Reversible Tracheal Obstruction in the Fetal Sheep: Effects on Tracheal Fluid Pressure and Lung Growth with Implications in Fetal Surgery

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The Title of the thesis by Ezat Hashim which is to be used for the **spine** of the thesis is the following:

Fetal Tracheal Obstruction: Effects on Lung Fluid Pressure and Growth

Contents

Abstract

Congenital diaphragmatic hernia (CDH) continues to carry a high morbidity and mortality. A number of treatment modalities including extracorporeal membrane oxygenation (ECMO) and *in utero* repair have minimally improved the mortality rate of this condition, mostly due to insufficient lung mass at birth and persistent pulmonary hypertension postnatally.

Experimental fetal tracheal ligation has been shown to increase lung growth *in utero* and to reduce the hernial contents in CDH. The purpose of this study was to determine the effect of reversible fetal tracheal occlusion on lung development. Nine fetal sheep were divided into two groups. Group 1 had intratracheal balloons placed and the balloons left inflated for 21-28 days. Group 2 consisted of littermates that served as controls. They had either uninflated balloons placed or were left unoperated. Tracheal pressure measurements were periodically recorded and the amniotic fluid pressure served as a reference. The animals were sacrificed near term and the lungs, heart and liver were weighed and corrected for body weight. Standard morphometry was used to compare the lungs further and the lung DNA and protein content were measured. Tracheal damage from the balloon catheter was also assessed.

Tracheal pressure was 3.85 (± .49 S.E.) mm Hg in experimental animals while it was an average of -0.27 (± .27 S.E.) mm Hg in controls ($p<.0001$). Tracheal occlusion increased lung weight and volume by 2-3 times ($p<.0001$ and $p=.0006$, respectively) while heart and liver weights remained similar to controls. Airspace fraction and radial alveolar counts were raised ($p=.044$ and $p=.0002$, respectively) and alveolar number per body weight was doubled ($p<.0001$). The alveolar number per lung volume was preserved, however, as was the DNA and protein content per unit weight of lung tissue. The chronic indwelling balloon catheter caused some mucosal and submucosal damage at the balloon site and proximal to it.

These results show that tracheal occlusion leads to an elevation of intratracheal pressure that is associated with a tremendous increase in lung growth over a short period in the third trimester fetal sheep.

The techniques used in this experiment may be easily modified for use with endoscopic surgical equipment.

Abrégé

La hernie diaphragmatique continue d'enregistrer un taux élevé de morbidité et de mortalité. On doit ce taux élevé à une masse pulmonaire insuffisante à la naissance et à une hypertension pulmonaire postnatale persistante. Bon nombre de traitements, parmi lesquels comptent la circulation extracorporelle par membrane (ECMO) et la correction chirugicale *in utero* , n'ont qu'en très faible partie réussi à en faire chuter le taux de mortalité et de morbidité.

Il y a toutefois une chirugie expérimentale chez le fœtus animal qui a affiché des résultats concluants dans la stimulation de la croissance des poumons *in utero* et dans la réduction du contenu de la hernie diaphragmatique : il s'agit de la ligature de la trachée.

Cette étude vise à déterminer les effets de l'occlusion réversible de la trachée sur le développement des poumons. Pour ce faire, deux groupes de fœtus de moutons du troisième trimestre de la gestation ont été étudiés. On introduisit une sonde à ballonnet dans la trachée des fœtus du premier groupe. Une fois gonflé, le ballonnet fut laissé dans la trachée de 21 à 28 jours. Le second groupe étant un groupe de contrôle, on répéta la même opération chez certains des fœtus, sans toutefois gonfler les ballons, tandis que le reste des fœtus ne subit aucune opération. On enregistra périodiquement la pression du liquide intra-trachéal chez les deux groupes, en se servant de la pression du liquide amniotique comme référence. On interrompit la grossesse peu avant terme afin de peser le corps, les poumons, le coeur et le foie des fœtus. Pour comparer les poumons des fœtus des deux groupes, on utilisa des examens morphométriques standard. De plus, on mesura l'ADN ainsi que le contenu en protéines trouvés dans les poumons et l'on enregistra les dommages trachéaux causés par le cathéter.

Chez les animaux expérimentaux, la pression trachéale était de 3,85 (± ,49 écart type) mm Hg; chez les animaux du groupe de contrôle, elle était en moyenne de -0,27 (± ,27 écart type) mm Hg. L'occlusion de la trachée permit une augmentation de 200 à 300 p. cent du poids et du volume pulmonaires ($p<,0001$ et $p=,0006$). Quant au poids du coeur et du foie, ils demeura sensiblement le même que celui enregistré chez les animaux du groupe de contrôle. La fraction alvéolaire et la

numération radiale alvéolaire («radial alveolar count») augmentèrent (p=,044 et p=,0002) et le nombre d'alvéoles doubla (p<,0001). Le nombre d'alvéoles par volume pulmonaire demeura toutefois inchangé De même, l'ADN et le contenu en protéine par poids pulmonaire restèrent inchangés mais furent augmentés par rapport au poids corporel de l'animal. On nota enfin que le cathéter interne avait causé des lésions au niveau de la muqueuse et de la sous-muqueuse sans toutefois affecter le cartilage.

Ces résultats démontrent que l'occlusion trachéale permet d'augmenter la pression intratrachéale, ce qui est associé à la stimulation de la croissance des poumons sur une courte période dans la mesure où ce traitement est effectuée au cours du troisième trimestre de gestation du fœtus de mouton. Les techniques utilisées au cours de cette expérimentation peuvent facilement être modifiées pour accomoder les équipements de chirurgie endoscopique.

Acknowledgements

The author would like to thank the following individuals for their help leading to the creation of this thesis.

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Dedication

To Sacha, with whom I was able to spend a most splendid and joyous year.

Introduction

The estimated incidence of congenital diaphragmatic hernias (CDH) is about 1 in 2400 births. The condition arises early in fetal development when there is failure in the separation of the thoracic and abdominal spaces. The consequence of this is the persistence of a passage in one or both of the diaphragms through which abdominal contents such as spleen, liver and intestines may enter the thoracic space and interfere with lung development during a critical period. Most newborns who become symptomatic immediately at birth have a high mortality rate, as high as 50% for those cases diagnosed *in utero*. The lung pathology appears to be secondary to compression of the developing lung by intrathoracic herniated abdominal contents. With the advent of prenatal diagnosis of CDH, it was hoped that immediate postnatal support and repair would decrease the mortality. However, morbidity and mortality remain high despite conventional management, including the use of extra corporeal membrane oxygenation (ECMO). Therefore, *in utero* repair is considered a reasonable option in this disease and has been attempted at a limited number of centers around the world, most notably Harrison and associates at UCSF. However, for a variety of reasons, their initial attempts at *in utero* repair of CDH had only a 4 in 14 survival rate. In short, postnatal care often comes too late and direct fetal repair is not the easy answer.

It has been noted clinically that newborns with CDH who are stabilized on a respirator have a slow but steady reduction of the hernial contents back into the abdomen. The positive pressure ventilation seems to push the hernial contents from the chest cavity. If the baby is allowed to breathe spontaneously, the negative pressures generated will quickly revert the hernia to its intrathoracic position.

Fetuses born with tracheal atresia are found to have lungs that are hyperplastic at both the microscopic and macroscopic levels. From this we propose a new approach at *in utero* correction of CDH by using temporary obstruction of the trachea as a vehicle for reducing the

hernia and preventing pulmonary hypoplasia. In this way we hope to avoid the acute hemodynamic changes associated with operative reduction of the hernial contents *in utero*. Furthermore, this method is to be taken one step further and allow the development of fetoscopic treatment of CDH *in utero*, thereby lessening the risk of premature labor associated with hysterotomy.

Lung and Diaphragm Development

Development of the diaphragm [1] [2]

The development of the diaphragm is critical in separating the embryonic abdominal and thoracic cavities . The diaphragm develops from four embryologic structures: the septum transversum, the pleuroperitoneal membranes, the dorsal mesentery of the esophagus and the body wall.

