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Lung Growth, Structural Remodeling, Surfactant Levels, and Lung Function After Reversible Fetal Lamb Tracheal Occlusion in Congenital Diaphragmatic Hernia

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Abstract

The effects of reversible fetal tracheal occlusion (TO), and antenatal glucocorticoids on lung growth, structure, surfactant levels, and function were assessed in a lamb hypoplastic lung model of congenital diaphragmatic hernia (CDH). CDH, CDH+TO, CDH+TO+release of the tracheal occlusion one week before delivery (TR), and unoperated twin controls were compared. TO+/-TR partially normalized the hypoplastic lungs of CDH: they accelerated growth of both lungs and led to structural maturity. Only TO thinned the medial area of small pulmonary arteries closer to control values. Despite TO, TR, and glucocorticoids, lungs from lambs with CDH have dysfunctional type II cells with decreased surfactant levels. Nonetheless, CDH+TO lambs showed normal oxygenation, ventilation, and compliance over untreated CDH, with a clear survival advantage over an eight hour resuscitation. TR one week before delivery had no added benefit in terms of lung function. It appears that surfactant independent mechanisms such as pulmonary growth and structural changes are of foremost importance in relating to improved compliance, oxygenation, and ventilation of CDH+TO animals.

Abrégé

Cette étude vise à déterminer à l'aide d'un modèle ovin d'hypoplasie pulmonaire causée par une hernie diaphragmatique congénitale (HDC) les effets de l'occlusion réversible de la trachée foetale (OT) et du traitement anténatal avec des glucocorticoides sur la croissance des poumons, le développement structural, le niveau de surfactant et la fonction pulmonaire. Quatre groupes expérimentaux sont comparés: 1. la HDC; 2. la HDC+OT; 3. la HDC+OT+le relâchement de l'occlusion trachéale (RO) une semaine avant la naissance; 4. le groupe contrôle (des jumeaux nonopérés). L'OT+/-RO normalisent en partie l'hypoplasie pulmonaire de la HDC: ils accélèrent la croissance des poumons et induisent une maturation structurale. Seule l'OT peut amincir l'aire de la média des petites artères pulmonaires. Malgré l'OT, le RO et les glucocorticoides, les poumons affectés par la HDC ont des cellules de type II dysfonctionnelles avec un niveau faible de surfactant. Néanmoins, au cours d'une resuscitation de huit heures, les agneaux avec l'HDC+OT ont démontrés une oxygénation, une ventilation et une compliance normales comparés aux agneaux avec une HDC. Le RO une semaine avant la naissance n'a aucun avantage en ce qui concerne la fonction pulmonaire. Il nous semble que des mécanismes indépendants du surfactant, tel que la croissance pulmonaire et les changements structuraux pulmonaires, ont une importance primordiale en ce qui concerne l'amélioration de la compliance, de l'oxygénation et de la ventilation des poumons affectés par la HDC et traités avec l'OT.

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Abbreviations

ABG= arterial blood gas

BAL= bronchoalveolar lavage fluid

CDH= congenital diaphragmatic hernia

CVP= central venous pressure

DBP= diastolic blood pressure

DLCO= diffusing capacity

ECMO= Extra-Corporeal-Membrane-Oxygenation

EM= electron microscopy

ET= endotracheal

FBM= fetal breathing movements

FEV_1= forced expiratory volume in 1 second

FiO_2= percentage of inspired oxygen

FRC= functional residual capacity

Hct= hematocrit

Hb= hemoglobin

HR= heart rate

I:E ratio= inspiratory time to expiratory time ratio

i.m.= intramuscular

i.v.= intravascular

LW/BW= lung weight over body weight ratio

MAP= mean arterial blood pressure

mRNA= messenger ribo-nucleic acid

MTBD= mean terminal bronchiole density

$NaHCO_3$= sodium bicarbonate

PA= pulmonary artery

$PaCO_2$= partial pressure of arterial carbon dioxide

PaO_2= partial pressure of arterial oxygen

PC= phosphatidylcholine

PEEP= positive end-expiratory pressure

PIP= peak inspiratory pressure

PPHN= persistent pulmonary hypertension of the newborn

RV= residual volume

RR= respiratory rate

SaO_2= arterial oxygen saturation

SBP= systolic blood pressure

SEM= standard error of the mean

SP-A= surfactant-protein A

SP-B= surfactant-protein B

SP-C= surfactant-protein C

SP-D= surfactant-protein D

Tinsp= inspiratory time

TLC= total lung capacity

TO= tracheal occlusion

TR= release of tracheal occlusion

US= ultrasound

VC= vital capacity

Introduction

Congenital diaphragmatic hernia (CDH) affects 1 in 2000-4000 live human births [1, 2]. The herniation of intestines through the diaphragmatic defect that occurs during fetal development results in: lung hypoplasia [3, 4]; pulmonary hypertension with increases in medial and adventitial thickness of peripheral pulmonary arteries [5-7]; a primary or secondary surfactant deficiency [8-12]; and low compliance postnatally that may be due in part to a lack of surfactant [13-15] and also to an increased lung collagen density [16]. This combination of factors is lethal in around 20-40% of those babies born with CDH despite optimal care [17-19], with the most important predictor of survival being the degree of lung hypoplasia [20, 21].

Given a prenatal diagnosis of CDH, the mother could be faced with difficult choices:

1. Termination of pregnancy. Or,

2. Standard postnatal care that may or may not require Extra-Corporeal-Membrane-Oxygenation (ECMO), followed by repair of CDH once hemodynamically stable for "good prognosis" CDH [1]. Although complete repair of the diaphragmatic defect in utero works for fetuses without liver herniation into the chest, it does not improve the survival rate over standard postnatal care. Of those with good prognosis CDH, there is a 75% survival for in utero repair versus 86% survival for standard postnatal care [22]. Or,

3. In utero intervention for "poor prognosis" CDH.

Poor prognosis CDH includes babies diagnosed by obstetrical ultrasound (US) before 25 weeks gestation, a lung-head ratio <1, and liver herniation into the chest [23, 24]. Complete in utero repair of CDH does not work for fetuses with liver herniation because reduction of the liver back into the abdomen causes fatal kinking of the umbilical vein [25]. For these fetuses, a "Plug the Lung Until it Grows (PLUG)" strategy, where the fetal trachea is occluded in utero, allows for lung growth, thus correcting the lung hypoplasia, and slowly reducing the viscera before birth [26, 27]. In a recent retrospective study of 34 human fetuses diagnosed with poor-prognosis CDH, it was found that the survival rate was 38% in the group treated by standard postnatal therapy (n=13), 15% in

the open tracheal occlusion group (n=13), and 75% in the group with tracheal occlusion by fetoscopy (n=8) [28]. Those treated with tracheal occlusion by fetoscopy had lung enlargement before birth demonstrated by ultrasound examination of the lungs, and had good postnatal respiratory function as determined by low percentage of inspired oxygen (FiO_2) requirements, and rapid extubations. In addition, it is believed that fetoscopic surgery is less invasive than open fetal surgery, and that it lessens the risk of myometrial irritability and preterm labour [29-33].

At first glance, it would seem that fetal tracheal occlusion (TO) could solve the problem of lung hypoplasia. Unfortunately, TO consistently causes a decrease in type II pneumocytes and a dramatic surfactant deficit [34-37]. Thus, TO either damaged type II cells or delayed their maturation while accelerating lung growth. However, even though lung growth after TO occurs at the expense of type II cell differentiation, this effect is reversible with the release of tracheal occlusion (TR) one week before birth in a normal, non-CDH fetal lamb lung model [38].

In the attempt of improving the outcome of CDH, research with CDH animal models has shown that a limited number of prenatal interventions such as experimental fetal TO, and prenatal glucocorticoids have independently proved beneficial in terms of improving oxygenation, ventilation, and compliance, and have shed some light on the pathophysiology of CDH [39-42].

Given the high mortality of CDH from pulmonary hypoplasia and its associated pulmonary hypertension, we sought to combine the beneficial effects of fetal TO to accelerate lung growth, then, TR one week before delivery to restore type II cell density, with the addition of antenatal glucocorticoids, to increase the surfactant synthetic ability of type II cells.

The **overall goal** of our study was to maximize existing prenatal interventions that can safely accelerate in utero lung growth, structural, biochemical, and functional maturation in a CDH model. Our **objective** was to analyze the effect of minimally invasive fetoscopic TO with or without TR one week before birth, with one dose of antenatal glucocorticoids one day before delivery, on lung growth, lung structural maturation, pulmonary artery remodeling, surfactant levels, and lung function, as

measured by oxygenation, ventilation, and compliance, in the hypoplastic lung model of lambs with a surgically created CDH.

Our **hypothesis** was that CDH with TO will allow for lung growth, structural maturation and pulmonary artery remodeling, with the addition of TR and antenatal glucocorticoids favoring increased type II cell density with increased surfactant levels, and improved lung function over CDH lungs.

Review of the Literature

I. Overview

The transition from fetus to neonate at birth is truly amazing; in the first few minutes of extrauterine life, the newborn must replace the liquid in the lungs with air, and establish the pulmonary circulation. In most newborns, the transition occurs smoothly, but it is known that anomalies in size, structure, and maturation of the lung are leading causes of infant mortality. As many as one-third of all early neonatal deaths are associated with lung hypoplasia [43].

Interestingly, the observation that babies born with laryngeal atresia, causing a natural form of tracheal occlusion, had large, hyperplastic lungs led to experimental fetal TO to counteract lung hypoplasia [42, 44]. To understand the mechanism of fetal TO causing lung growth, we must first grasp the normal physiology controlling the structural and biochemical maturation of fetal lungs, then examine lung hypoplasia in CDH and how it is affected by TO, by release of the occlusion before birth, and by antenatal glucocorticoids.

II. Normal Lung Development

The five lung development stages in the human [45, 46] and the lamb [47] are (human/ lamb), where term is >37 weeks gestation in humans, and >145 days gestation in the lamb:

a. 0-6 weeks/ 0-40 days: the embryonic stage where the lung is developing as a single bud off the anterior portion of the foregut and thus forming the proximal airways with the appearance of segmental bronchi.

b. 7-16 weeks/ 40-80 days: the pseudoglandular stage where the entire bronchial/conducting airways are formed.

c. 17-24 weeks/ 80-120 days: the canalicular stage where the peripheral airways (acinus) and distal pulmonary circulation, ie: future airspace volume, are formed. In the sheep, maximal lung growth occurs between 112 and 124 days gestation.

d. 24-36 weeks/ 120-145 days: the terminal sac stage where there is formation of gas-exchange units with alveolar development and type II cell differentiation. The alveolar surface area increases through the development of alveolar sacs, and the distance

between the alveolar epithelium and capillaries decreases, making it possible for gas exchange to occur. Thus, the lungs of a baby born before 24 weeks would not be expected to support extrauterine life.

e. 36 weeks- 3 to 8 years postnatally/ 145 days: the alveolar, postnatal stage where there is an increase in the number and size of alveoli. It is important to note that in humans, only 1/3-1/2 of alveoli are present at birth [45, 46], while in the lamb, most of the alveoli are present at birth [47].

Normal diaphragm development allows for normal lung growth by separating the thoracic and abdominal cavities, and maintaining their respective organs in their proper domains. The diaphragm is derived from four components: a. the septum transversum ventrally that forms the central tendon b. the pleuroperitoneal membranes dorsolaterally c. the mesentery of the esophagus dorsally that forms the crura, and d. the intercostal muscle group that forms the posterior-lateral muscle [1]. Closure of the pleuroperitoneal canal to form the pleuroperitoneal membrane normally occurs during the eighth week of gestation in humans [48], and by day 28 of gestation in lambs [49]. It is thought that the posthepatic mesenchymal plate is important in leading to the closure of the canals [48]. If there is failure of the pleuroperitoneal canals to close, such as in CDH, then, the thoracic and abdominal cavities communicate freely. This becomes a problem once the viscera return into the abdomen during the tenth week of gestation in humans, where they will herniate into the chest preventing lung growth through competition for space.

III. The Importance of Lung Liquid

Experiments have shown that fetal lung liquid is essential to lung growth and that fetal lung liquid volume must be maintained within normal physiological ranges for proper lung development [50-54].

During fetal life, the pulmonary epithelial cells secrete a fluid that fills the potential airspaces. This luminal fluid is called lung liquid and is not inhaled amniotic fluid. Influx of amniotic fluid into the trachea is apparently a very rare event [50]. The lung liquid has its own distinct composition that is relatively stable throughout gestation, such as high chloride and low protein contents [50]. Lung liquid is important because it maintains the developing lung expanded, and this expansion is crucial for lung growth. It

is not known exactly when lung liquid secretory function starts, but by mid-gestation, lung liquid is secreted at mean rates of 5 ml/hr/kg as determined by lamb fetal lung development experimental models [50]. The lung liquid secretion increases with gestational age. On the other hand, the lung liquid secretion is decreased with parturition, β-adrenergic agents, epinephrine, prostaglandin E2, vasopressin, fetal hypoxemia, elevated cortisol and thyroid hormones [52]. This makes physiological sense as in the days shortly before birth, there is a surge of fetal hormones such as cortisol and epinephrine, and, during labor, there is relative fetal hypoxemia, thus preparing the fetus for extrauterine air breathing by decreasing the secretion rate of lung liquid in anticipation of clearance of lung liquid. There is no change in lung liquid secretion rates by fetal behaviour (awake or sleeping), or by fetal breathing movements.

Even though fetal breathing movements (FBM) do not affect the rate of lung liquid secretion, FBM of normal incidence and intensity are necessary for maintaining normal lung volumes and thus normal lung growth [51]. Experiments have shown that if FBM are abolished, for example, with phrenic nerve ablation or by using muscle relaxants, this causes lung hypoplasia [50, 52]. Normally, FBM last for 20-30 minutes/hour and occur during rapid-eye-movement sleep which is present for 30-50% of time during late gestation. When the fetus is not practicing FBM, it is apneic. What exactly stimulates the fetus to alternate between apneic states and FBM states is not well understood.

The role of the diaphragm, and the laryngeal constrictor muscles is important in the mechanics of FBM and in the maintaince of lung volume [50]. For example, during normal fetal apnea, the laryngeal constrictor muscle is tonically active creating an airway resistance, and thus lung liquid accumulates as the rate of lung liquid secretion is greater than lung liquid efflux. During FBM, the laryngeal constrictor relaxes, and allows for a small net rate of fluid efflux. Experiments have shown that the fetal lung filled with liquid has an elastic recoil resulting in negative intrapleural pressures (0.2-0.7 mm Hg below amniotic fluid pressure) [50-53]. The fetal intra-tracheal pressure is slightly greater than amniotic pressure by 1-3 mm Hg because of the high laryngeal resistance and pulmonary recoil pressure [50]. Thus, lung volume is maintained during apnea by the contraction of

the laryngeal constrictor muscle, and lung volume is maintained during FBM by contraction of the diaphragm opposing the lung recoil. Apparently, the volume of tracheal fluid moved with each FBM is less than 1-2% of the total luminal volume; this may be due to the high resistance of the laryngeal constrictors, and the viscosity of the lung liquid [50]. By the resistance to outflow of tracheal fluid, and negative intrapleural pressure, there is thus a tendency to maintain the "status quo" of lung liquid volume inside the lungs and positive intra-tracheal pressure. As such, lung liquid volume is around 30 mL/kg, and, interestingly, is roughly equal to functional residual capacity (FRC) postnatally [52].

The greatest increase in lung growth in fetal lambs (limited data for humans) occurs during the canalicular stage of development where lung liquid secretion increases rapidly, thus increasing lung liquid volume, and there is maximal intra-tracheal pressure [50, 54].

How does the normally sustained expansion of the fetal lung by lung liquid volume affect the growth and maturation of the fetal lung? Moessinger performed an in utero experiment in fetal lambs where he decreased right fetal lung volumes by drainage of lung fluid, and increased left fetal lung volumes by ligation of the mainstem bronchus and compared them to controls [43]. What he found was that the left lung (occluded) was significantly hyperplastic and contained more DNA than controls, while the right lung (drained) was significantly hypoplastic and contained less DNA. However, indices of lung maturation such as pulmonary surfactant showed no difference between the hypoplastic, hyperplastic, and control groups. He thus concluded that lung cell multiplication is influenced by local distension with lung fluid while the maturation of surfactant is under systemic control.

Although lung liquid is important for fetal lung growth, part of it must be removed for normal gas exchange at birth. There are different ways fetal lung liquid is removed at birth: compression of the thorax during vaginal delivery, absorption of lung liquid into the pulmonary capillaries, clearance by pulmonary lymphatics, and most importantly, transepithelial movement of alveolar fluid by the amiloride-sensitive sodium transport by lung epithelium [55].

In a normal newborn, in order to overcome the surface tension of the alveoli and the viscosity of the remaining lung fluid, the first breath must generate great negative intrathoracic pressures of 60-80 cm H_2O [56]. The volume of the first breath is around 40 mL, but not all of it is expired, thus establishing FRC. Subsequent breaths will then require less inflation pressures as more alveoli remain inflated. Apparently, the alveoli open up in a serial sequence. A normal FRC is established within the first few hours of life [56].

IV. The Importance of Pulmonary Artery Remodeling

At birth, the pulmonary vasculature is remodeled rapidly to allow an abrupt reduction in pulmonary vascular resistance and a 10 fold increase in pulmonary blood flow [57-59]. Adaptation involves the entire arterial pathway, but especially important are the resistance arteries just proximal to the respiratory unit. In the fetus, pre-acinar branching of both conventional (arteries that accompany airways and branch with them) and supernumerary arteries (arteries which branch off the conventional ones at right angles and supply alveoli immediately adjacent to the broncho-vascular tree) is completed by mid fetal life, and is closely associated with the bronchial branching pattern [60]. Vascular development in the acinar region proceeds concurrently with alveolar growth and multiplication. Normally, at birth, no muscle is present in arterial walls beyond the level of the terminal bronchioles; however, muscular arteries of the fetus have thicker walls than arteries of the same size in adults [61]. Soon after birth, there is an immediate increase in external diameter and thus a drop in wall thickness of the vessels below 200 μm, while the larger vessels take up to 4 months to fall to adult thickness [62]. New conventional arteries are added up to 18 months, and new supernumerary vessels up to 8 years [63]. In the first 2 months, there is a rapid increase in arterial size, and arterial number, with a decrease in medial artery thickness especially in the first 10 days [64]. There is gradual remodeling with deposition of connective tissue [65]. Intra-acinar arteries become more muscular during childhood as they increase in size also [66].

If there is failure of pulmonary artery remodeling, pulmonary hypertension occurs with its clinical manifestation of worsening right-to-left shunting, poor oxygenation, hypercapnea, and acidosis [57, 67]. The structural changes of the pulmonary arteries with

muscular extension into intra-acinar arteries, medial thickening, and adventitial thickening with excessive connective tissue deposition contribute to the severity of pulmonary hypertension [68]. Some believe that the thick walled pulmonary arteries of idiopathic persistent pulmonary hypertension of the newborn (PPHN) become excessively muscularised during fetal life, and are unable to adapt at birth [57, 59].

Interestingly, for normal fetuses, in arteries <100 μm, wall thickness and adventitia has been reported to be significantly increased in those <34 weeks gestation, and this thickness decreased in those >34 weeks gestation [7]. Moreover, it has been found that endothelin-1 (a potent vasoconstrictive and pro-mitogenic peptide) levels are detected at 12 weeks gestational age in human fetuses, and rise to a peak at 24 weeks gestational age, upon which there is a dramatic decrease until birth, and this low level is maintained into normal adulthood [69]. These two findings may indicate that pulmonary artery remodeling is especially important in the latter part of gestation.

V. The Importance of Type II Cells and Surfactant

The type II cells, which only make up 5% of the lung epithelium, are extremely important as they produce and secrete surfactant [70]. They also are the precursor cells to type I cells that make up most of the lung's alveolar lining [70]. Type II cells are detected at around 12 weeks gestation in humans, with maturation as evidenced by lamellar bodies appearing at 24 weeks gestation [71, 72]. Surfactant produced by type II cells consists of phospholipids and surfactant proteins. Disaturated phosphaditylcholine (PC) is detectable in amniotic fluid at around 30 weeks, even though it can be identified earlier in lung tissue [72]. Surfactant protein A (SP-A) first appears in significant quantities at around 30 weeks also, and then increases in parallel with the surfactant lipids [72]. Thus, SP-A is considered to be an indicator of fully differentiated type II cells [73, 74]. Surfactant protein B (SP-B) and surfactant protein C (SP-C) appear earlier, with their messenger ribo-nucleic acid (mRNA) detected at 13 weeks [72]. In normal animals, surfactant lipids and apoproteins increase in an approximately coordinated manner in late gestation [75-78].

In the fetal lamb, the window of type II cell maturation may correspond to 125-135 days when there is a gradual increase in surfactant flux. Thereafter, there is a rapid

increase from 135 days until birth [79]. It is important to note that the final stages of fetal lamb surfactant system maturation have been designated: immature (99-119 days gestation); transitional (120-134 days gestation); and mature (135 days to term) [75]. Virtually all lambs delivered before 130 days gestation develop respiratory distress syndrome, while all lambs delivered after 136 days maintain spontaneous breathing [75].

There are two surfactant pools in the lung: one is the extracellular pool, surfactant secreted into the alveolar spaces and isolated by bronchoalveolar lavage (BAL), and the second is the intracellular pool that consists of lung tissue lamellar bodies in alveolar type II cells [80]. When the extracellular surfactant pool is analyzed in normal animals, it is composed of 90% lipids of which the most common is PC, and 10% surfactant-specific proteins SP-A, SP-B, SP-C, and SP-D [80]. The phospholipid composition of the lamellar bodies is very similar to the composition of the extracellular compartment [80]. Lamellar bodies contain SP-B and SP-C which constitute 28% and 22% of total lamellar body protein, whereas SP-A accounts for only 1% of lamellar body protein [81]. Extracellular surfactant from BAL indicates a different pattern of surfactant protein distribution where SP-A accounts for 50%, SP-B for 8%, and SP-C for 4% of the protein [81]. When compared to lung tissue SP-A content, the BAL SP-A content is higher. This suggests that SP-A secreted into BAL is derived from sources other than lamellar bodies, such as from other organelles within type II cells or Clara cells [81, 82].

The phospholipid composition of BAL and lung tissue surfactant is mainly PC, of which more than half is fully saturated [76]. The saturated PC consists almost entirely of dipalmitoyl species and it is dipalmitoyl-PC that is largely responsible for the activity of pulmonary surfactant in reducing surface tension [76, 83]. Phosphatidylglycerol is the second most abundant phospholipid in surfactant and accounts for up to 12% of the total [76]. On the other hand, sphingomyelin is only a minor component of surfactant [76]. The unique composition of surfactant phospholipids has allowed the development of assays for measurement of surfactant in amniotic fluid. Thus, the ratio of PC (lecithin) to sphingomyelin (L/S ratio) and the amount of phosphatidylglycerol are used clinically to assess the extent of fetal lung maturity [76].

