# Morphology of the developing fetal lung – the rabbit experimental model

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Fetal lung development may become impaired by congenital malformations or in utero events. These may be mimicked in animal models, useful in the study of the pathophysiology or therapy of these diseases. Our group has focused on prenatal management of congenital diaphragmatic hernia, because this condition still leads to 30% mortality related to the consequences of lung hypoplasia and pulmonary hypertension. We have been using for that purpose the rabbit model. In this paper we demonstrate that surgical induction of diaphragmatic hernia in the pseudoglandular phase of lung development provokes lung hypoplasia. This is reflected in morphometric changes in airways and vessels, predominantly in the ipsilateral lung. We also discuss the effects on morphometry of pressure or immersion fixation of unrespirated lungs, showing that either protocol can disclose one or more structural alterations in the hypoplastic lung.

**Keywords**: pulmonary hypoplasia, congenital diaphragmatic hernia; airway morphometry

## 1. Introduction

Fetal lung development is a very complex process that follows a well-orchestrated time schedule with a relative high similarity through different species as shown in Fig. 1. This process can be impaired by *in utero* events, including fetal malformations involving lung development, the presence of chronic oligohydramnios or interrupted by preterm birth. As a consequence, structural changes in the lung may arise, including reduction of airway epithelial surface, thickening of the interstitial tissue, and alterations in the vascular bed, which in turn can cause significant disturbances of post-natal lung function.

The condition our group has focussed most on is congenital diaphragmatic hernia (CDH). The experimental study of this condition described herein, can serve as a template for the study of other lung development disorders. CDH occurs in 1:3,000 births and is thought to arise from failure of fusion of the pleuroperitoneal canal in the 9$^{th}$ week (embryologic stage; Fig. 1). Typically the diaphragmatic defect is unilateral and left sided. It is assumed that viscera herniate into the thorax, where they compete for space with the developing lungs. This leads to variable degrees of lung hypoplasia. In essence, CDH lungs have markedly less and smaller alveoli, thickened alveolar walls and increased interstitial tissue [1-3]. Parallel to airway changes, pulmonary vasculature is abnormal, with a reduced number of vessels, adventitial thickening, medial hyperplasia and peripheral extension of the muscle layer into the smaller intra-acinary arterioles [4,5]. As a consequence of these changes, neonates suffer from variable degrees of ventilatory insufficiency and pulmonary hypertension, with a mortality that still exceeds 30 % [6].

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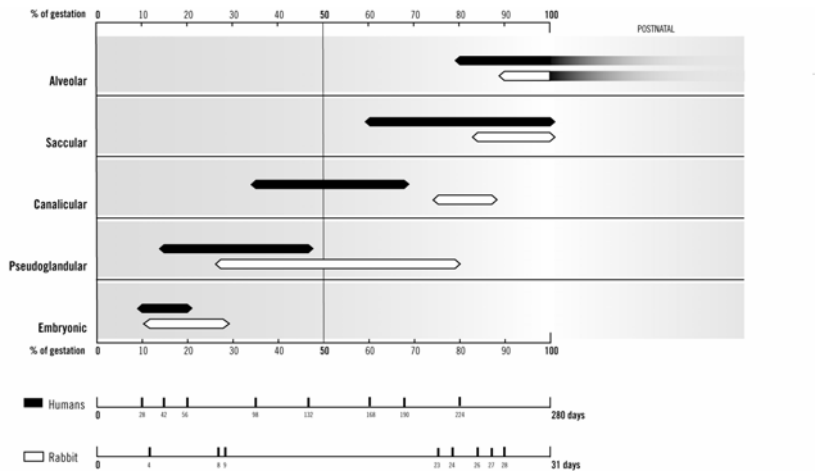


**Figure 1.** Comparison of lung development stages in the human and rabbit with their relationship towards gestational length (Adapted from Toelen et al., 2007, in press [7]).

