ group (0.0307 ± 0.0052). The p-value for the difference between means in CDH+ and CDH+TO was 0.021. The low p-value indicated that TO induced lung growth in CDH affected lungs. In addition, the p-value for the difference between Control and CDH+TO was 0.218, indicating no difference between these groups. Taken together, TO induced lung growth in the nitrofen-affected CDH lungs and brought them to the level of non-nitrofen (controls) status. See table 4.

B: 3 **Lung Histology**

H&E staining revealed that, lungs of pups from the nitrofen-fed animals have less alveolar structures and thicker interstitium than control, non-nitrofen animals (see figure 4). These changes were present in both CDH positive and negative lungs supporting the previous studies that nitrofen-induced changes are independent of CDH. Lungs from the TO group were more mature than control lungs. Comparing CDH+ to CDH+TO group, lungs were comparatively more mature in the latter group having thinner interstitium and more alveolar ducts. Thus, TO reversed nitrofen-induced changes in the lung.

B: 4 **Results from IHC**

In CDH+ lungs, Shh was detected as a brown staining at all time points. Due to background staining it was difficult to draw a clear-cut line between day 20 and 21 slides. Although Shh staining in the control lungs was less intense than CDH+ lungs,

no clear conclusion could be drawn from these slides owing to background staining (see figure 5 and 6). Furthermore we were unable to detect any real difference in Shh staining between CDH positive and CDH negative groups. Thus we were unable to verify the previously reported down-regulation (or delayed peaking) of Shh in CDH lungs. Although more intense staining was detected in the epithelium, we detected some staining in the mesenchyme as well. As Shh is predominantly expressed in epithelium the finding we observed could be due to excessive background staining. Despite multiple attempts at IHC staining, varying exposure time to the antibody and using various resources available at the MCH research institute we could not achieve good staining of target areas without background staining.

Since we were unable to detect any real difference in Shh expression between nitrofen-fed (CDH + and CDH -ve) and control (non nitrofen) lungs, we did not pursue IHC staining for TO groups. This would have been unnecessary and useless, as we do not have any reference points where we could compare CDH+TO lungs.

B.5 **Results from qRT-PCR**

The 21 DGA fetal rat lungs were examined for Shh-mRNA detection via qRT-PCR. The expression levels of Shh were calculated as the ratio of Shh-mRNA to the housekeeping gene-mRNA. The values are shown as exponentials (E) in table 5 and expressed with graphs in figure 7. Transcripts of mRNA of Shh were elevated in nitrofen-affected CDH+ and CDH- negative lungs when compared to controls (n = 12 in each group). The mean for CDH+ group was 6.13E-02 (613×10^{-4}) with a 95% confidence interval from 5.04E-02 (504×10^{-4}) to 7.21E-02 (721×10^{-4}). The mean for CDH- group was 5.93E-02 (593×10^{-4}) with 95% confidence interval around the mean from 4.60E-02 (460×10^{-4}) to 7.26E-02 (726×10^{-4}). Conversely, values for the control group were quite different from these two with a mean of 2.59E-02 (259×10^{-4}) and 95% confidence interval from 1.38E-02 (138×10^{-4}) to 3.80E-02 (380×10^{-4}). Since the confidence interval for the control group does not include the confidence interval values of either CDH+ or CDH- groups, the results were statistically significant (P value < 0.05). Furthermore, the expression levels of Shh mRNA of CDH+ group were not statistically different from CDH- group, as the confidence

intervals of these two groups intersect each other. Thus nitrofen-affected lungs showed a higher expression irrespective of the presence or absence of CDH.

The TO group (tracheal occlusion on control group) had a mean expression level of Shh-mRNA equal to 1.55E-02 (155×10^{-4}) which was not significantly different from the mean value in control lungs, 2.59E-02 (259×10^{-4}). Thus TO in the lungs not affected by nitrofen, does not alter the expression levels of Shh. Conversely, the mean expression level of Shh–mRNA in the CDH + TO group was 1.28E-02 (128×10^{-4}) which was significantly different from the CDH+ lungs. Thus, TO in the nitrofen-affected CDH lungs decreases the expression levels of Shh-mRNA to the control levels.

DISCUSSION

Our results from the rabbit model demonstrate that lungs from animals whose trachea were occluded before birth were larger compared to control group. These findings are in accordance with previously reported studies that TO invariably leads to accelerated lung growth and larger lungs. Lung histology from these animals revealed that lungs from the TO group had decreased MTBD compared to the control group. Thus TO in this animal model led to lung growth and lung maturation. Surgical creation of CDH in the fetal rabbit proved difficult, with 0/6 survival. The learning phase could be a possible cause of the high mortality and is expected when using such a fragile model. Discussions with other investigators, who have successfully used this model, make us hopeful that this difficulty could be overcome and the rabbit model remains within reach. The rat model proved reliable for the creation of CDH. In this animal model we were able to show that nitrofen caused CDH in 75 % of the pups when dams were gavaged with 100mg of the agent on day 9.5 post conception. This is in accordance with the reported figure of 50-80 % (Guilbert et al, 2000). After some initial failures we were able to achieve a survival rate of 40 % for TO. This is consistent with other reports ((Kitano et al, 1999 and Yoshizawa et al, 2003). The success rate was somewhat lower in the nitrofen-affected fetuses than the controls but still enough to be worthwhile. Nitrofen-fed CDH+ animals and CDH negative animals showed significantly smaller lungs compared to the control group. Thus lungs were smaller irrespective of CDH. This may be, as previously reported, due to the overall deleterious effect of nitrofen on these pups. Lungs from the TO group were bigger compared to the control group. The difference between these two groups was statistically significant. This again reflects the previously observed phenomenon that TO leads to lung growth in experimental animals. The enhanced maturation of TO lungs compared to control lungs was evident by the histological findings of thin interstitium and more alveolar spaces in the former group. This shows the overall beneficial effect of TO on the lungs. TO in the CDH+ lungs led to better lung indices, with LW/BW significantly larger than non TO CDH+ lungs.

IHC for Shh protein expression in the rat lung proved difficult and unreliable. The major concern was the excessive background staining. Although we detected Shh in the

rat lung specimens, due to the background staining it was not possible to draw any conclusion about its expression pattern. We employed all the techniques reported in the literature and suggested by our collaborators but were unable to solve the problem of background staining. Attempts at reducing the concentration of antibody, duration of exposure of the lung specimen to the antibody and cutting (with microtome) the specimens in thinner sections either lead to no staining or the same level of excessive background staining. Discussing this problem with investigators from McGill University and from the University of Toronto revealed that those investigators had faced similar problems with antibodies purchased from Santa Cruz Inc. The Toronto group developed their own anti-Shh antibody to tackle these problems.

qRT-PCR analysis showed that the levels of Shh mRNA are higher in nitrofen-affected lungs compared to non-affected controls at 21 days of gestation. This effect was not related to the presence of CDH because both CDH+ and CDH- lungs, in our experiments, showed similar levels of expression. Since nitrofen affects the maturation of the lungs, there was a delay in the expression of Shh leading to a peak expression levels later during gestation in comparison to control lungs. This was previously reported by Unger et al who studied Shh expression in mice in controls and nitrofen-exposed animals at different time points from 15 days of gestation to term (22 days). Our findings confirm that nitrofen may down-regulate Shh, by delaying its expression, in the rat lungs. TO in the control group did not alter the expression levels of Shh mRNA. This observation is consistent with the knowledge that Shh is a signaling protein involved in lung maturation and branching early on, therefore its expression may not be altered by TO in already mature lungs. However, we had thought that TO might have "de-differentiated" the lungs to the point of inducing branching beyond the period where this normally occurs. In nitrofen-affected lungs, TO led to a decrease in the expression of Shh-mRNA, bringing it back to levels similar to the control (non-nitrofen) lungs. We do not know what happened to Shh-mRNA levels between the TO on day 19 and sacrifice on day 21, since all animals sacrificed on day 20 were used for IHC, which did not yield interpretable results. Therefore we cannot say whether TO accelerated the peak of Shh-mRNA expression compared to the non-occluded nitrofen-treated animals. Ideally Shh-mRNA expression should have

been studied at earlier time points during gestation (day 19.5 and day 20) to have a clear picture of the expression pattern. Unfortunately this could not be done due to shortage of time. Our initial hypothesis that TO induced bronchial branching through Shh up-regulation in the hypoplastic CDH lungs could not be confirmed in this study. In the future a direct method of protein detection, such as IHC or Western blot, could be employed to verify the levels of Shh at different time points after TO. If employed as method of measurement, the IHC would need a careful consideration of the antibody source. In addition, direct morphometric methods (e.g. lung casting) could be used to evaluate bronchial branching. If branching is not enhanced by TO, then lung growth could occur through an accelerated alveolar development. Markers of that development such as lgl1 would be interesting to study in the future. Similarly, markers of vascular development such as VEGF could be evaluated.