Both SP-A and SP-B are essential for tubular myelin formation, and SP-B is critical for film formation [82]. The lamellar bodies containing lipids and surfactant proteins are exocytosed from the type II cells, and unravel to form tubular myelin and loose lipid arrays. This forms the alveolar surface film that decreases surface tension. The lowered surface tension allows for proper lung inflation during inspiration, and prevents alveolar collapse during expiration. Surfactant secretion is stimulated in the intact lung by hyperventilation and lung inflation [81].

Interestingly, in vitro, SP-A inhibits surfactant secretion and stimulates PC uptake by type II cells [81, 82]. However, in vitro SP-A function does not correlate with in vivo SP-A function as SP-A gene knock-out mice have normal lung function [84]. It is thought that SP-A is not an important regulator of surfactant metabolism in vivo under steady state condition [82]. Rather, in vivo, SP-A is considered a lung host-defense molecule, much like complement [76, 80, 85].

In type II cells, SP-B is localized to the lamellar bodies [86]. Inactivation of the SP-B gene results in disruption of lamellar bodies and normal processing of other surfactant components [84, 87]. Once SP-B and PC are in lamellar bodies, they have secretion kinetics that are parallel [86]. SP-B gene knock-out mice do not survive after delivery unless rescued with SP-B proprotein or managed with liquid perfluorocarbon ventilation [84]. The heterozygous mice have decreased lung compliance and air-trapping [84]. In both homozygous, and heterozygous mice, PC, SP-A, SP-C, and SP-D are present in normal amounts [84]. SP-B content is a predictor of lung function at birth [84]. From a clinical point of view, there is a significant incidence of abnormal structural variants of SP-B or low levels of SP-B amongst infants with respiratory distress syndrome [84].

VI. Lung Hypoplasia

Fetal lung hypoplasia is rarely idiopathic and is usually associated with an abnormality of the physical factors necessary for normal fetal lung growth. Hypoplastic lungs are defined as lungs with a low lung weight to body weight ratio (LW/BW) of <0.012 (67% of mean normal ratio) [4], a low radial alveolar count of <4.1 (75% of mean normal count) [4], or high mean terminal bronchiole density [88]. There are four main physical factors that can lead to lung hypoplasia [53]:

1. Adequate fetal intrathoracic space is not available [53]:

This occurs if there is a space-occupying lesion in the fetal thorax preventing normal lung expansion. Such is the case in congenital diaphragmatic hernia where there is a hole in the diaphragm allowing intestines to herniate into the thorax, preventing lung growth.

2. Adequate intrauterine space is not available [53]:

This occurs with oligohydramnios where there is reduced amniotic fluid either from premature rupture of membranes causing a leak of amniotic fluid, or from urinary tract abnormalities that reduce the flow of urine into the amniotic cavity. It is thought that the low amniotic fluid cannot "cushion" the fetal thorax from the weight of the uterine walls, and the fetus assumes a position of extreme trunk flexion with reduction in thoracic volume, upward displacement of the diaphragm, and restriction of fetal breathing movements, thus limiting lung growth. Another hypothesis is that pulmonary hypoplasia associated with oligohydramnios is a result of an increased loss of pulmonary fluid [89]. This concept developed from the observation that fetal rabbits which had amniotic fluid shunting (to simulate oligohydramnios) and ablation of fetal breathing movements by high cervical cord transection had a further decrease in lung growth than those fetal rabbits which had only high cervical cord transection [89].

3. Fetal breathing movements of normal frequency and intensity are not possible [53]:

Animal experiments where the fetal phrenic nerve is sectioned have hypoplastic lungs by abolishing fetal breathing movements. In addition, because of the lack of innervation of the diaphragm by the phrenic nerve, there is diaphragmatic muscle atrophy resulting in a passive upward movement of the diaphragm into the thorax because of lung recoil. This upward movement of the diaphragm limits normal lung growth.

4. Normal balance of lung liquid volume and pressure is not possible [53]:

When the fetal positive intra-tracheal pressure is decreased to amniotic fluid pressure levels by performing a tracheostomy in utero in fetal lambs, it is found that lung growth is decreased. In other experiments were the fetal lung liquid is chronically drained, there is resultant lung hypoplasia. Interestingly, a case report of a baby born with

laryngeal atresia, and tracheo-esophageal fistula (unchecked lung fluid drainage into the gastro-intestinal tract) that resulted in pulmonary hypoplasia demonstrates the importance of an adequate amount of distending lung fluid for lung growth [90].

VII. CDH Lung Development

Around 20-40% of neonates with CDH are unable to adapt to extrauterine life [17-19]. This is due in part to the associated lung hypoplasia and pulmonary hypertension as seen in human CDH and animal models of CDH [49, 91-94]. In the human, CDH is thought to occur when there is failure of closure of the pleuroperitoneal canal at 8-10 weeks gestation, corresponding to the pseudoglandular stage, or even earlier in the embryonic stage [18]. CDH lungs have a low lung weight to body weight ratio, low radial alveolar count, and a low number of airways. The hypoplastic lungs also appear structurally immature with thickening of alveolar walls and muscularization of the small pulmonary arteries. Even though the defect in the diaphragm is usually left-sided, both lungs are affected. The ipsilateral lung is the most hypoplastic, but the contralateral lung is also hypopolastic as a result of compression from a shifted mediastinum. Even though in the fetal lamb model of CDH, we are creating a defect later in gestation at the beginning of the canalicular stage, this nevertheless profoundly affects lung development.

The pulmonary hypertension seen in CDH is similar to idiopathic PPHN [93]. The mechanisms that may contribute to pulmonary hypertension in CDH are not fully known, but can be divided in terms of vasoactive mediators, and structural factors.

Even though inhaled nitric oxide is a selective pulmonary vasodilator that significantly improves oxygenation in newborns with PPHN from various causes, it is often not effective in CDH [95, 96]. The poor response of CDH to nitric oxide is not clear as there appears to be an intact nitric oxide pathway with normal pulmonary artery relaxation response [97, 98, 99]. However, the relaxation response of pulmonary veins to nitric oxide is abnormal [99].

Interestingly, human babies with CDH and significant PPHN have elevated endothelin-1 levels, a potent vasoconstrictive and pro-mitogenic peptide [69, 100-103]. Thus, endothelin-1 is suspected to be a pathological mediator of PPHN in patients with CDH [93]. Endothelin-1 is a vasoactive peptide released by the endothelium with both

constrictor and dilator activities. In the fetal lamb, even though endogenously produced endothelin-1 acts on at least two receptors, ET-A and ET-B, which mediate vasoconstriction and vasodilation, respectively, it primarily causes vasoconstriction [98, 104, 105]. Imbalance of the endothelin-1 receptor activation favoring pulmonary vasoconstriction is thought to account for PPHN in the fetal lamb CDH model [98]. Interestingly, in the rat nitrofen induced CDH model, if an endothelin-converting enzyme/neutral endopeptidase inhibitor is given during pregnancy, there is increased survival at birth over CDH rats without treatment, and this could support the hypothesis that endothelin could exert an action on the embryologic process of PPHN associated with CDH [106]. It may be that the hyperreactivity of the endothelin-1 pathway accounts not only for the functional pulmonary vasoconstriction in CDH, but also for the histological pulmonary vascular remodeling by stimulating smooth muscle cell proliferation, with increased wall thickness of small pulmonary arteries [98].

Structurally, human CDH lungs have a decreased number of arterial branches and an increased muscularisation of the arterial tree, with extension of the muscularisation into intra-acinar arteries [3, 21, 107-113]. On the other hand, CDH lungs have a normal capillary load because there is a proportional decrease in total alveolar surface area, and in total capillary surface area [110, 114]. For all sizes of arteries of human CDH lungs, there is increased medial and adventitial thickness; for those arteries with an external diameter of <75 μm, both medial and adventitial areas are increased, and for those arteries >75 μm, only the adventitial area is increased [5]. All sizes of veins of human CDH have an increased adventitial area [6, 115]. It is thought that connective tissue accumulation within the vessel wall contributes to alterations in vascular conductance and to persistence of hypertension. Furthermore, in human CDH, newly synthesized pro-collagen was detected in the media and adventitia of pulmonary arteries [116]. The importance of structural changes, even in the postnatal period, was shown further by the adventitial thinning of small pulmonary arteries <100 μm as one of the mechanisms by which ECMO treatment may lead to improvement in pulmonary hypertension associated with human CDH [7].

Even though different animal models have been validated for the study of PPHN, it is important to remember that humans and different animal species with significant PPHN show gradients of pulmonary artery structural changes. For example, in the experimental model of fetal lambs with PPHN where the ductus arteriosus is ligated in utero, there is an increase in vascular percent medial thickness only in small vessels of 50-100 μm diameter, with the rest of the larger pulmonary arteries showing minimal pulmonary vascular remodeling when compared to other animals, like the rat [117-119]. In addition, CDH pulmonary hypertension arterial changes seem more accentuated in human cases as opposed to the CDH lamb model. In the fetal sheep model of PPHN, there is lack of changes in connective tissue deposition, and only the small arteries are affected. This may explain why in the human CDH, the adventitial changes involve all sizes of arteries, while in the lamb CDH, only the small arteries (pulmonary arteries 50-100 μm in diameter) have increased adventitial areas when compared to controls.

Lungs of neonates with CDH have a poor compliance [120]. The compliance of the hypoplastic lung is decreased because of a primary or secondary surfactant deficiency, and because of an increased intrinsic lung stiffness caused by increased collagen content [8-17, 20]. This decrease in compliance creates a clear disadvantage in these neonates as during their first breaths, they are unable to establish a normal FRC. Their alveoli collapse and subsequent breaths will require higher inflation pressures that can lead to eventual respiratory distress. One study analyzed FRC and compliance of human neonates with CDH as a predictor of outcome. It found that the most accurate predictor of poor outcome (defined as death or oxygen dependency at 28 days of life) was a very low compliance (<0.18 mL/cm H_2O/kg) [120]. Functionally, hypoplastic CDH lungs exhibit poor gas exchange with low PaO_2, and high $PaCO_2$ when compared to controls [121]. In the fetal lamb with CDH, the lungs are profoundly surfactant deficient: BAL has shown decreased phospholipid, a decreased % PC, SP-A, and SP-B, even though the CDH fetal lamb model has been shown to have an increased type II cell density [9, 18, 37, 122].

Other studies showed that in human neonates with hypoplastic lungs from CDH, a minimum lung volume of 45% of the value predicted from age-matched controls is required for survival [20, 21]. Thereafter, there is postnatal lung growth and pulmonary

artery remodeling in CDH babies [123]. Just as alveolar size and number increase postnatally in the normal situation [61], this also occurs in the hypoplastic lungs of CDH babies. In one longterm follow-up study of 19 patients with repair of CDH with a follow-up of 6-18 years, their total lung capacity (TLC), vital capacity (VC), residual volume (RV), diffusing capacity (DLCO), and forced expiratory volume in 1 second (FEV_1) were all normal [124]. Conversely, in a more recent study of 60 patients with a repaired CDH at a mean follow-up of 29.6 years, obstructive or restrictive ventilatory impairment was found in 52% of the patients; 25% of the patients had both obstructive and restrictive impairment, but relatively few of the patients with abnormal lung function had clinical symptoms [125]. In addition, even though there is postnatal vascular remodeling with larger and less muscular arteries in CDH, thereby diminishing the pulmonary hypertension over time, longterm follow-up studies have consistently shown that CDH survivors have a persistent reduction of blood flow on the ipsilateral lung. Ventilation on the ipsilateral side is also lower, but the perfusion is worse [124, 125]. Thus, it appears that initially, a CDH baby needs a minimum lung volume and a minimum compliance to survive. Because the lungs grow postnatally, even if there is persistent abnormal perfusion on the ipsilateral lung, most survivors have few clinical respiratory symptoms beyond the second year of life [126].

VIII. Tracheal Occlusion to Correct Lung Hypoplasia

As mentioned earlier, the clinical observation that babies born with laryngeal atresia [127], a form of "natural" TO, had larger than normal lungs, led to the idea that in utero TO may cause lung growth and thus reverse lung hypoplasia. Indeed, prolonged increases in lung liquid volume and pressure caused by fetal TO greatly stimulate lung growth as proven by various animal models with normal lungs or with hypoplastic lungs.

Fetal TO reverses lung hypoplasia in CDH as shown by previous studies using the fetal ovine model [41, 42]. In the lamb CDH model, TO accelerates lung growth with a radial alveolar count comparable to normal lungs, and demonstrates thin alveolar walls with very little interstitial tissue compared to the thickened alveolar walls and increased interstitium of CDH lungs [37]. The air-space fraction and alveolar numerical density of CDH+TO are similar to controls. TO also reverses the increased muscularization of

pulmonary arteries of CDH [128, 129]. In the fetal lamb, DiFiore et al have shown that CDH+TO results in normal capillary load, normal thickness of capillary-alveolar interface, and no fully muscularised vessels <100 μm [128]. One important detail to note from the latter study is that both TO and CDH were done at the same operation, and thus, the lung was not hypoplastic to start off with.

An elevated pulsatility index has been described in fetal CDH lambs [130]. This is a Doppler measurement which reflects pulmonary vascular impedance as a measure of small vessel resistance to pulsatile flow [130]. Fetal TO reverses this high pulsatility index, resulting in a normal physiological response to changes in oxygen tension at term [130]. Similarly, CDH+TO in rats showed that in the preacinar arteries, both medial thickness and adventitial thickness were decreased to normal levels; while in the intra-acinar arteries, medial thickness, but not adventitial thickness, was decreased to levels even lower than normal [129].

The specific mechanisms responsible for lung growth from TO are not entirely known, but there are several theories. Neonatal lambs with CDH+TO have increased mean lung liquid volumes, and increased mean lung air volumes when compared to controls. TO results in intra-tracheal pressures of 4 mm Hg higher compared to control animals without TO [131]. Is the accelerated lung growth associated with in utero TO because of retained growth factors in the lung fluid, increased intra-tracheal and intra-alveolar pressures, or both ?

Some believe it is the elevated intra-tracheal pressure that produces large volume changes in liquid-filled lungs of fetal lambs. This volume increase creates a tension on the cells of the pulmonary system resulting in cell proliferation. The hypothesis of control of cell division by tension is seen in other systems, for example, the use of skin expanders where mechanical stress leads to increased cell proliferation [132, 133]. In vitro studies of fetal pulmonary cells in culture have shown that rhythmic stretching by as little as 5% can increase cell proliferation [134, 135]. It is thought that platelet-derived-growth-factor-β (PDGF-β) and its receptor are involved in mechanical strain-induced fetal lung cell division [136]. Distortion of the extracellular matrix by mechanical distension may affect type II cell function [70]. In addition, if the pressure inside the trachea is maintained at a

level higher than the pressure generated by TO alone (ie: 8.5 mm Hg intratracheal pressure in the pressurized group versus 4 mm Hg intratracheal pressure in the TO group), then there is a significant increase of lung growth in the pressurised group compared to TO alone [137]. This indicates that the amount of sustained intratracheal pressure correlates with lung growth. In addition, the duration of TO also correlates with lung growth as 3 weeks of TO [131] produces larger lungs than 1 week of TO [34].

On the other hand, in the experiment by Nardo [138], it was found that increases in lung liquid volumes may be more important than increases in intratracheal pressures. Normal fetal lungs were occluded in utero and compared to controls. It was found that tracheal pressures increased from the normal control value of 3 mm Hg to 4.3 mm Hg in those with TO within one day and remained at this level for the duration of the occlusion period. Lung liquid volume increased progressively from 25 mL/kg on day 0 to 100 mL/kg on day 7 of TO, but did not increase further afterwards (this may be due to the physical boundary of the chest wall preventing any further increases in lung liquid volume). DNA and protein contents were markedly increased in the TO group especially by day 2 of TO. Thus, they concluded that the mechanisms responsible for lung growth by TO are most active on day 2 of TO and returned to control levels by day 10 of TO. The TO lungs had a stable protein/DNA ratio indicating that lung hyperplasia, and not hypertrophy had occurred. Interestingly, the increase in DNA content correlated with the increase in lung liquid volume, but not with the increase in intra-tracheal pressure. Thus, they felt that it was the increase in lung liquid volume rather than increases in intra-tracheal pressures that was responsible for the increased lung growth with TO.

However, this discussion becomes like "the chicken or the egg" debate. In a closed space, like the fetal thoracic cavity, an increase in volume will lead to an increase in pressure. We feel that initially the pulmonary epithelial cells produce lung liquid which is trapped because of the tracheal occlusion, and this accumulation of fluid leads to an increase in pressure over a certain period of time. This increase in pressure will create a mechanical stress leading to increased cell proliferation and increased alveolar count. These increased number of pulmonary cells produce even more lung liquid, thus

increasing lung volumes and lung fluid. The cycle of growth induced by tension continues as long as tracheal pressure remains elevated.

Are there particular growth factors associated with accelerated lung growth? In an experiment by Papadakis [139], fetal lambs had TO, but their lung liquid volume was aspirated and replaced with an equal amount of saline. It was found that replacement of lung liquid with saline inhibits the lung growth seen after TO. Even though there may have been momentary drops in intra-tracheal pressures during the lung fluid exchanges, and thus decreasing the growth stimulus, their findings suggest that there are specific growth factors in the lung liquid that are "trapped" with TO that may be important in lung cell proliferation.

Growth factors that may be involved in fetal lung growth include: insulin-like growth factors (IGF-I and IGF-II), epidermal growth factor (EGF), PDGF, transforming-growth-factor-β1 (TGF-β1), and TGF-β2 [140, 141]. The precise interactions between these growth factors is not yet elucidated. As stated earlier, PDGF is important in mechanical strain fetal lung growth [136]. IGF-I gene expression is reduced in the fetal lamb CDH model and restored to normal by TO [142, 143]. Another group has found that IGF-I mRNA expression is actually increased in lung tissue from human CDH autopsies [144]. Moreover, TO induces IGF-II mRNA expression, while lung hypoplasia caused by abolition of fetal breathing movements decreases IGF-II mRNA [145, 146]. EGF is known to accelerate type II cell maturation to a greater extent than steroids [147]. Structurally related to EGF, TGF-α causes remodeling of the developing lung during postnatal alveolarisation [148]. In addition, only TGF-β2, and not TGF-β1, mRNA and protein are increased in CDH+TO [149]. Interestingly, both TGF-β1 and TGF-β2 inhibit the expression of SP-A in human fetal lung explants, and this may suggest that increases in TGF-β2 due to TO may result in delayed type II cell maturation [150-152]. Recently, keratinocyte growth factor (KGF), which is involved in normal lung organogenesis and is a potent mitogen of type II cells, has been found to be downregulated in CDH lambs, while it is upregulated in CDH lambs treated with TO [153].

IX. Is Tracheal Occlusion the Cure for Lung Hypoplasia?

At first glance, it would seem that TO could solve the problem of lung hypoplasia. Unfortunately, TO may have a pitfall: TO of normal fetal lungs consistently causes a decrease in type II pneumocytes and a dramatic surfactant deficit [34-37]. Thus, TO either damages type II cells or delays their maturation while accelerating lung growth. Perhaps it is the exaggerated lung expansion and mechanical stress induced by TO that favors the conversion of type II cells into type I cells, as it is known that type II cells can differentiate into type I cells under situations of lung injury or alveolar stretch [70, 154].

In addition, TO of fetal hypoplastic lungs also have a low type II cell number. For example, fetal lambs with CDH+TO for 19 days have a decreased type II cell density, as identified by SP-B immunohistochemistry [37]. Even though one paper did not find any difference between CDH and CDH+TO in terms of levels of saturated PC in lung tissue homogenate, the saturated PC as a percentage of total amount of phospholipid was lower in the CDH+TO group [41]. Additional stidies have found that CDH+TO lungs have reduced lung lavage phospholipids [155], reduced surfactant lipid synthesis from isolated type II cells [155], and reduced disaturated PC in lamellar bodies isolated from homogenised lung tissue when compared to CDH [37].

Paradoxically, CDH+TO lungs have better oxygenation and ventilation than CDH [41, 42, 155] with the compliance of CDH+TO being better than CDH alone, but not quite reaching normal neonatal lamb values [42, 155]. The compliance of CDH+TO lung is increased 3.5 times when compared to CDH alone. By adding surfactant to CDH+TO, these lungs demonstrate even better oxygenation [156].

X. One Step Further: Release of Tracheal Occlusion Before Birth

Fortunately, even though lung growth after TO occurs at the expense of type II cell differentiation, this effect is reversible with the release of tracheal occlusion (TR) before birth in the lamb model [38]. Thus, TO of normal fetal lungs for some weeks, followed by TR before birth allows for beneficial effect on lung growth, and also allows for the type II cells to recover to normal density with appropriate surfactant production. For example, BinSaddiq et al found that TR 2 days before birth was not sufficient to reverse the deleterious effects of TO on type II cells, but that TR 7 days before birth

restored type II cell density and surfactant gene expression almost back to normal levels of the control group [38]. Thus, TR at least one week before delivery restores type II cell number, as detected by SP-C in-situ hybridization, and SP-C mRNA expression in normal, non-hypoplastic fetal lamb lungs [38].

Another study by Papadakis et al was done where CDH, CDH+TO for 2 weeks+TR 2 weeks before delivery, normal lungs+TO for 4 weeks, and controls were compared [35]. Similarly to BinSaddiq et al, Papadakis et al found that normal lungs+TO have a decreased type II cell density as identified by anti-SP-B immunohistochemistry. In contrast to other studies [9, 18, 37, 122], Papadakis et al found no difference in type II cell density between CDH, CDH+TO+TR, and controls [35]. Interestingly, their treatment of TO+TR to a hypoplastic CDH lung model did not produce lung growth as CDH and CDH+TO+TR groups had similar lung weight/ body weights and radial alveolar counts [35]. This may be explained if their technique of TO was not completely occlusive, and thus ineffective at promoting lung growth. Thus, their conclusions regarding CDH versus CDH+TO+TR in terms of type II cell density and maturity may be loosely interpreted.

XI. The Effect of Glucocorticoids on Normal Fetal Lungs

Glucocorticoids have a dramatic effect on the fetal lung of several animal species and humans: they increase lung tissue and alveolar surfactant; they increase compliance and maximal lung volume; they decrease vascular permeability; and they produce more mature parenchymal structure. This translates to improved respiratory function, and increased survival [157].

More specifically, glucocorticoids enhance SP-B and SP-C mRNA in fetal lungs [78, 158]. On the other hand, glucocorticoids have a complex effect on SP-A gene expression that is species-specific, and dependent on the gestational age, and the dose given [74, 158]. At lower doses, there is a stimulatory effect on SP-A mRNA, and at higher doses, an inhibitory effect [74, 158]. To further cloud the picture, surfactant protein mRNA is unaltered in Glucocorticoid Receptor knock-out mice; this would indicate that the glucocorticoid receptor is not required for surfactant protein gene expression in the perinatal and postnatal periods [158]. However, Corticotropin Releasing

Factor knock-out mice have a 44% reduction in SP-B mRNA [158]. In addition, steroids stimulate type II cell choline-phosphate cytidyltransferase, the enzyme responsible for PC synthesis [159].