Given the clinical repercussions, much experimental research has been dedicated to the study of CDH as well as methods and effects of antenatal reversion of lung hypoplasia. When choosing an animal model for that study, several factors play a role. First, its lung development should mimic that of humans as close as possible. Second, the pathology of interest must be inducible in the antenatal period and properly mimiced. For CDH three kind of models exist, a teratogenic rodent model, for instance following nitrofen exposure, [8], a surgical model, described in fetal lambs or rabbits [9] and a genetic model, like the Fog2 mice [10]. We have extensive experience with pregnant rabbits for a variety of conditions, including the lung. Of relevance to CDH is that rabbits have a pulmonary development that largely mimics that of the human lung as shown in Fig. 1. Alveolization occurs prior to birth so that at term, rabbit lungs are in the terminal air sac stage. In addition, rabbits are relatively inexpensive, non-seasonal in their mating habits, have a short gestational period and a large litter size. The only disadvantage of this model may be the rather small size of the fetuses, making the surgical procedures more difficult (fetal weight at surgery = 15 grams; at term 35-45 grams).

There were two research questions in this study. Lung development in neonates with CDH is asymmetrical, i.e. that the ipsilateral lung is more affected by hypoplasia than the contralateral one [11]. In this paper we aimed to test whether this was also the case in our *surgical* animal model, which to our knowledge was never documented formally before. For that purpose we quantified lung developmental changes in lungs of fetal rabbits undergoing surgical induction of DH, studying differences between both lungs, as well as their basal and apical parts.

We also used two different fixation methods prior to morphometry. *In utero* the lung is filled with fluid, to become only expanded by air after the first postnatal breath. Standard protocols of a human (or other large species) air-filled lung involve typically fixation by immersion in formalin, while the lungs are being perfused with the fixative at a given pressure (e.g. 25 cm $H_2O$) through the cannulated trachea [12]). This way airways are theoretically fixed under equal pressure all over, avoiding impact on morphometric measurements by partial collapse of the lung. Many fix lungs of small species also under pressure, providing this is technically feasible and would be reliable [13, 14]. Lung specimens in the rabbit weigh at term 0.5-1.5 gram, making airway pressure fixation a meticulous and time consuming job, which is also prone to errors at the different steps. Studies in rat and mice almost by need perform lung morphometry on non-perfused lungs [15-17]. We focus on lungs pathology in the prenatal period, and as such harvest these prior to birth, i.e. where no respiration took place yet. We hypothesized that immersion fixation without pressure would not impact lung morphometric measures in a specimen that was never ventilated.

## 2. Material and methods

Time-mated pregnant white rabbits (hybrid of Dendermonde and New Zealand White) were transported to the animal facility at least a few days prior to first surgery. Animals were housed in separate cages at normal room temperature and daylight, with free access to food and water. They were treated according to current guidelines on animal well-being, and any experiment was approved by the Ethics Committee for Animal Experimentation (Faculty of Medicine of the Katholieke Universiteit Leuven).

For this experiment, 14 does underwent feto-maternal surgery under general anesthesia and in sterile conditions. At 23 d of gestation (pseudoglandular stage; term=32 d) left sided diaphragmatic hernia (DH) was induced, as described in detail elsewhere [13]. We operated on the two ovarian-end fetuses; because they are in general larger and away from the cervical end. At term, the does were first euthanized and fetuses were delivered by caesarean section at least 20 minutes later, to ensure all fetuses were dead and no respiration of air would take place. Fetuses that were found macerated, were recorded as non-survivors and excluded from further analysis. First a pair of fetuses, i.e. one ovarian-end DH fetus as well as a size matched control, were harvested for the present study. If also a second DH-fetus survived, it was, together with a size matched control, harvested and processed, so that its organs could be snap-frozen for later molecular work, which was not part of this study.

In two out of the 14 does the DH fetuses did not survive, resulting in 12 DH fetuses and a similar number of size-matched controls (CTR). DH and control fetuses were assigned randomly to two groups, undergoing lung formalin fixation either under pressure of 25 cm $H_2O$ (DH n=5; CTR n=5) or without, 0 cm $H_2O$ (DH n=7; CTR n=7). Fetuses were first weighed using a scale measuring accurately up to 0.001g (HF 2000; A&D Instruments, Haasrode, Belgium). At necropsy the liver was removed, weighed and subsequently the lungs were removed "en bloc". Lungs assigned to the 0 cm$H_2O$ group were first separated from the trachea. The right and left lungs were weighed and then immersed in 6% neutral buffered formalin solution for 24 hours. Lungs assigned to the 25 cm $H_2O$ group were kept on the trachea, serving for cannulation and perfusion fixation. After leak proof tying of the trachea to the cannula, the lung specimen was immersed in a formalin bath. Simultaneously the trachea was irrigated with formalin at 25 cm $H_2O$. This pressure is created by tilting the level of formalin in an additional container 25 cm above the fluid surface of the formalin bath. A pump is used to maintain this container filled at that level. Keeping the lungs on the windpipe precluded measurement of left and right lung weights under exact the same conditions in both pressure groups. Therefore gross anatomical findings will only be displayed for the 0 cm $H_2O$ fixation pressure group.