CONCLUSION

The rabbit model remains within reach for *in vivo* studies of CDH-affected lungs. TO in the rabbit model induces lung growth in non-CDH lungs. Surgical creation of CDH in this animal model proved difficult and needs further refinements. The rat model is reliable for the creation of CDH by nitrofen. Although TO proved difficult in this model, with a lower success rate in the nitrofen-affected animals, it was still enough to be worthwhile. Nitrofen-induced changes in the rat lungs are independent of CDH. Tracheal occlusion in this animal model is associated with more mature lungs compared to controls. IHC for Shh was not reliable and may be dependent on the source of the antibody. Shh-mRNA expression is delayed in the nitrofen-affected lungs. Again, this delay in Shh-mRNA expression is independent of CDH. TO, in the nitrofen-affected lungs, brings Shh-mRNA expression levels to those of the control lungs. Whether this means that Shh expression itself is reversed to normal cannot be determined from our experiments since our attempts at immunohistochemistry were unsuccessful. Whether tracheal occlusion leads to increase bronchial branching could not be proven from the present study. In the future morphometric assessment of branching (such as lung casting) can be employed to differentiate increased bronchial branching from alveolization. Similarly other markers of lung development could be studied, e.g. VEGF for vascular growth and lgl1 for alveolar development.

SUMMARY

BACKGROUND: The mechanism of pulmonary hypoplasia in congenital diaphragmatic hernia (CDH) is poorly understood. The herniation of abdominal organs into the chest was thought to produce lung hypoplasia by compression. Nitrofen-induced CDH, in rodent models, suggests that hypoplasia precedes the herniation. The understanding of these mechanisms is important for therapeutic intervention to be successful. The sonic hedgehog (Shh), a signaling protein of the hedgehog pathway is essential for the lung maturation and bronchial branching in the respiratory system of rats. Shh is down-regulated in the nitrofen-induced CDH lungs in mice. The aim of this study was to see if tracheal occlusion (TO), which induces lung growth in animal models of CDH, could up-regulate Shh expression in the rat model.
METHODS: To create CDH in fetal rats, time-dated pregnant rats were gavaged 100 mg of nitrofen in olive oil on day 9.5 post conception. Control rats received olive oil only. Tracheal occlusion was performed on day 19 of gestation (term = 22 days). Animals were sacrificed on day 20 and 21 and lungs were dissected out for studies. Lung growth was measured by lung to body weight ratio (LW/BW) and histology. The expression of Shh was measured by immunohistochemisty (IHC) and by quantitative real time polymerase chain reaction (qRT-PCR). TO-induced changes were compared both in the normal controls and nitofren-affected lungs for growth and Shh expression.
RESULTS: TO induced lung growth in nitrofen-affected lungs in comparison to non-occluded controls. IHC failed to yield interpretable results because of technical problems with the antibody used. Higher levels of Shh-mRNA expression were observed in nitrofen-affected (CDH+ & CDH-) lungs in comparison to controls on day 21. TO in nitrofen-treated lungs, lowered the Shh-mRNA levels to control levels.
CONCLUSIONS: The nitrofen-induced hypoplasia of CDH is reversible with TO. Shh-mRNA expression in nitrofen-affected lungs is delayed and peaks later during gestation. Tracheal occlusion decreases Shh-mRNA expression and brings it closer to control levels. Our study however does not confirm that Shh is up-regulated by tracheal occlusion. A direct method of protein detection (such as IHC and Western blot) may be employed in the future to confirm if TO leads to a normalized expression of Shh in this animal model.

REFERENCES

1. **Acosta JM, Thebaud B, Castillo C, Mailleux A**: Novel mechanisms in murine nitrofen-induced pulmonary hypoplasia: FGF-10 rescue in culture. *Am J Physiol Lung Cell Mol Physiol. 281: 250-7, 2001.*
2. **Allan DW, Greer JJ**: Pathogenesis of nitrofen-induced congenital diaphragmatic hernia in fetal rats. *J Appl Physiol. 83:338-347, 1997.*
3. **Arman E, Haffner-Krausz, Gorivodsky M**: RFgfr2 is required for limb outgrowth and lung-branching morphogenesis. *Proc Natl Acad Sci USA. 96:11895-9, 1999.*
4. **Asabe K, Tsuji K, Handa N**: Immunohistochemical distribution of surfactant apoprotein-A in congenital diaphragmatic hernia-II. *Asian J Surg. 26:205-9, 2003.*
5. **Askanazi SS, Perlman M**: Pulmonary hypoplasia: lung weight and radial alveolar count as criterion of diagnosis. *Arch Dis Child. 54: 614-618, 1979.*
6. **Bahlmann F, Merz E, Hallermann C**: Congenital diaphragmatic hernia: ultrasonic measurement of fetal lungs to predict pulmonary hypoplasia. *Ultrasound Obstet Gynecol. 14:162-8, 1999.*
7. **Baptista MJ, Melo-Rocha G, Pedrosa C**: Antenatal vitamin A administration attenuates lung hypoplasia by interfering with early instead of late determinants of lung underdevelopment in congenital diaphragmatic hernia. *J Pediatr Surg. 40: 658-65, 2005.*
8. **Bellusci S, Furuta Y, Rush MG, Henderson R**: Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. *Development. 124: 53-63, 1997.*
9. **Bellusci S, Grindley J, Emoto H, Itoh N, Hogan BL**: Fibroblast growth factor 10 (FGF-10) and branching morphogenesis in the embryonic mouse lung. *Development. 124: 4867-78, 1997.*
10. **Bellusci S, Henderson R, Winnier G**: Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. *Development. 122: 1693-702, 1996.*

11. **Bernard KW, Parham GL, Winkler WG**: Q fever control measures: recommendations for research facilities using sheep. *Infect Control. 3: 461-5, 1982.*

12. **Besnard V, Wert SE, Hull WM**: Immunohistochemical localization of Foxa1 and Foxa2 in mouse embryos and adult tissues. *Gene Expr Patterns. 5:193-208, 2004.*

13. **Brandsma AE, ten Have-Opbroek AA, Vulto IM**: Alveolar epithelial composition and architecture of the late fetal pulmonary acinus: an immunocytochemical and morphometric study in a rat model of pulmonary hypoplasia and congenital diaphragmatic hernia. *Exp Lung Res. 20: 491-515, 1994.*

14. **Brandsma AE, Tibboel D, Vulto IM**: Inhibition of T3-receptor binding by Nitrofen. *Biochim Biophys Acta. 1201: 266-70, 1994.*

15. **Bratu I , Flageole H, Laberge JM, Kovacs L, Faucher D, Piedboeuf B**: Lung function in lambs with diaphragmatic hernia after reversible fetal tracheal occlusion. *J Pediatr Surg. 39:1524-31, 2004.*

16. **Bratu I, Flageole H, Laberge JM, Piedboeuf B**: Surfactant levels after reversible tracheal occlusion and prenatal steroids in experimental diaphragmatic hernia. *J Pediatr Surg. 36: 122-7, 2001.*

17. **Bratu I, Flageole H, Laberge JM, Chen MF, Piedboeuf B**: Pulmonary structural maturation and pulmonary artery remodeling after reversible fetal ovine tracheal occlusion in diaphragmatic hernia. *J Pediatr Surg. 36: 739-44, 2001.*

18. **Bucher U, Reid L**: Development of the intrasegmental bronchial tree: the pattern of branching and development of cartilage at various stages of intra-uterine life. *Thorax 16: 207-218, 1964.*

19. **Burri PH:** Fetal and postnatal development of the lung. Annu *Rev Physiol. 46:617-28, 1984.*

20. **Cardoso WV, Williams MC, Mitsialis SA**: Retinoic acid induces changes in the pattern of airway branching and alters epithelial cell differentiation in the developing lung in vitro. *Am J Respir Cell Mol Biol. 12: 464-76, 1995.*

21. **Cardoso WV**: Molecular regulation of lung development. *An Rev Physiol. 63:471-94, 2001.*

22. **Cerna A, Janega P**: Changes in sialic acid expression in the lung during intrauterine development of the human fetus. *Acta Histochem. 104: 339-42, 2002.*

23. **Chang R, Andreoli S, Ng YS**: VEGF expression is downregulated in nitrofen-induced congenital diaphragmatic hernia. *J Pediatr Surg. 39: 825-8, 2004.*

24. **Chapin CJ, Ertsey R, Yoshizawa J, Hara A**: Congenital diaphragmatic hernia, tracheal occlusion, thyroid transcription factor-1, and fetal pulmonary epithelial maturation. *Am J Physiol Lung Cell Mol Physiol. 289: 44-52, 2005.*

25. **Chen C, Chen H, Sun J**: Smad1 expression and function during mouse embryonic lung branching morphogenesis. *Am J Physiol Lung Cell Mol Physiol. 288: 1033-9, 2005.*

26. **Chung UI, Schipani E, McMahon AP**: Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. *J Clin Invest. 107: 295-304, 2001.*

27. **Cilley RE, Zgleszewski SE, Krummel TM**: Nitrofen dose-dependent gestational day-specific murine lung hypoplasia and left-sided diaphragmatic hernia. *Am J Physiol. 272:362-71, 1997.*

28. **Compernolle V, Brusselmans K, Acker T**: Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. *Nat Med. 8: 702-10, 2002.*

29. **Correia-Pinto J, Baptista MJ, Pedrosa C**: Fetal heart development in the nitrofen-induced CDH rat model: the role of mechanical and nonmechanical factors. *J Pediatr Surg. 38: 1444-51, 2003.*

30. **Cortes RA Killer RL**: Survival of severe congenital diaphragmatic hernia has morbid consequences. *J Pediatr Surg.* 40: 36-45, 2005.