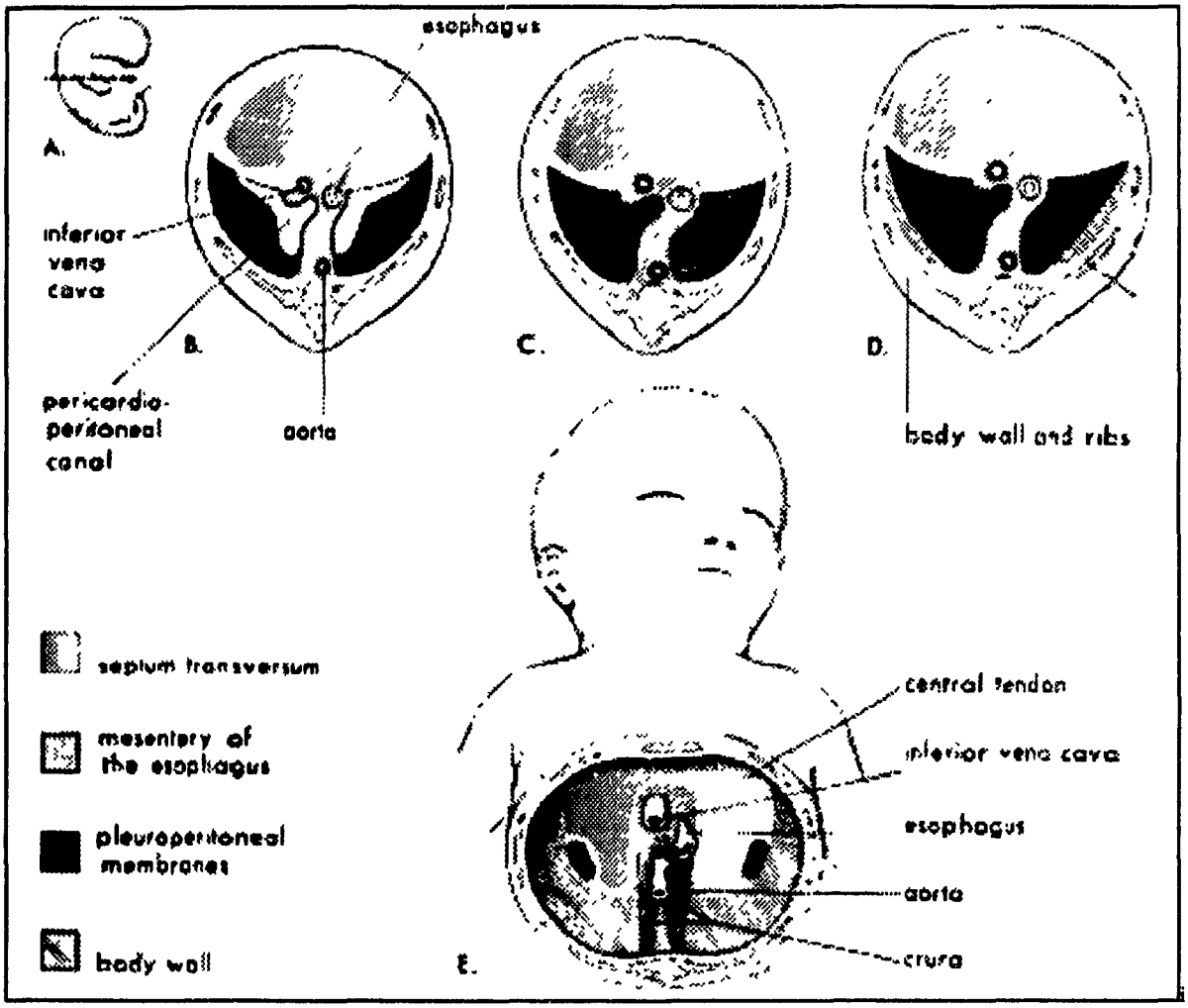

Figure 1: Drawings illustrating development of the diaphragm. *A*, Sketch of a lateral view of an embryo at the end of the fifth week (actual size) indicating the level of the section. *B* to *E* show the diaphragm as viewed from below *B*, Transverse section showing the unfused pleuroperitoneal membranes. *C*, Similar section at the end of the sixth week after fusion of the pleuroperitoneal membranes with the other two diaphragmatic components *D*, Transverse section through a 12-week embryo after ingrowth of the fourth diaphragmatic component from the body wall. *E*, View of the diaphragm of a newborn infant, indicating the probable embryologic origin of its components [1].

The **septum transversum** develops from a mass of mesoderm that eventually becomes a thick incomplete partition (figure 1). This septum fuses dorsally with the primitive mediastinum and pleuroperitoneal membranes. Before head folding begins, the septum transversum lies adjacent to the third, fourth and fifth cervical somites which send out myoblasts and nerves which follow the migration of the septum as the diaphragmatic muscle tissue and phrenic nerves.

The **pleuroperitoneal membranes** develop on either side of the esophagus and fuse with its mesentery as well as the septum transversum. In the developing diaphragm these membranes cover much area but in the newborn they constitute a minor part of the diaphragm (figure 1). Defective formation and/or fusion of the pleuroperitoneal membrane results in a congenital diaphragmatic hernia. This occurs 8 times more commonly on the left side, probably because the right side usually closes earlier. The fusion of these membranes is usually complete by the sixth week and if this does not happen by the tenth week (at which time the intestines pass from the umbilical cord to the abdomen), abdominal contents may herniate into the thoracic cavity.

The **dorsal mesentery of the esophagus** fuses with the septum transversum and pleuroperitoneal membranes and constitutes the median portion of the diaphragm and eventually becomes the crura of the diaphragm when muscle fibers grow into it.

The **body wall** contributes to the posterolateral portion of the diaphragm. This occurs as the pleural cavities enlarge and excavate the costodiaphragmatic recesses. Because of this development, the peripheral part of the diaphragm receives its sensory innervation from the lower six or seven intercostal nerves.

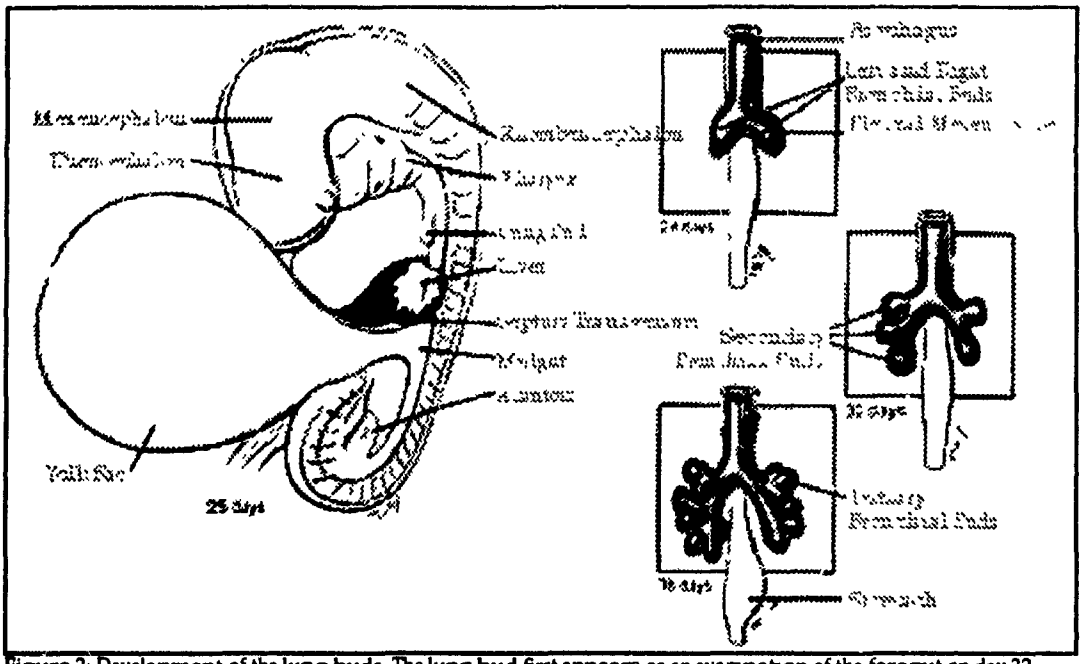

Figure 2: Development of the lung buds. The lung bud first appears as an evagination of the foregut on day 22 and bifurcates into two primary bronchial buds between day 26 and 28 Early in the 5th week, the right bronchial bud branches into three secondary bronchial buds while the left bronchial bud branches into two By the 6th week, secondary bronchial buds branch into tertiary bronchial buds (10 on the right and 8 on the left) to form the bronchopulmonary segments [2].

Development of the bronchi and the lungs

The lungs begin as an outpouching of the primitive gut and is called the **lung bud** (figures 2) which eventually segments into the **bronchial buds**. By the fifth week, the bronchial buds form the primitive primary bronchi as they grow laterally into the primitive pleural cavities . Each primary bronchus develops secondary bronchi and this continues so that by the seventh week there are ten tertiary (segmental) bronchi on the right lung and eight or nine on the left. The surrounding mesenchyme around each segmental bronchus develops to form a bronchopulmonary segment. By 24 weeks, there are 17 orders of branches and the respiratory bronchioles are present.