Paraffin-embedded lungs were cut into 5-µm sections. As the fetal rabbit lung is rather small, a section through the *entire* lung was made [18, 19]. The slides were stained with Hematoxylin and Eosin for airway morphometry and with Elastica van Gieson (Hart's method) using Weigherts's solution (resorcinol-fuchsin) for vascular morphometry [20].

### 2.1. Main outcome measures.

2.1.1. Gross analysis: fetal body weight (FBW), fetal liver weight (FLW), left lung weight (LLW), wet right lung weight (RLW), total fetal lung weight (TLW) were measured in wet conditions. From FBW and lung weights the lung-to-body weight ratios (Left LBWR, Right LBWR, LBWR) were calculated.
2.1.2. Microscopy: All measurements were performed with a Zeiss AXIOPLAN light microscope (Carl Zeiss, Oberkochen, Germany) at a magnification of x200. The total surface of each lung section was first virtually divided in an apical and basal part, each of them divided in up to 10 random non-overlapping fields, in total up to 20 fields per lung, hence providing observations in left and right lung, as well as basal and apical lung parts.
2.1.3. Airway morphometry. The parameters of interest were measured in the lung parenchyma focusing on the respiratory airways as shown in Fig. 2a-c. Two special oculars were used, one with a grid, another with a ruler, as shown in Fig. 2a and c, respectively.
*Lm – mean linear intercept.* The linear intercept is an index directly related to alveolar size. The number of cross-sections of an alveolar wall with half of the ruler was counted, as shown in Fig. 2c. Each

alveolar wall was counted as two crossings. This was counted first in the horizontal direction, then in the vertical direction, turning the ocular by 90°. Two numbers per field were obtained, their average calculated and used in the equation: *Lm = (0,57/average intercepts) x 1000 (µm).* The coefficient 0, 57 is calculated from the length of the ruler and the coefficient of shrinkage = 0,612 [21].
*MTBD – mean terminal bronchiolar density.* The number of terminal bronchioles in a given high power field is inversely related to the number of alveoli supplied by each bronchiole, as shown in Fig. 2a, c. For this parameter a grid of 10x10 squares was used. Only the bronchioles, which are in the central part of the grid (6x6 squares), were counted. All bronchioles crossing or only touching the left and lower borders of this "6x6" square were excluded. All bronchioles crossing or touching the right or upper borders even outside were included. The sum of all bronchioles was calculated, from which the average number per field, which was used in the equation: *MTBD = average / 0, 23 (br/mm$^2$).* The coefficient 0, 23 is calculated from the surface of the "6x6" square and the coefficient of shrinkage = 0,612 [22].
*Lmw – mean wall transection length.* This is an index of the thickness of alveolar septae [23]. To calculate this parameter we checked how many of 25 hidden points per field fall on air-space or on tissue, and we counted the number of tissue hidden points. Figure 2a shows the hidden points falling on air-space (*) or on tissue (#). From the total number of points (not less than 500 per lung) we calculated the % of tissue, which is used in the equation: *Lmw = (Lm x % of tissue) / 100 (µm).*