31. **Crawford D.C, Wright V.M, Drake D.P**: Fetal diaphragmatic hernia: The value of fetal echocardiography in the prediction of postnatal outcome. *Obstet Gynaecol. 96: 705-10, 1989.*

32. **Davey MG, Hedrick HL, Bouchard S, Mendoza JM**: Temporary tracheal occlusion in fetal sheep with lung hypoplasia does not improve postnatal lung function. *J Appl Physiol. 94: 1054-62, 2003.*

33. **De Jong PA, Nakano Y**: Estimation of lung growth using computed tomography. *Eur Respir J. 22: 235-8, 2003.*

34. **De Lorimer AA, Tierney DF, Parker HR**: Hypoplastic lungs in fetal lambs with surgically produced congenital diaphragmatic hernia. *Surgery 62: 12-17, 1967.*

35. **Demayo F, Minoo P, Plopper C.G**: Mesenchymal-epithelial interactions in lung development and repair: are modeling and remodeling the same process? *Am J Physiol Lung Cell Mol Physiol. 283: 510-7, 2002.*

36. **Demiryurek AT, Wadsworth RM, Kane KA, Peacock AJ**: The role of endothelium in hypoxic constriction of human pulmonary artery rings. Am Rev *Respir Dis. 147: 283-90, 1993.*

37. **De Paepe ME, Johnson BD, Papadakis K**: Temporal pattern of accelerated lung growth after tracheal occlusion in the fetal rabbit. *Am J Pathol. 152: 179-90, 1998.*

38. **De Paepe ME, Mao Q, Luks FI**: Expression of apoptosis-related genes after fetal tracheal occlusion in rabbits. *J Pediatr Surg. 39:1616-25, 2004.*

39. **De Rooij JD, Hosgor M, Ijzendoorn Y, Rottier R, Tibboel D**: Expression of angiogenesis-related factors in lungs of patients with congenital diaphragmatic hernia and pulmonary hypoplasia of other causes. *Pediatr Dev Pathol. 7:468-77, 2004.*

40. **Desai TJ and Cardoso WV**: Growth factors in lung development and disease: friends or foe? *Respir Res. 3:2, 2002.*

41. **Dietert RR, Etzel RA, Chen D**: Workshop to identify critical windows of exposure for children's health: immune and respiratory systems work group summary. *Environ Health Perspect. 108:483-90, 2000.*

42. **DiFiore JW, Fauza DO, Slavin R, Peters CA**: Experimental fetal tracheal ligation reverses the structural and physiological effects of pulmonary hypoplasia in congenital diaphragmatic hernia. *J Pediatr Surg. 29: 248-56,*

1994.

43. **DiFiore JW, Fauza DO, Slavin R, Wilson JM**: Experimental fetal tracheal ligation and congenital diaphragmatic hernia: a pulmonary vascular morphometric analysis. *J Pediatr Surg. 30: 917-23, 1995.*
44. **Enns GM, Cox VA, Goldstein RB, Gibbs DL**: Congenital diaphragmatic defects and associated syndromes, malformations, and chromosome anomalies: a retrospective study of 60 patients and literature review. *Am J Med Genet. 79:215-25, 1998.*
45. **Flageole H, Evrard VA, Piedboeuf B, Laberge J-M**: The plug-unplug sequence: An important step to achieve type 2 pneumocyte maturation in the fetal lamb model. *J Pediatr Surg. 33: 299-303, 1998.*
46. **Flecknoe S, Harding R, Maritz G, Hooper SB**: Increased lung expansion alters the proportions of type I and type II alveolar epithelial cells in fetal sheep. *Am J Physiol Lung Cell Mol Physiol. 278: 1180-5, 2000.*
47. **Fauza DO, Tannuri U, Ayoub AA, Capelozzi VL**: Surgically produced congenital diaphragmatic hernia in fetal rabbits. *J Pediatr Surg. 29: 882-6, 1994.*
48. **Francis BM**: Teratogenicity of bifenox and nitrofen in rodents. *J Environ Sci Health B. 21: 303-17, 1986.*
49. **Geggel RL, Murphy JD, Langleben D**: Congenital diaphragmatic hernia: arterial structural changes and persistent pulmonary hypertension after surgical repair. *J Pediatr. 107: 457-64, 1985.*
50. **George DK, Cooney TP, Chiu BK, Thurlbeck WM**: Hypoplasia and immaturity of the terminal lung unit (acinus) in congenital diaphragmatic hernia. *Am Rev Respir Dis. 136: 947-50, 1987.*
51. **Gilbert SF**: Developmental biology. *7th edition, 2003. Published by Sinauers associates Inc. Sunderland, USA.*
52. **Gosche JR, Saleem Islam and Scott C. B**: Congenital diaphragmatic hernia: searching for answers. *Am J Surg. 190: 324-32, 2005.*
53. **Greer JJ, Cote D, Allan DW**: Structure of the primordial diaphragm and defects associated with nitrofen-induced CDH. *J Appl Physiol. 89:2123-9,*

2000.

54. **Groenman F, Unger S, Post M**: The molecular basis for abnormal human lung development. *Biol Neonate. 87:164-77, 2005.*
55. **Guilbert TW, Gebb SA, and Shannon JM**: Lung hypoplasia in the nitrofen model of congenital diaphragmatic hernia occurs early in development. *Am J Physiol Lung Cell Mol Physiol. 279: 1159-71, 2000.*
56. **Hara A, Chapin CJ, Ertsey R, Kitterman JA**: Changes in fetal lung distension alter expression of vascular endothelial growth factor and its isoforms in developing rat lung. *Pediatr Res. 58: 30 –7, 2005.*
57. **Harrison M.R, Adzick N.S, Estes J.M**: A prospective study of the outcome of fetuses with congenital diaphragmatic hernia. *JAMA. 271: 382-4, 1994.*
58. **Harrison MR, Adzick NS, Bullard KM:** Correction of congenital diaphragmatic hernia in utero VII: a prospective trial. *J Pediatr Surg 32:1637-42, 1997.*
59. **Harrison MR, Killer RL**: A randomized trial of fetal endoscopic tracheal occlusion for severe fetal congenital diaphragmatic hernia. *N Engl J Med. 349: 1916-24, 2003.*
60. **Harrison MR, Adzick NS, Nakayama DK, deLorimier AA**: Fetal diaphragmatic hernia: pathophysiology, natural history, and outcome. *Clin Obstet Gynecol, 29:490-501, 1986.*
61. **Hashim E, Laberge JM, Chen MF, Quillen EW Jr**: Reversible tracheal obstruction in the fetal sheep: effects on tracheal fluid pressure and lung growth. *J Pediatr Surg. 30: 1172-7, 1995.*
62. **Hassett MJ, Glick PL, Karamanoukian HL, Rossman JE**: Pathophysiology of congenital diaphragmatic hernia. XVI: Elevated pulmonary collagen in the lamb model of congenital diaphragmatic hernia. *J Pediatr Surg 30:1191-4, 1995.*
63. **Haugen SE, Linker D, Eik-Nes S**: Congenital diaphragmatic hernia: determination of the optimal time for operation by echocardiographic monitoring of the pulmonary arterial pressure. *J Pediatr Surg. 26: 560-2, 1991.*

64. **Hedrick HL, Crombleholme TM, Flake AW**: Right congenital diaphragmatic hernia: Prenatal assessment and outcome. *J Pediatr Surg. 39: 319-23, 2004.*

65. **Hedrick MH, Estes JM, and Sullivan KM**: Plug the lung until it grows (PLUG): a new method to treat congenital diaphragmatic hernia in utero. *J Pediatr Surg. 29: 612-7, 1994.*

66. **Hislop AA, Wigglesworth JS, Desai R**: Alveolar development in the human fetus and infant. *Early Hum Dev. 13: 1-11, 1986.*

67. **Hurt SS, Smith JM, Hayes AW**: Nitrofen: a review and perspective. *Toxicology. 29: 1-37, 1983.*

68. **Hyatt BA, Shangguan X, Shannon JM**: FGF-10 induces SP-C and Bmp4 and regulates proximal-distal patterning in embryonic tracheal epithelium. *Am J Physiol Lung Cell Mol Physiol. 287: 1116-26, 2004.*