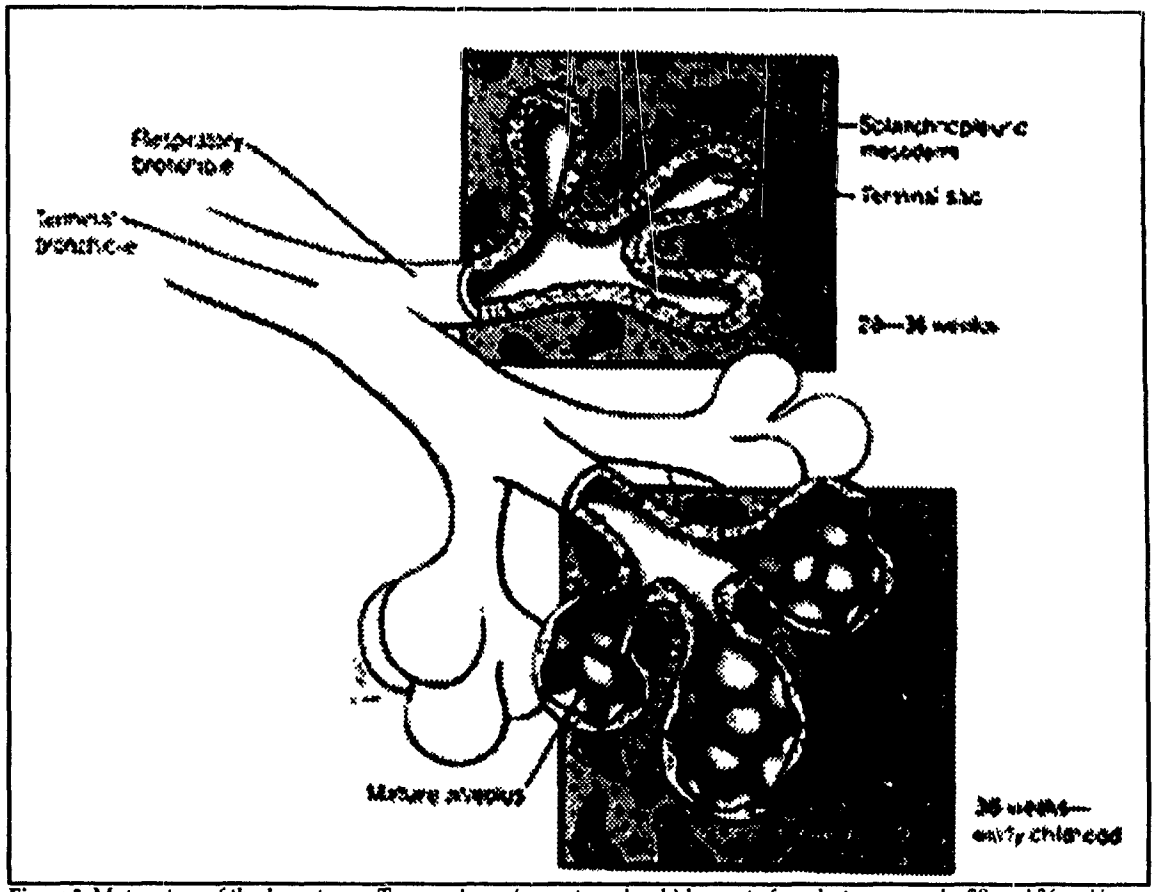

Figure 3: Maturation of the lung tissue. Terminal sacs (primitive alveoli) begin to form between weeks 28 and 36 and begin to mature between 36 weeks and birth. Only 5 to 20 percent of all terminal sacs produced by the age of eight, however, are produced prior to birth [2]

Lung development is divided into 4 stages (figure 3):

1. the **pseudoglandular period** stretches from **week 5 to 17** and the developing lung histologically resembles a gland. By the end of this period, all the major elements of the lung have formed except those involved in gas exchange and therefore respiration is not possible at this time.

2. the **canalicular period** between **week 16 to 25** is when the airway lumina become much larger and lung tissue becomes highly vascular. By the 24th week, the terminal bronchioles have given rise to respiratory bronchioles with primitive alveoli called terminal

sacs. Gas exchange is possible with the presence of the highly vascular terminal sacs.

3. the **terminal sac period** from **24 weeks to birth** is characterized by the multiplication of terminal sacs and their epithelial lining becoming very thin Type 1 alveolar cells. By about 25 to 28 weeks, there is enough development of the vascular network that adequate gas exchange may take place with survival of premature infants. At 23 to 24 weeks type 2 alveolar cells begin to secrete surfactant and by 28 to 32 weeks the amount of surfactant is sufficient to prevent alveolar collapse.

4. the **alveolar period** takes place during the **late fetal period to 8 years of age**. There is further maturation of the alveolar structure with continued wall thinning for better gas exchange as well as a continued multiplication of the number of alveoli from more primitive ones until around 8 years of age. About one eighth to one sixth the adult number of alveoli are present in the newborn.

Hutchins, et al [3] investigated the development of the human tracheobronchial tree by reconstructing the tree from histologic sections corresponding to different gestational ages. The authors describe the development of the tree as an epithelial bud pushing through and compressing a mesenchymal front. When the advancing front becomes less compressible, the epithelial bud grows laterally, causing bifurcations and even trifurcations to develop. They support the idea that progressive branching of the tracheobronchial tree depends on the direct physical investment of the growing epithelial bud by mesenchyme.

Histologically [4], the alveolar epithelial cells that are important in gas exchange are known as Type 1 and 2 alveolar cells. Another

group of cells are called undifferentiated, presumably the progenitors of the above cells. The Type 2 pneumocyte are characterized by their thick, rounded shape and cytoplasmic lamellar bodies that are rich in surfactant. These cells tend to be distributed along the junctions of the alveolar septa, ideally placed to distribute surfactant without impeding gas exchange. Type 1 cells are highly specialized squamous cells that cover a large alveolar surface area, having only a thickness of 0.2 microns. They tend to intimately hug the underlying connective tissue and vascular network and are therefore important in gas exchange.

Clinical Consequences of CDH

The most frequent and most serious diaphragmatic hernia is the posterolateral type known as a Bochdalek hernia [5]. They tend to occur on the left side and are frequently associated with intrathoracic stomach, liver, intestines and other abdominal organs. These ectopic organs occupy pleural space and can impede lung development both ipsilateral and contralateral to the hernia. The pulmonary hypoplasia is evident grossly and the lung histology is more consistent with a more immature lung tissue. Physiologically, there may be pulmonary hypertension both from a decreased vascular bed and increased muscularization of arterioles. This may cause a persistent fetal circulation postnatally. If the consequent poor gas exchange cannot be treated, death may quickly ensue.

Pulmonary hypoplasia has been described with conditions other than CDH [6] [7] [8] including anencephaly, renal malformations, cystic lung lesions and tracheoesophageal fistula. Askenazi and Perlman [9] attempted to define pulmonary hypoplasia in terms of radial alveolar count (RAC) and lung weight to body weight ratios (LW/BW) in humans. They find that in CDH, both LW/BW ratio and RAC are reduced. In a human postmortem study by George, et al [10] the effect of CDH on lung development was examined. The affected lungs correlated to younger gestational ages and the radial alveolar count was consistent with lungs of less alveolar complexity. These effects were seen to a greater extent on the lung ipisilateral to the CDH.

Whenever the question of fetal lung maturity arises, a frequent index is the amniotic fluid lecithin-to-sphingomyelin ratio [11]. Surfactant levels in CDH are under debate. Hisanaga, et al [12] present a report of 2 cases of CDH found to have low L/S ratios. However, these are case reports and the authors admit that CDH may occur with low, normal or high L/S ratios as cited in previous literature [13]. Furthermore, their explanation for low levels as resulting from a lower number of Type 2 alveolar cells is not necessarily true since the amount produced per cell as well as the total number of cells may vary from case to case. In a more recent study Sullivan et al [14] present a series of L/S ratios in prenatally diagnosed CDH and found that there

was no significant difference from controls after 36 weeks gestation. Before 36 weeks, however, lower ratios are found in the CDH group.

The association of nonpulmonary anomalies with CDH has been documented epidemiologically by Puri and Gorman [7]. Their analysis includes a 10 year period from 1973 to 1982 during which CDH occured with an incidence of 1 in 2097 births. Of the 36 cases seen, 56% were found to have lethal nonpulmonary anomalies at autopsy. The authors believe the impact that fetal surgery may have on cases of CDH would be minimal because of the associated anomalies. They admit, however, that selection of cases for *in utero* correction is essential and may affect the outcome in these cases. Others, however, have not found such a high incidence of associated nonpulmonary anomalies [15].

Harrison, et al [15] offer the notion of a 'hidden mortality' involving CDH. In this 6 year retrospective study, the mortality of CDH babies actually treated at a tertiary referral center was recalculated to include those babies that never made it to a large center. This reassessment showed that CDH occurred in 1 in 5455 live births and that the 'true' mortality increased from 30% to 66%. More than half the children with CDH died before they could be treated.

Extracorporeal membrane oxygenation (ECMO) is a recently developed tool for managing children who have an inadequate capacity for gas exchange through lung ventilation. The system involves shunting the patient's blood to a gas exchanging membrane and then circulating it back to the patient. Because of risks involved in the procedure, it is reserved for severely ill children who may benefit from its use over a short duration. The system is ideal for stabilizing children severely affected by CDH. A review of the experience with ECMO at the Montreal Children's Hospital has recently been completed [16]. Of 9 patients with high-risk CDH placed on ECMO, 7 (78%) had a good perinatal outcome. The authors stress that their success was due to a prolonged delay before CDH repair which may give time for the pulmonary vasculature to open up. However, their long term follow up (unpublished) show an 80% morbidity associated with survival in these high-risk patients.