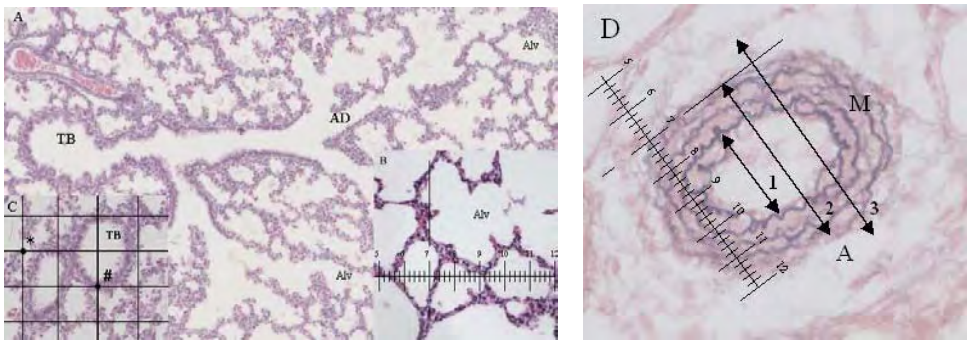


**Figure 2**. Histologic photomicrographs of peripheric respiratory airways: the distal part of the respiratory tree (a, mag.x200); terminal bronchiole (c, mag.x400); alveoles (b, magn.x400). A segment of the grid used to count the terminal bronchioles and the hidden points is drawn on Figure 2c. *- a point on the air-space (not counted), # - a point on tissue (counted). A segment of the ruler used to measure Lm is drawn on Figure 2b. TB- terminal bronchiole, AD- alveolar duct, Alv- alveole. On a cross section of a fetal pulmonary peripheral artery stained with Elastica van Gieson (d, mag x400) adventitial (1), external (2) and internal diameter (3) were measured along the shortest axe. A – adventitita, M – media.

2.1.4. Vascular morphometry. Figure 2d shows how the vascular morphometry was measured. In 10 random non-overlapping fields all peripheric muscularized vessels with external diameter ED≤100 µm were measured, which in rabbits correspond to the pre- and intra-acinar arteries [24] and are believed to be the resistance arteries [25]. The adventitial diameter (AD, µm), external diameter (ED, µm) and internal diameter (ID, µm) were measured along the shortest axis of the vessel [26]. From these the proportionate medial (%MT) and adventitial (%AT) thickness can be calculated using the equation: %MT = (ED – ID) / ED x 100 and %AT = (AD – ED) / E*D x 100*. These two parameters actually nullify the effects of vasodilatation, vasoconstriction and tissue shrinkage [26].

2.2. Statistical analysis.

Morphometric measurements were done by two observers (X.R. and E.V.), who were blinded to the nature of the experimental fetal procedure, and their observations were averaged. All data are presented as mean ± standard error of the mean (SEM). Differences between fetuses from the same does (DH to

internal normal) or within left and right lung, or apical and basal part of the lung of the same fetuses, were analysed pair wise. Observations between different fixation pressure groups were compared unpaired wise. We used GraphPadPrism version 4.0 (GraphPadPrism Software, San Diego California USA). P values less than 0.05 were considered as statistically significant.

## 3. Results

### 3.1. Gross anatomy of control fetuses of 0 cm $H_2O$and morphometry at 25 cm $H_2O$.

The gross anatomic findings between the two fixation pressure groups were comparable. Accurate weighing of the lungs without trachea prior to fixation was only possible in the 0 cm $H_2O$ group, as shown in Fig. 3. In the normal fetuses of that group, the mean wet left lung weight was 69 % of the right. We then compared morphometry findings in left and right lung of fetuses without hernia which were fixed under 25 cm $H_2O$. All airway and vascular indices in the left and right lung were comparable. Neither a difference was found between the apical and basal part of the lungs, except for the right lung, where the MTBD was 10% higher in the apical part than in the basal part (p=0.04).