69. **Ingham PW, McMahon AP**: Hedgehog signaling in animal development: paradigms and principles. *Genes Dev. 15: 3059-87, 2001.*

70. **Inanlou MR, Kablar B:** Abnormal development of the diaphragm in mdx: MyoD-/-(9th) embryo leads to pulmonary hypoplasia. *Int J Dev Biol. 47:363-71, 2003.*

71. **Iritani I**: Experimental study on embryogenesis of congenital diaphragmatic hernia. *Anat Embryol (Berl). 169: 133-9, 1984.*

72. **Jaillard SM, Pierrat V, Duboi A:** Outcome at 2 years of infants with congenital diaphragmatic hernia: a population-based study. *Ann Thorac Surg. 75: 250-6, 2003.*

73. **Jeffery PK:** The development of large and small airways. *A J Respir Crit Care Med 157: 174-180, 1998.*

74. **Jeffery TK, Upton PD, Trembath RC**: BMP4 inhibits proliferation and promotes myocyte differentiation of lung fibroblasts via Smad1 and JNK pathways. *Am J Physiol Lung Cell Mol Physiol. 288: 370-8, 2005.*

75. **Jesudason EC**: Challenging embryological theories on congenital diaphragmatic hernia: future therapeutic implications for paediatric surgery. *Ann R Coll Surg Engl. 84: 252-9, 2002.*

76. **Joe P, Wallen LD**: Effects of mechanical factors on growth and maturation of the lung in fetal sheep. *Am J Physiol Lung Cell Mol Physiol 272: 95-105,1997.*

77. **Kanai M, Kitano Y, von Allmen D, Davies P**: Fetal tracheal occlusion in the rat model of nitrofen-induced congenital diaphragmatic hernia: tracheal occlusion reverses the arterial structural abnormality. *J Pediatr Surg. 36: 839-45, 2001.*

78. **Kaufman, MH**: The Atlas of Mouse Development. *Academic Press, Orlando, FL. 1992, ISBN 0-1240-2035-6.*

79. **Keijzer R, Liu J, Ge X, Verbeken E**: Dual-hit hypothesis explains pulmonary hypoplasia in the nitrofen model of congenital diaphragmatic hernia. *Am J Pathol. 156: 1299-306, 2000.*

80. **Keller RL, Hawgood S, Neuhaus JM, Harrison MR**: Infant pulmonary function in a randomized trial of fetal tracheal occlusion for severe congenital diaphragmatic hernia. *Pediatr Res. 56: 818-25, 2004.*

81. **Kikkawa Y, Motoyama EK, Gluck L**: Study of the lungs of fetal and newborn rabbits. Morphologic, biochemical, and surface physical development. *Am J Pathol. 52:177-210, 1968.*

82. **Kinsella J, Truog W, Walsh WF, Goldberg RN, Bancalari EM**: Randomized, multicenter trial of inhaled nitric oxide and high-frequency oscillatory ventilation in severe, persistent pulmonary hypertension of the newborn. *J Pediatr 131:51-62, 1997.*

83. **Kitano Y, Flake AW, Quinn TM**: Lung growth induced by tracheal occlusion in the sheep is augmented by airway pressurization. *J Pediatr Surg* 35:216-222, 2000.

84. **Kitano Y, Von Allmen D, Kanai M**: Fetal lung growth after short-term tracheal occlusion is linearly related to intratracheal pressure. *J Appl Physiol. 90: 493-500, 2001.*

85. **Kitano Y, Davies P, von Allmen D, Adzick NS, Flake AW**: Fetal tracheal occlusion in the rat model of nitrofen-induced congenital diaphragmatic hernia. *J Appl Physiol. 87: 769-75, 1999.*

86. **Kitano Y, Kanai M, Davies P, von Allmen D**: Lung growth induced by

prenatal tracheal occlusion and its modifying factors: a study in the rat model of congenital diaphragmatic hernia. *J Pediatr Surg. 36: 251-9, 2001.*

87. **Kitano Y, Adzick NS**: New developments in fetal lung surgery. *Curr Opin Pediatr. 11: 193-9, 1999.*
88. **Kitaoka H, Burri PH, Weibel ER**: Development of the human fetal airway tree - analysis of the numerical density of airway end tips. *Anat Rec 244: 207-213, 1996.*
89. **Kluth D, Kangah R, Reich P**: Nitrofen-induced diaphragmatic hernias in rats: an animal model. *J Pediatr Surg. 25: 850-4, 1990.*
90. **Kluth D Keijzer R, Hertl M, Tibboel D**: Embryology of congenital diaphragmatic hernia. *Semin Pediatr Surg. 5: 224-33, 1996.*
91. **Kluth D, Losty PD, Schnitzer JJ**: Towards understanding the developmental anatomy of congenital diaphragmatic hernia. *Clin Perinatol. 23: 655-671, 1996.*
92. **Kobayashi H, Puri P**: Plasma endothelin levels in congenital diaphragmatic hernia. *J Pediatr Surg 29:1258-61, 1994.*
93. **Kotecha S**: Lung growth: implications for the newborn infant. *Arch Dis Child Fetal Neonatal Ed. 82: F69-74, 2000.*
94. **Langston C, Kida K, Reed M**: Human lung growth in late gestation and in the neonate. *Am Rev Respir Dis 129:607-13, 1984.*
95. **Larsen WJ**: Human Embryology. *3rd Ed. New York: Churchill Livingstone, 2001.*
96. **Lassus P, Turanlahti M, Heikkila P**: Pulmonary vascular endothelial growth factor and Flt-1 in fetuses, in acute and chronic lung disease, and in persistent pulmonary hypertension of the newborn. *Am J Respir Crit Care Med. 164:1981-7, 2001.*
97. **Lebeche D, Malpel S, Cardoso WV:** Fibroblast growth factor interactions in the developing lung. *Mech Dev. 86:125-36, 1999.*
98. **Leinwand MJ, Tefft JD, Zhao J, Coleman C**: Nitrofen inhibition of pulmonary growth and development occurs in the early embryonic mouse. *J Pediatr Surg. 37: 1263-8, 2002.*

99. **Levy M, Maurey C, Dinh-Xuan AT, Vouhe P**: Developmental expression of vasoactive and growth factors in human lung: Role in pulmonary vascular resistance adaptation at birth. *Pediatr Res. 57:21R-25R, 2005.*

100. **Liao SL, Luks FI, Piasecki GJ**: Late-gestation tracheal occlusion in the fetal lamb causes rapid lung growth with type II cell preservation. *J Surg Res. 92:64-70, 2000.*

101. **Lipsett J, Cool JC, Runciman SI:** Morphometric analysis of preterm fetal pulmonary development in the sheep model of congenital diaphragmatic hernia. *Pediatr Dev Pathol. 3:17-28, 2000.*

102. **Litingtung Y, Lei L, Westphal H, Chiang C**: Sonic hedgehog is essential to foregut development. *Nat Genet. 20:58-61, 1998.*

103. **Losada A, Tovar JA, Xia HM**: Down-regulation of thyroid transcription factor-1 gene expression in fetal lung hypoplasia is restored by glucocorticoids. *Endocrinology. 141:2166-73, 2000.*

104. **Luks FI, Roggin KK, Wild YK, Piasecki GJ**: Effect of lung fluid composition on type II cellular activity after tracheal occlusion in the fetal lamb. *J Pediatr Surg. 36:196-201, 2001.*

105. **Luks FI, Wild YK, Piasecki GJ, De Paepe ME**: Short-term tracheal occlusion corrects pulmonary vascular anomalies in the fetal lamb with diaphragmatic hernia. *Surgery. 128:266-72, 2000.*

106. **Manson JM**: Mechanism of nitrofen teratogenesis. *Environ Health Perspect. 70:137-47, 1986.*

107. **McDonald JA**: Lung Biology in Health and Disease; *Volume 100. New York, Marcel Dekker, 1997.*

108. **Mercurio AR, Rhodin JAG:** An electron microscopic study on the Type 1 pneumocyte in the cat: differentiation; *Am J Anat 146:255-272, 1976.*

109. **Moore KL**: Development of body cavities, primitive mesenteries and the diaphragm In "*The Developing Human: Clinically Oriented Embryology" pp159-169 WB Saunders Company, Philadelphia, 1988.*

110. **Morishige WK, Joun NS**: Influence of glucocorticoids on postnatal lung development in the rat: possible modulation by thyroid hormone.

Endocrinology 111:1587-94, 1982.