Glucocorticoids also increase lung compliance and maximal lung volumes by surfactant-independent means in preterm rabbits [160]. The lung structural remodeling and maturity achieved by glucocorticoids is shown by changes in collagen:elastin ratio [161], and by morphometric changes with decreased perilobar connective tissue, decreases in alveolar wall thickness and an increase in aerated parenchyma in animal species [162-164]. In addition, glucocorticoids have an anti-oxidant effect in lambs [165], reduce protein leak from pulmonary vasculature [157], and accelerate clearance of fetal lung liquid before birth [157].

Interestingly, while glucocorticoid induced structural maturity occurs within 48 hours after treatment, it can take longer for surfactant pool sizes of the alveolar and lung tissue to increase [166-168]. Thus, it appears that the endogenous synthesis and secretion of PC is a slow process. This slow synthesis rate may explain the long delay between prenatal glucocorticoid treatment and increased surfactant pools. This implies that the improved pulmonary function at birth of preterm animals after antenatal glucocorticoids is initially from lung structural changes.

Moreover, antenatal steroid treatment further improved type II pneumocyte number and function in normal fetal lambs with TO and TR [169].

XII. The Effect of Glucocorticoids on CDH Fetal Lungs

Prenatal glucocorticoid treatment in a CDH lamb model with high dosing fetal intravenous (i.v.) steroids twice a day for three days before delivery improved lung morphology with thinning of the interstitium, and more mature alveoli with thinner walls [40]. There was significant decrease of glycogen in the contralateral lung, but not the ipsilateral lung, where a decreased glycogen level is an indication of pulmonary biochemical maturation [39]. Even though the concentrations of lung tissue disaturated PC did not change, there were significant improvements in oxygenation and compliance compared to CDH without prenatal glucocorticoids [39]. In another study with CDH lambs and antenatal maternal intramuscular (i.m.) glucocorticoids, the authors did not

find any improvement in gas exchange, and no difference in lung lavage phospholipid concentrations between the steroid treated and the saline treated CDH [170]. However, there was a marked improvement in compliance of CDH lambs treated with one dose of glucocorticoids once a day for two days before delivery [170]. Clearly, the steroid protocol, ie: whether it is given to the ewe or the fetus directly, the route, the dosage, the timing, and the duration will affect the intensity of the outcome. Interestingly, in rats with nitrofen-induced CDH, glucocorticoids also have an effect of increasing SP-A and SP-B mRNA, with correction of the biochemical, morphometric, and compliance abnormalities of CDH [171-173].

As for pulmonary artery remodeling, to our knowledge, there are no documented reports of the effect of antenatal steroids on the CDH pulmonary media and adventitia in the fetal lamb model. However, in the rat nitrofen-induced CDH model, antenatal maternal dexamethasone treatment reverses CDH pulmonary arterial structural changes with resultant normal medial thickness and area, and normal adventitial thickness and area [174-175].

XIII. Prenatal Interventions and Survival Protocols for Human CDH Fetuses

Presently, the mean survival rate for live-born babies with CDH is 60-80%, with some improvement in recent years, using the concept of gentle ventilation and permissive hypercapnea [18, 19, 176]. This is because the baby is born with a "fait accompli": pulmonary hypoplasia. Consequently, the only hope for improvement is to address the pulmonary hypoplasia by intervening before the lung is irreversibly affected. This can only be accomplished during intra-uterine life.

In utero repair of the diaphragmatic defect in human fetuses with CDH was attempted in 14 patients as reported in 1993 by the University of California in San Francisco group [25]. Five died intraoperatively, and 9 were successfully repaired. However, of those 9, 5 died post-operatively, and only 4 survived. The major lesson learned was that direct diaphragmatic repair is not possible if the liver is up in the chest since reduction of the liver back into the abdominal cavity causes fatal umbilical vein kinking.

Clearly, not all human fetuses with CDH are the same. Prenatal ultrasonographic predictors have divided human fetuses with CDH into two groups: good and poor prognosis [23, 24]. The poor prognosis CDH includes those fetuses diagnosed before 25 weeks gestation, with a lung-head ratio less than 1, and with a significant portion of the liver up in the chest as evidenced by Doppler flow of the umbilical vein or hepatic vein. The good prognosis CDH did not have any of those features. If the diagnosis of CDH was made at or before 25 weeks gestation, survival was 56% versus 100% if made after 25 weeks. As these were all left-sided CDH, the lung-head ratio is defined as the right lung area to head circumference as measured by prenatal ultrasound (US). If the lung-head ratio is less than 0.6, there are no survivors; if the lung-head ratio is 0.6-1.35, there is a 61% survival rate; and if the lung-head ratio is greater than 1.35, there is 100% survival. In addition, if the liver was up in the chest, survival was 56%, while if the liver was down, then survival was 100%.

Moreover, as published in 1997, for good prognosis human fetuses with CDH without liver herniation and favorable lung-head ratio, in utero correction of the diaphragmatic defect did not improve survival over standard postnatal care (75% versus 86%, respectively) [22].

Candidates for fetal intervention are those "poor prognosis" CDH human fetuses such as liver up in the chest, low lung-head ratio, and early diagnosis [28]. For these, in utero fetal TO has proven beneficial, particularly when a fetoscopic approach is used, by allowing for prenatal lung growth such that extra-uterine existence is possible. Thus, for poor prognosis isolated left sided CDH fetuses, survival rate was 38% in the group treated by standard postnatal therapy; 15% in the open TO group; and 75% in the fetoscopic TO group [28].

There is a trend toward the use of maternal glucocorticoids for the prenatal treatment of fetuses diagnosed with CDH [177]. One protocolized approach included antenatal assessment, antenatal steroids, planned delivery, use of prophylactic surfactant, gentle ventilation, and ECMO if indicated [177].

XIV. Prenatal Interventions and Survival Protocols for Lamb CDH Fetuses

In order to understand the pathophysiology of CDH and how medical and surgical interventions can alter the course of this disease, an appropriate animal model must be in place. For CDH, there are two main established animal models that resemble the human CDH condition: the surgically created CDH in the fetal lamb at the early canalicular stage, and the nitrofen-induced CDH in the fetal rat at the embryonic stage [94]. Of course, each model has its limitations: the lamb CDH model is a relatively "late" onset CDH, while nitrofen is a teratogen, and affects other organ systems also. Thus, the larger lamb CDH model is mainly used to study surgical and medical interventions, and measure ventilatory parameters, while the smaller rat CDH model is mainly used for embryological and molecular studies.

The following is a review of resuscitation studies of fetal lamb CDH with various treatment modalities and how these affected pulmonary parameters. The number in parenthesis is the date that particular paper was published. Most of these studies were done under the assumption that the fetal lamb CDH model is surfactant deficient, and that while TO promotes lung growth, it further depresses the surfactant pool by decreasing the number of type II cells. Thus, the treatment modalities have the goal of either increasing lung growth and/or increasing surfactant pool.

a. CDH+glucocorticoids

1. (1996) The resuscitation was conducted for 30 minutes after birth, and compared CDH versus CDH+fetal i.v. glucocorticoid high dosing twice a day for three days before delivery. The CDH+glucocorticoid group showed improved lung morphology with thinning of the interstitium, and more mature alveoli with thinner walls [38]. There was significant decrease of glycogen in the contralateral lung, but not the ipsilateral lung, where a decreased glycogen level is an indication of pulmonary biochemical maturation. Even though the concentrations of lung tissue disaturated PC did not change, there were significant improvements in oxygenation and compliance in the CDH+glucocorticoids when compared to CDH without prenatal glucocorticoids [39].

2. (1999) The resuscitation was conducted for 2 hours after birth, and compared CDH, CDH+maternal i.m. glucocorticoids at 24 hours before delivery,

CDH+maternal i.m. glucocorticoids at 24 hour and 48 hours before delivery, and normal controls without any treatment. The authors did not find any improvement in gas exchange, and no difference in lung lavage phospholipid concentrations between the glucocorticoid treated and the saline treated CDH. However, all CDH animals, regardless of whether or not they received glucocorticoids, had low phospholipid concentrations when compared to controls. Nevertheless, there was a marked improvement in compliance for the CDH+maternal i.m. glucocorticoids at 24 hour and 48 hours before delivery, but not quite reaching normal control values [170].

b. CDH+surfactant

1. (1994) The resuscitation was conducted for 30 minutes after birth, and compared CDH versus CDH+surfactant before the first breath. Exogenous prophylactic surfactant improved gas exchange with higher PaO_2, lower $PaCO_2$, and higher pH when compared to untreated CDH. In addition, the exogenous prophylactic surfactant increased lung volumes, FRC, and compliance [15].

2. (1996) The resuscitation was conducted for 4 hours after birth, and compared CDH, CDH+surfactant before the first breath, and normal controls without any treatment. Exogenous prophylactic surfactant in CDH increased pulmonary blood flow, and decreased pulmonary vascular resistance to normal levels. This resulted in lower right-to-left shunting with higher PaO_2, lower $PaCO_2$, and higher pH when compared to untreated CDH [178].

3. (1996) The resuscitation was conducted for 4 hours after birth, and compared CDH versus CDH+surfactant rescue after 30 minutes of ventilation. Surfactant rescue had no effect on PaO_2, $PaCO_2$, or pH. Thus, if surfactant is to be effective in CDH, it should be administered as early as possible, even before the first breath [14].

c. CDH+TO

1. (1994) The resuscitation was conducted for 2 hours after birth, and compared CDH versus CDH+TO. CDH+TO had larger lungs and mature alveolar structure with increased compliance that translated to improved oxygenation and ventilation when compared to CDH hypoplastic lungs [42].

2. (1994) The resuscitation was conducted for 1 hour after birth, and compared CDH versus CDH+TO. Similarly, CDH+TO had larger lungs with higher PaO_2, higher pH, and lower $PaCO_2$ when compared to CDH hypoplastic lungs [41].

3. (1996) The resuscitation was conducted for 30 minutes after birth, and compared CDH, CDH+TO, and normal controls without any treatment. CDH+TO resulted in significant lung growth with similar lung weight over body weight to controls. The lungs of CDH+TO had better oxygenation and ventilation than CDH, and was comparable to controls. However, the compliance of CDH+TO was intermediate between the low compliance of CDH and the high compliance of control lungs. In addition, because the BAL showed a reduction in total phospholipids with a decrease in surfactant synthesis by isolated type II in the CDH+TO group, it was thus assumed that the improved oxygenation, ventilation, and compliance of CDH+TO versus CDH may only be a transient phenomenon, as this was only a 30 minute survival study [155].

d. CDH+TO+surfactant

1. (1997) The resuscitation was conducted for 4 hours after birth, and compared CDH+in utero direct repair of the diaphragmatic defect, CDH+TO, and CDH+TO+surfactant before the first breath. CDH+TO and CDH+TO+surfactant had larger lungs than CDH with in utero repair. There was no difference between the three groups in terms of pH and $PaCO_2$. Over the course of the 4 hours, PaO_2, pulmonary blood flow, and pulmonary vascular resistance worsened for the CDH+TO group when compared to the other two groups. It was thus concluded that CDH+TO induces lung growth at the expense of type II cell maturation. CDH+TO resulted in surfactant deficient lungs, that over the course of a longer resuscitation of 4 hours, deteriorate. By adding surfactant to CDH+TO, this deterioration was prevented [156].

e. hypoplastic lungs+TO+TR

1. (1999) In order to mimic the "CDH hypoplastic lung", pulmonary hypoplasia was induced in fetal lambs by prolonged drainage of lung liquid. This method of inducing lung hypoplasia was selected because it avoided the need for surgical creation of CDH and its subsequent repair needed for the planned prolonged resuscitation. The resuscitation was conducted for 8 weeks, and consisted of three groups: 1. the group with

lung liquid drainage from day 112 of gestation until term (149 days); 2. the group with lung liquid drainage from day 112, then TO at 137 days, then TR at 147 days, and delivery at 149 days; and 3. controls were normal lambs delivered at 149 days. The lambs with lung liquid drainage from day 112 of gestation until term had hypoplastic lungs, and died within 4 hours of birth. The ones that had lung liquid drainage+TO+TR were hypoxic for the first week and were hypercapneic at 2 days of life. Pulmonary diffusing capacity, gas volumes, and respiratory compliances were not different between control and lung liquid drainage+TO+TR lambs. Minute ventilation was not different between the two groups; but tidal volumes were lower and respiratory frequencies were higher in lung liquid drainage+TO+TR lambs than controls for 2 weeks after birth. The authors concluded that 10 days of TO in the presence of initial lung hypoplasia prevented death at birth and returned most aspects of pulmonary function to normal by 1-2 weeks after birth [179].

XV. What Remains To Be Done: CDH+TO+TR+glucocorticoids

Given the high mortality of CDH from pulmonary hypoplasia, we sought to combine the beneficial effects of fetal tracheal occlusion to accelerate lung growth with release of the tracheal occlusion one week before birth to restore type II cell density, and antenatal glucocorticoids to increase the surfactant synthetic ability of the remaining number of type II cells. We elected to resuscitate our experimental and control animals for up to 8 hours, as this would be longer than any of the other CDH studies, to further observe pulmonary function trends over time.

Materials and Methods

Animal model:

CDH Creation:

Animal protocols were approved by the McGill University Animal Care Committee. At 80 days gestation, a left sided CDH was created in the fetal lamb as described previously [180]. Briefly, the time-dated pregnant mixed breed ewe was fasted 24 hours pre-operatively. On the day of surgery, anaesthesia was induced in the ewe with i.v. thiopental and maintained with halothane-oxygen mixture while being ventilated on a respirator. After wool clipping, and initial cleansing of the abdomen with proviodine soap and alcohol, the ewe was prepped with proviodine solution, and draped, with strict asepsis being maintained. A midline laparotomy was performed, the uterus was exposed and the gravid horn of the uterus was delivered into the incision. The fetal left hemithorax was identified by palpating the fetal parts through the uterine wall, using the tail, spine, shoulder blade, and costal margin as landmarks. The uterine wall was then sutured with 4-0 silk to the underlying fetal skin as anchor points at the fetus' left shoulder blade and fetus' left costal margin. A small incision in the uterus was made between the sutures at a point 2/3 of the way down from the shoulder stitch to the costal stitch. A left thoracotomy was performed on the fetus. After retracting the fetal left lung superiorly into the chest with a cotton Q-tip, the diaphragm was identified, and was picked up and nicked in its central white tendinous part with a 23G needle tip. The hole in the diaphragm was further enlarged with a fine scissors and spread with a small mosquito forceps, until the hole was 1-1.5 cm in diameter. At this point, at least 2 stomachs (the sheep has 3 stomachs) were gently pulled up into the left chest. The fetal thorax was closed by re-approximating the ribs with interrupted 4-0 silk suture. The fetal chest wall muscle and skin were closed with a running 4-0 silk suture. Prior to closure of the hysterotomy in 2 layers with 2-0 Dexon suture, the uterus was instilled with warm saline, cefazolin 500 mg, gentamicin 150 mg, and liquamycin 200 mg. The fascia of the ewe was closed with #1 Prolene interrupted suture, the subcutaneous tissue reaproximated with a running 3-0 Vicryl suture to obliterate the dead space, the skin was closed with a running subcuticular 3-0

Vicryl suture, and opsite was sprayed on the insicion. The ewe received liquamycin 400 mg i.m. each day for three days post-operatively.

Tracheal Occlusion:

Endoscopic TO was achieved using a detachable balloon system (GVB12 Latex Goldvalve Balloon with a maximum diameter 14 mm, length 22.5 mm, volume 2.5 mL; and CCOXLS co-axial catheters, Yocan Medical Systems, Ontario, Canada). The balloon was inflated with 1.5 mL of saline colored with methylene blue, after being positioned by fetal tracheoscopy (2.7 mm Semi Flexible Mini-Endoscope, Karl Storz, Germany) at 108 days gestation [88, 181, 182]. Briefly, the ewe was prepared as described above for the operation. After delivering the gravid uterus into the incision, the fetal head and mouth were palpated through the uterine wall. The fetal head position was maintained by the surgical assistant to allow for neck extension and optimal endoscope angle of entry. A small 1 cm hysterotomy was made over the fetal snout through which the endoscope was introduced into the fetal mouth. A purse-string suture with 2-0 Dexon was tightened around the hysterotomy to maintain the seal between the uterine wall and endoscope such that there was minimal amniotic fluid loss. Low flow, low pressure irrigation with warm saline, through the first instrument channel of the endoscope, was used to enhance visibility, to obtain sufficient workspace, and to dilate the vocal cords. The endoscope was then gently advanced in the trachea. The second instrument channel was used to position the balloon in the trachea. At first, we positioned the balloon 2 cm above the right upper lobe orifice; however, this proved later on to make tracheal release more difficult if the balloon was too low in the thoracic trachea. We then decided to position the balloon higher up in the cervical trachea at approximately 2 cm below the vocal cords allowing for easy access for future ultrasound (US) guided release. Once properly positioned, the balloon was inflated with 1.5 mL of saline colored with methylene blue, and thus creating the TO. The balloon was detached from its catheter. The balloon has a valve preventing leaking of the filling fluid. The tracheoscope was then removed, the uterus was instilled with cefazolin, gentamicin, and liquamycin, the purse-string closed, and another layer of hysterotomy closure with 2-0 Dexon suture was made over the purse-

string. The rest of the ewe abdominal closure and post-operative care was done as detailed earlier.

Tracheal Release:

TR was done at 129 days gestation either by US-guided (ALOKA SSD-630, probe 3.5 MHz, Instruments for Science and Medicine Inc., Quebec, Canada) percutaneous, or trans-uterine deflation of the fetal intra-tracheal balloon, with a 22G spinal needle; or, by performing fetal tracheoscopy as detailed earlier and deflating the fetal intra-tracheal balloon directly with the micro-scissors via the endoscopic instrument channel. Briefly, the US-guided percutaneous deflation of the fetal intra-tracheal balloon was as follows. The fetal trachea was visualized using US, and we confirmed that it was not a vascular structure by having no doppler flow signals. We then searched the trachea for the balloon by measuring an increase in the mean tracheal diameter. The balloon location could also be visualized by the fact that the valve of the balloon appeared as an echogenic point. Once the tracheal balloon was clearly visualized, the maternal abdomen was prepped and draped, and under sterile conditions, a 22 G spinal needle was placed percutaneoulsy under US guidance through the maternal anterior abdominal wall, into the uterus, and through the anterior fetal trachea. Balloon deflation was confirmed by aspiration of methylene blue stained tracheal fluid, and the percutaneous needle was removed. If percutaneous deflation was not possible because of difficult visualization, or potentially unsafe because of needle trajectory, then maternal laparotomy was performed and US guided needle deflation of the fetal tracheal balloon was done through the uterine wall. If this failed, fetal tracheoscopy was performed as described earlier, and the balloon was deflated with the mini-scissors through the endoscope instrument channel.

All ewes received 250 mg medroxyprogesterone i.m. at 129 days to prevent preterm labour [183]. Progesterone is not known to influence lung development or the response to future glucocorticoid treatment [168].

All ewes also received one dose of 0.5 mg/kg betamethasone i.m. at 135 days to accelerate fetal surfactant production since the lambs are slightly premature [75, 184-187]. The rationale for the glucocorticoid dose and route was to give as much as needed

for an effect on lamb lung function, but also as little as possible to prevent preterm labour in the ewe. Rebello et al have shown that either fetal or maternal treatment with 0.5 mg/kg of betamethasone improved postnatal lung function if given 24 hours before delivery [185].

At 136 days (term=145 days), the fetus was delivered by caesarian, and sacrificed at birth for phase I of the study, and resuscitated for 8 hours for phase II of the study.

Four groups were compared for the phase I study: CDH (n=7), CDH+TO (n=6), CDH+TO+TR (n=6), and unoperated twin controls (n=16). All groups were given antenatal glucocorticoids.

Phase I outcome measures were:

1. Lung growth: LW/BW
2. Lung morphometry: mean terminal bronchiole density (MTBD)
3. Pulmonary artery remodeling: pulmonary artery medial and adventitial areas
4. Type II cell density assessed by electron microscopy (EM)
5. Capillary load assessed by EM
6. Lung tissue and BAL content of SP-A and SP-B
7. BAL content of PC

Four groups were compared for the phase II study: CDH (n=5), CDH+TO (n=5), CDH+TO+TR (n=5), and unoperated twin controls (n=4). All groups were given antenatal glucocorticoids.

Phase II outcome measures were:

1. Lung growth: LW/BW
2. Survival time
3. Arterial blood gas trends: PaO_2, $PaCO_2$, pH
4. Oxygenation and ventilatory parameters
    a. modified ventilatory index (MVI) [188]

        $MVI = (RR \times PIP \times PaCO_2)/1000$

    b. ventilatory efficiency index (VEI) [189]

        $VEI = 3800/((PIP-PEEP) \times RR \times PaCO_2)$

    c. alveolar-arterial oxygen gradient ($AaDO_2$) [188]

$$AaDO_2=[((713 \text{ X } FiO_2) - PaCO_2)/0.8] - PaO_2$$

5. Compliance

Phase I. Lung lavage and processing:

The body weight was measured, and after harvesting the lungs, the total lung weight was measured. Then the right lung and left lung were separated by transecting the left mainstem bronchus, and their respective weights were measured. The total, right and left LW/BW were determined.

After the left mainstem bronchus stump was oversewn on the right lung, the trachea on the right lung and the left bronchus on the left lung were cannulated. BAL was performed on each lung. Each lung was filled twice with saline by gravity until fully distended and the fluid was drained by gravity. Efficiency of this procedure for PC retrieval was expected to be approximately 60% [190]. The two alveolar washes were pooled, and the total volume of lavage fluid was recorded. The lavage fluid was centrifuged at 500g for 10 minutes, to eliminate cellular debris that remained in the lavage fluid. The remaining supernatant was centrifuged at 40 000g for 15 minutes. The final pellet was analyzed for BAL SP-A and BAL SP-B. The final supernatant and final pellet were analyzed for total BAL PC, and total BAL protein. Samples of lung tissue (approximately 1 g each) of the peripheral upper and lower lobes were frozen in liquid nitrogen and stored at -80 °C for analysis of SP-A, SP-B, and protein.

The right middle lobe and lingula were then tied off and fixed with 3% glutaraldehyde at a pressure of 25 cm H_2O for 15 minutes, and processed for electron microscopy (EM).

The remainder of the right and left lungs were separately perfused via cannulation of the trachea or left mainstem bronchus, with 10% formaldehyde at a pressure of 25 cm H_20 for 48 hours, after which time, 2-3 mm thick random transverse 2 cm long slices were taken of both upper and lower lobes, and then embedded in paraffin.

Phase I. Lung Morphometry for Lung Structural Maturity:

Sections 4-5 μm thick of the paraffin embedded lung were stained with hematoxylin and eosin, and morphometric evaluation was performed using the mean terminal bronchiole density (MTBD) method. This method was chosen over the radial

alveolar count because of decreased intra and inter-observer variation. The number of terminal bronchioles seen in a given field is inversely proportional to the number of alveoli surrounding each bronchiole [88, 181, 182]. Thus, a structurally more mature lung has a smaller mean terminal bronchiole density. Forty random (10 from each upper and lower lobes), non-overlapping fields were examined per animal at 100X magnification.