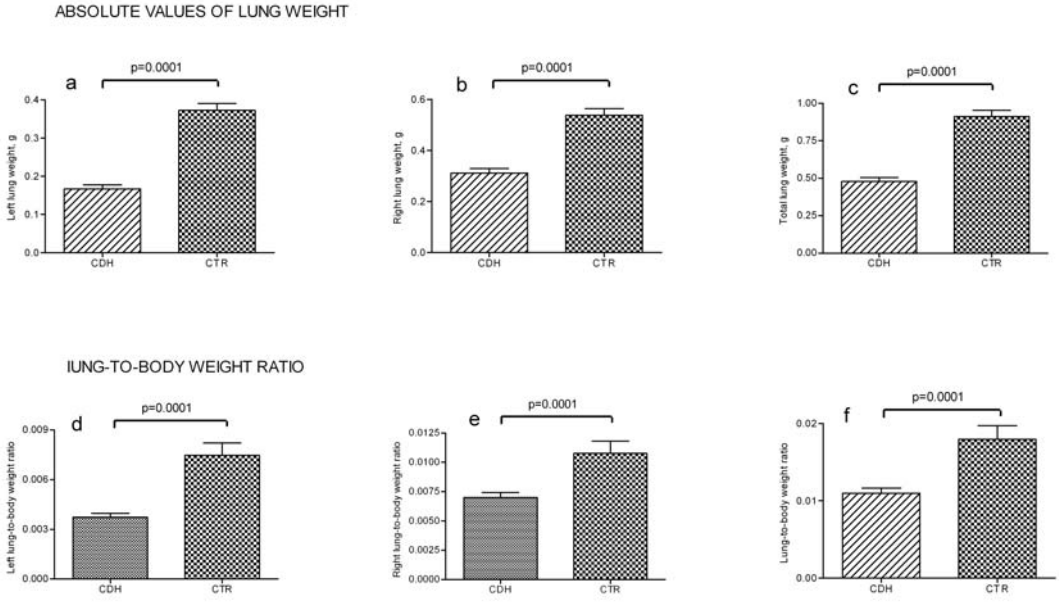


**Figure 3.** Graphical display of gross anatomical findings: lung weights and lung-to-body weight ratios in CDH and CTR fetuses (0 cm$H_2O$; * $p<0.05$).

### 3.2. Gross anatomy of fetuses with CDH and morphometry at 25 cm $H_2O$.

Figure 3 shows also gross anatomy findings of CDH fetuses from the 0 cm $H_2O$ group. In DH fetuses the mean wet left lung weight was 55 % of the right The total lung weight in CDH fetuses was significantly lower than in their control littermates (p=0.0001), as shown in Fig. 3. As a result, the left LBWR and right LBWR as well as the total LBWR in CDH (LBWR (CDH)= 0.011±0.0009 compared to (CTR)= 0.0206±0.0012; p=0.0001) were significantly smaller than in CTR (p=0.0001). This number is compatible with lung hypoplasia.

Morphometric indices were first studied at 25 cm $H_2O$. Within the same lung of a fetus with CDH, morphometric indices were comparable for apical and basal lung areas. Also measurements in ipsilateral

and contra-lateral CDH lungs were comparable, with the exception of MTBD scores. MTBD in the ipsilateral lung, was 70 % higher than in the in the contralateral one (p=0.01). When compared to *normal* littermates, alveolar size (Lm) and wall thickness (Lmw) were not significantly different. The basal parts of left (ipsilateral) CDH lungs showed a trend for higher MTBD, but that did not reach significance (difference: 1.826; CI: -0.02 to 3.67; p= 0.051). In the apical part of the left CDH lung the MTBD was more than twice as high as in CTR (difference: 2.261; CI: 0.06 to 4.47; p=0.047). Overall, the ipsilateral lung has twice the density of terminal bronchioles than normal littermates (difference: 2.043; CI: 0.19 to 3.9; p=0.0378). In contrast, right CDH lungs had MTBD scores comparable to normal lungs (difference: -0.17; CI: -0.9 to 0.5; p=0.5).

The medial thickness of vessels in CDH lungs was not different in left or right lung, neither apical or basal areas. However, medial thickness was significantly larger in ipsilateral CDH lungs when compared to normal fetuses, (difference: 10.24; CI: 3.44 to 17.03; p=0.023). The difference of medial thickness in contralateral lung vessels to the normal did not reach significance (difference: 2.5; CI: -9.2 to 14.2; p=0.4).

### 3.3. Effect on morphometry of fixation without pressure.

We first looked to the normal lungs, and focused our attention to the left ones, because, in case of CDH, they are the most affected. Fixation without pressure did not cause a significant decrease of linear intercept of normal left lungs, but results in a significantly higher MTBD (p=0.02) as well as Lmw (p=0.01), as shown in Fig.4. In left CDH lungs, the morphometric indices were comparable between pressure and immersion fixed lungs.

The experimental relevance of our study lays obviously in observations in CDH fetuses and whether these differences to normal littermates are changing when changing fixation protocol. In general, this part of the experiment demonstrated that the architectural changes present in hypoplastic lungs, still become obvious, but through other morphometric indices, when a non pressure fixation protocol is used. Figure 4 shows that the trends in differences for most if not all critical morphometric parameters are maintained. Under both fixation protocols the medial thickness was increased. Under pressure, ipsilateral DH lungs had a twice as high MTBD count when compared to normal. The difference in MTBD between CDH and normal lungs became smaller when omitting fixation (no longer significant). Instead there was a trend for a larger difference for Lmw (not significant; p=0.07) and a significant difference for Lm (p=0.035), both parameters not being different at 25 $cmH_2O$ fixation pressure. Those changes were most marked in the basal part of the lungs (data not shown).