111.**Moya FR, Thomas VL, Mysore MR**: Fetal lung maturation in congenital diaphragmatic hernia. *Am J Obstet Gynecol. 1995 Nov; 173(5): 1401-5.*

112.**Mullor JL, Sánchez P, Ruiz I Altaba A**: Pathways and consequences: Hedgehog signaling in human disease. *Trends Cell Biol. 12:562-9, 2002.*

113.**Munson L, Chassy LM, Asa C**: Efficacy, safety and reversibility of bisdiamine as a male contraceptive in cats. *Theriogenology. 62:81-92,2004.*

114.**Muratore CS, Uttar S and Jaksic T**: Nutritional morbidity in survivors of congenital diaphragmatic hernia. *J Pediatr Surg. 36:1171-6, 2001.*

115.**Muratore CS, Wilson JM**: Congenital diaphragmatic hernia: where are we and where do we go from here? *Semin Perinatol. 24:418-28, 2000.*

116.**Nardo L, Hooper SB, Harding R**: Stimulation of lung growth by tracheal obstruction in fetal sheep: relation to luminal pressure and lung liquid volume. *Pediatr Res. 43:184-90, 1998.*

117.**National Toxicology Program**: Bioassay of nitrofen for possible carcinogenicity. *Natl Cancer Inst Carcinog Tech Rep Ser. 26:1-87, 1978.*

118.**Nobuhara KK, Fauza DO, DiFiore JW**: Continuous intrapulmonary distension with perfluorocarbon accelerates neonatal (but not adult) lung growth. *J Pediatr Surg 33: 292-298, 1998.*

119.**Ohi R, Suzuki H, Kato T**: Development of the lung in fetal rabbit with experimental diaphragmatic hernia. *J Pediatr Surg 11:955-959, 1976.*

120.**Okazaki T, Sharma HS, McCune SKl**: Pulmonary vascular balance in congenital diaphragmatic hernia: Enhanced endothelin-1gene expression as a possible cause of pulmonary vasoconstriction. *J Pediatr Surg 33:81-84, 1998.*

121.**Ontario Congenital Anomalies Study Group:** Apparent truth about congenital diaphragmatic hernia: a population-based database is needed to establish benchmarking for clinical outcomes for CDH. *J Pediatr Surg. 39:661-5, 2004.*

122.**O'Toole SJ, Irish MS, Holm BA, Glick PL:** Pulmonary vascular abnormalities in congenital diaphragmatic hernia. *Clin Perinatol. 23:781-94, 1996.*

123.**O'Toole SJ, Karamanoukian HL, Irish MS**: Tracheal ligation: the dark side of in utero congenital diaphragmatic hernia treatment. *J Pediatr Surg. 32:407-10, 1997.*

124.**Oyewumi L, Kaplan F, Sweezey NB**: Lgl1, a mesenchymal modulator of early lung branching morphogenesis, is a secreted glycoprotein imported by late gestation lung epithelial cells. *Biochem J. 376:61-9, 2003.*

125.**Paci M, de Franco S**: Septum transversum diaphragmatic hernia in an adult. *J Thorac Cardiovasc Surg. 129: 444-5, 2005.*

126.**Pascal de Lagausie, Anthony de Buys-Roessingh, Ferkdadji L**: Endothelin receptor expression in human lungs of newborns with congenital diaphragmatic hernia. *Pathol. 205:112-8, 2005.*

127.**Pepicelli CV, Lewis PM, and McMahon AP**: Sonic hedgehog regulates branching morphogenesis in the mammalian lung. *Curr Biol. 8:1083-6, 1998.*

128.**Perl AK, Whitsett JA**: Molecular mechanisms controlling lung morphogenesis. *Clin Genet. 56:14-27, 1999.*

129.**Portnoy J, Curran-Everett D, Mason RJ**: Keratinocyte growth factor stimulates alveolar type II cell proliferation through the extracellular signal-regulated kinase and phosphatidylinositol 3-OH kinase pathways. *Am J Respir Cell Mol Biol. 30: 901-7, 2004.*

130.**Pringle KC:** Human fetal lung development and related animal models. *Clin Obstet Gynecol. 29:502-13, 1986.*

131.**Prodhan P, Kinane TB**: Developmental paradigms in terminal lung development. *Bioessays. 24:1052-9, 2002.*

132.**Quinn TM, Sylvester KG, Kitano Y**: TGF-beta2 is increased after fetal tracheal occlusion. *J Pediatr Surg. 34:701-4, 1999.*

133.**Ramirez MI, Rishi AK, Williams MC**: TGT3, thyroid transcription factor I, and Sp1 elements regulate transcriptional activity of the 1.3-kilobase pair promoter of T1_, a lung alveolar type I cell gene. *J Biol Chem 272: 26285-26294, 1997.*

134.**Reale FR, Esterly JR**: Pulmonary hypoplasia: a morphometric study of the lungs of infants with diaphragmatic hernia, anencephaly, and renal

malformations. *Pediatrics. 51:91-96, 1973.*

135.**Robnett-Filly B, Goldstein RB, Sampior D, Hom M**: Morgagni hernia: a rare form of congenital diaphragmatic hernia. *J Ultrasound Med. 22:537-9, 2003.*

136.**Roth-Kleiner M, Post M**: Similarities and dissimilarities of branching and septation during lung development. *Pediatr Pulmonol. 40:113-34, 2005.*

137.**Rottier R, Tibboel D:** Fetal lung and diaphragm development in congenital diaphragmatic hernia. *Semin Perinatol. 29:86-93, 2005.*

138.**Roubliova XI, Verbeken EK, Wu J, Vaast P**: Effect of tracheal occlusion on peripheric pulmonary vessel muscularization in a fetal rabbit model for congenital diaphragmatic hernia. *Am J Obstet Gynecol. 191:830-6, 2004.*

139.**Ruiz I Altaba A, Palma V, Dahmane N**: Hedgehog-Gli signaling and the growth of the brain; *Nat Rev Neurosci. 3:24-33, 2002.*

140.**Sekine K, Ohuchi H, Fujiwara M, Yamasaki M**: Fgf10 is essential for limb and lung formation. *Nat Genet. 21:138-141, 1999.*

141.**Shehata SM, Mooi WJ, Okazaki T**: Enhanced expression of vascular endothelial growth factor in lungs of newborn infants with congenital diaphragmatic hernia and pulmonary hypertension. *Thorax. 54:427-31, 1999.*

142.**Siebert J.R, Haas J.E, Beckwith J.B**: Left ventricular hypoplasia in congenital diaphragmatic hernia. *J Pediatr Surg 19: 567-570, 1984.*

143.**Silver MM, Thurston WA, Patrick JE**: Perinatal pulmonary hyperplasia due to laryngeal atresia. *Hum Pathol 19:110-3, 1988.*

144.**Skandalakis, JE, Gray SW, Symbas P**: The trachea and lungs: Embryology for Surgeons. *Williams and Wilkins, pp 414-450, 1994.*

145.**Skari H, Bjornland K, Haugen G, Egeland T**: Congenital diaphragmatic hernia: a meta-analysis of mortality factors. *J Pediatr Surg. 35:1187-97, 2000.*

146.**Smith NP, Jesudason EC, Featherstone NC:** Recent advances in congenital diaphragmatic hernia. *Arch Dis Child. 90:426-8, 2005.*

147.**Smith NP, Jesudason EC, Losty PD**: Congenital diaphragmatic hernia. *Paediatr Respir Rev. 3:339-48, 2002.*

148.**Solomon HM, Wier PJ, Johnson CM, Posobiec LM**: Benzofuranyl ureas

with potent cardiovascular teratogenicity in rats. *Teratology. 61:211-221, 2000.*

149.**Sparrow MP, Lamb JP**: Ontogeny of airway smooth muscle: structure, innervation, myogenesis and function in the fetal lung. *Respir Physiol Neurobiol. 137:361-72, 2003.*

150.**Stefanutti G, Filippone M and Tommasoni N:** Cardiopulmonary anatomy and function in long-term survivors of mild to moderate congenital diaphragmatic hernia. *J Pediatr Surg. 39:526-31, 2004.*

151.**Stege G, Fenton A, Jaffray B**: Nihilism in the 1990s: The True Mortality of Congenital Diaphragmatic Hernia. *Pediatrics 112:532-535, 2003.*

152.**Stolar CJ, Dillon PW, Stalculp SA**: Extracorporeal membrane oxygenation and congenital diaphragmatic hernia: modification of the pulmonary vasoactive profile. *J Pediatr Surg. 20:681-3, 1985.*

153.**Stecca B, Ruiz I Altaba A**: The therapeutic potential of modulators of the Hedgehog-Gli signaling pathway. *J Biol. 1: 9, 2002.*

154.**Sutherland MF, Parkinson MM, Hallett P**: Teratogenicity of three substituted 4-biphenyls in the rat as a result of the chemical breakdown and possible metabolism of a thromboxane A2-receptor blocker. *Teratology. 39: 537-45, 1989.*

155.**Sweed Y, Puri P**: Congenital diaphragmatic hernia: Influence of associated malformations on survival. *Arch Dis Child 69:68-70, 1993.*

156.**Sydorak RM, Harrison MR**: Congenital diaphragmatic hernia: advances in prenatal therapy. *Clin Perinatol. 30:465-79, 2003.*

157.**Sylvester KG, Rasanen J, Kitano Y, Flake AW**: Tracheal occlusion reverses the high impedance to flow in the fetal pulmonary circulation and normalizes its physiological response to oxygen at full term. *J Pediatr Surg. 33:1071-4, 1998.*