Phase I. Lung Morphometry for Pulmonary Artery Evaluation:

Four-5 μm thick sections of the paraffin embedded lung were stained with Gomori's aldehyde fuchsin stain where the elastic fibers stain deep purple, and counter-stained with Halmi's stain, and where collagen stains green, and muscle fibers stain red. Pulmonary arteries were distinguished from pulmonary veins on the basis of structure and position [191]. Arteries that were approximately round, meaning that the maximal external diameter did not exceed the minimal external diameter by >50%, and had both an external and internal elastic lamina were analyzed. At least 15 arteries per lung equally distributed between upper and lower lobes were examined at 100-200X, with a total of at least 30 random arteries per animal, using the ImageProPlus Version 4.0 for Windows Image Analyzer (Media Cybernetics, Silverspring, MD, USA). The external diameter was defined as the diameter between the external lamina and expressed in μm [191]. Pulmonary arteries were divided in 5 groups according to size: 0-75 μm, 76-100 μm, 101-150 μm, 151-250 μm, and 251-500 μm. The medial area was defined as the area contained by the external elastic lamina minus the area contained by the internal elastic lamina, and expressed in μm^2. The adventitial area was defined as the area contained by the entire artery minus the area contained by the external elastic lamina, and expressed in μm^2. The lumen area was defined as the area contained by the internal elastic lamina. Please see Figure 1 for details. In uninjected arteries, medial area is thought to be a better measurement than percentage of wall thickness because medial area does not vary with the degree of distension of the artery, whereas the percentage of wall thickness does [192, 193].

Phase I. Type II cell density:

Type II cell density was determined by EM as the number of type II cells per cm^2 of tissue alveolar surface area as previously described [38], with some modification.

Results were expressed as the number of type II cells over the cellular surface area of the alveoli, excluding the alveolar air-space in order to avoid bias that could arise from alveolar distension produced by TO. Cellular surface area was determined from EM images using the NIH Image Program, version 1.61 for the Macintosh. Type II cells were recognized by their characteristic lamellar bodies and glycogen inclusions [194].

Phase I. Capillary Load:

Lung capillary load was determined by EM as the vessel luminal surface area to tissue surface area, expressed as a percentage.

Phase I. Lung tissue SP-A, tissue SP-B, BAL SP-A, BAL SP-B, and BAL PC Content:

Ovine SP-A and SP-B were separately determined by enzyme-linked immunosorbent assay (ELISA) of the lung homogenate and of the BAL as described previously, and using purified sheep SP-A and bovine SP-B as controls [195]. SP-A and SP-B concentrations were normalized to total protein content in the lung homogenate, and to the PC content of the BAL. Tissue and BAL protein were determined by the Lowry assay [196] using bovine serum albumin as controls.

BAL PC contents were determined using an enzymatic phospholipid assay kit (Boehringer and Mannhein, Diagnostic Kit PL MPR #691844), as directed by the manufacturer. BAL PC content was normalized to animal body weight.

Phase II. Resuscitation for 8 hours:

At 136 days gestation, the fetal lamb was delivered by caesarian in steps. The maternal sheep underwent general anaesthesia with halothane 0.5-2% (titrated to effect) and pentobarbital 6-32 mg/kg i.v (titrated to effect). A laparotomy was made, and the uterus exposed. A hysterotomy big enough to allow only for the fetal head and neck to emerge was made. A latex glove filled with saline was placed over the lamb's snout to avoid spontaneous breathing. While the fetus was still under placental circulation, cannulation of the carotid artery, jugular vein, and trachea were done as follows. A transverse incision was made in the fetal neck at roughly the midpoint between the suprasternal notch and the thyroid cartilage to allow for a limited dissection of the right jugular vein, right common carotid artery (pre-ductal), and trachea. Once the right jugular vein was identified, it was distally ligated with 2-0 silk tie, and a 5.5 Fr triple lumen

catheter was inserted down to the 10 cm mark, and secured with 2-0 silk tie. The triple lumen was flushed with 1 U/mL heparinised saline. Once the right common carotid was identified, it was distally ligated with 2-0 silk tie, and an 18G Jelco catheter was inserted, and secured with 2-0 silk tie. Blood was withdrawn from the arterial line for fetal arterial blood gas (ABG). Then, the arterial line was flushed with 1U/mL heparinised saline. After the cricoid cartilage was identified, the trachea was opened between its third and fourth rings with a transverse incision. If the animal was in the CDH+TO group, the intra-tracheal balloon was palpated before opening the trachea; the trachea distal to the balloon was encircled with umbilical tape which was gently tightened to prevent the subsequent deflated balloon remnant from lodging down the bronchus; the trachea was incised just distal to the balloon but proximal to the umbilical tape, and the balloon was then punctured with the edge of a number 11 blade, upon which the balloon remnant was removed with fine pick-ups. The tension on the umbilical tape was released, and excess lamb lung liquid was suctioned into a suction trap. A 4 mm uncuffed clamped (by putting a 5 mL syringe plunger on its end) endotracheal (ET) tube was inserted into the trachea down to the 2 cm mark. The ET tube was secured with umbilical tape around the trachea. The neck incison was closed with interrupted 2-0 silk sutures. Based on an average birth weight of 3 kg, paralysis was achieved by pancuronium bromide 0.3 mg i.v. (0.1 mg/kg) given through the jugular venous line. Analgesia was achieved by ketamine 18 mg i.v. (6 mg/kg) through the jugular venous line. We also administered sodium bicarbonate ($NaHCO_3$) 6 mmol i.v. (2 mmol/kg) through the jugular venous line.

The hysterotomy was enlarged to allow for the entire fetus to be delivered. The umbilical cord was clamped with umbilical tape and cut, and the latex glove was removed.

After the cord was clamped, the lamb was immediately, and gently bagged with oxygen, weighed, and placed under the radiant overhead warmer with a warming blanket, and then dried.

Before the mother was euthanised, placental blood was collected and stored on ice for later neonatal lamb blood transfusion, if needed. The mother was given an anaesthetic overdose with Pentothal i.v. for euthanasia.

I. Resuscitation Protocol:

Simultaneously, the lamb was connected to a Sechrist Infant Ventilator Model IV-100B. Initial ventilator settings were: peak inspiratory pressure (PIP) 25 cm H_2O, peak end-expiratory pressure (PEEP) 5 cm H_2O, FiO_2 1.0, respiratory rate (RR) 120 breaths per minute, inspiratory time (Tinsp) 0.25 sec, and inspiratory time to expiratory time ratio (I:E) 1:1. The range of permitted ventilatory settings were as follows: PIP 15-35 cm H_2O, PEEP 3-7 cm H_2O, FiO_2 0.21-1.0, RR 10-120 breath per minute, Tinsp minimum 0.25 sec, and I:E ratio aimed for optimal ratio of 1:2 or 1:3, but not lower than 1:1. The goal was to maximize oxygenation, and ventilation, while avoiding barotrauma. The principle of permissive hypercapnea ventilation was used to minimize barotrauma. Ventilatory settings were changed accordingly if $PaCO_2$ >65 mm Hg or $PaCO_2$ <40 mm Hg; if PaO_2 <40 mm Hg or PaO_2 >100 mm Hg; and if pH<7.4 or pH>7.5. We calculated the pH deficit up to pH 7.4, and bolused with 2 mmol/kg of $NaHCO_3$ i.v. to increase the pH by 0.1 unit.

Heart rate (HR), post-ductal oxygen saturation (SaO_2) via the pulse oxymeter on the lamb's tail, systolic (SBP), diastolic (DBP), and mean arterial (MAP) blood pressures, and central venous pressures (CVP) were monitored continuously. A rectal temperature probe was inserted to monitor rectal temperature continuously with a goal of 38-39 °C achieved with the overhead warmer, and heating blankets. Blood glucose, electrolytes (sodium, potassium, calcium), hematocrit (Hct), and pre-ductal arterial blood gas (ABG) analysis were done using a portable clinical analyzer and EG7+ cartridges (i-STAT, Sensor Devices Inc., Waukesha, WI, USA), for the initial fetal blood sample before umbilical cord clamping, and every 15 minutes for the first hour of life, then every 30 minutes for the second hour of life, then every hour until the end of the 8 hour resuscitation. Urine output was regularly checked as a a percutaneous cystostomy was performed by inserting an 18G Jelco catheter suprapubically.

If the MAP was <40 mm Hg, or there was a drop of blood pressure >50% of baseline, then, we ruled out a tension pneumothorax, and checked the Hct. If the MAP was <20 mm Hg, we administered an Epinephrine bolus of 0.1 mg i.v. If we suspected a tension pneumothorax because of an acute, dramatic decrease in cardiopulmonary

dynamics, we quickly visualized the thorax for hyper-inflation, and auscultated the lung fields for decreased breath sounds. We decompressed the pneumothorax by inserting a 10-12Fr chest tube into the fifth intercostal space in the right (most common side for barotrauma in our CDH experiment) mid-axillary line, and leaving it to straight drainage. If hemodynamics did not improve, then a chest tube was placed on the contralateral side. If this still did not result in any improvement, then a tension pneumomediastinum (in lambs, the mediastianal lobe is part of the normal anatomy of the right lung) was suspected, and the only way to decompress it was by performing a subxyphoid incision, and incising its pleura. If a low MAP was accompanied by a Hct of <0.30, then we transfused 10 mL/kg i.v. of placental blood, and if the Hct was >0.30, then we bolused 10 mL/kg i.v. of Normal Saline solution, with a maximum bolus 20 mL/kg/hr.

Ionized calcium levels were checked with each blood gas, and if the ionized calcium was less than 0.9 mmol/L, then 1mL/kg i.v. of 10% calcium gluconate was given. Potassium levels were also checked, and if the potassium was less than 3 mmol/L, then 1 mEq i.v. potassium was given slowly. We also monitored for hypoglycemia (glucose <2.0 mmol/L) or hyperglycemia (glucose >20 mmol/L); however, this never became an issue.

The jugular venous line was a triple lumen: one lumen was used for a continuous infusion, using an intravenous syringe pump, of Dextrose 5% in Water at 4 mL/kg/hr, mixed in with ketamine at 2 mg/kg/hr, and with 0.12 mEq/mL potassium chloride; the second lumen was used for the $NaHCO_3$ i.v. 0.5 mmol/kg/hr infusion at 1 mL/hr, and for any additional sodium bicarbonate boluses; the third lumen was used for CVP readings and it was continuously flushed with 0.5 mL/hr of 1 U/mL heparin in normal saline to keep it patent. Pancuronium bromide i.v. 0.1 mg/kg/hr in Dextrose 5% in Water drip was given at 1 mL/kg/hr piggy-backed to the ketamine drip. The arterial line from the right common carotid artery was continuously flushed with 0.5 mL/hr of 1 U/mL heparin in normal saline to keep it patent. A single dose of antibiotics was administered within 60 minutes after birth: ampicillin 50 mg/kg i.v. and gentamicin 2.5 mg/kg i.v. through the jugular venous line.

II. Compliance:

Respiratory mechanics were determined every 60 minutes, starting at 1.5 hours, from measurements of tracheal pressure, flow, and volume using a pulmonary function machine (Hewlett Packard 7754B system) and pneumotachometer. Compliance was calculated as the change of volume divided by the change of pressure in the expiratory phase of the respiratory cycle during no flow state. Compliance was then expressed over the body weight.

III. Termination of resuscitation:

Resuscitation ended once eight hours of life had progressed, or cardiac dysfunction occurred despite optimization of cardio-respiratory parameters. Death before the anticipated eight hour resuscitation would usually be preceded by:

i. bradycardia (heart rate<80) for 30 minutes

ii. persistent hypotension with MAP <20 mm Hg despite blood transfusion, fluid bolus, bilateral chest tubes and subxyphoid incision for relief of tension pneumothorax, and epinephrine bolus

iii. pH<6.8 on three arterial blood gases done 60 minutes apart, in spite of alkalinisation, and hyperventilation

IV. Neonate lamb sacrifice:

Once termination of resuscitation was determined, the lamb received a lethal dose of i.v. pentothal for euthanasia. The neck and chest were opened, and the position of the diaphragmatic hernia was noted and whether or not there was herniation of viscera. The lung condition and level of gross barotrauma was noted. The trachea was dissected, and the lungs were removed en block with the heart. Total lung weight was measured. Then the right lung and left lung were separated by transecting the left mainstem bronchus, and their respective weights were measured. The total, right and left LW/BW were determined. Specimens were taken from the right middle lobe and lingula for dry-to-wet lung weight analysis. For this, a small piece of lung tissue was resected, weighed, and left to air dry for a minimum of one week. When completely dessicated, the tissue was re-weighed, and the dry-to-wet ratio was thus obtained [162]. To calculate dry right and dry left lung weights, the wet right and wet left lung weights were multiplied by the dry-to-

wet ratio. The total dry lung weight was calculated as the average between the right and left dry-to-wet ratio multiplied by the total wet lung weight.

Phase I and Phase II. Statistical Analysis:

Statistical analysis was performed using the statistical software SPSS version 9. Results from Phase I and Phase II were processed separately. Results between the four groups were compared using ANOVA with Duncan post-hoc testing, and right and left lung comparisons within each group were made with paired t-test. P values of <0.05 were considered statistically significant. Data is presented as mean +/- standard error of the mean (SEM), and where #=different from control ($p<0.05$), and *=different from CDH ($p<0.05$).

Phase I animals included for analysis were: CDH (n=7), CDH+TO (n=6), CDH+TO+TR (n=6), and controls (n=16). Phase II animals included for analysis were: CDH (n=5), CDH+TO (n=5), CDH+TO+TR (n=5), and controls (n=4). Because of the small number of animals in each group, it was difficult to calculate a "true" power of the study. However, based on previous studies as described under "Prenatal Interventions and Survival Protocols for Lamb CDH", we expected to observe significant differences between CDH and controls, as well as between CDH and CDH+TO+/-TR in both Phase I and Phase II of the study. To note is that there were two sets of controls; one set for Phase I, and the other set for Phase II of the study. They essentially have the same prenatal treatment, but Phase I controls were sacrificed at birth, and the Phase II controls were sacrificed after an 8 hour resuscitation. Thus, we could assume that the characteristics of a Phase I control at birth would also hold true for a Phase II control at birth in terms of baseline lung morphometry, pulmonary artery structure, and surfactant levels at birth. Moreover, the baseline characteristics of Phase I CDH+/-TO+/-TR animals at birth would also hold true for their Phase II counterparts at birth.

During the resuscitation Phase II study, the average over the eight hours (as shown in Tables 8 and 9) was the statistical average where the sum of all measurements for a particular group was divided by the number of measurements, regardless of time. Thus, they were not weighted for their corresponding time over the 8 hour period.

Results

General Results:

The mortality rate for Phase I was 33%, and for Phase II, 34%. This is acceptable, as the quoted mortality for fetal lamb CDH experiments is around 50% [9]. It is important to note that in our analysis, only animals with documented herniated viscera up in the left chest at autopsy were considered to be "CDH+/-TO+/-TR". If there was no diaphragmatic hole or no herniated viscera at the time of autopsy, then these animals would be excluded from analysis. In Phase I, 3 animals were excluded, and in Phase II, 1 animal was excluded from analysis. Thus, Phase I animals included for analysis were: CDH (n=7), CDH+TO (n=6), CDH+TO+TR (n=6), and controls (n=16). Phase II animals included for analysis were: CDH (n=5), CDH+TO (n=5), CDH+TO+TR (n=5), and controls (n=4).

In terms of technique, all of our TO using the detachable balloons were reliably inflated and snug against the tracheal wall at autopsy. For this study, we did not examine the trachea histologically, but from a previous study by Benachi et al [197], there can be erosions of the tracheal epithelium due to the balloon of TO. However, as similarly reported previously, macroscopically, no trauma could be seen to the pharynx, vocal cords, or tracheal cartilage [182]. Even though TO achieved by intra-tracheal balloon causes mild epithelial changes such as unfolding of the epithelium, limited epithelial defects, and local inflammatory changes, these changes disappear following TR [198].

For Phase I of our study, TR was successfully achieved by US percutaneous needle deflation in 3 of the 6 lambs; these were the last three cases. In the first three cases, the balloon had been placed 2 cm above the right upper lobe orifice; this was too low for proper US detection and safe deflation percutaneously. Thus, in one lamb, the ewe had a laparotomy, and the balloon was deflated with a 22 G spinal needle using US guidance, but through the uterine wall. In the other two lambs, we had to directly visualize the fetal intra-tracheal balloon with the fetal tracheoscope, and deflated the balloon with the micro-scissors via the endoscope instrument channel. We remedied this problem: when the balloon was placed 2 cm below the vocal cords, the balloon was easily seen by ultrasound and could be safely deflated percutaneously. In Phase II, TR was successfully achieved by US percutaneous needle deflation in 4 of the 5 lambs. In the

case of the unsuccessful percutaneous TR, the balloon had been optimally placed in the fetal cervical trachea, but, the experimental fetus was not in optimal position: there were twins in the same uterine horn, and the experimental animal was underneath the control. It was thus physically impossible for our spinal needle to reach that depth in order to puncture the intra-tracheal balloon. We elected to do a maternal laparotomy, and the balloon was deflated with a 22 G spinal needle using US guidance, but through the uterine wall. In all eleven TRs (Phase I and II), all balloons were reliably deflated at autopsy. There were no fetal losses, premature labor, or other complications because of the added procedure of TR one week before delivery.

Phase I Results:

The CDH lungs (Figure 2) from our experimental fetal ovine model were markedly hypoplastic as evidenced by lung weight of 1.26% of body weight (Figure 3) and by the elevated mean terminal bronchiole density (Figure 4). Figure 2 shows the thoracic cavity of a CDH lamb; one can appreciate the hole in the diaphragm through which a Kelly forceps is passed for demonstration purposes, and through which small bowel herniate into the left chest, preventing ipsilateral lung growth, and causing a mediastinal shift with an effect on contralateral lung growth. Treatment of CDH by TO with or without TR resulted in lungs that were intermediate in size with a weight of 2.03-2.28% of body weight. This was significantly larger than CDH but still somewhat smaller than the controls with a lung weight of 2.98% of body weight.

Even though the CDH+TO+/-TR groups did not produce lungs the size of controls, these lungs were structurally mature as indicated by a low mean terminal bronchiole density when compared to the immature lungs of CDH, despite the fact that herniated viscera were still present in the left chest at birth. The lung growth after TO was never sufficient to displace the abdominal viscera out of the thoracic cavity, in contrast with descriptions by others where the TO was performed at the same time as the CDH was created [42]. At 100X magnification, CDH lungs had numerous terminal bronchioles near the pleural edge, indicating an immature lung, and thickened, non-distendable interstitium (Figure 5A). In contrast, at 100X magnification, CDH+TO (Figure 5B), and CDH+TO+TR (Figure 5C) had similar pulmonary architecture to controls (Figure 5D)

with few terminal bronchioles near the pleural edge, a thinned out interstitium, and fully distended alveoli.

The percentage increase of right lung growth from the baseline CDH hypoplastic lung in the CDH+TO group was 85%, and in the CDH+TO+TR group was 59%. Similarly, the % increase of left lung growth from the CDH hypoplastic lung in the CDH+TO group was 72%, and in the CDH+TO+TR group was 63%. Even though the left lung was the more hypoplastic in the CDH+/-TO+/-TR groups, both right and left lungs grew proportionally as evidenced by the stable right to left lung ratios of CDH+/-TO+/-TR (Table 1). There was no difference between right and left lung MTBD, except for CDH where the right lung MTBD was 5.35+/-0.56 versus left lung MTBD 7.05+/-0.53 ($p<0.05$).

The adventitial area was only statistically different for arteries <75 μm in size, where the CDH+/-TO+/-TR had large adventitial areas when compared to controls (Table 2). In terms of medial area, CDH and CDH+TO+TR had thickened areas in pulmonary arteries <75 μm in size when compared to controls. CDH+TO had intermediate medial areas in between the high area of CDH and the low area of controls (Table 3). Figure (6) shows the actual contributions, stacked on top of each other, of the lumen area, medial area, and adventitial area for pulmonary arteries <75 μm. It can be seen that for pulmonary arteries <75 μm, controls had the smallest overall vessel area, CDH+TO had an intermediate area, while CDH and CDH+TO+TR had the largest areas. In Figure (7A), at 100X magnification, one can appreciate the thickened connective tissue impinging on the already thickened small pulmonary arteries of CDH. This is in contrast to the stretched out parenchyma of CDH+TO (Figure 7B) that appears to have a tethering effect of holding the thinned out small pulmonary arteries open (at 100X magnification). Figure (7C) shows the appearance of CDH+TO+TR lungs where the parenchyma is thinned out, but the small pulmonary arteries appear to have a thickened area (at 100X magnification). Figure (7D) shows the normal alveolar architecture and arterial appearance of controls (at 100X magnification).

As for the capillary load, there was no difference between controls and CDH+/-TO+/-TR in terms of the ratio of vessel surface area to tissue surface area as seen on electron microscopy (Table 4).

Type II cell density was abnormally increased in CDH, and CDH+TO+/-TR restored type II density to normal levels (Figure 8).

BAL SP-A and BAL SP-B levels were similar in all four groups, indicating no differences in these surfactant apoproteins secreted in utero by day 136 of gestation (Table 5). In addition, BAL protein concentrations were similar in the four groups, indicating that CDH+/-TO+/-TR did not compromise the integrity of the fetal lung vascular epithelium. However, BAL PC was very low in CDH and remained low after TO+/-TR. Similarly, lung tissue SP-B was low in CDH+/-TO+/-TR. On the other hand, a different pattern of results was observed for lung tissue SP-A content. CDH lambs exhibited lung SP-A tissue concentrations comparable to those in control fetuses, while low tissue SP-A content was observed with CDH+TO+/-TR (Table 5).

Phase II Results:

The CDH lungs were hypoplastic as evidenced by wet lung weight of 1.11% of body weight (Figure 9). Treatment of CDH with TO+/-TR resulted in significant lung growth with a % wet lung weight to body weight (1.88% and 2.39%) that was comparable to controls (1.73%). Similar to our Phase I results, even though the left lung was more hypoplastic in the CDH+/-TO+/-TR groups, both right and left lungs grew proportionally as evidenced by the stable right to left lung ratios of CDH+/-TO+/-TR (Table 6).

Table (7) shows the dry-to-wet ratio expressed as a percentage. The total lung dry-to-wet ratio was the highest for CDH at 20.7%, and this was statistically different from CDH+TO at 16.8% dry-to-wet ratio. The right lung dry-to-wet ratio was the lowest for CDH+TO at 16.1%, and this was statistically different from controls at 19.2%, or CDH at 19.9%. The left lung dry-to-wet ratio was the highest for CDH at 21.5%, and this was statistically different from CDH+TO at 17.5% dry-to-wet ratio. This signifies that CDH+TO lungs had a higher water content than CDH lungs. Moreover, for the right lung, CDH+TO lungs had a higher water content not only than CDH lungs, but also than

control lungs. It is not surprising that TO lungs would contain more water at birth, but it is interesting to see that it persists even after 8 hours of resuscitation.