Harrison [17] [18] has reviewed the clinical experience of *in utero* correction of CDH at the University of California, San Francisco (UCSF). Their extensive experience on the subject is unparalleled. An analysis of the natural history in unrepaired CDH show that factors portending worse outcome were polyhydramnios, associated anomalies and evidence of herniated liver into the chest. A total of 61 cases of CDH were diagnosed *in utero* of which 14 very severe cases were eventually subjected to *in utero* surgery. The outcome of these attempts is as follows: five intraoperative deaths stemming mostly from technical problems with herniated liver and uncontrolled uterine contractions; three postoperative fetal deaths; two neonatal deaths mostly from poorly controlled uterine contractions and preterm delivery ; four survivors. The authors document the difficulties with herniated liver, amniotic fluid leak and control of uterine contractions. Reducing herniated liver often resulted in bleeding and kinking of umbilical vessels. Subsequent pregnancies in women who tried were uneventful. They also underscore the importance of early repair between 22 and 30 weeks gestation. Too early, the fetal tissues are too delicate for manipulation and too late, the risk of preterm labor is increased.

Thus, CDH may manifest a spectrum of clinical presentations. Herniation that is detected early in gestation is associated with a more dismal prognosis. Initial hopes that early detection and treatment could affect the outcome have not been met since survivors are left with long term complications.

Many of the problems in conventional management arise from the fact that the lungs are hypoplastic and immature with hypertrophied vascular elements. In an attempt to reverse these changes before birth, *in utero* correction has been attempted in highly selected cases. These attempts have been associated with a high complication rate mainly from uncontrolled labor and technical problems during surgery. However, there may be other ways to induce lung growth *in utero*. In order to explore the possibilities, a review of the factors influencing lung growth is necessary.

Biochemical and Physical Factors Influencing Lung Development

Kitterman [19] offers an excellent review of fetal lung development. Many of the concepts have been derived from the fetal sheep model because of its widespread use in this field of study. The fetal lungs are fluid producing organs until a few days before term with the amount of lung fluid that the potential airspaces contain approximating postnatal functional residual capacity (FRC). For lungs to function efficiently immediately after birth as gas exchanging organs, the fluid production must decrease to form an adequate gas-fluid interface. In the fetal sheep, at about 2 days before term the rate of fluid production decreases from approximately 4.5 ml/kg/h to negligible amounts.This decrease correlates well with a rise in fetal cortisol levels.

Pulmonary fluid production is downregulated by cortisol and alpha-adrenergic agents. Cortisol appears to attain its action through modification of alpha-adrenergic receptors late in gestation. Infusion of prostaglandin E2 also decreases tracheal fluid production in fetal sheep.

The lungs grow proportionally to the fetal body so that lung weight expressed as a fraction of body weight remains constant. Lung growth is mainly due to an increase in cell number as opposed to cell size since the concentration of DNA per gram of lung tissue does not change. A number of physical criteria must be met with for normal lung growth to occur: adequate intrathoracic space, adequate intrauterine space and amniotic fluid volume, normal fetal breathing movements and, finally, the balance between fluid volume and pressure within the trachea and future airspaces must be within normal.

Biochemical Factors

Biochemical development appears largely influenced by hormonal factors. Cortisol, adrenergic agents, insulin, sex hormones and thyroid hormone are all implicated in this.

The importance of surfactant synthesis cannot be understated. These phospholipids decrease the surface tension of the gas-fluid interface along the alveolar surface, preventing atelectasis. Early studies

in the fetal sheep found that surfactant levels increase in a gradual fashion until 135 days gestation after which the increase accelerates rapidly up until term (140-145 days gestation) [20] This dramatic increase is associated with a parallel increase in fetal cortisol. Rethmeier, et al [21] investigated the changes in lung surfactant composition with gestational age in the fetal sheep. They found that tracheal L/S ratios were significantly higher and more consistent than amniotic fluid ratios. Furthermore, there was a sudden increase in the ratio around day 130 of gestation and 2 days before term, lung fluid production dramatically decreased.

Kitterman, et al [22] found excellent correlation between surfactant levels and fetal serum cortisol levels. They also found an inverse relationship between cortisol levels and lung DNA content. Cellular growth was inhibited but differentiation was enhanced by the steroid. Functional lung maturity, according to them, is attained largely in the last few days of gestation corresponding to a rise in fetal cortisol levels. In a later study, the near-term rise in surfactant levels is confirmed [23].

Tracheal fluid production must be altered before birth in preparation for air breathing. Kitterman, et al [24] investigated the effect of atropine and cervical vagosympathetic trunk sectioning on fetal sheep lung fluid production near term. It appeared that lung fluid production progressively decreased independently of the above factors but correlated inversely with the level of fetal plasma cortisol. That is, a rise in fetal cortisol corresponded to a decrease in lung fluid production. This decreased production was noticeable about 48 hours before term.

Crone, et al [25] determined the temporal relationship between cortisol and lung development. In this experiment, hypophysectomy was performed on fetal sheep early in the third trimester and lungs were compared between groups receiving exogenous cortisol or ACTH and those that did not. Their findings show that hypophysectomy gave very immature lungs with thick interalveolar walls and low airspace fraction. The addition of cortisol or ACTH for 3 to 4 days before term can reverse these changes toward normal. Type 1 pneumocyte count increased while Type 2 cells decreased with this treatment.

The effect of steroids on lung **structure** in fetal lambs was investigated by Kendall, et al [26]. They found that an infusion of cortisol into the fetuses late in gestation accelerated many aspects of pulmonary development including the morphology of gas-exchange areas and differentiation of Type 2 pneumocytes.

The importance of thyroid hormones in lung development has been studied. Cunningham, et al [27] found that **midgestation** fetal thyroidectomy in the sheep caused arrested development of lung tissue at the canalicular phase. These fetuses consequently had low L/S ratios and immature surfactant profiles at birth. Erenberg and associates [28] found similar results while Thornburn [29] demonstrated that **late third trimester** thyroidectomy in fetal sheep had no discernible effect on lung development. Because of this, he postulated that thyroid hormone is important in the **initiation** of surfactant synthesis.

Insulin and sex hormones appear to have a more limited role. Insulin may inhibit the formation and release of pulmonary surfactant in fetal lung while male hormones may decrease surfactant levels relative to the female [19].

Physical Factors

Since lung growth proceeds by lung tissue growing into the future thoracic cavity, this physical process may be affected by other confounding physical elements that may either increase or decrease lung growth. Laryngeal stenosis, CDH, cystic lung lesions and fetal pleural effusions may all affect lung development at critical stages. With the possibility of manipulating fetal organ growth through *in utero* techniques, there has been renewed interest in determining the factors involved in this process.

Human Examples

The association between laryngeal stenosis and lung growth has been known for many years. Wigglesworth, et al [30] have documented two human cases of laryngeal atresia associated with hyperplastic lung

growth, a finding that is used to diagnose the condition *in utero* [31]. The morphometric analysis in these cases showed an increase in lung volume over normal as well as increases in lung surface area and alveolar number. When the number of cells were estimated through measurements of DNA content, there did not appear to be an increased number of cells. They concluded that **lung liquid retention** promotes alveolar distention by *redistribution* of cells rather than an increase in cell population. This explanation is not supported by experimental data in animal studies [32] [33].

Increased fluid **retention** through laryngeal atresia and tracheal stenosis appears then to result in hyperplastic lungs. The effects of uncontrolled lung fluid **leak** is illustrated in a case report by Walton, et al [34]. Laryngeal atresia found in association with a tracheoesophageal fistula resulted in hypoplastic lung growth, presumably the result of unchecked lung fluid drainage into the fetal gastrointestinal tract.

Human pulmonary hypoplasia has been studied in detail by Nakamura, et al [35]. The authors conclude that different causes of pulmonary hypoplasia have different effects on pulmonary measurements. In particular, causes that have an influence before 16 weeks gestation (such as early diaphragmatic herniation) result in both reduced bronchiolar branching and delayed acinar development while those after this period result only in delayed acinar development.

Haidar, et al [36] examined hypoplastic human fetal lungs to distinguish any changes in the epithelial cells. They found no statistically significant difference between control and hypoplastic lungs when percentages of Type 2 cells were compared. However, this study was a post-mortem one and was restricted to oligohydramnios-associated pulmonary hypoplasia.

Laboratory Experiments

As is apparent in nature, the effect of lung fluid dynamics on pulmonary development has also been observed in various experimental models. Increased fetal lung fluid loss appears to result in pulmonary hypoplasia while increased lung fluid retention causes pulmonary hyperplasia. Tracheal ligation prevents the egress of lung

fluid in the fetus while fetal tracheostomies and orolaryngeal stents have been used to increase the pulmonary fluid loss. The resultant pulmonary changes have been shown in many studies.

Carmel, et al [37] performed early experiments of rabbit fetuses where the fetal tracheas were ligated several days prior to term. The original intent was to prevent amniotic fluid from being aspirated, a mechanism that was believed to be important in lung development. Ligation was found to create lungs that were large for fetal weight with thinner alveolar septations. The authors concluded that the fetal lung produced intrinsic fluid and that prevention of lung fluid escape forced the respiratory tract to expand against a hydrostatic gradient.

The effect of fetal tracheal ligation was also noted by Lanman and associates [38] who performed this on fetal sheep and obtained lung weights substantially greater than normal. The purpose of his study was to assess the influence of tracheal fluid or surfactant on the precipitation of labor. The effect on the lung was apparently an incidental finding.