158.**Taira Y, Miyazaki E, Ohshiro K**: Administration of antenatal glucocorticoids prevents pulmonary artery structural changes in nitrofen-induced congenital diaphragmatic hernia. *J Pediatr Surg. 33: 1052-1056, 1998.*

159.**Taleporos P, Salgo MP, Oster G**: Teratogenic action of bis (dichloroacetyl)

-diamine on rats: patterns of malformations produced in high incidence at time-limited periods of development. *Teratology. 18:5-15, 1978.*

160.**Teramoto H, Yoneda A, Puri P**: Gene expression of fibroblast growth factors 10 and 7 is downregulated in the lung of nitrofen-induced diaphragmatic hernia in rats. *J Pediatr Surg. 38:1021-4, 2003.*

161.**Thebaud B, Tibboel D, Rambaud C**: Vitamin A decreases the incidence and severity of nitrofen-induced congenital diaphragmatic hernia in rats. *Am J Physiol. 277:423-9, 1999.*

162.**Thebaud B, Barlier-Mur AM, Chailley-Heu B**: Restoring effects of vitamin A on surfactant synthesis in nitrofen-induced congenital diaphragmatic hernia in rats. *Am J Respir Crit Care Med. 164:1083-9, 2001.*

163.**Thebaud B, Mercier JC, Dinh-Xuan AT**: Congenital diaphragmatic hernia. A cause of persistent pulmonary hypertension of the newborn which lacks an effective therapy, *Biol Neonate. 74:323-36, 1998.*

164.**Thibeault DW, Haney B**: Lung volume, pulmonary vasculature, and factors affecting survival in congenital diaphragmatic hernia; *Pediatrics. 101:289-95, 1998.*

165.**Thurlbeck WM**: Prematurity and developing lung. *Clin perinatol. 19:497-519, 1992.*

166.**Timothy P. S, Patricia R. C, Kenneth MM**: Survival in Early- and Late-Term Infants with Congenital Diaphragmatic Hernia Treated with Extracorporeal Membrane Oxygenation. *PEDIATRICS 110: 590-596, 2002.*

167.**Tschanz SA, Burri PH**: Postnatal lung development and its impairment by glucocorticoids. *Pediatr Pulmonol Suppl. 16:247-9, 1997.*

168.**Ulich TR, Yi ES, Longmuir K, Yin S**: Keratinocyte growth factor is a growth factor for type II pneumocytes in vivo. *Clin Invest 93:1298-1306, 1994.*

169.**Unger S, Copland I, Tibboel D**: Down-regulation of sonic hedgehog expression in pulmonary hypoplasia is associated with congenital diaphragmatic hernia. *Am J Pathol. 162:547-55, 2003.*

170.**Van Meurs KP, Robbins ST:** Congenital diaphragmatic hernia: long-term outcome in neonates treated with extracorporeal membrane oxygenation.

J Pediatr. 122:893-9, 1993.

171. **Walterhouse DO, Lamm ML, Villavicencio E**: Emerging roles for hedgehog-patched-Gli signal transduction in reproduction. *Biol Reprod. 69:8-14, 2003.*

172. **Weaver M, Dunn NR, Hogan BL**: Bmp-4 and fgf10 play opposing roles during lung bud morphogenesis. *Development. 127:2695-704, 2000.*

173. **Wigglesworth JS, Desai R**: Effect on lung growth of cervical cord section in the rabbit fetus. *Early Hum Dev. 3:51-65, 1979.*

174. **Wijgerde M, Ooms M, Hoogerbrugge JW, Grootegoed JA**: Hedgehog signaling in mouse ovary: Indian hedgehog and desert hedgehog from granulosa cells induce target gene expression in developing theca cells. *Endocrinology. 146(8): 3558-66, 2005.*

175. **Wild YK, Piasecki GJ, De Paepe ME**: Short-term tracheal occlusion in fetal lambs with diaphragmatic hernia improves lung function, even in the absence of lung growth. *Journal of Pediatric Surgery. 35:775-779, 2000.*

176. **Wilson JM, DiFiore JW, Peters CA**: Experimental fetal tracheal ligation prevents the pulmonary hypoplasia associated with fetal nephrectomy: possible application for congenital diaphragmatic hernia. *J Pediatr Surg. 28:1433-9, 1993.*

177. **Wu J, Ge X, Verbeken**: Pulmonary effects of in utero tracheal occlusion are dependent on gestational age in a rabbit model of diaphragmatic hernia. *J Pediatr Surg. 37:11-7, 2002.*

178. **Wu J, Yamamoto H, Gratacos E**: Lung development following diaphragmatic hernia in the fetal rabbit. *Hum Reprod. 15:2483-8, 2000.*

179. **Yoshizawa J, Chapin CJ, Sbragia L, Ertsey R**: Tracheal occlusion stimulates cell cycle progression and type I cell differentiation in lungs of fetal rats. *Am J Physiol Lung Cell Mol Physiol. 285:344-53, 2003.*

180. **Zeltner TB, Caduff JH, Gehr P**: The postnatal development and growth of the human lung. *Morphometry Respir Physiol. 67:247-267, 1987.*

181. **Zoetis T, Hurtt ME**: Species comparison of lung development. *Birth Defects Res B Dev Reprod Toxicol. 68:121-4, 2003.*

Table 1: Stages of lung development

	Embryonic	Pseudo-glandular	Canalicular	Saccular	Alveolar
Rat (term=22)	E 9.5 to 12.5	E 12.5 to 16.5	E 16.5 to 17.5	E 17.5 to P 4	P 4 to P 14
Rabbit (term=31)	DGA 5 to 19	DGA 19 to 24	DGA 24 to 27	DGA 27 to 29	DGA 29 to 31
Human (term= 42 weeks)	Week 3 to 7	Week 7 to 16	Week 16 to 26	Week 26 to 36	Week 36 to postnatal year2

Legend: The five stages of lung development as described in the literature.

E = embryonic days of gestation in rats. P = Postnatal days in rats

Table 2: Comparison of LW/BW and MTBD in TO vs. Control groups in rabbits

N = 5	LW/BW		MTBD	
	Control	TO	Control	TO
Mean	0.021	0.032	10.31	7.725
Standard Deviation	0.003	0.007	0.089	0.491
P-value	0.004		0.006	

Legend: TO induced growth in fetal rabbit lungs with higher values for LW/BW and low MTBD in comparison to controls. The difference for these indices between the Control and the TO group was statistically significant.
LW/BW: Lung weight to body weight ratio.
MTBD: Mean terminal bronchial density.

Table 3: LW/BW in the controls, CDH+ and CDH- groups of fetal rats

LW/BW	Control (n = 17)	CDH+ (n =15)	CDH- (n =19)
Mean	0.0379	0.0307	0.0327
Standard deviation	0.0034	0.0052	0.0062

Legend: P-value for the difference between the Control and the CDH+ group was 0.001 and between the Control and the CDH-groups, 0.003. No significant difference between CDH+ and CDH- groups.

Table 4: Comparison of LW/BW amongst various groups in rats

	Control (n = 17)	TO (n = 8)	CDH + (n = 15)	CDH+ plus TO (n = 16)
Mean	0.0379	0.0544	0.0307	0.0361
Standard deviation	0.0033	0.0077	0.0052	0.0022