Even though CDH+TO lungs had greater lung water content than CDH, the lung growth achieved by CDH+TO was indeed significant when comparing dry lung weights. Figure (10) shows the dry total lung weight over body weight ratios, where CDH lungs are hypoplastic at 0.23%. Treatment of CDH with TO+/-TR resulted in significant lung growth with dry lung weight of body weight (0.36% and 0.43%) that were comparable to controls (0.32%). These results parallel the wet total lung weight over body weight ratios results shown in Figure (9).

Figure (11) shows the highest PaO_2 achieved over the resuscitation. The highest PaO_2s were the best for CDH+TO (239 mm Hg) and controls (217 mm Hg), while CDH (89 mm Hg) and CDH+TO+TR (64 mm Hg) had poor highest PaO_2s. As shown in Figure (12), the lowest $PaCO_2$s were the best for CDH+TO (29 mm Hg) and controls (22 mm Hg), while CDH (54 mm Hg) and CDH+TO+TR (47 mm Hg) had poor lowest $PaCO_2$s.

Table (8) shows the mean values averaged over the eight hour resuscitation for pH, $PaCO_2$, PaO_2, and $AaDO_2$. It can be seen that for pH and $PaCO_2$ averaged over eight hours, three groups arise: one group with acidosis and hypercarbia corresponding to CDH animals; one intermediate group with middle values for pH and $PaCO_2$ corresponding to CDH+TO+TR; and the third group with normal acid-base status and normalized $PaCO_2$ corresponding to CDH+TO and controls. For PaO_2 and $AaDO_2$ averaged over eight hours, two groups arise: one with hypoxia and high $AaDO_2$ gradient corresponding to the CDH and CDH+TO+TR groups; and the other with normal PaO_2 levels and lower $AaDO_2$ gradient corresponding to the CDH+TO and control groups. Table (9) shows the mean values averaged over the eight hour resuscitation for VEI, MVI, and compliance. For VEI, two groups arise from the results: one with the smallest VEI corresponding to CDH and CDH+TO+TR; and the other with a high VEI corresponding to CDH+TO and controls. Interestingly, for the MVI and compliance, each experimental group was significantly different from each other. The descending rank of averaged MVI, starting with the highest, was: CDH, then CDH+TO+TR, then CDH+TO, and then controls. The

ascending rank of averaged lung compliance, starting with the lowest, was: CDH, then CDH+TO+TR, then controls, and then CDH+TO.

Figures (13-19) show the trends over the eight hour resuscitation for lung compliance, MVI, VEI, $PaCO_2$, pH, PaO_2, and $AaDO_2$ gradient. [Note: Because of space constraints, I did not add the symbols # or * to signify statistical significance for the graphs representing trends over eight hours. I did add the upper bar of the SEM for each mean value represented on the graph. Thus, if one imagines the lower error bar of the SEM of one point in time that corresponds to the mean of a particular group, and if that bar does not overlap with the upper band of the SEM of another group at the same time, then those two points are statistically different at that time. Basically, if there is no overlap of the error bars of the SEM at one particular time, then those two means are statistically different with $p<0.05$]. It can be seen that for compliance trends (Figure 13), CDH lungs are the least compliant throughout the length of the resuscitation, and that CDH+TO are the most compliant, and at certain times, even more so than normal controls. On the other hand, CDH+TO+TR show intermediate compliance values.

For MVI trends (Figure 14), CDH lungs have the highest values, meaning a more difficult ventilation, and CDH+TO had low MVI values throughout. Even though CDH+TO+TR initially had high MVI, ventilation improved after 180 minutes into the resuscitation. Towards the end of the resuscitation, CDH+TO+TR, CDH+TO, and controls all had low MVI values. The VEI trend (Figure 15) is another measure of ease of ventilation where a higher value signifies an easier ventilation. Figure (15) demonstrates further that after 180 minutes of resuscitation, a dramatic change in VEI was seen in those CDH lambs treated with TO+/-TR. While VEI improved for CDH+TO+TR to intermediate values, the most impressive improvement was seen in CDH+TO lambs that followed the same curve as the normal controls. In contrast, untreated CDH lambs had a persistently low VEI throughout the eight hour resuscitation. The pH trends (Figure 16) clearly showed that after 180 minutes of resuscitation, CDH lambs dipped into acidosis, while CDH+TO+/-TR and controls achieved normal acid-base balance. Similarly, $PaCO_2$ trend values (Figure 17) for CDH showed persistent hypercapnia, while CDH+TO and controls showed a trend towards normocapnia from the start. Initially, CDH+TO+TR

lambs also showed hypercapnia, but after 180 minutes, showed improvement towards intermediate values of $PaCO_2$.

Interestingly, while pulmonary ventilation showed significant trends in pH, $PaCO_2$, VEI, MVI, and compliance, pulmonary oxygenation did not show the same dramatic differences in trends between groups. There were no clear differences between the PaO_2 trends, except for the few sporadic high PaO_2s of CDH+TO (Figure 18). The $AaDO_2$s (Figure 19) were only beginning to show differences after 300 minutes, with CDH+TO having lower $AaDO_2$ when compared to CDH or CDH+TO+TR.

Table (10) shows the number of lambs that survived the entire eight hour resuscitation, and whether they had chest tubes or subxyphoid incisions for relief of tension pneumothoraces. All controls, CDH+TO, and CDH+TO+TR survived 8 hours. None of the controls developed tension pneumothoraces. In contrast, two CDH lambs did not survive the eight hour resuscitation: one died at 5 hours, and the other at 7 hours. All CDH animals required chest tubes at a median time of 1.5 hours into the resuscitation. Three CDH lambs required a subxyphoid incision to drain the mediastinal lobe, two of which were the ones that died at 5 and 7 hours. For CDH+TO animals, three required chest tubes at a median 5 hours into the resuscitation, and two needed a subxyphoid incision. For CDH+TO+TR, three lambs also required chest tubes at a median of 4 hours of resuscitation, and one required a subxyphoid incision. Of those animals that developed tension pneumothoraces, all had PIP of 35 cm H_20. Thus, the development of tension pneumothorax was an indication of iatrogenic barotrauma because of the need to change ventilatory settings in view of persistent acidosis, hypercapnea, or hypoxia.

Conclusion

Our results show that both right and left lungs were severely hypoplastic in this animal model of CDH. TO with or without TR partially normalized the hypoplastic lungs of CDH: this resulted in an accelerated, harmonious growth and structural maturation of both lungs despite the persistence of the CDH at autopsy. Furthermore, lung growth was not significantly affected by TR one week before delivery. CDH lungs were structurally immature with numerous terminal bronchioles near the pleural edge and thickened interstitium and alveolar walls. In contrast, CDH+TO+/-TR allowed for normalization of pulmonary architecture with thinned out interstitium, and normal appearing alveoli with few terminal bronchi near the pleural edges. Thus, lung growth achieved by TO+/-TR appeared to follow normal structural development.

The current study confirms previous observations that CDH hypoplastic lungs have thickened pulmonary artery media and adventitia in arteries <75 μm in diameter [5]. We further showed that these fetal anatomic changes are not "fixed" and nonreversible components of the pulmonary vascular bed, as some may have postulated [98]: TO reversed the increased pulmonary artery medial area, but not the adventitial area in arteries <75 μm in diameter of our present fetal lamb CDH model. Similarly, DiFiore et al have shown that CDH+TO in the fetal lamb reversed the increased muscularization of pulmonary arteries: for vessels of diameters <100 μm, 29% of them were fully muscularized in CDH, whereas none were muscularized in CDH+TO and controls [128]. It is important to note that DiFiore had created the CDH and TO at the same operation, and had injected and inflated the pulmonary artery with barium-gelatin; our CDH and TO were created at different times, allowing lung hypoplasia to occur before applying TO, and the pulmonary artery was not inflated. This would explain the apparent discrepancy between non-inflated and inflated pulmonary artery diameter size and how it relates to muscularization of the media. It is interesting to note that for normal neonate lambs, nearly 100% of inflated arteries with a diameter of 51-100 μm, at <24 hours from birth, have been reported to be muscularized [199]. Obviously, different methods of injection, inflation, and fixation of the lung or pulmonary arteries will affect the relative reported size of the artery; what is important is the trend between the groups.

Like others [110, 114], we found no difference between control or CDH+/-TO+/-TR in terms of composition at the level of the acinus when comparing capillary load. In the fetal lamb, there is an initial increase in alveolar surface area followed by an increase in endothelial surface area as indicated by a change in the ratio of capillary alveolar surface area during the last third of gestation [47]. After 120 days gestation, there is a close relationship between pulmonary capillary formation and alveolar development in the fetal lamb [47]. Our results show that TO with or without TR in a hypoplastic CDH model maintains that proportional relationship between capillary and alveolar development even though the main growth stimulus is initiated by alveolar stretch or bronchial pressure.

We further show that the effect of TO on pulmonary artery remodeling may be especially significant in the latter part of gestation, as one week of TR before delivery prevents thinning of the small pulmonary artery (<75 μm) medial area. By promoting harmonious lung growth that include alveoli and capillaries and by allowing thinning of the medial area in small pulmonary arteries, TO would undoubtedly decrease the pulmonary hypertension seen postnatally with CDH.

Our results reveal that despite TO, TR, and prenatal glucocorticoids, lungs from lambs with CDH have dysfunctional type II cells with decreased BAL PC and decreased lung tissue SP-B. Moreover, when compared to normal lambs treated with TO, CDH animals treated with TO appear to respond differently in terms of type II cell numbers and surfactant composition.

In normal fetal lungs, TO induces the lungs to grow at the expense of type II cell density [34-37], likely due to signaling related to alveolar stretch. It may be that TO, through mechanical distension, promotes proliferation of respiratory epithelial cells, thus suppressing differentiation of mature type II cell phenotype while allowing for type I cell growth [70, 154]. This would explain the decreased type II cell density and surfactant immaturity associated with TO in normal lungs. However, CDH lungs do not behave like normal lungs since type II cell density does not necessarily correlate with the amount or composition of surfactant. For example, type II cell density is elevated in CDH, yet the type II cells produce low levels of BAL PC and low levels of lung tissue SP-B.

Furthermore, despite normal type II cell density, CDH+TO+/-TR lambs still produce low levels of BAL PC and low levels of lung tissue SP-B. Since BAL SP-A and BAL SP-B were similar in the four groups, the surfactant protein levels initially secreted in the alveolar spaces by the fetus are not altered in CDH+/-TO+/-TR. It should be noted that fetal alveolar pools are relatively small and that most of the surfactant is secreted at birth [75]. Since tissue SP-B is low in CDH+/-TO+/-TR, this indicates that they would have reduced stores of SP-B. Unlike normal lungs, for CDH lungs, TR offers no added benefit over TO in terms of surfactant production: there is still poor quality surfactant with low BAL PC and low lung tissue stores of SP-B. SP-B is essential for normal respiration, and SP-B and PC are predictors of lung function at birth [84]. Since these are reduced with CDH, the use of prophylactic exogenous surfactant before the first breath at birth may be useful in the therapy of CDH even after prenatal intervention such as TO and maternal steroid administration.

In normal animals, surfactant lipids and apoproteins increase in an approximately coordinated manner in late gestation [75-78]. In our control animals, as expected, SP-A, SP-B, and PC contents were elevated appropriately in a parallel manner. However, it appears that, in the experimental animals, the normal physiological expression of surfactant components was altered. For example, even though all groups had normal levels of BAL surfactant proteins, CDH lambs possessed high levels of lung tissue SP-A which were comparable to control levels. In contrast, CDH lambs treated with TO+/-TR displayed low levels of tissue SP-A. Thus, for CDH lambs, overall relative SP-A content was elevated. On the other hand, CDH+TO+/-TR had uniformly low levels of lung tissue SP-A, tissue SP-B, and BAL PC content. In CDH, the loss of normal coordinated production of surfactant lipids and proteins suggest a disruption of the normal physiological controls which induce surfactant production during development. In the normal situation, despite a parallel, coordinated surfactant production, the genes for surfactant proteins may be independently regulated through the net effect of humoral influences [75]. Further, SP-A can be secreted separately from other surfactant components [82]. It is important to note that while SP-A is essential for tubular myelin formation, SP-A gene ablation ("knock-out") studies show that SP-A deficient mice have

normal lung function [84]. SP-A is important for the innate host defense system of the lung [76, 84]. The manner in which the relative SP-A deficiency in CDH+TO+/-TR animals affects the lung remains to be determined.

Since PC and SP-B levels are predictors of lung function at birth [76, 84], we would have anticipated that our present resuscitation study would have demonstrated a decline in pulmonary function in CDH lambs treated with TO+/-TR compared to controls. Others have persistently shown that CDH+TO have initial good physiological function if resuscitated <4 hours [41, 42, 155, 156]; yet, there was a fear that the surfactant deficiency in CDH+TO would lead to progressive deterioration in lung function with longer periods of ventilation. We prove this not to be true. Several conclusions can be drawn from our resuscitation (Phase II) study:

1. TO with or without TR reversed lung hypoplasia induced by CDH, and resulted in lung weights that were comparable to controls.
2. CDH animals treated with TO with or without TR were all able to survive the eight hours of resuscitation, while only 3 out of 5 CDH animals survived eight hours with significant acidosis, hypercapnea, hypoxia, and barotrauma.
3. Marked improvement in ventilation was noted after 180 minutes for controls, and CDH+TO+/-TR, that resulted in improvements in pH and $PaCO_2$. CDH+TO values were clearly superior to CDH values and reached normal values, while CDH+TO+TR values remained intermediate. As to the physiological mechanism responsible for the improvement in ventilation after 180 minutes for controls and CDH+TO+/-TR, we can only speculate that the initial low ventilation-perfusion mismatch was reversed as compliance increased and more ventilatory units were recruited relative to the pulmonary blood flow.
4. Oxygenation was improved in CDH animals treated with TO, but not those treated with TO+TR. Perhaps pulmonary artery remodeling and thinning of the media of small pulmonary arteries, that is only achieved with TO until delivery, were important factors in lessening pulmonary hypertension, leading

to decreased right-to-left shunting, and improvements in oxygenation for CDH+TO to levels comparable to controls.

5. The compliance of CDH animals was low, and treatment with TO allowed for normal compliance levels. CDH+TO+TR had intermediate compliance values.
6. Release of the tracheal obstruction one week before delivery has no added benefit in terms of lung function in this model.

The improvement in lung function of CDH+TO is most likely from surfactant-independent mechanisms that include an appropriate lung growth to allow a minimum lung volume needed for adequate gas exchange [21], a normal pulmonary architecture to allow for compliant lungs [120], and pulmonary artery remodeling to prevent persistent pulmonary hypertension [59]. The concept that surfactant independent mechanisms may improve lung compliance arose from the observation that while glucocorticoid-induced increases in lung compliance normally occur within 48 hours after treatment, it can take longer before significant alterations in alveolar and/or tissue surfactant lipids become apparent [163]. It has become evident that glucocorticoids increase lung compliance through surfactant-independent as well as surfactant-dependent mechanisms [74, 76, 157, 163]. The former include structural changes related to alterations in collagen/elastin ratio, morphometric alterations associated with decreased perilobar connective tissue, and reduction in alveolar wall thickness resulting in marked elevation in aerated parenchyma [163]. The conclusion that our CDH+/-TO+/-TR+glucocorticoid lamb models are likely surfactant deficient may appear incompatible with our present experimental observations or previous observations that CDH+TO [41, 42, 155] or CDH+glucocorticoids [39, 170] lambs have increased oxygenation, ventilation indices, and survival compared to CDH alone. However, these observations would be reconciled if compliance were increased in these models through surfactant-independent mechanisms. As indicated above, compliance is influenced by the lung's intrinsic connective tissue qualities, as well as by surfactant. An increase in compliance contributes to distension and recruitment of alveoli and opening up of previously closed vessels for gas exchange, resulting in improved lung function and decreased pulmonary hypertension. The increased lung volumes would stretch the parenchyma and the resultant radial traction on the extra-alveolar vessels

would lead to a lower vascular resistance [200]. Thinning of the interstitium and more mature alveoli with thinner walls have been reported for CDH+glucocorticoids [40]. In addition, our study confirms that TO+/-TR, in a CDH hypoplastic lung, accelerates lung growth, leading to a more normal parenchymal structure in terms of terminal bronchiole density, while maintaining an appropriate capillary load. Despite a surfactant deficiency in CDH+TO+glucocorticoids, the normalization of lung architecture and size led to normalization of lung function.

While our resuscitation model was limited as we could not provide our sickest CDH lambs with high-frequency-ventilation, inhaled nitric oxide, or extra-corporeal-membrane-oxygenation as would happen in the human condition [19, 177, 201], we did inadvertently show that pulmonary hypoplasia complicated by iatrogenic lung injury from high ventilatory pressures resulted in significant barotrauma, and decreased survival. Postnatally, in babies with CDH, there is a tendency to use lower PIP (<25 mm Hg), and high-frequency-ventilation or ECMO instead of inducing barotrauma [19].

To achieve a reduction in the high mortality and morbidity associated with human CDH, it will be necessary to optimize numerous aspects of care:

1. Improve lung growth prenatally since a minimum lung volume of 45% of the value predicted from age-matched controls is required for survival of CDH babies treated with extra corporeal membrane oxygenation [21].
2. Improve compliance further (hence, decreases pulmonary hypertension) by giving maternal glucocorticoids and exogenous surfactant to the baby before the first breath.
3. Avoid barotrauma as discussed above.
4. Decrease pulmonary hypertension. Pharmacological manipulations, including the use of inhaled nitric oxide, have had limited success in the treatment of the pulmonary hypertension associated with CDH. It now appears that gentle ventilation and the use of ECMO, if necessary, will allow for spontaneous improvement over time in many patients. Improving compliance at birth may be another step to minimize pulmonary hypertension, but the most severely affected fetuses may require TO to provide them with a sufficient number of alveoli and an adequate pulmonary capillary bed.

For poor prognosis CDH (herniated liver, lung:head ratio <1, <25 weeks gestational age at diagnosis), fetal TO is feasible and can promote prenatal lung growth that improves survival from 38% up to 75% [28, 202]. TO can be done with tracheal clipping, either by an open or fetoscopic approach [28, 202]. We have proposed an alternative method where the trachea is obstructed with a detachable balloon placed by fetal tracheoscopy thereby eliminating the risk of laryngeal nerve and tracheal trauma associated with tracheal clipping. Fetal tracheoscopy is conducted through a single port and thus may reduce the risk of amniotic fluid leak and premature rupture of membranes. Even though TR one week before delivery does not provide added benefit over TO until term, in the event that TO causes excessive uncontrolled lung growth resulting in fetal hydrops [203], this method can be reversed by US-guided percutaneous needle puncture. Once babies with CDH survive the neonatal period without iatrogenic lung injury, it is expected that postnatal lung growth and remodeling will occur [123]. A baby with CDH needs a minimum lung volume to survive; despite persistent signs of ipsilateral lung hypoplasia after postnatal lung growth and remodeling, most survivors have few clinical respiratory symptoms [125].

Summary

In our fetal lamb CDH model, TO+/-TR+glucocorticoids allowed for accelerated lung growth and normalized pulmonary architecture. Only CDH treated with TO allowed for thinning of the pulmonary artery media area for arteries <75 μm in external diameter. There was poor quality surfactant in CDH, despite TO, TR, and glucocorticoids as shown by low lung tissue SP-B, and low BAL PC. This implies that exogenous prophylactic surfactant may be needed at birth in babies with CDH. The second phase of our study confirmed that CDH lungs were hypoplastic as these lambs showed low pulmonary compliance, acidosis, hypercapnia, hypoxia, pulmonary barotrauma, and decreased survival if resuscitated for eight hours. TO reversed lung hypoplasia induced by CDH, and normalized lung compliance, MVI, VEI, and arterial blood gases. It was only after 180 minutes of resuscitation that the beneficial effects of TO on lung function could be observed. Even though release of the tracheal occlusion did not hinder lung growth or MVI, values for VEI, pulmonary compliance and arterial blood gases remained intermediate suggesting that TR one week before delivery was not beneficial over TO until term in this lung model. The improvements in lung function of CDH animals treated with TO were primarily from surfactant-independent mechanisms via pulmonary growth and structural changes with thinning of the interstitium, thinner alveolar walls, and pulmonary artery remodeling.