Alcorn, et al [39] observed the effect of 3-4 weeks of tracheal ligation on fetal sheep lung development. The result was a doubling of lung tissue weight with respect to normal. Basic morphometry indicated a larger airspace fraction in ligated lung samples. Electron microscopy showed a decrease in the number of Type 2 alveolar cells that could be found. In the same study, chronic tracheal drainage was also performed and had the opposite effect: lungs were half the size of normal with smaller airspace fraction and an increase in the number of Type 2 alveolar cells. The authors suggested that lung fluid may act as an internal stent over which lung tissue is able to grow.

Fewell and associates [40] found similar results of performing *in utero* tracheostomies on fetal sheep. This experiment resulted in smaller, less developed lungs with altered DNA tissue levels. However, when looking at tissue and airway surfactant levels, no significant alteration was found. It appeared that cellular maturity is affected independently of lung architectural development.

Fisk, et al [41] confirm the hypoplastic effect of fetal tracheostomy on fetal lung development. Furthermore, the authors found no statistically significant alteration in fetal breathing patterns except for a

marginal increase in apneic periods. Apneic periods are known to be associated with less fluid egress from the lungs [42] and therefore this response may represent attempts by the fetus in retaining more lung fluid to potentiate its own lung development.

In their rabbit model, Adzick, et al [43] conclude that pulmonary hypoplasia associated with oligohydramnios was a result of an ***increased loss of pulmonary fluid*** causing a loss of the internal stenting forces over which lung growth develops. In this study, the effect of oligohydramnios was reversed by *in utero* tracheal ligation and caused the lungs to grow about double the size of the control animals.

Moessinger and associates [44] performed an interesting experiment whereby in the same fetal preparation, the right lung was chronically drained while at the same time the left mainstem bronchus was ligated. Again, the ligated lungs were larger while the drained lungs were small. However, when examining the surfactant levels per unit tissue DNA, there was no difference between hyperplastic, hypoplastic and control lungs. This illustrates nicely that *fetal lung cellular multiplication is under the influence of local distention while the biochemical maturation of surfactant production is under systemic control.*

The regulation of the flow of lung fluid in fetal sheep was analyzed by Harding, et al [45]. The overall flow of fluid from the lungs was 14.4 ml/h in control animals. However in animals whose laryngeal motor innervation was interrupted, the flow decreased by 25%, lending importance to the laryngeal mechanism in lung fluid flow. Others [46] have measured flow rates of 9.6 ml/h through tracheostomy ports and 5.1ml/hr through the intact upper aerodigestive mechanism. The same authors analyzed the lung fluid electrolyte composition and found only a change in potassium concentration with gestational age. Kitterman, et al measured lung fluid production at 4.5 cc/kg/hr.

In similar experiments, Fewell [42] concluded that the larynx provides substantial resistance to the inspiration of amniotic fluid by the fetus but does not impede the egress of lung fluid in the opposite

direction during breathing. However, egress of this fluid is impeded during periods of apnea. Tracheal pressure was 1.5-3.0 mm Hg relative to amniotic fluid pressure. Other investigators [47] have reported that intratracheal pressure is normally between 1.8-2.0 mm Hg and that this pressure is not affected by transient paralysis of the fetus with tubocurare.

Additional modulators of lung growth such as placental mass reduction [48] and phrenic nerve sectioning [49] [50] are cited in the literature but their effect is less than that of pulmonary fluid dynamics. Lung growth seems to be greatly influenced by the amount of lung fluid that is retained in the future airspaces. This fluid normally has an intrinsic pressure in the range of 2 mm Hg relative to amniotic fluid pressure. The regulating factors of tracheal fluid production appear to be under hormonal control but physical factors that decrease the space into which the lung may grow may severely limit lung development. Cystic lung lesions, oligohydramnios, and CDH may all physically impede lung growth. Models examining CDH have been developed and used extensively due to its relatively high incidence in children.

Congenital Diaphragmatic Hernia

Different causes of lung hypoplasia result in different patterns of lung development and therefore much experimental work has been done concentrating specifically on models of CDH. The rabbit, mouse [51] and rat [52] have all been used in this, but by far the most extensive work has been using the sheep model.

Harrison's group at UCSF has performed a series of experiments to determine if *in utero* correction of the CDH would result in a reversal of the pulmonary pathology toward normal [53] [54]. In their sheep model, an intrathoracic balloon was introduced into the fetus in order to simulate the space-occupying abdominal organs in CDH. In some animals the balloon was deflated 3 weeks before term, mimicking *in utero* correction. All uncorrected animals died at birth whereas all corrected animals survived. The corrected lungs showed reversal of lung pathology toward normal. It was from these experiments and others that *in utero* correction was eventually attempted on humans.

Pringle and his group [55] present their data on early creation of CDH in the fetal sheep. In this excellent study, CDH was created at around 75 days gestation and some of these were repaired at around 110 days gestation. The lungs between the different groups were then compared at different stages of development. They confirm the stunted pulmonary development in uncorrected CDH along with increased Type 2 cell incidence in this group. Repair of the CDH *in utero* caused these changes to return towards normal. The authors argue that CDH should be created as early as possible before any potential airspaces have developed. They also note the paucity of alveolar wall capillaries, a finding consistent with pulmonary hypertension.

Hashimoto, et al [56] concentrated on the effect of CDH on Type 2 alveolar cell morphology. In their fetal sheep model, detailed cytological morphometric measurements were made on the Type 2 pneumocytes in animals with CDH, in those without CDH, and in those with *in utero* correction of CDH. They found that Type 2 cells were more abundant in the hypoplastic lungs of uncorrected CDH animals.

The hemodynamic consequences of CDH has been investigated by Olivet, Rupp, et al [57]. In their fetal sheep model, early CDH was created and the animals were delivered near term by cesarean section. The fetuses were then cannulated to measure hemodynamic parameters. There was good evidence for arterial hypoxemia, pulmonary hypertension and elevated pulmonary vascular resistance in animals with CDH when compared with controls.

Since one of the primary consequences of CDH is pulmonary hypertension, it is important that *in utero* correction reverse these changes. Adzick, et al [58] investigated the pulmonary vascular changes associated with *in utero* correction of CDH in the sheep model. Early CDH was created at 60 days gestation and repair was attempted in some of these animals at around 105 days gestation. There was increased muscularisation of the pulmonary arteries associated with CDH. The arterial changes reverted toward normal when CDH was corrected *in utero*.

DiFiore, et al recently reported an experiment where tracheal ligation was used to reverse the pulmonary hypoplasia seen in CDH [33]. Diaphragmatic hernias were created in fetal sheep at 90 days gestation and the effects of tracheal ligation were measured using morphometric and physiologic parameters near term. The ligated animals had lungs almost 5 times larger than unligated animals. They were also 3 times larger than normal control lungs. In fact, the lung growth was so spectacular that the tissue pushed the hernial contents back into the abdomen and continued to grow through the hernial orifice into the abdomen. The ligated lungs were more mature when compared to unligated ones. Morphometric parameters equaled or exceeded control animals. The authors suggest that increased intratracheal pressure may stimulate local pulmonary pressure receptors that in turn activate lung growth mediators.

It becomes obvious that if lung fluid dymanics can be altered in a controlled fashion then perhaps conditions associated with pulmonary hypoplasia may be better managed. Thus far, researchers have increased fetal pulmonary development by using tracheal ligation, which is not clinically applicable since it would probably produce more harm than good in the human fetus. The mechanism of action of tracheal ligation on lung development is also poorly understood and in vivo studies on the effect on tracheal fluid pressure has not been well documented. Because of these shortcomings, the present project set out to fulfill the following goals:

1) prevent fetal lung fluid egress in an easily reversible manner
2) measure the changes in tracheal fluid pressure following tracheal occlusion
3) be able to perform the same fetal preparation using endoscopic equipment.

After investigating the various options, it was decided to use the fetal sheep model primarily due to its size (for potential use with endoscopic equipment) and the extensive background literature that is available with this animal. A standard Swan-Ganz catheter was used as

the tracheal obturator by inflating the catheter balloon in the fetal trachea. The balloon was inflated with saline in order to minimize osmotically-driven balloon shrinkage or expansion. The Swan-Ganz catheter also served as a tracheal pressure-monitoring catheter since the pressure port distal to the balloon reflected lung fluid pressure while the proximal port reflected amniotic fluid pressure which could be used as a baseline reading.

Materials and Methods

Time-dated mixed breed pregnant ewes were operated on at 108-118 days gestation. Rapid induction intubation was performed with Thiopental 1 gram intrajugular and the anesthetic state was maintained with Halothane gas. The animal was placed in slight right decubitus position for easy access to port exit sites along the animal's left side. Intravenous access was established and maintained with lactated Ringer's solution. Ancef 1 gram, Gentamicin 150 mg and Liquamycin (tetracycline) 400 mg were given intravenously. The entire abdomen and left flank and axilla were meticulously clipped, cleaned with proviodine soap then alcohol, and finally prepped with proviodine solution and draped.

A lower midline laparotomy was made and the uterine horns were examined. An adequate incision was made to avoid compromising uterine blood flow when mobilizing the uterus extra-abdominally. Once the fetal number and position was established, a standard Swan-Ganz catheter was tunneled from the left upper flank\lower chest region into the abdominal cavity.