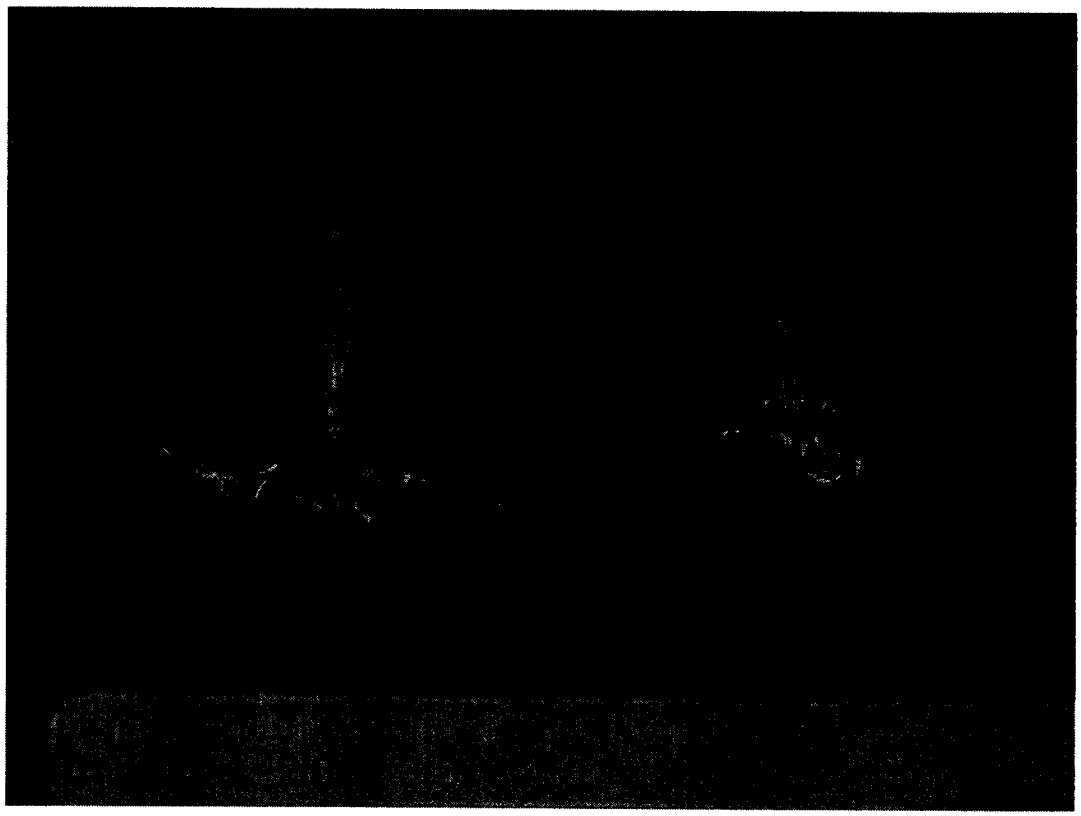
Legend: The lung weight to body weight ration (LW/BW) before and after tracheal occlusion (TO) of various groups is compared in this table. The P value for the difference between the control and the TO groups was 0.001 and for the CDH+ and the CDH+ plus TO group was 0.021. There was no significant difference between control and CDH+ TO animals.

Table 5: mRNA expression levels of Shh in Nitrofen-treated lungs vs. Controls at 21 days of gestation.

Category	Mean value	Standard deviation	Confidence interval	
			Lower value	Higher value
CDH+ (n =12)	6.13E-02 (613×10^{-4})	1.09E-02 (109×10^{-4})	5.04E-02 (504×10^{-4})	7.21E-02 (721×10^{-4})
CDH- (n =12)	5.93E-02 (593×10^{-4})	1.33E-02 (133×10^{-4})	4.60E-02 (460×10^{-4})	7.26E-02 (726×10^{-4})
Controls (n =12)	2.59E-02 (259×10^{-4})	1.21E-02 (121×10^{-4})	1.38E-02 (138×10^{-4})	3.80E-02 (380×10^{-4})
CDH+ plus TO (n=8)	1.28E-02 (128×10^{-4})	1.15E-02 (115×10^{-4})	1.05E-02 (105×10^{-4})	2.95E-02 (295×10^{-4})
TO (n= 12)	1.55E-02 (155×10^{-4})	1.01E-02 (101×10^{-4})	1.23E-02 (123×10^{-4})	2.40E-02 (240×10^{-4})

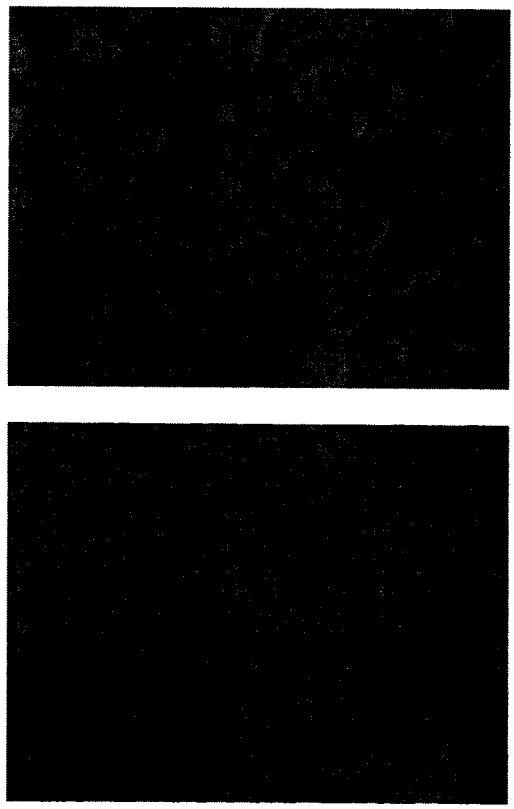
Legend: Nitrofen-treated lungs (CDH + & CDH-) had higher Shh-mRNA expression levels in comparison to the Control group. These values were extrapolated from a standard curve based on house keeping gene and are expressed as exponentials (E).

Figure 1: TO-induced lung growth in fetal rabbits



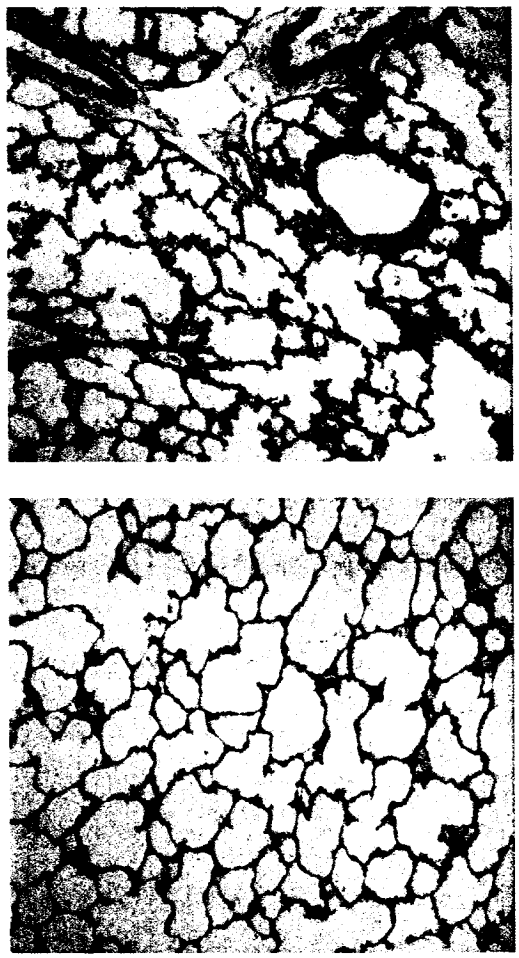
Legend: TO, at 27 DGA in fetal rabbits, resulted in larger lungs (left) compared to non-occluded control (right) evidenced by naked eye examination.

Figure 2: Lung histology of fetal rabbit at different stages of development.



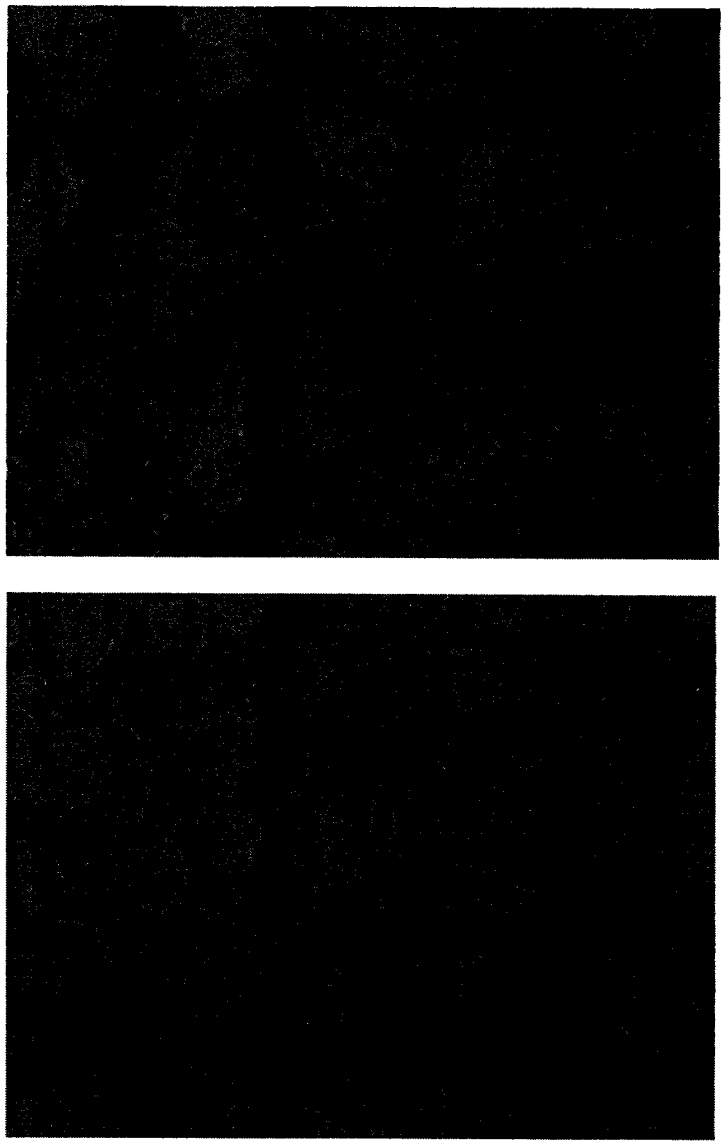
Legend: Lung histology (H&E staining) of fetal rabbit lungs at two different stages of lung development. The pseudo-glandular stage of lung at 23 days of GA (Above) and canalicular stage at 27 days of GA (below). Note thinned interstitial tissue and increase branching of alveolar spaces in the canalicular stage compared to thick interstitium and closed bronchioles of the pseudo-glandular stage.