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Tables:

Table (1). Phase I. Right and Left Lung Growth

	RLW/BW %	LLW/BW%	RLW/LLW
CDH	0.83+/-0.10 #	0.43+/-0.04 #	2.01+/-0.24 #
CDH+TO	1.54+/-0.24 *	0.74+/-0.15 * #	2.18+/-0.14 #
CDH+TO+TR	1.32+/-0.15 * #	0.70+/-0.12 * #	1.97+/-0.15 #
CONTROL	1.82+/-0.07 *	1.17+/-0.04 *	1.56+/-0.04 *

Legend Table (1):
BW=body weight, RLW=right lung weight, LLW=left lung weight
Data are given as mean +/-SEM
#=different from control, $p<0.05$; *=different from CDH

Table (2). Pulmonary Artery Adventitial Area

PA Adventitial Area (μm^2) For All Artery Sizes					
	PA size (μm):				
	0-75	**76-100**	**101-150**	**151-250**	**251-500**
CDH	4996+/- 493 #	9871+/- 999	18528+/- 2239	38185+/- 4142	52979+/- 11539
CDH+TO	4487+/- 274 #	9540+/- 929	15609+/- 1552	39362+/- 5439	89571+/- 24469
CDH+TO+TR	5304+/- 289 #	10688+/- 1402	24623+/- 5457	30218+/- 3192	138630+/- 28408
CONTROL	3139+/- 163	8205+/- 854	14066+/- 1104	33887+/- 3905	94698+/- 21410

Legend Table (2):
PA= pulmonary artery
Data are given as mean+/- SEM
#= different from control, $p<0.05$

Table (3). Pulmonary Artery Medial Area

	PA Medial Area (μm^2) For All Artery Sizes				
	PA size (μm):				
	0-75	**76-100**	**101-150**	**151-250**	**251-500**
CDH	1365+/- 101 #	2676+/- 220	4584+/- 493	11101+/- 1166	32100+/- 7770
CDH+TO	1265+/- 52	2774+/- 176	4955+/- 485	11726+/- 1805	26181+/- 2609
CDH+TO+TR	1336+/- 63 #	2973+/- 283	5182+/- 752	9215+/- 816	39010+/- 7803
CONTROL	1124+/- 34 *	2543+/- 106	3981+/- 185	8903+/- 617	30272+/- 6059

Legend Table (3):
PA= pulmonary artery
Data are given as mean +/-SEM
#= different from control, $p<0.05$; *= different from CDH, $p<0.05$

Table (4). Capillary Load

	Vessel/Tissue Surface Area (%)		
	Total	**Right Lung**	**Left Lung**
CDH	10.08+/- 1.01	8.18+/- 0.89	11.97+/- 1.26
CDH+TO	9.61+/- 1.10	9.56+/- 1.98	9.67+/- 1.18
CDH+TO+TR	11.18+/- 1.46	12.65+/- 2.91	9.70+/- 0.33
CONTROL	11.02+/- 1.07	12.04+/- 1.79	10.0+/- 1.19

Legend Table (4):
Data are given as mean +/- SEM
There is no statistical difference between the four groups

Table (5). Surfactant Composition

	BAL SP-B	BAL SP-A	BAL protein	BAL PC	Lung Tissue SP-B	Lung Tissue SP-A
CDH	119 +/-50	34 +/-8	1.21 +/-0.79	38.71 +/-22.07 #	17.65 +/-4.26 #	0.25 +/-0.06
CDH+TO	129 +/-54	50 +/-14	2.66 +/-1.04	51.54 +/-7.52 #	11.12 +/-3.11 #	0.05 +/-0.01 * #
CDH+TO+TR	323 +/-191	69 +/-48	2.77 +/-0.61	31.17 +/-8.62 #	14.11 +/-2.60 #	0.09 +/-0.03 * #
CONTROL	182 +/-45	28 +/-8	1.59 +/-0.30	233.87 +/-45.03 *	45.29 +/-5.60 *	0.19 +/-0.03

Legend Table (5):
BAL SP-B= bronchoalveolar lavage fluid Surfactant Protein-B ng/mg PC
BAL SP-A= bronchoalveolar lavage fluid Surfactant Protein-A µg/mg PC
BAL protein= bronchoalveolar lavage fluid protein µg/µL
BAL PC= bronchoalveolar lavage fluid phosphatidylcholine µg/g body weight
Lung Tissue SP-B= lung tissue Surfactant Protein-B ng/mg protein
Lung Tissue SP-A= lung tissue Surfactant Protein-A µg/mg protein
Data are given as mean +/- SEM
#= different from control, $p<0.05$; *= different from CDH, $p<0.05$

Table (6). Phase II. Right and Left Lung Growth

	RLW/BW %	LLW/BW%	RLW/LLW
CDH	0.79+/-0.08	0.32+/-0.04	2.5+/-0.1 #
CDH+TO	1.66+/-0.23 *	0.69+/-0.18 *	2.7+/-0.4 #
CDH+TO+TR	1.30+/-0.12 *	0.55+/-0.08 *	2.4+/-0.2 #
CONTROL	1.05+/-0.03 *	0.66+/-0.02 *	1.5+/-0.1

Legend Table (6):
BW=body weight, RLW=right lung weight, LLW=left lung weight
Data are given as mean +/-SEM
#=different from control, $p<0.05$; *=different from CDH

Table (7). Dry-to-Wet Lung Weight Ratios

	Total Dry-to-Wet %	Right Dry-to-Wet %	Left Dry-to-Wet %
CDH	20.7+/-1.3	19.9+/-0.7	21.5+/-1.9
CDH+TO	16.8+/-1.3 *	16.1+/-1.5 * #	17.5+/-1.3 *
CDH+TO+TR	19.2+/-0.4	17.9+/-0.4	20.5+/-0.4
CONTROL	19.0+/-0.9	19.2+/-1.1	18.7+/-0.8

Legend Table (7):
Data are given as the mean+/-SEM
#=different from control, $p<0.05$, *=different from CDH, $p<0.05$

Table (8). Average pH, PaCO2, PaO2, and AaDO2 Over the Eight Hour Resuscitation

	pH	PaCO2 (mm Hg)	PaO2 (mm Hg)	AaDO2 (mm Hg)
CDH	7.11+/-0.02 #	103+/-3 #	49+/-3 #	707+/-5 #
CDH+TO	7.39+/-0.02 *	55+/-4 *	87+/-9 *	635+/-18 *
CDH+TO+TR	7.32+/-0.02 * #	82+/-4 * #	41+/-2 #	744+/-5 #
CONTROL	7.40+/-0.02 *	48+/-3 *	78+/-7 *	646+/-25 *

Legend Table (8):
Data are given as the mean+/-SEM
#=different from control, $p<0.05$; *=different from CDH, $p<0.05$

Table (9). Average MVI, VEI, and Compliance Over the Eight Hour Resuscitation

	MVI †	100xVEI	Compliance †
CDH	401+/-15	1.27+/-0.06 #	0.27+/-0.01
CDH+TO	165+/-21	18.48+/-3.16 *	0.87+/-0.03
CDH+TO+TR	284+/-20	4.32+/-0.84 #	0.46+/-0.01
CONTROL	93+/-12	19.65+/-2.99 *	0.72+/-0.03

Legend Table(9):
Data are given as mean+/-SEM
#=different from control, $p<0.05$; *=different from CDH, $p<0.05$, †=all are different from each other, $p<0.05$
MVI=modified ventilatory index=(RRxPIPxPaCO2)/1000
100xVEI=100xventilatory efficiency index=100x3800/((PIP-PEEP)xRRxPaCO2)
Compliance= mL/cm H2O/kg

Table (10). Survival and Barotrauma

	Survived 8 hours (N)	Age at death (hours)	Chest tube (N)	Median age at chest tube (hours)	Subxyphoid incision (N)	Age at subxyhpoid incision (hours)
CDH (n=5)	3	5, 7	5	1.5	3	1, 1.5, 1.5
CDH+TO (n=5)	5	-	3	5	2	1.5, 6
CDH+TO+TR (n=5)	5	-	3	4	1	1.5
CONTROL (n=4)	4	-	0	-	-	-

Figures:

Legends for Figures:

Figure 1. Pulmonary artery morphometry.

The external diameter is defined as the diameter between the external lamina and expressed in μm. The medial area is defined as the area contained by the external elastic lamina minus the area contained by the internal elastic lamina, and expressed in μm^2. The adventitial area is defined as the area contained by the entire artery minus the area contained by the external elastic lamina, and expressed in μm^2. The lumen area is defined as the area contained by the internal elastic lamina.

Figure 2. Thoracic cavity of a CDH lamb.

One can appreciate the hole in the diaphragm through which a Kelly forceps is passed for demonstration purposes, and through which small bowel herniate into the left chest, preventing ipsilateral lung growth, and causing a mediastinal shift with an effect on contralateral lung growth. B=bowel, D=diaphragm, H=heart, K=Kelly forceps, L=Left Lung, R=Right Lung.

Figure 3. Phase I. Lung weight/body weight.

Data is presented as mean +/- standard error of the mean (SEM), and where #=different from control ($p<0.05$), and *=different from CDH ($p<0.05$).

Figure 4. Mean Terminal Bronchiole Density (MTBD).

Data is presented as mean +/- standard error of the mean (SEM), and where *=different from CDH ($p<0.05$).

Figure 5. Pulmonary structure.

At 100X magnification, CDH lungs (A) had numerous terminal bronchioles (TB) near the pleural edge (arrow), indicating an immature lung, and thickened, non-

distendable interstitium. In contrast, at 100X magnification, CDH+TO (B), and CDH+TO+TR (C) had similar pulmonary architecture to controls (D) with few terminal bronchioles near the pleural edge, a thinned out interstitium, and fully distended alveoli.

Figure 6. Pulmonary arteries with an external diameter less than 75 μm.

This figure shows the actual contributions, stacked on top of each other, of the lumen area, medial area, and adventitial area for pulmonary arteries <75 μm. It can be seen that for pulmonary arteries <75 μm, controls had the smallest overall vessel area, CDH+TO had an intermediate area, while CDH and CDH+TO+TR had the largest areas. Data is presented as mean +/- standard error of the mean (SEM), and where #=different from control ($p<0.05$), and *=different from CDH ($p<0.05$).

Figure 7. Pulmonary arteries.

In (A), at 100X magnification, one can appreciate the thickened connective tissue impinging on the already thickened small pulmonary arteries (arrow) of CDH. This is in contrast to the stretched out parenchyma of CDH+TO (B) that appears to have a tethering effect of holding the thinned out small pulmonary arteries (arrow) open (at 100X magnification). (C) shows the appearance of CDH+TO+TR lungs where the parenchyma is thinned out, but the small pulmonary arteries (arrow) appear to have a thickened area (at 100X magnification). (D) shows the normal alveolar architecture and arterial (arrow) appearance of controls (at 100X magnification).

Figure 8. Type II cell density.

Data is presented as mean +/- standard error of the mean (SEM), and where *=different from CDH ($p<0.05$).

Figure 9. Phase II. Lung weight/body weight.

Data is presented as mean +/- standard error of the mean (SEM), and where *=different from CDH ($p<0.05$).

Figure 10. Phase II. Dry lung weight/body weight.

Data is presented as mean +/- standard error of the mean (SEM), and where *=different from CDH ($p<0.05$).

Figure 11. Highest PaO_2.

Data is presented as mean +/- standard error of the mean (SEM), and where #=different from control ($p<0.05$), and *=different from CDH ($p<0.05$).

Figure 12. Lowest $PaCO_2$.

Data is presented as mean +/- standard error of the mean (SEM), and where #=different from control ($p<0.05$), and *=different from CDH ($p<0.05$).

Figure 13. Lung compliance over eight hours.

Data is presented as mean + standard error of the mean (SEM), and if there is no overlap of the error bars of the SEM (both upper SEM shown and lower "imaginary" SEM) at one particular time, then those two means are statistically different with $p<0.05$.

Figure 14. MVI. Modified ventilatory index over eight hours.

Data is presented as mean + standard error of the mean (SEM), and if there is no overlap of the error bars of the SEM (both upper SEM shown and lower "imaginary" SEM) at one particular time, then those two means are statistically different with $p<0.05$.

Figure 15. 100XVEI. Ventilatory efficiency index over eight hours.

Data is presented as mean + standard error of the mean (SEM), and if there is no overlap of the error bars of the SEM (both upper SEM shown and lower "imaginary" SEM) at one particular time, then those two means are statistically different with $p<0.05$.

Figure 16. pH over eight hours.

Data is presented as mean + standard error of the mean (SEM), and if there is no overlap of the error bars of the SEM (both upper SEM shown and lower "imaginary" SEM) at one particular time, then those two means are statistically different with $p<0.05$.

Figure 17. $PaCO_2$ over eight hours.

Data is presented as mean + standard error of the mean (SEM), and if there is no overlap of the error bars of the SEM (both upper SEM shown and lower "imaginary" SEM) at one particular time, then those two means are statistically different with $p<0.05$.

Figure 18. PaO_2 over eight hours.

Data is presented as mean + standard error of the mean (SEM), and if there is no overlap of the error bars of the SEM (both upper SEM shown and lower "imaginary" SEM) at one particular time, then those two means are statistically different with $p<0.05$.

Figure 19. $AaDO_2$ over eight hours.

Data is presented as mean + standard error of the mean (SEM), and if there is no overlap of the error bars of the SEM (both upper SEM shown and lower "imaginary" SEM) at one particular time, then those two means are statistically different with $p<0.05$.