The fetus was examined by palpation through the uterine layers and the position of the fetal trachea determined. A small hysterotomy was made over this site, being careful to avoid any cotyledons. Once the amniotic cavity was entered, Babcock clamps were used to tether the edge of the uterine wound to the fetal skin in order to minimize amniotic fluid spillage and fetal exposure. At this point, the catheter was brought into the uterus through a separate site by pushing the catheter tip through the uterine layers and making a small nick with electrocautery from the inner aspect of the uterus. The fetal neck was reexamined for tracheal position and a tracheostomy was performed by incising the fetal skin, exposing the trachea and making a small stab wound between two tracheal rings about two fingerbreadths below the larynx. The Swan-Ganz catheter was then inserted into the trachea through this site up to the level of the catheter thermistor. For experimental animals, the Swan-Ganz catheter balloon was inflated using 0.4-0.7 cc of a 1-to-1 radiocontrast and normal saline mixture. The amount of balloon inflation was determined to be adequate by pulling

back intermittently on the catheter during inflation and when resistance was felt, inflation was stopped. Tracheal fluid also stopped leaking at this point. In controls with uninflated balloons, a 6-0 Maxon suture was used to create a snug tracheostomy fit around the catheter to prevent tracheal fluid leakage. The overlying skin was then reapproximated with 3-0 silk suture and the catheter was anchored to this area using the same suture. A similar suture was then used to anchor the proximal (CVP) port near the tracheostomy site.

The fetus was then placed back into the uterus and antibiotics (Ancef 500 mg, Gentamicin 150 mg and Liquamycin 200 mg) were added to the amniotic fluid. The hysterotomy was closed with either a running 2-0 chromic or automatic stapler, being careful to include all uterine layers in the closure. A 2-0 chromic pursestring suture was used around the catheter uterine entrance site. The repairs were then tested for leaks before placing the uterus back into the abdomen. Control animals were either untouched controls or those with catheters but with deflated balloons.

The abdominal wound was closed with 2-0 Prolene suture for the fascia and 3-0 Dexon in a subcuticular fashion. The catheter was fixed at the ewe exit site with a nonabsorbable suture and placed in a pouch sutured to the ewe's flank. The ewes recieved 400 mg of Liquamycin intramuscularly each day for 3 days postoperatively.

At weekly postoperative intervals , the ewes were examined with ultrasound to check for fetal viability. With the ewes quietly standing, the pressure ports from the Swan-Ganz catheters were attached to pressure transducers and pressure readings were taken at 10 Hertz and averaged over 2 second blocks for a 30-40 minute period. Data acquisition was accomplished by driving the pressure signal through a data translation DT2821 analog-to-digital converter mounted on a 80486 computer. Because the catheter CVP port reflected the amniotic fluid environment and the distal port the tracheal fluid environment, simultaneous pressure readings of the respective environments could be taken. These readings were stored on disk for later analysis. The data points were then reexamined and a stable interval of 10-30 minutes was selected. The difference between the

tracheal and amniotic fluid pressure was calculated and then averaged for each data acquisition session. The averaged readings were eventually grouped and an ANOVA was used to compare experimental and control pressure readings.

The ewes were kept on this protocol for 3-4 weeks until sacrifice. At sacrifice, a 1 gram Thiopental injection was followed by a lethal bolus of KCl intravenously. Laparotomy was performed and the uterus mobilized. Hysterotomy was followed by a quick exploration of the amniotic cavity and the fetal torso and hindlimbs were placed extraabdominally. The umbilical cord was palpated for pulsations and 0.25 gram of Thiopental was injected into the umbilical vein. The umbilical cord was then clamped and cut and the fetal head was kept intrauterine until no umbilical cord pulsations could be felt. This was to avoid air breathing by the fetus. The catheter was clamped and cut near the tracheostomy site in order to preserve balloon inflation for later examination.

The fetus was then taken and body weight was measured. The trachea, lungs and heart were removed as one complex and the liver was removed separately. The catheter balloon inflation was confirmed by palpation of the trachea before removing it. Tracheal fluid was collected for a separate study by tipping the organ complex over a container until fluid could no longer be passively poured out. The organs were then placed in bags filled with saline and then covered in ice for transport to the Pathology Department for finer dissection.

The heart was separated from the lungs near the hilum. Extraneous tissue was dissected free from the trachea and lungs. The trachea was cut 4 cm proximal to the right upper lobe branch point and the lungs were then weighed. The ventricular portion of the heart was weighed after separation from the atria along the atrioventricular groove. The liver weight was also recorded.

The right middle lobe and left lingula were tied and cut for snap freezing. This was accomplished by wrapping tissue in tinfoil, placing this in a container of isopentane and immersing this container in liquid nitrogen. The containers were eventually transferred to a freezer set at -70°C. These specimens were later analyzed for DNA and protein

content using standard methods [59] [60]. Small segments of lung tissue were also minced and placed in glutaraldehyde for electron microscopy.

The rest of the lung was fixed by instillation of 10% buffered formaldehyde through the trachea at 25 cm hydrostatic pressure for 24-48 hours. The upper tracheal segment corresponding to the balloon site was fixed by immersion in the same formaldehyde solution. The fixed lungs were then taken and lung volumes were measured using the volume displacement method [61]. The lungs were cut in sagittal sections 3-5 mm thick and random 1 cm by 1.5 cm blocks were taken from the sections, representing each lobe, and embedded in paraffin. Paraffin blocks were also made of relevant tracheal segments. Hematoxylin and eosin stained slides were made from each paraffin block.

Lung tissue sections were examined morphometrically using a computerized image analysis system (MCID, Imaging Research Inc., Brock University, St. Catherines, Ontario). The system was programmed to measure lung airspace fraction and count alveolar spaces. To simplify the counting process, an alveolar space was counted if it was wholly enclosed by connective tissue or partially enclosed with the remainder of the boundary being the edge of the computer monitor. Fifteen readings were taken for each lung lobe for a total of sixty readings per lung. The data was then transferred to a spreadsheet program and values for alveolar surface area and alveolar number were calculated using the method of Weibel [62] [63]. The total alveolar number per unit volume of lung was calculated using the following formula:

$$N = ((n)^{3/2})/(ß(£)^{1/2})$$

where

N = **number** of alveoli/unit volume
n = **number** of alveoli counted/unit area
ß = 1.55 (a constant)
£ = **airspace fraction**

Total alveolar surface area was calculated using the following formula:

$$SA = (NK)/((£V/N)^{2/3})$$

where

SA = **total** alveolar surface area/fetus
K = 4.8 (a shape constant)
N = **total** alveolar number/fetus
V = **total** lung volume/fetus
£ = **airspace fraction**

Radial alveolar counts (RAC) were taken by hand using crosshairs incorporated into the microscope objective [64] [65] [35]. A total of 10 readings from each upper and lower lobe were taken by 2 independent observers at different times. The total number of readings per animal was thus 40. Tracheal segments were examined simultaneously by 2 observers and various pathologic changes were noted.

In order to allow for comparison between experimental and control groups, data was corrected for fetal body weight where appropriate. Statistical analysis was done using a Student t-test or ANOVA when appropriate. A Scheffe test was applied when a small number of data was obtained. Values for means were expressed in terms of standard error. P-values were considered significant when less than 0.05.

Results

The first 5 ewes that were operated aborted prior to term. Most of these animals miscarried 10 days postoperatively with fetuses that were dead and showed evidence of severe autolysis and infection. The protocol was modified to include Liquamycin (for chlamydia), to cannulate only a single fetus per ewe, and to expose as little of the fetus as possible during the procedure. The final protocol was as presented in the materials and methods section above.

Having done this, the subsequent 4 ewes went successfully to term. Because of the high rate of twin and triplet gestation, a total of 4 experimental and 5 control fetuses was obtained.

Pressure Measurements

Pressure measurements from all 4 experimental animals were taken while 2 of the 5 control animals had readings taken. Two additional sets of measurements from a viable experimental and control fetus were obtained before miscarriage. Fetal viability was confirmed using ultrasound at the time of the pressure recordings. Each fetus had an average of 3 different recordings over the span of the experiment for a total of 15 recordings for the experimental group and 9 recordings for the control group. The mean tracheal pressure for experimental animals was 3.85 ± .49 mm Hg while the control measurements were -0.27 ± .27 mm Hg. The groups were statistically different with a p-value of <.0001.

Gross Parameters

At sacrifice, the experimental lungs were found to be much larger than control specimens with tissue growth bulging through intercostal spaces and costodiaphragmatic recesses. The tissue appeared more translucent and boggy. No unusual hemorrhage was noted. The catheter ports were checked and found to be in good location in all cases. A small dilatation of the trachea was usually present at the balloon site (figures 4 and 5).