Figure 3: Lung histology of fetal rabbit after TO



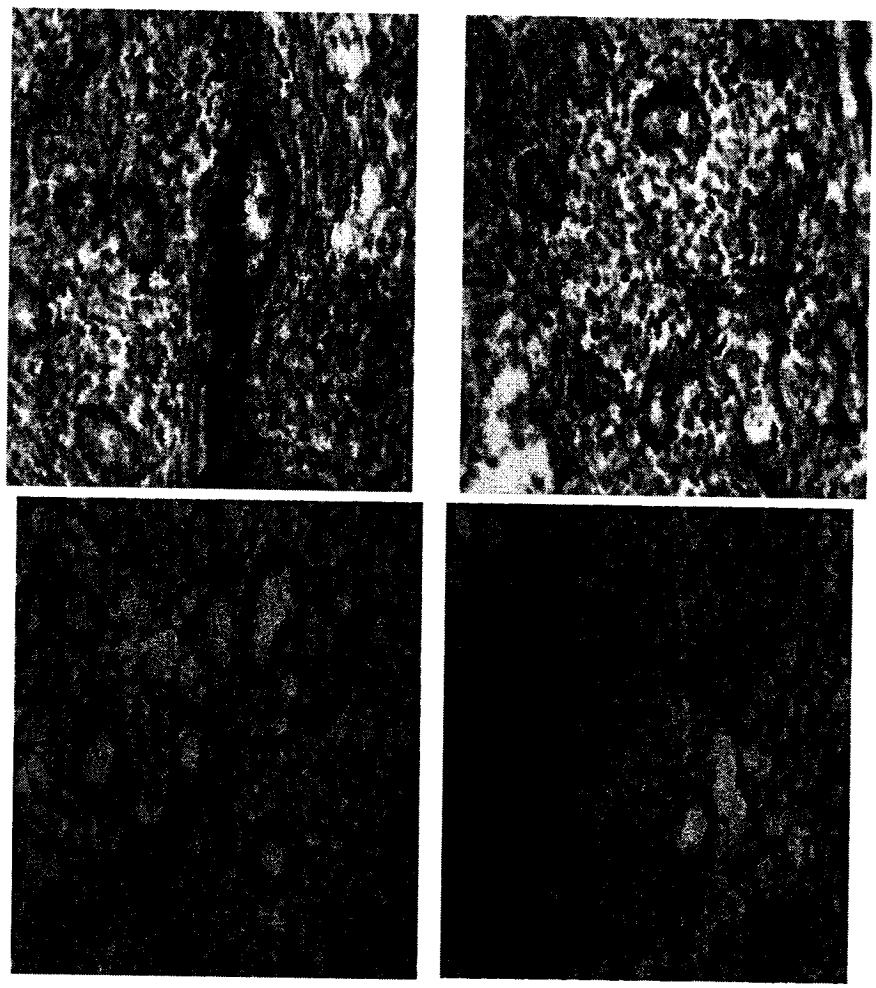
Legend: Lung histology (H&E staining) of control group (above) vs. TO (below) at 30 DAG. The TO lung shows more alveoli compared to control group. The inter-alveolar septae are thinner in the TO group compared to the control group.

Figure 4: Rat lung histology of the CDH+ vs. control lungs at E 21



Legend: Nitrofen-affected CDH lungs (above) show thicker interstitial septae than control lungs (below). Magnification x40.

Figure 5: IHC for Shh in fetal rat lungs at different stages of lung growth

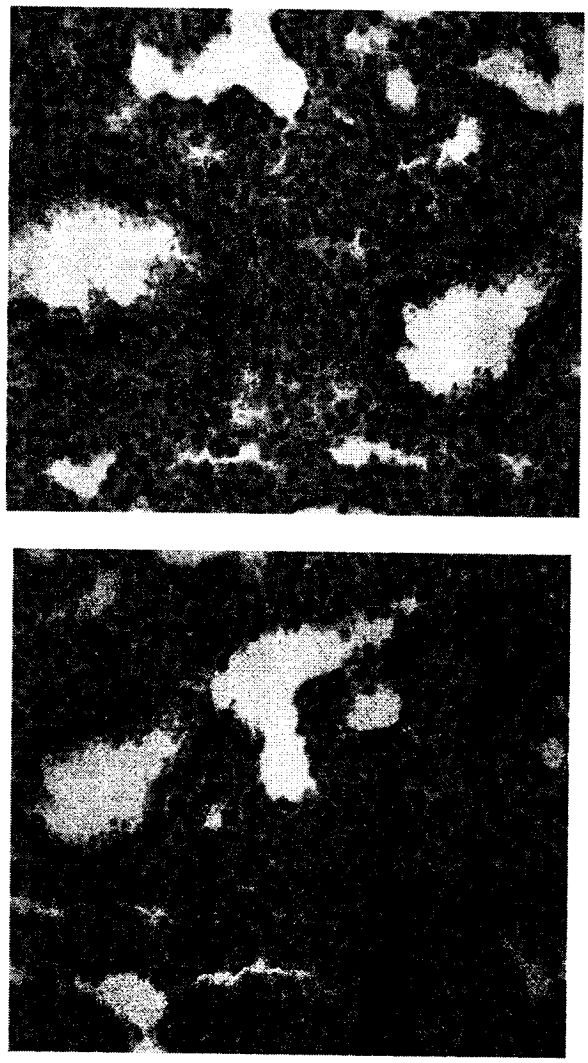


CDH+: Day 20 (above)
Day 21 (below)

Control: Day 20 (above)
Day 21 (below)

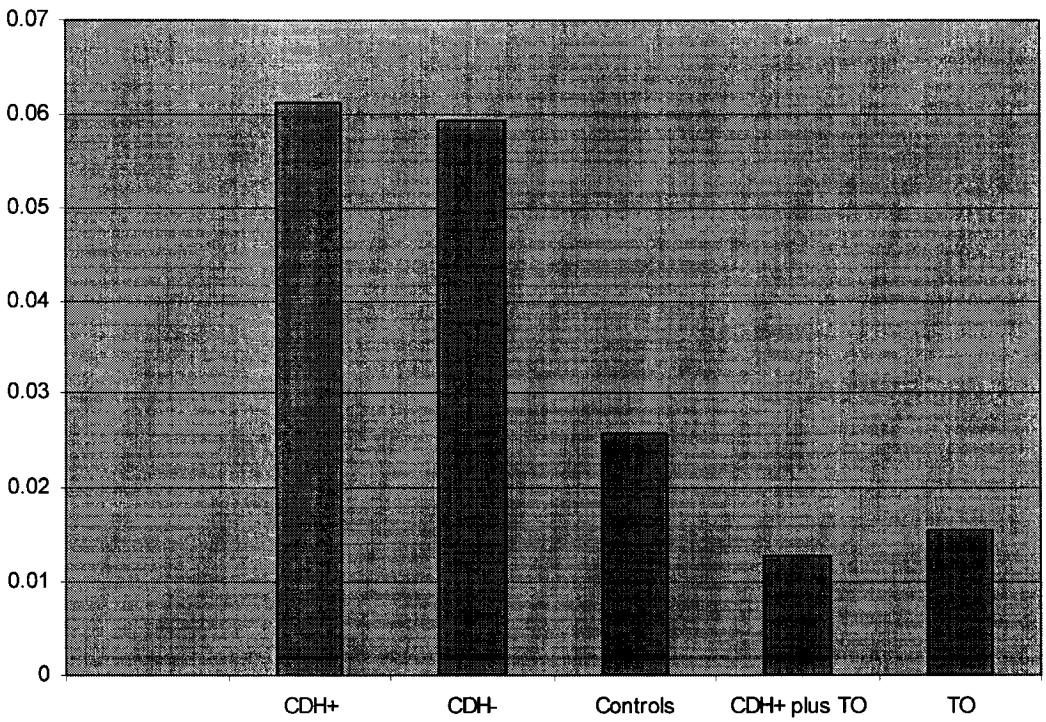
Legend: IHC staining for Shh (brown pigment) of CDH+ and control fetal rat lungs at day 20 and 21 of gestation. Magnification is x40.

Figure 6: IHC for Shh in fetal rat lungs at 20 days of gestation.



Legend: IHC for Shh (brown pigment) in day 20 specimens of CDH+ (above) and control (below) fetal rat lungs. Magnification: x60.

Figure 7: mRNA expression levels of Shh in fetal rat lungs at 21 days of gestation



Legend: Nitrofen-affected (CDH+ and CDH-) lungs show higher levels of expression in comparison to controls. TO lowers the mRNA to the control levels.