Figure (1). Pulmonary Artery Measurements

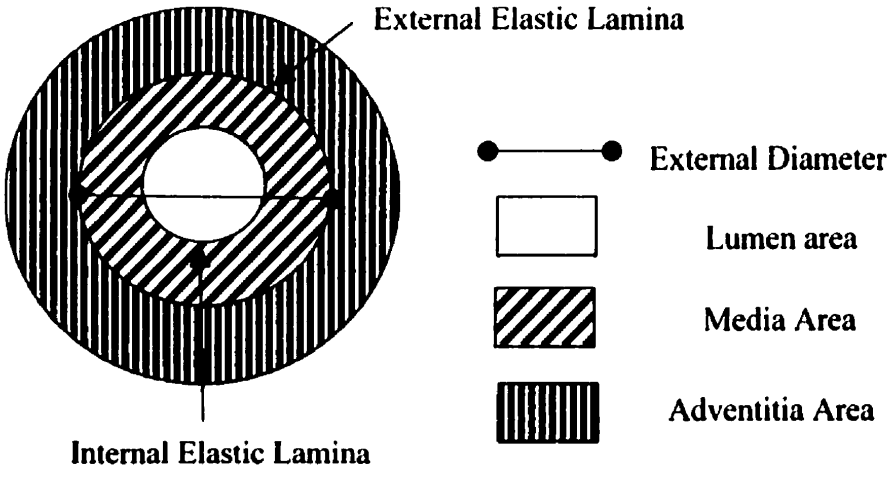

FIG 2

Figure 3. Phase I. Lung Weight/Body Weight

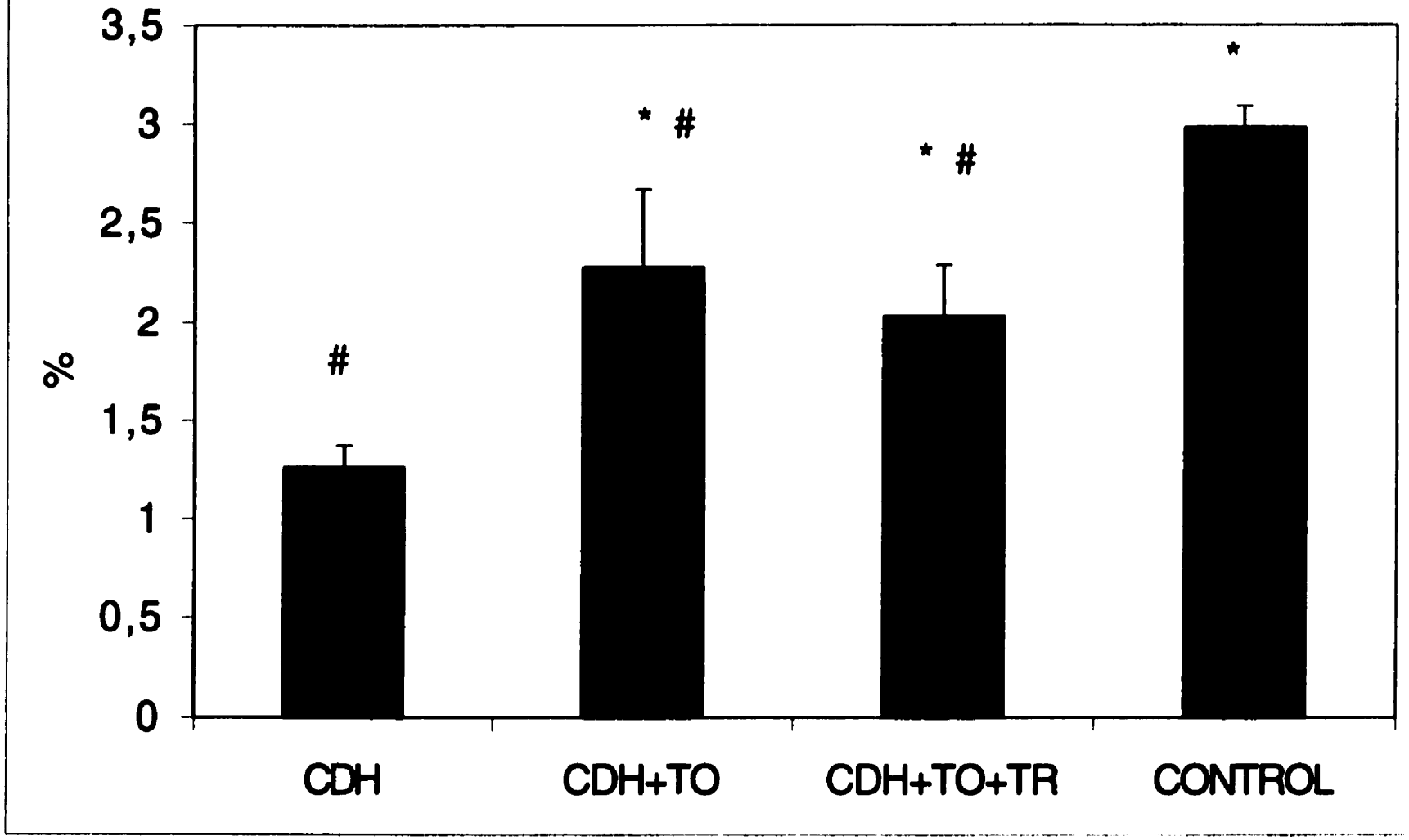

Figure 4. Mean Terminal Bronchiole Density

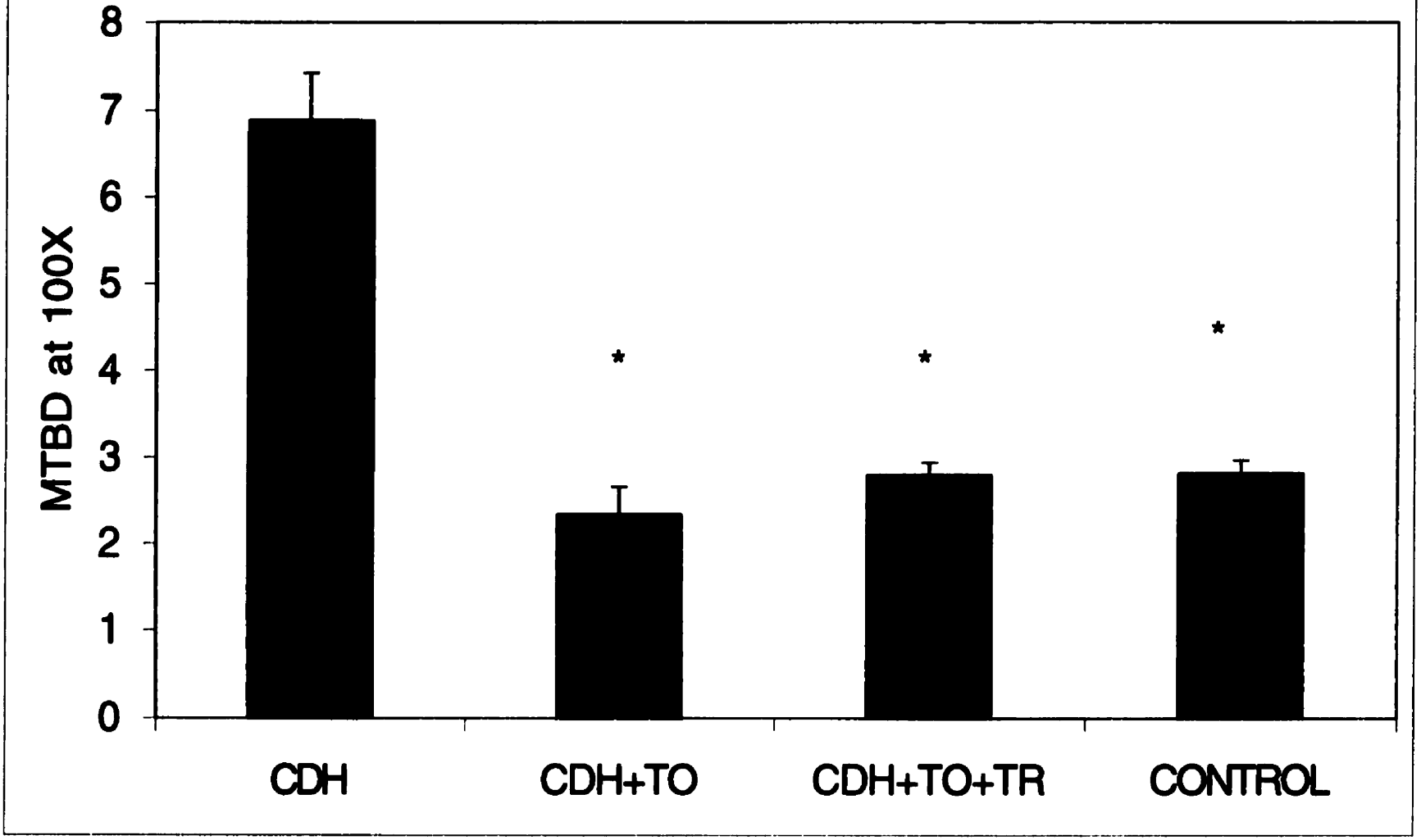

FIG 5

Figure 6. Pulmonary Arteries Less Than 75 μm

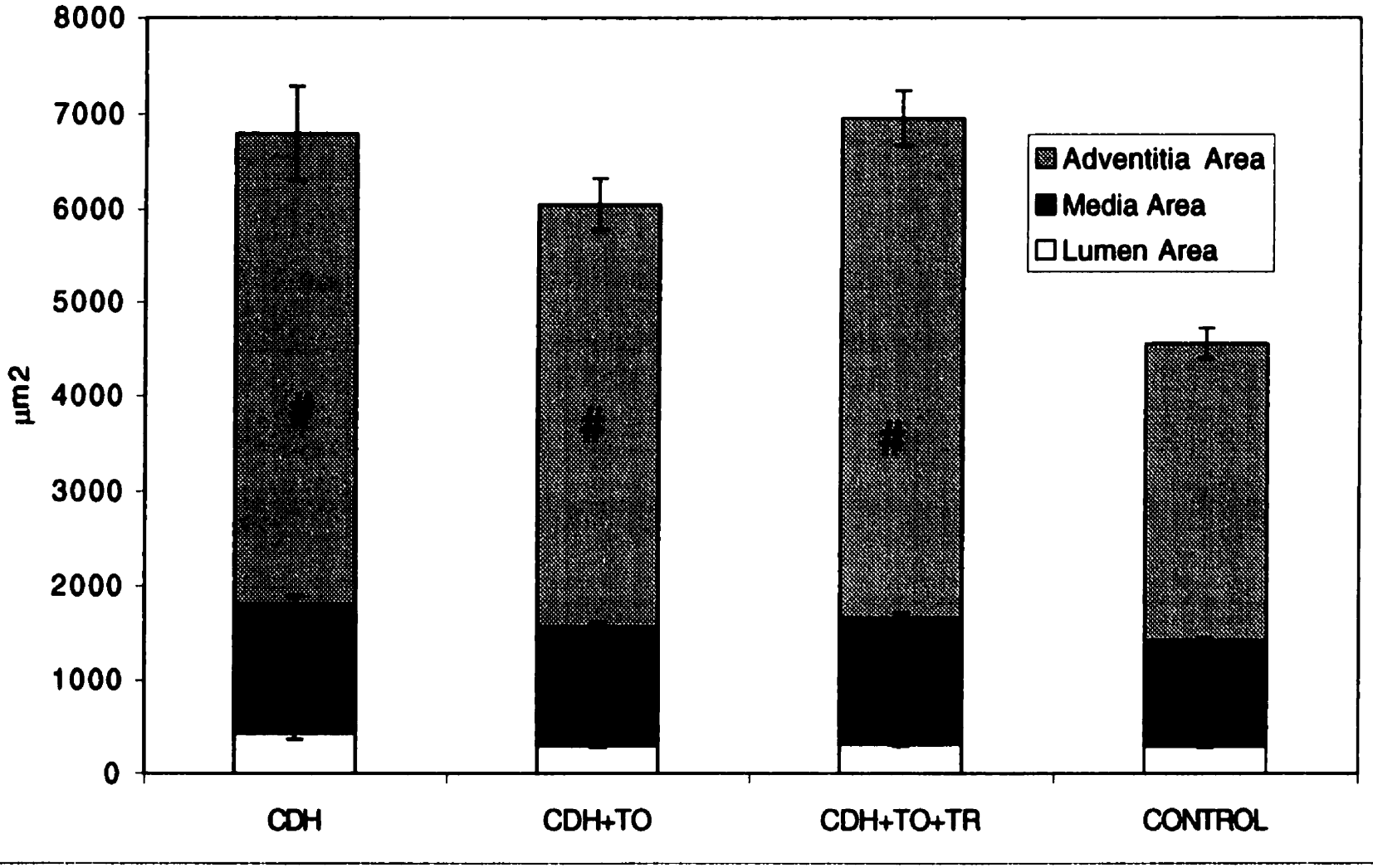

FIG. 7

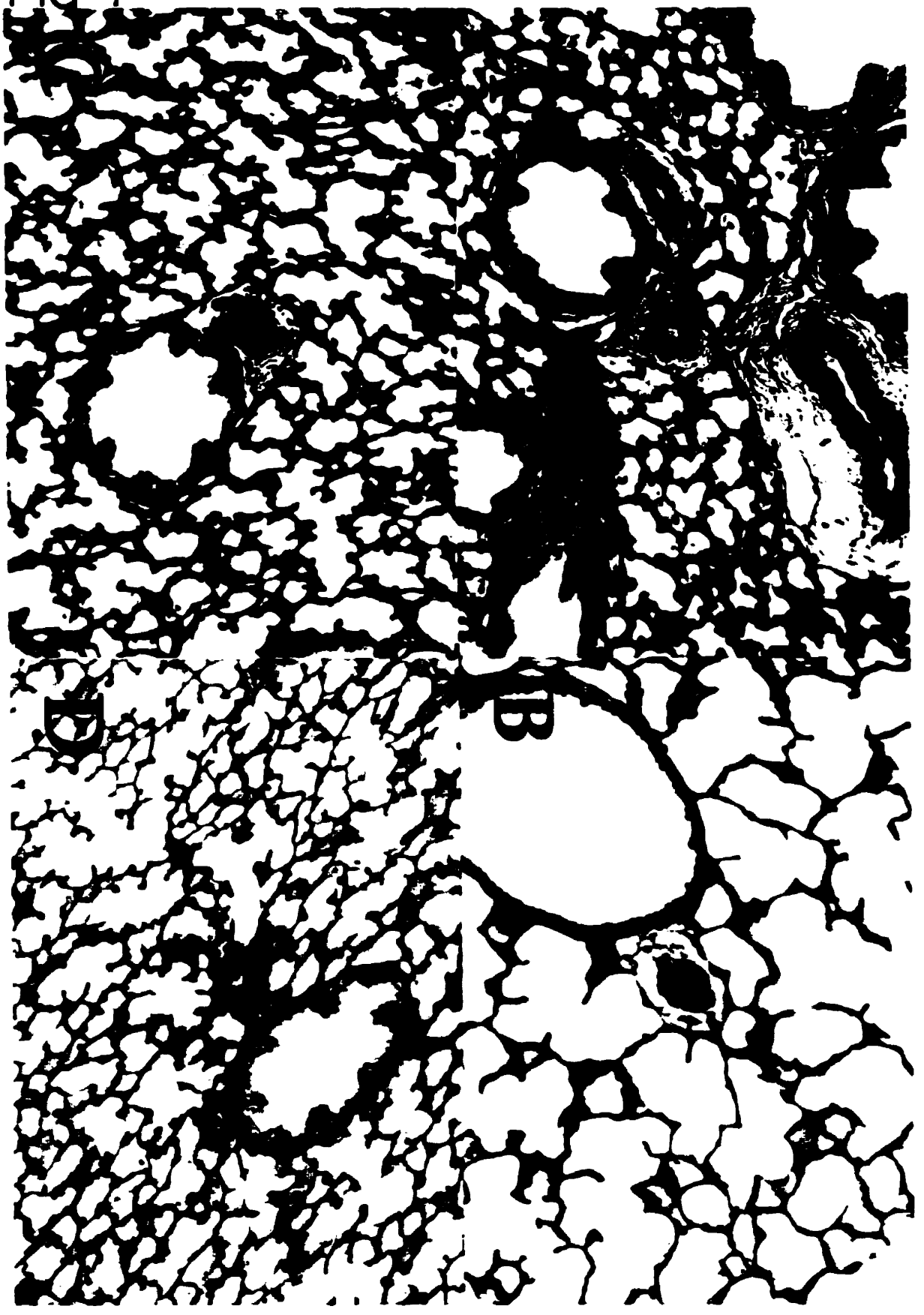

Figure 8. Type II Cell Density

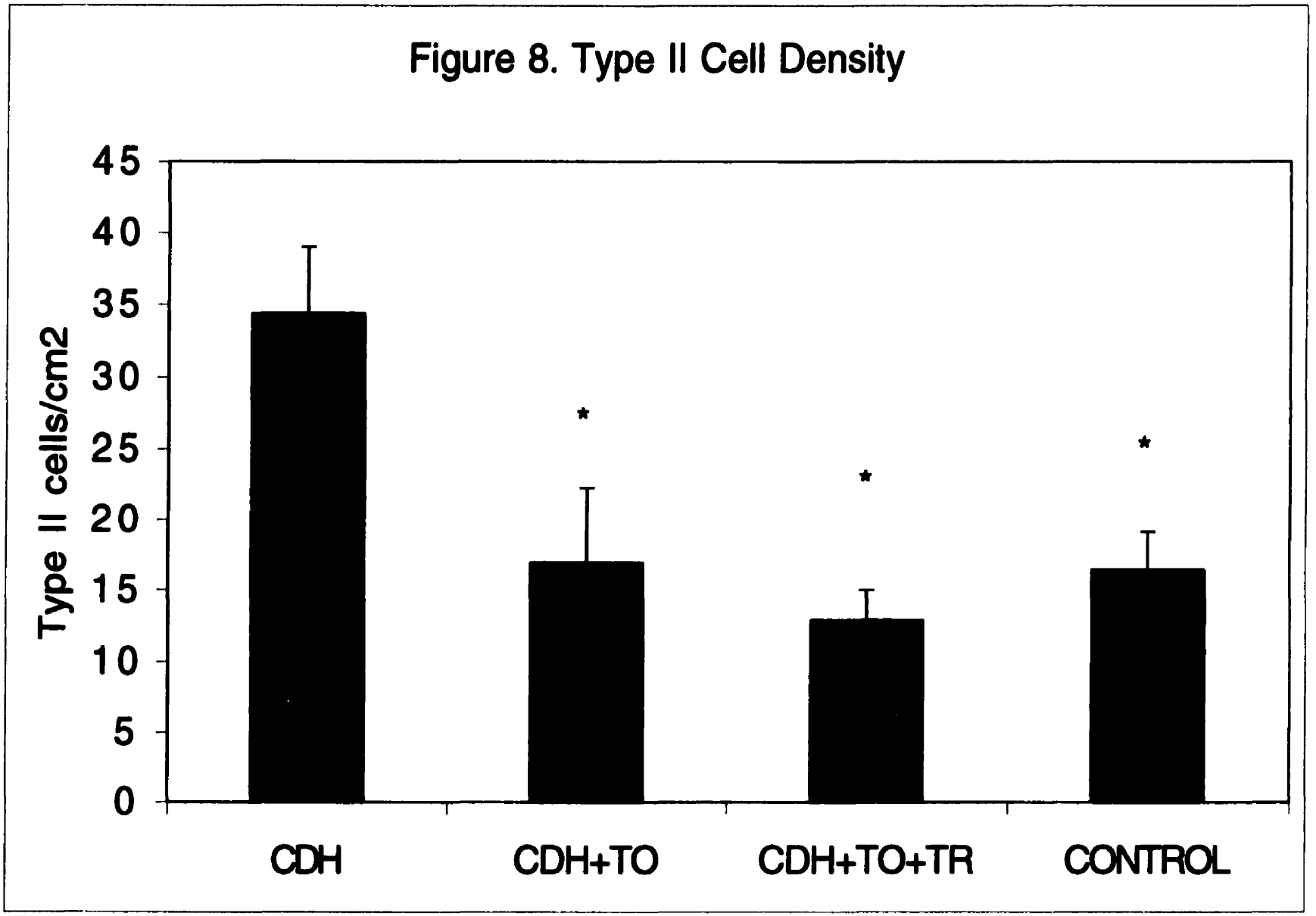

Figure 9. Phase II. Lung Weight/Body Weight

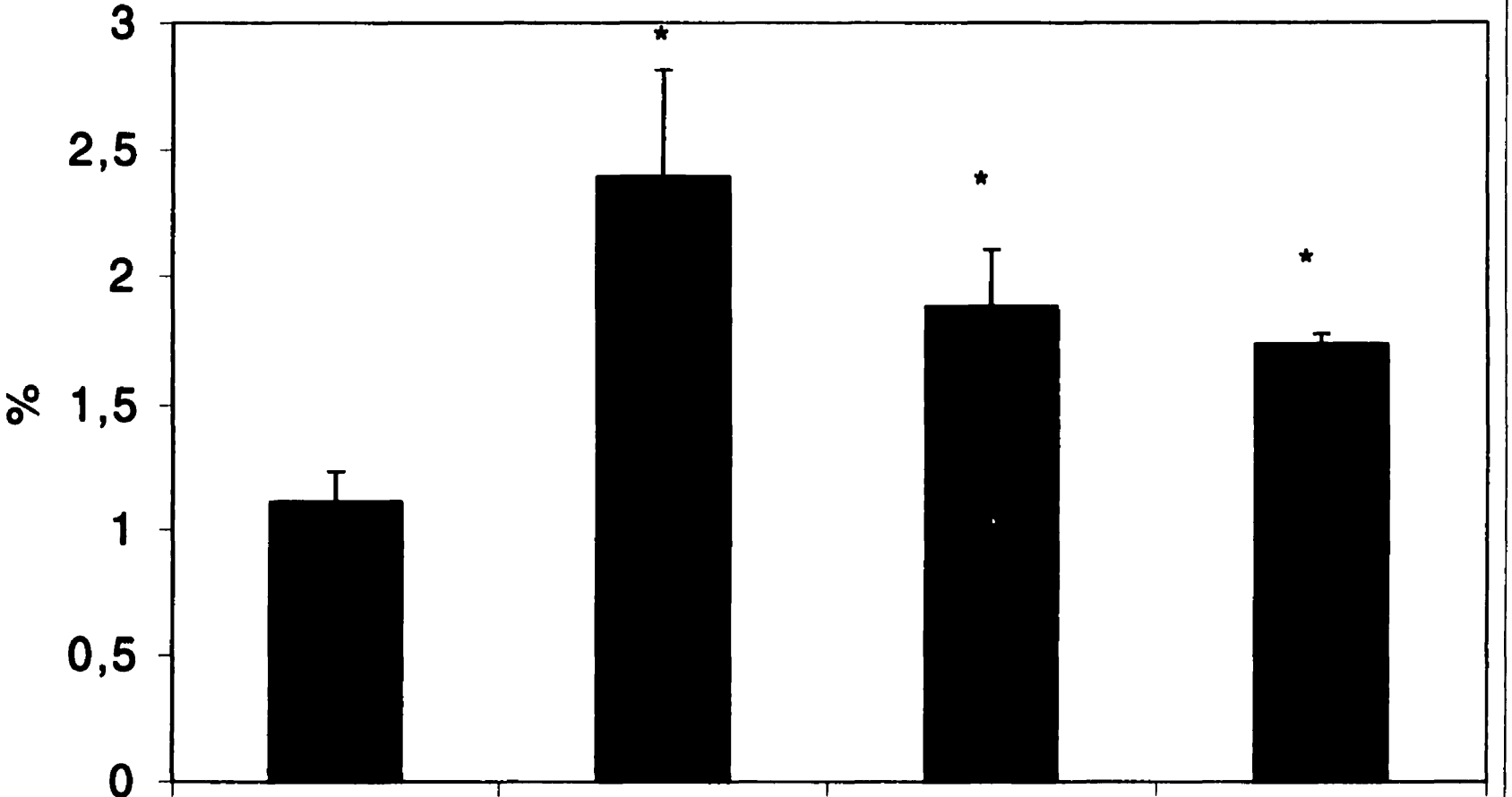

Figure 10. Dry Lung Weight/Body Weight

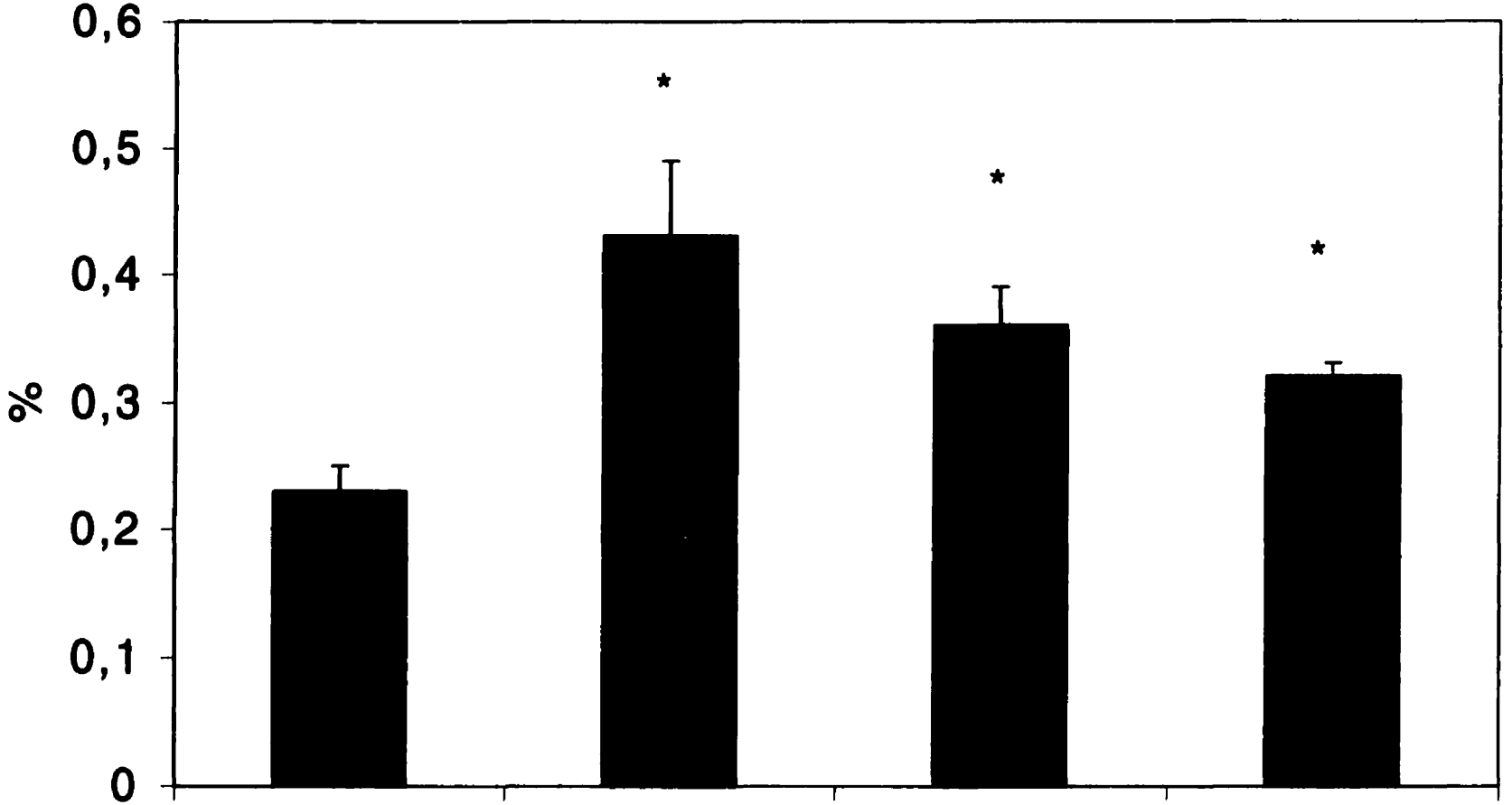