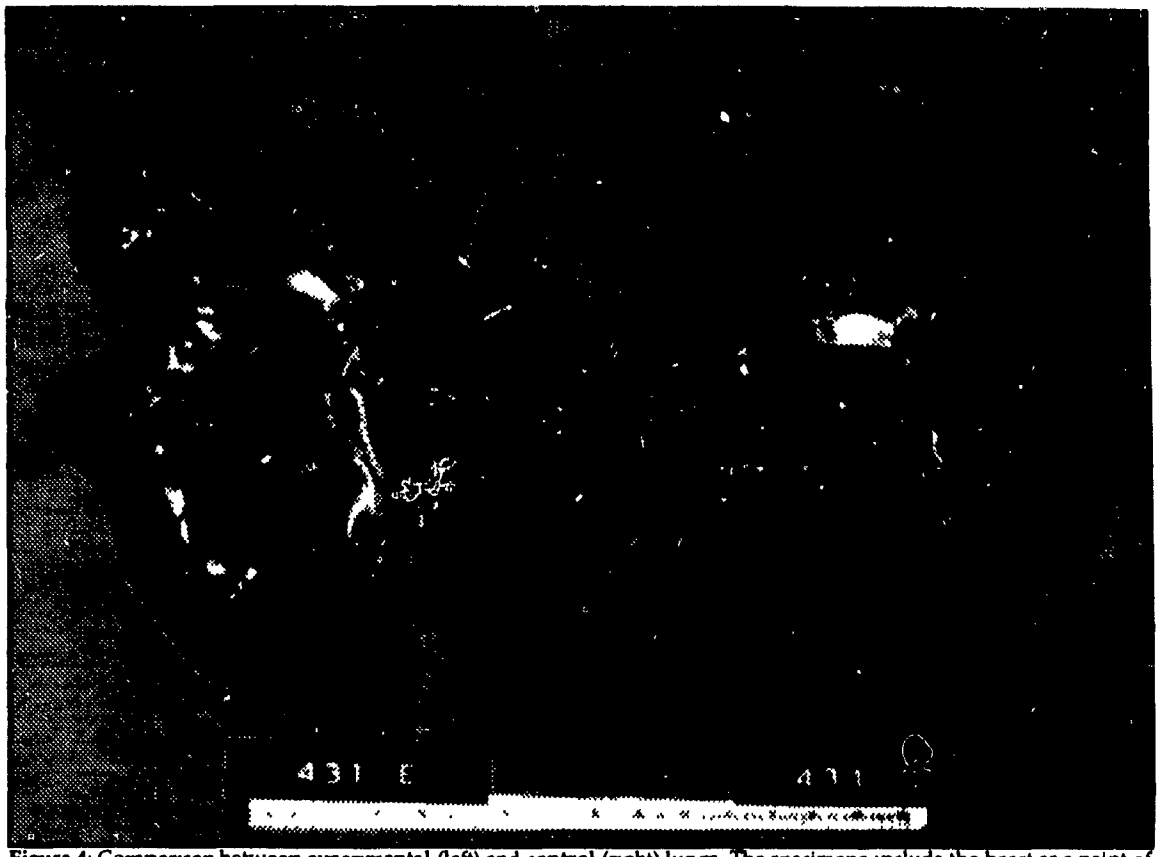

Figure 4: Comparison between experimental (left) and control (right) lungs. The specimens include the heart as a point of reference for size

Fetal lung, liver and heart weight were compared and showed a statistically significant difference only with respect to the lung (table 1). Post-fixation lung volumes were also significantly different. Pulmonary parameters for experimental animals were 2 to 3 times the control values with respect to weight and volume. This data indicates that the procedure used on experimental animals selectively affects the lung and does not have an obvious effect on other large organs. Among the controls, there was no difference between the animals left untouched and those with an uninflated balloon catheter in place.

Measurement	Experimental (n=4)	Control (n=5)	p-value
LW/BW (%)	6.76 ± 43	2.62 ± .20	<0.0001
LV/BW (ml/g)	.15 ± .017	058 ± 005	0 0006
HW/BW (%)	478 ± .027	468 ± .021	0.78
Li/BW (%)	2.29 ± 0.31	1.88 ± 0.14	0.24

Table 1: Summary of gross measurements on different organ systems The abbreviations are as follows: **LW** (Lung Weight), **BW** (Body Weight); **LV** (Lung Volume), **HW** (Heart Weight), **Li** (Liver Weight)

Morphometric Measurements

Histologically, there appeared to be slightly greater alveolar number and thinner septations in the experimental lungs, otherwise they appeared to have normal architecture when compared to control specimens. The arteries appeared normal and capillaries containing red blood cells were noted in the alveolar septae.

The image analyzer was programmed to measure airspace fraction and count the number of alveoli per unit area. The airspace fraction was found to be greater in experimental animals (p=0.044). We find that the alveolar numerical density does not vary statistically between the two groups (table 2). However, because the size of the lungs are different between control and experimental groups, a calculation of alveolar number per unit weight of fetus shows a doubling of the number of alveoli in experimental animals (table 2). This supports the notion that the lung tissue that develops as a result of the tracheal occlusion does so while preserving the ratio between parenchyma and airspace volume.

Measurement	Experimental (n=4)	Control (n=5)	p-value
Airspace Fraction	.72 ± .03	.64 ± .02	044
SA/BW(cm^2/g)	110.8 ± 10.8	43.4 ± 2.9	.0003
Alv/LV(#*10^6/ml)	7.31 ± .81	9.37 ± .92	0.14
Alv/BW(#*10^6/g)	1.10 ± .040	.536 ± .049	<.0001
RAC	7.86 ± .34	5.33 ± .19	.0002

Table 2: Summary of morphometnc measurements of lung tissue Abbreviations are as follows: **SA** (Total Alveolar Surface Area), **BW** (Fetal Body Weight); **Alv** (Total Alveolar Number); **LV** (Total Lung Volume); **AWT** (Alveolar Wall Thickness), **RAC** (Radial Alveolar Count)

The total alveolar surface area was derived and normalized for fetal body weight (table 2). This result showed highly significant differences between the 2 groups with the experimental animal lungs having more than twice the surface area of control values. Experimental animals also had a thinner average alveolar wall thickness than the control group.

The values for the radial alveolar count were plotted in a similar manner and showed a significant difference. This measure is an index of alveolar complexity and estimates the number of gas exchanging units distal to the terminal bronchiole.

DNA and Protein Analysis

No statistically significant differences were found in the analysis of DNA or protein levels per unit weight of lung tissue. Also the ratio of protein to DNA levels was preserved between the 2 groups implying that lung growth had occurred through cell multiplication and not hypertrophy of existing lung elements. When looking at this data in terms of fetal body weight, the amount of lung DNA and protein is found to be 2-3 times greater in experimental animals. The results are summarized in table 3.

Measurement	Experimental (n=4)	Control (n=5)	p-value
Protein (mg/g)	26 ± 7	20 ± 4	0.48
DNA (mg/g)	2.8 ± .2	2.8 ± .2	0.97
Protein/DNA	10.0 ± 3.6	7.3 ± 1.2	0.45
Protein/BW (mg/g)	1.68 ± .41	0.52 ± .11	0.019
DNA/BW (mg/g)	0.186 ± .017	0.073 ± .009	0.0004

Table 3: Summary of DNA and protein analysis of lung tissue. Values were derived per gram of wet weight of lung. A ratio of the two measures is also compared. When comparing the DNA and protein in terms of fetal body weight (BW), the experimental lungs were significantly different from control lungs.

Tracheal Histology

Tracheal sections were examined at sites below, above and at the balloon occlusion site. The area of the tracheostomy was also examined. The **normal tracheal histology** showed a ciliated pseudostratified columnar mucosa, either flattened or undulated, overlying a loose submucosal layer containing lymphatic and vascular components. The cartilage was crescentic in shape with the posterior open ends of the crescent being joined by a layer of trachealis muscle.

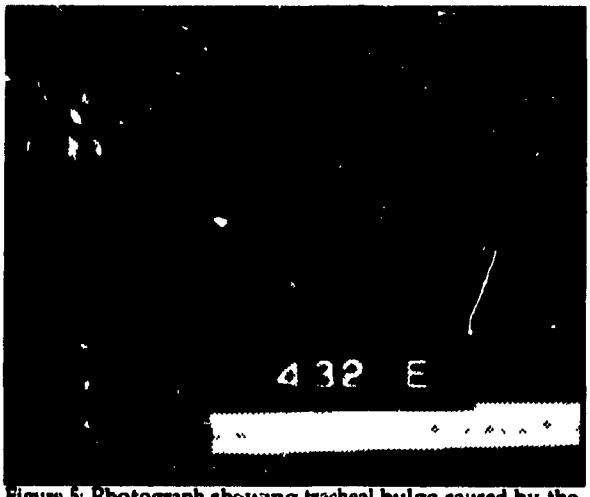

Figure 5: Photograph showing tracheal bulge caused by the intratracheal balloon.

Changes noted in **cannulated control animals** include a flattening of the mucosa just below the tracheostomy site with fibrotic changes and thinning of the submucosa. This could be attributed to an intratracheal rubbing action by the Swan-Ganz catheter.

In **experimental animals**, the mucosa was found to be affected significantly. Squamous metaplasia was apparent at the balloon site with areas of frank epithelial necrosis and sloughing. On one occasion, the squamous changes were found in areas above the balloon site while at the balloon site there was simple flattening of the mucosa. Submucosal changes ranged from granulation tissue infiltration, polymorphonucleocyte or lymphocyte infiltration to focal areas of fibrosis and calcification. Changes below the balloon site were either not apparent or minimal with areas of mucosal thinning. The effect on the tracheal cartilage was minimal, being restricted to perichondrial thickening and increased separation of the posterior free ends of the

cartilage. At this posterior end, however, the trachealis muscle was found to be thickened at and below the balloon site. At the tracheostomy site, a squamous epithelial tract could be seen connecting the skin to the tracheal mucosa.

To summarize, balloon occlusion of the fetal trachea is associated with a rise in tracheal fluid pressure from zero to 4 mm Hg. The effect on growth after 3 weeks of occlusion is primarily a doubling of size and volume. A doubling of the alveolar surface area and alveolar number is seen as well as an increase in the radial alveolar count. Finally, the DNA and protein content of the lung is also increased by the same magnitude.

Discussion

The effect of balloon occlusion of the fetal trachea appears comparable to the effect of laryngeal atresia and tracheal ligation in terms of lung morphology and morphometry. The present study is the first to show the *in vivo* changes in tracheal pressure associated with tracheal obstruction.

The tracheal pressure measurements in experimental animals was found to be in the range of 4 mm Hg while the control animals were fluctuating around zero relative to amniotic fluid pressure. Control values in other studies [66] [47] [42] give readings of 1.25-3.0 mm Hg relative to amniotic fluid pressures. However, in most of these studies [47] [42], the relative placement of the tracheal and amniotic fluid catheters is unclear. In the remaining study [66] the amniotic fluid pressure was derived from the difference between the maternal intrabdominal pressure and the intrauterine pressure. In contrast, the present study maintained a controlled relative distance between the two catheters by fixing the amniotic fluid pressure port near the fetal tracheostomy site. The difference in pressure between the two ports is what was calculated as the tracheal fluid pressure.

Values from experimental animals show that tracheal obstruction is indeed related to an elevation in tracheal fluid pressure by a magnitude of 4 mm Hg. DiFiore, et al obtain pressures of 0 mm Hg for control animals and 6 mm Hg for experimental tracheally ligated animals [33]. However, in their study pressures were measured only at the time of fetal delivery and relative to air. The present in vivo preparation is probably a better reflection of the true changes.

The rise in intratracheal pressure associated with tracheal obstruction is not surprising. As was mentioned earlier, the fetal lungs act as fluid secreting organs up until near term [43] [23] [40] [44] [21] and thereby contibute to the amniotic fluid volume. The active transport across the pulmonary epithelium eventually diminishes near term in coordination with rising fetal cortisol levels [24], preparing the lungs for air-breathing. Thus, it is expected that tracheal ligation or obstruction would result in fluid secretion in an enclosed space and

increase the intratracheal pressure. The pressure rise would continue until a plateau is reached, 4 mm Hg in the present study. What is surprising is that such a small change in pressure is associated with a large difference in overall lung development.

The gross changes associated with tracheal occlusion are comparable to those seen in other studies in terms of lung growth and control values [58] [43] [39] [33] [32] [19]. Lung weight and volume are increased by a factor of 2 to 3 with both tracheal obstruction and tracheal ligation. These lungs are obviously large for gestational age and appear histologically normal. As was shown in the present study, the effect of organ growth appears to be confined to the lung, since the heart and liver are not affected. This is consistent with what one sees in human congenital laryngeal atresia [30]. This finding is important since rapid lung enlargement might have impaired heart development by compression.

Histologic and morphometric analysis were used to compare lung architecture. From this it is evident that lung growth in response to tracheal obstruction results in an increased number of lung units and not simply an increase in the size of preexisting units. Airspace fraction was slightly greater in experimental lungs, a characteristic found in more mature lungs [67]. Alveolar surface area and alveolar number did not differ significantly between the two groups when expressed in terms of lung weight or volume. When expressed in terms of fetal body weight, however, tracheal obstructed lungs show a 2 to 3-fold increase in these values relative to controls. These changes are essentially proportional to those occuring at the macroscopic level. Most values obtained were consistent with those from other studies [33] [32] the exception being with alveolar number, probably a reflection of the different method in counting alveoli. Traditionally, counting alveoli has been done by a human observer that arbitrarily designates what constitutes an alveolus. This is useful for disregarding artifact and counting alveoli that are incompletely surrounded by parenchyma.The present study used an automated image analyzer to count alveoli by the strict definition of an alveolus being wholly enclosed by lung

parenchyma. The scanned histology image was reviewed by the primary investigator before counting in order to ensure exclusion of gross artifact and the tedium of counting was then accomplished by the computer. The advantage of this is consistency in the counting protocol. The disadvantage is that partially enclosed alveolar spaces are not counted individually which would result in an underestimation of the alveolar number. However, as long as counting is done in a similar fashion between control and experimental lungs, valid comparisons can be made.

The radial alveolar count (RAC) was found to be significantly different between tracheal obstructed lungs and control lungs. This shows that alveolar complexity beyond the terminal bronchiole increases with this intervention. Cooney and Thurlbeck [65] evaluated the use of the RAC as a measure of alveolar development. They state that measurement is independent of shrinkage but depends highly on lung inflation due to recruitment of alveoli. They add that statistical strength is increased when using 40 counts per case as we did in this study. The authors support the use of the RAC for measuring alveolar complexity and development. In a similar study, the same authors [64] conclude that both the radial count method and estimates of alveolar surface area correlated better with gestational age than other morphometric parameters.

In order to assess whether the tissue growth is due to hyperplasia or hypertrophy, the tissue DNA and protein content may be used [68] [33] [44] [32] [69]. Increased mitosis will naturally lead to an increase in tissue DNA levels. Increased production of interstitial matrix and cytoplasm will lead to elevated tissue protein levels. A ratio of tissue DNA to protein levels would be expected to remain constant if the balance between the number of cells and the amount of extracellular matrix remains constant. In this study, both tissue DNA and protein levels between experimental and control lungs remain unchanged when expressed per gram of lung tissue. The ratio of DNA to protein was also unchanged. However, because experimental lungs are much larger, these values in terms of fetal body weight are significantly

different, indicating that both cell number and quantity of interstitium are elevated after tracheal obstruction.

Analysis of the changes to the trachea show that damage occurs along the tracheal segment between the tracheostomy site and the Swan-Ganz catheter balloon. The mucosa adjacent to the catheter tubing exhibits squamous metaplasia probably from chronic rubbing by the catheter against the mucosa. At the balloon site the damage was more severe, ranging from squamous metaplasia to frank necrosis and sloughing. It is comforting to note that even with the crude obturator used in this study, these changes were confined to the mucosa and submucosa while the cartilage was left relatively unaffected. It would nevertheless be worthwhile to design a clinically applicable obturator that would minimize tracheal damage.

It is evident from this study and others [43] [32] [33] [37] [39] that the prevention of fluid egress from the potential airspaces in the developing fetus results in accelerated lung growth. One proposed mechanism [33] of action is the modulation of local growth factors via airspace pressure receptors. An increase in lung fluid pressure may augment a local growth cascade. The effect on growth has been shown to confine itself strictly to areas of lung fluid retention [44] indicating the importance of fluid retention in this mechanism. Systemically circulating growth factors would probably be ineffective without tracheal obstruction. Another possibility is the augmentation of response to certain growth factors by pressure-induced modulation of membrane bound receptors.

Whereas pressure tends to cause necrosis of tissue (as is frequently seen with abcesses and tumor growth), tension has been shown to increase the mitotic activity in various tissues. As described by Laplace's Law, wall tension increases proportionally to intraluminal pressure. Epithelial cell division in response to tension has been investigated by several authors. Curtis and Seehar [70] have shown that increased mechanical stress applied to cultured fibroblasts leads to an increase in mitotic activity by speeding up the cell cycle. The authors

explain the effect through the possible involvement of the cellular microfilament system in the control of the cell cycle. Squier [71] observed the effect of applied tension in vivo on mouse skin and confirms increased mitotic activity in response to this intervention. Liu, et al [72] studied the effect of mechanical stretch on proliferation of fetal rat lung cells. They conclude that mechanical forces act directly to stimulate fetal rat lung cell growth and that this effect is not mediated by prostaglandins or leukotrienes.

With fetal surgery becoming a reality in the clinical setting, methods of minimizing the surgical complications are being investigated. One of the gravest complications is preterm labor that is often difficult to control medically. The uterine instability is believed to be secondary to the operative hysterotomy wound [8]. It would seem logical, then, that reducing the size of the wound may decrease this dreaded compication. Modern technology has allowed 'minimally invasive surgery' with laparoscopic equipment and this has recently been used in the experimental setting in fetal surgery [73] [74] [75] [76].

Conclusion

This study has shown that fetal tracheal obstruction in the third trimester is associated with both an inceased pulmonary fluid pressure and lung growth. These pulmonary responses to blocking the fetal trachea occurs over a relatively short time period (within 3 weeks in this study) and the response in lung growth is comparable to that seen with fetal tracheal ligation. Future studies will involve the analysis of electron microscopy images obtained from this present work. The effect of accelerated lung growth on pulmonary vessel distribution and physiology will be investigated. There will also be application of a similar fetal preparation but with occlusion closer to term and for a shorter duration. Immunohistochemistry will be used to screen for putative growth factors that may be involved in this mechanism. Laparoscopic instruments will also be used for the fetal instrumentation.

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