Figure 11. Highest PaO2

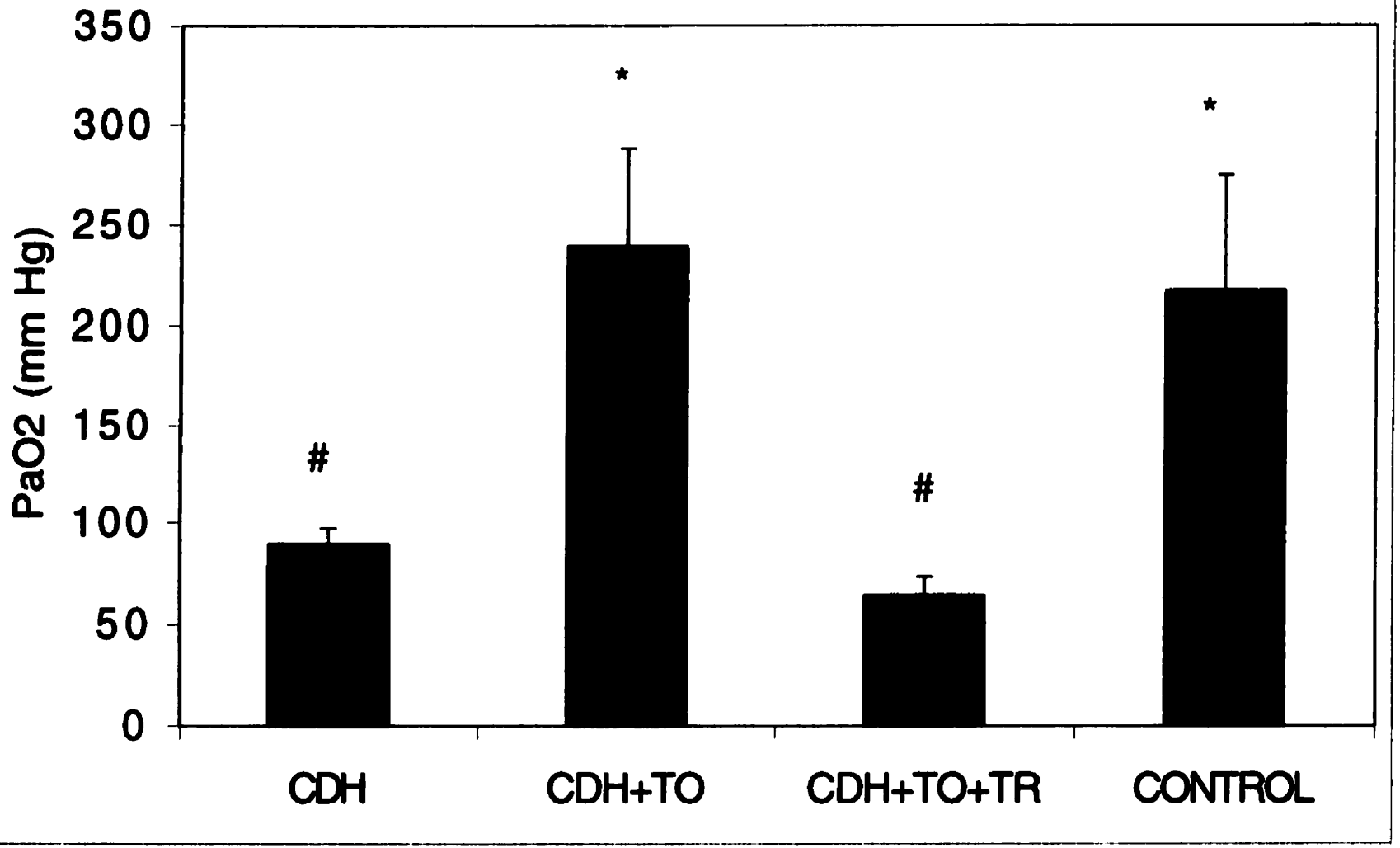

Figure 12. Lowest PaCO2

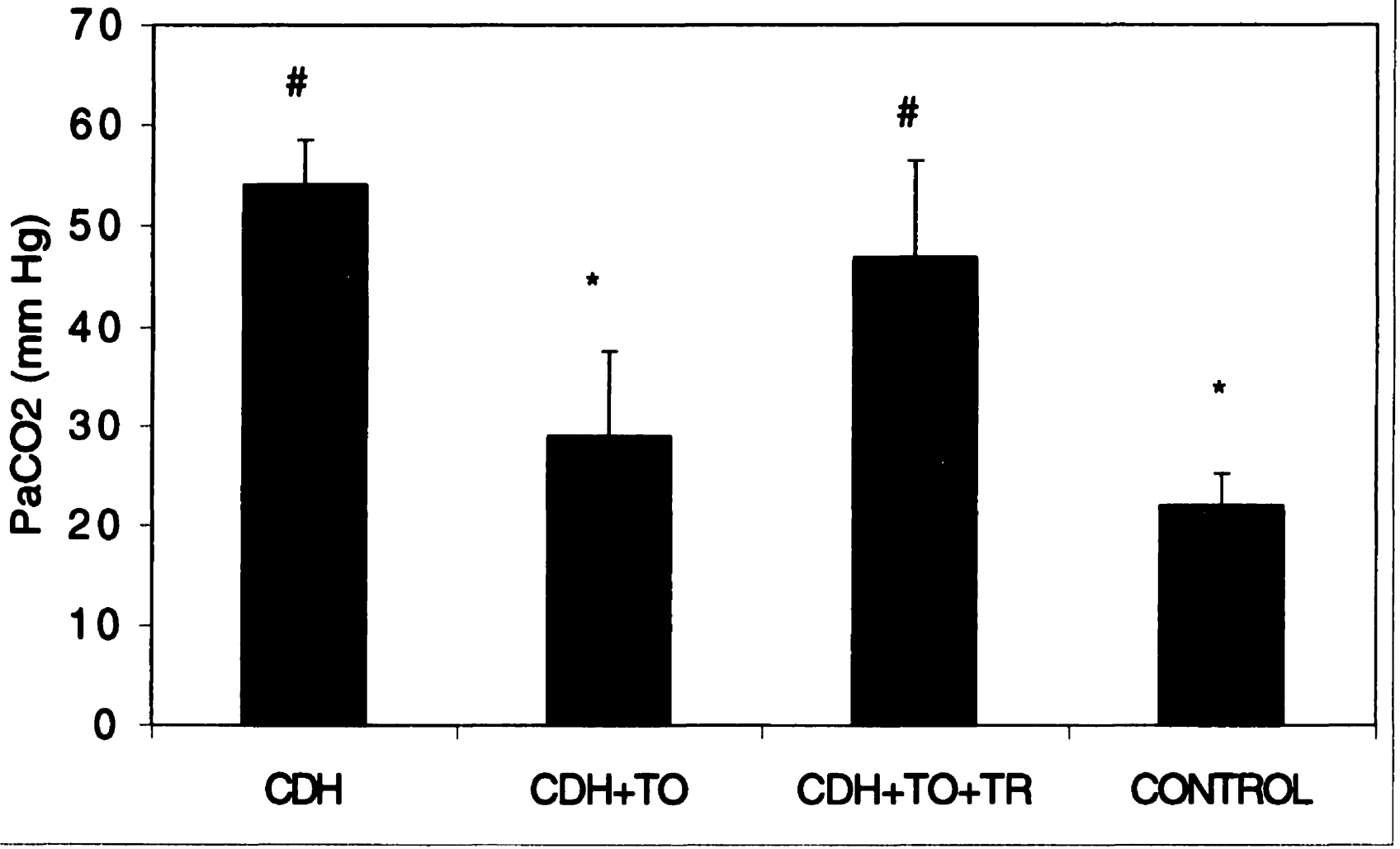

Figure 13. Lung Compliance

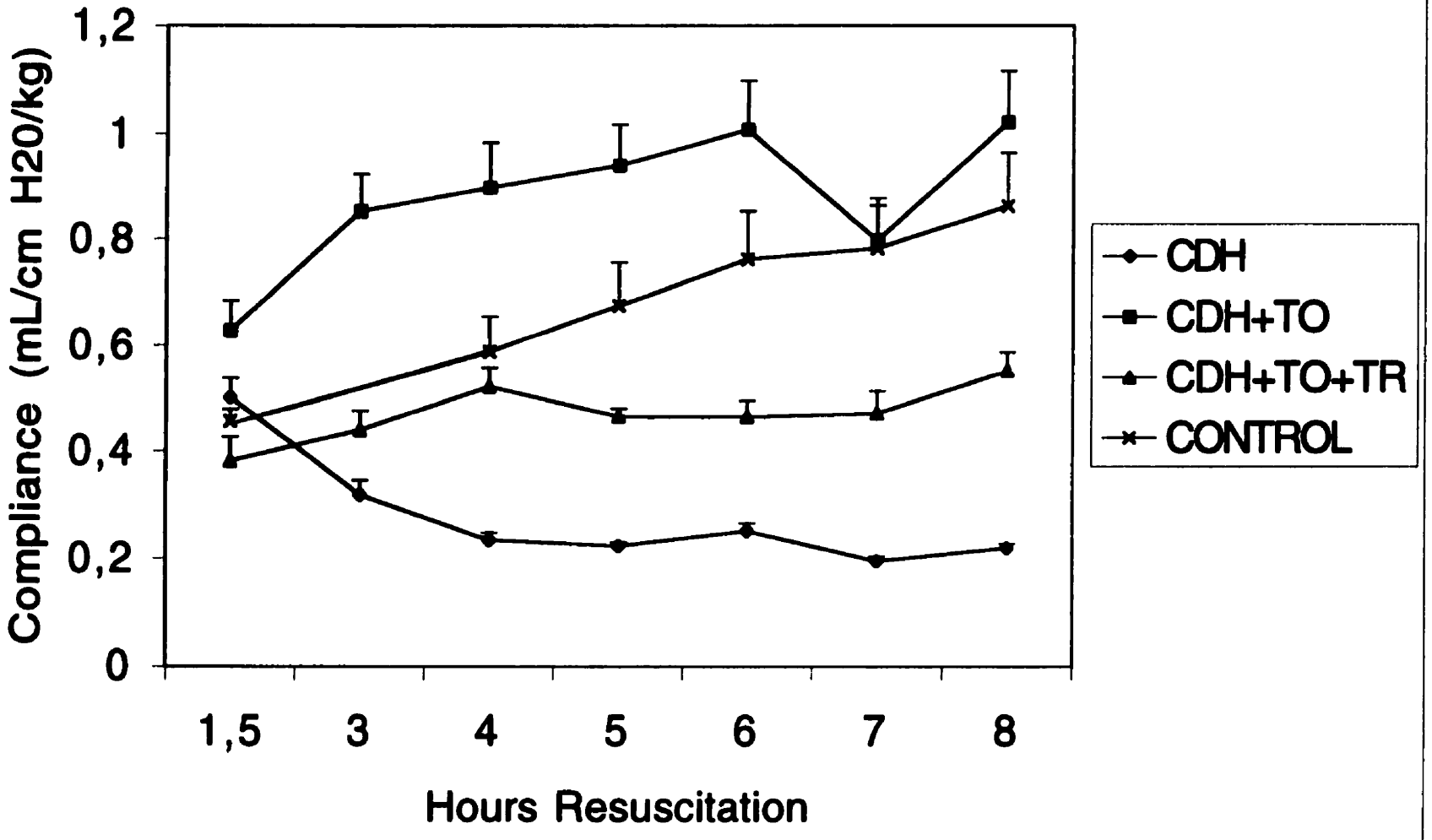

Figure 14. MVI

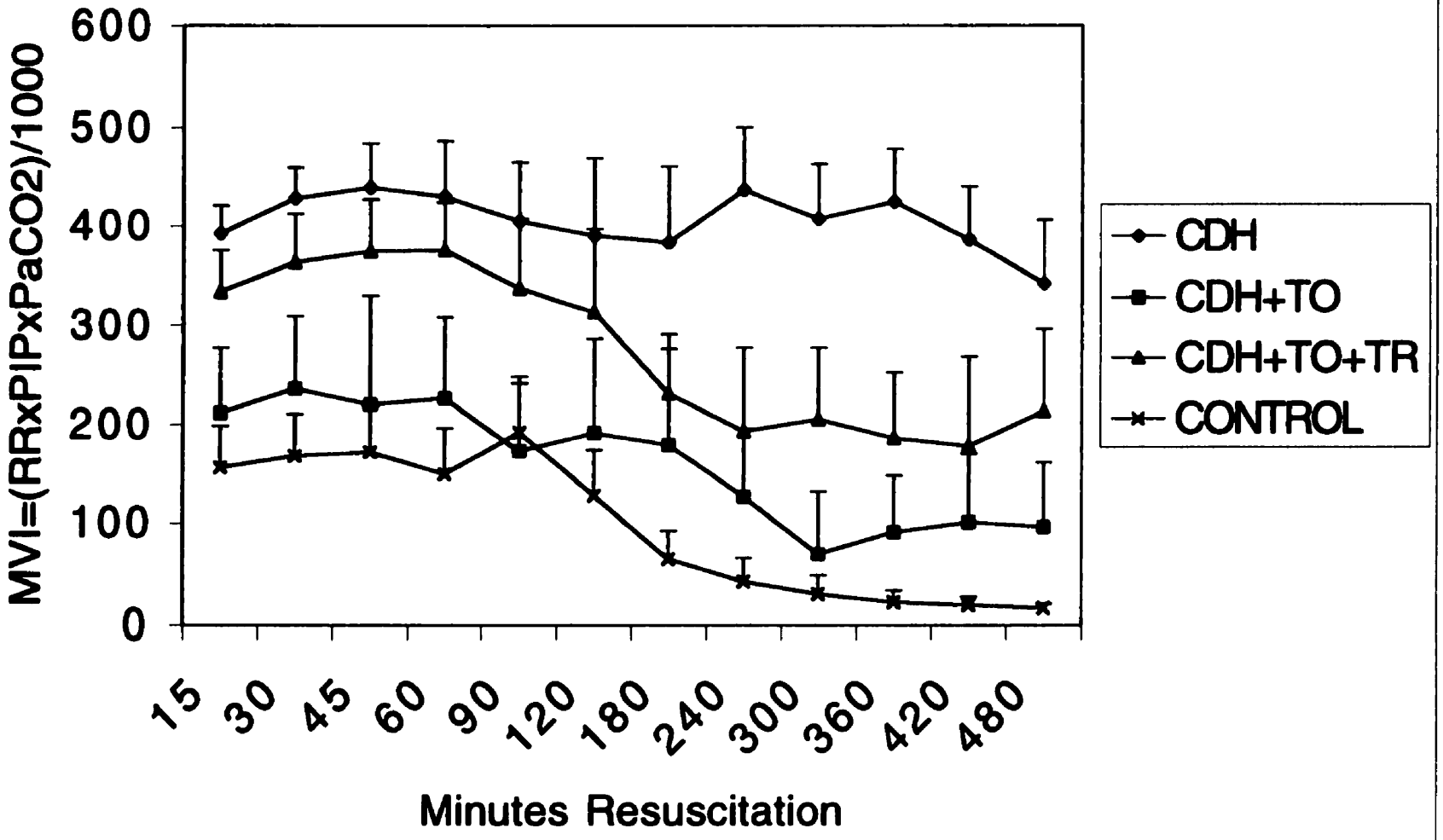

Figure 15. 100xVEI

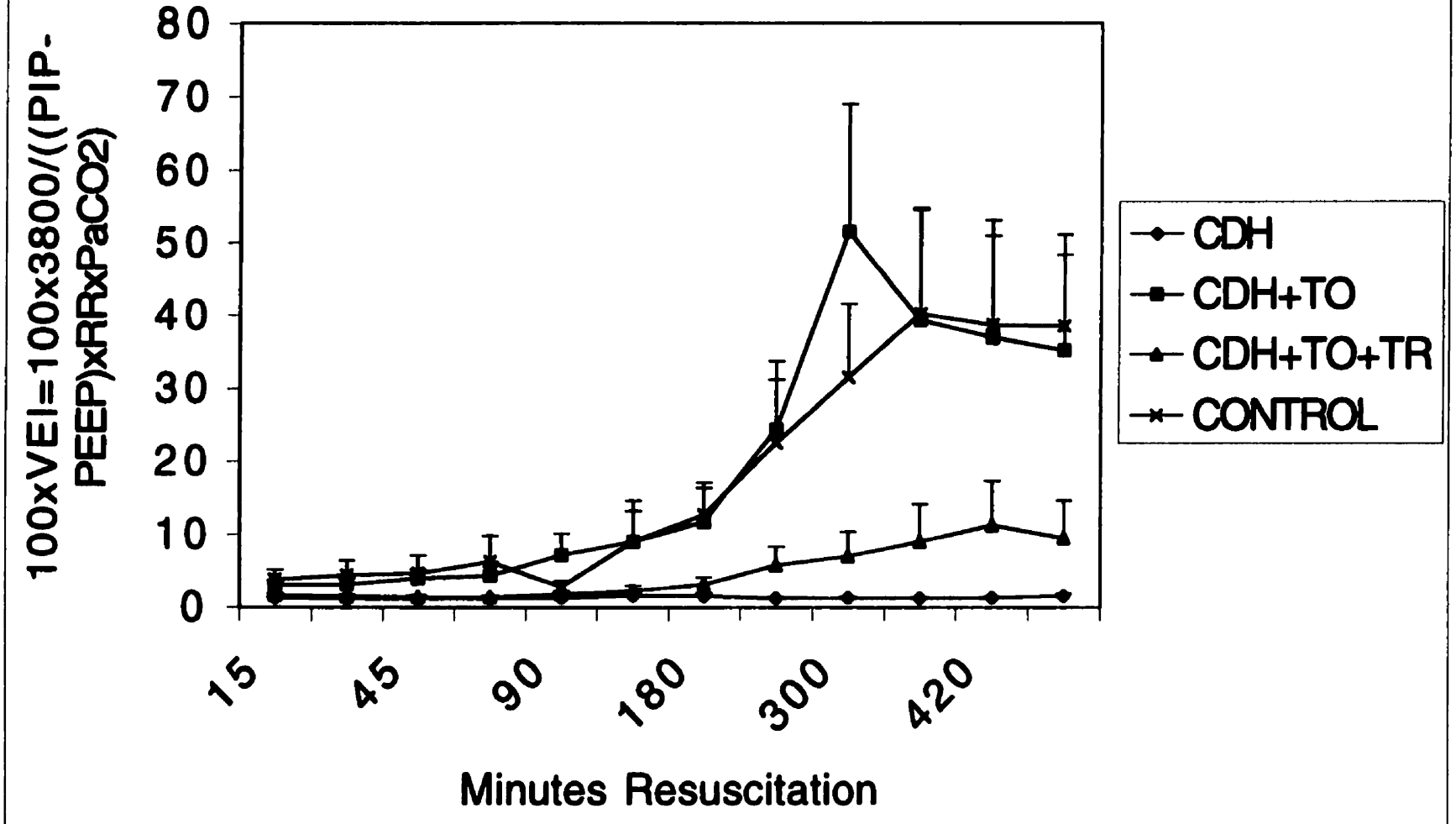

Figure 16. pH

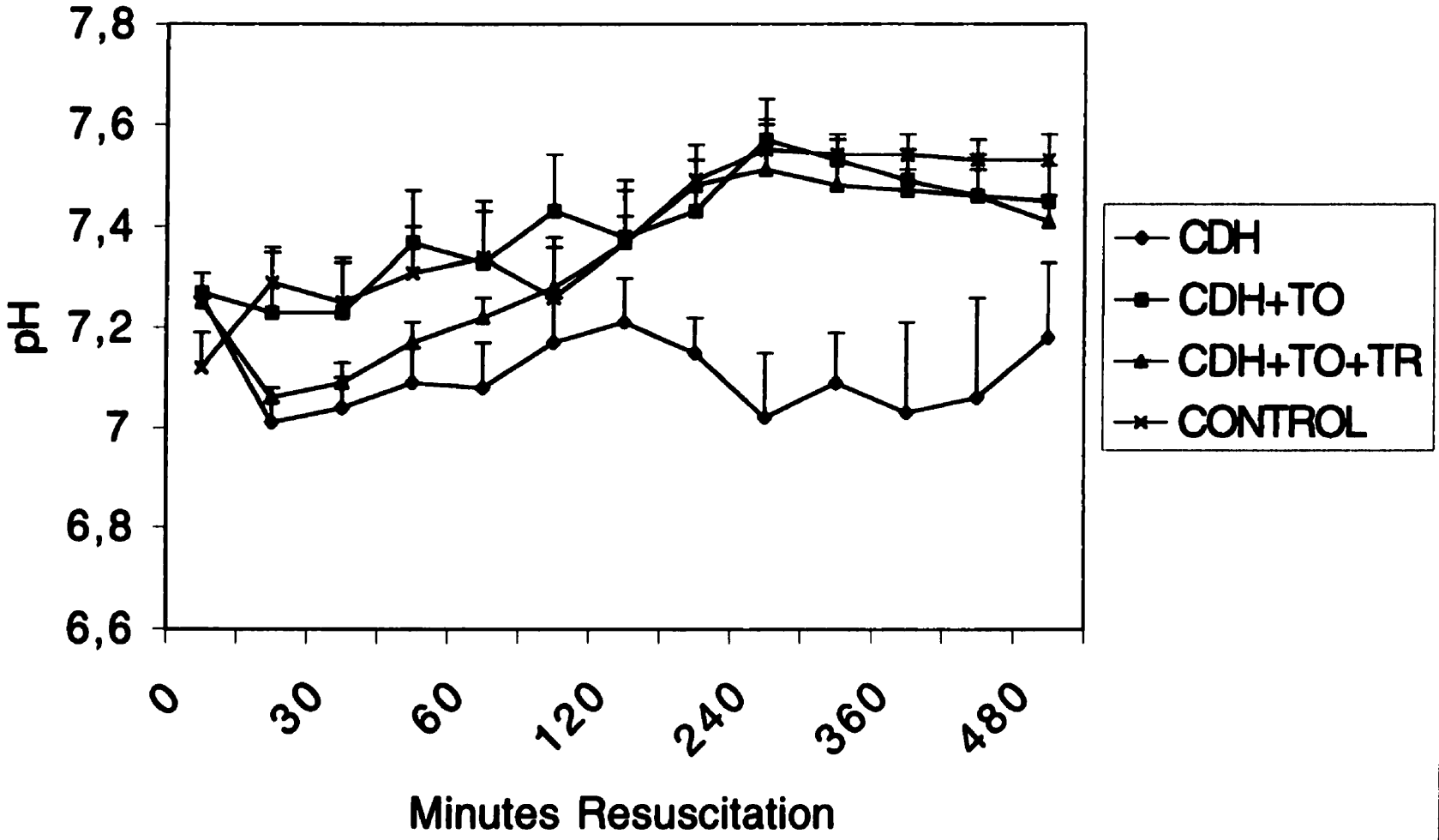
7,8
7,6
7,4
7,2
7
6,8
6,6
pH
0
30
60
120
240
360
480
Minutes Resuscitation
CDH
CDH+TO
CDH+TO+TR
CONTROL

Figure 17. PaCO2

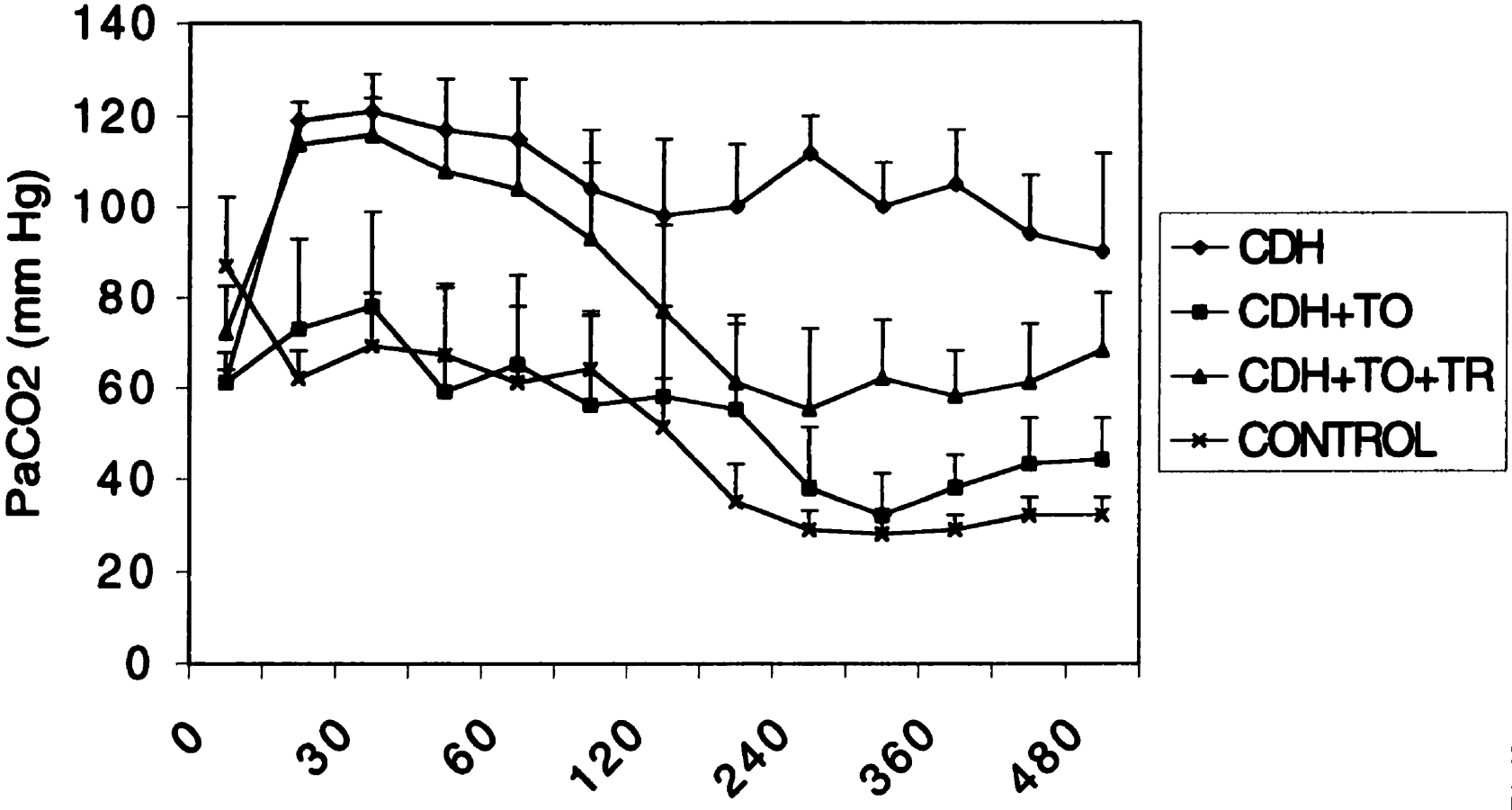

Figure 18. PaO2

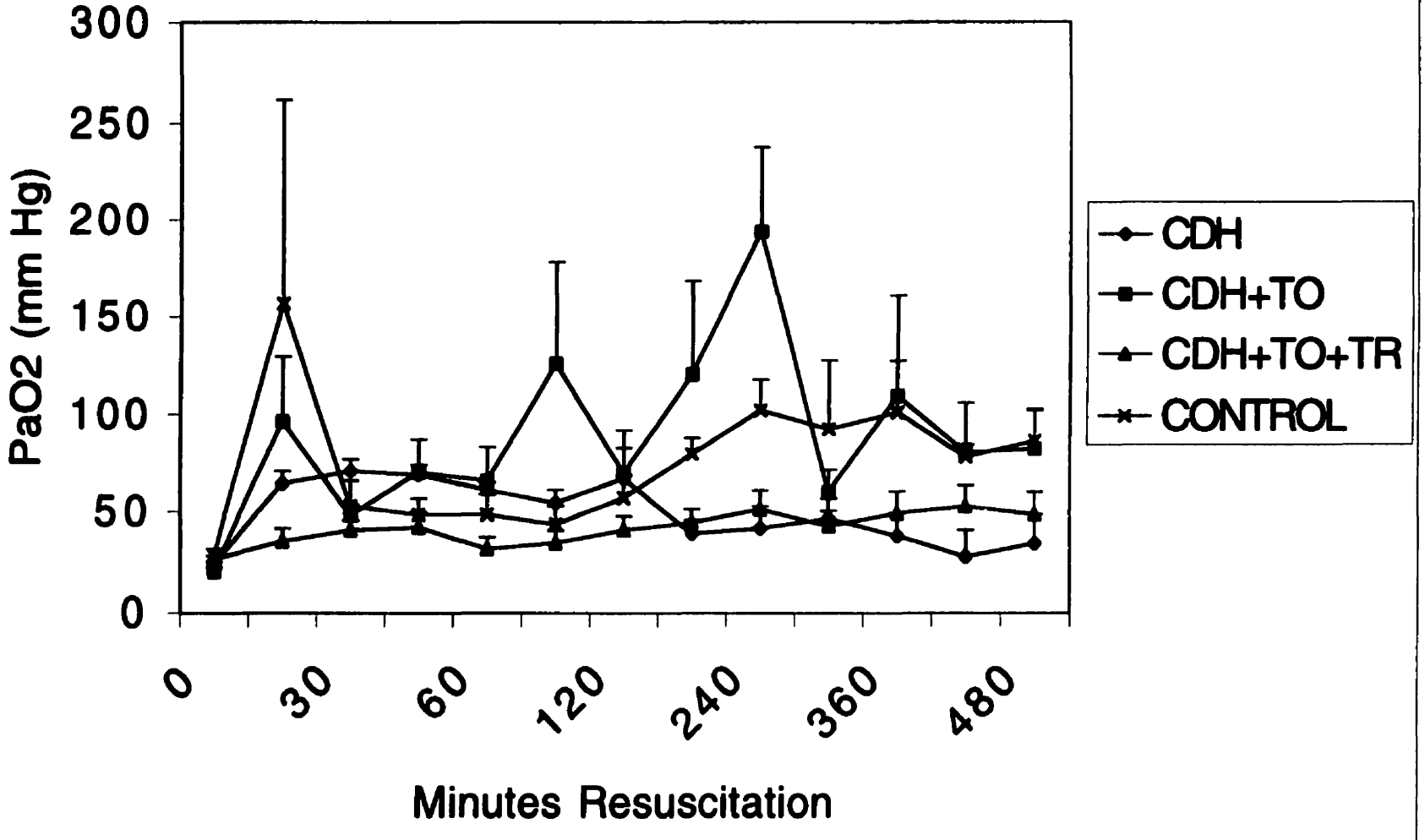

Figure 19. AaDO2