In right lungs, which are apparently morphologically unaffected by CDH under a pressure protocol, immersion fixation now revealed a significant increase in MTBD. The Lm and Lmw were not significantly different under both fixation protocols.

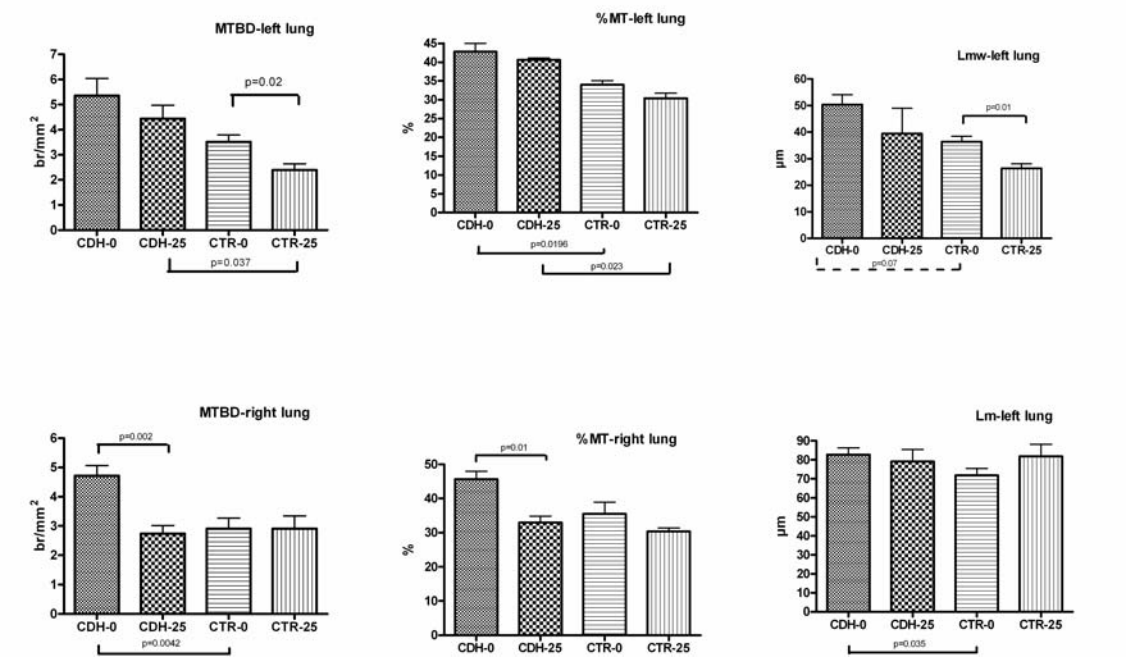


**Figure 4.** Graphical display of airway and vascular morphometric parameters of left/right lungs fixed under 25 cm or 0 cm $H_2O$ in CDH and CTR groups. On top of the graphs are shown significant differences between surgical groups (effect of hypoplasia); under the X-axis are significance levels marked for effects of pressure protocol, both with their p value.

## 4. Discussion

Surgical induction of DH decreases lung weight significantly to levels compatible with lung hypoplasia on both sides. The left lung is more affected by this process, certainly when the effect is expressed in terms of proportional lung weight. The predominant effect on the ipsilateral lung is only partially translated in airway morphometric findings when lungs are expanded during fixation. Ipsilateral lungs score more "hypoplastic" than the contralateral ones when counting the number of terminal bronchioles, which is inversely related to the number of alveoli. Recently, we also carried out ventilation experiments, demonstrating that these structural changes parallel alterations in lung tissue mechanics [27]. Less well studied but clinically very relevant, induction of DH also induces vascular changes, as shown by a marked increase in medial thickness in the smaller "resistance" arteries [28]. The rabbit model of CDH in this respect features all needed requirements, even when the lesion is induced as late as the pseudoglandular phase of lung development.

Lung morphometry has been typically done under pressure, to nullify effects of airway collapse. In those circumstances, morphometric measurements reflect not only airway architecture, but also their compliance. We demonstrate in the first part of our experiments, that, under 25 cm $H_2O$, rabbit CDH-lungs have an increased medial thickness in ipsilateral lungs. They also had significantly decreased number of alveoli (or increased MTBD), with a trend for larger differences in the basal part of the ipsilateral lung, although that did not reach significance. Again the apical part of the lung had an increased MTBD. Alveolar wall thickness tended to increase, neither was the linear intercept any different.

The second goal of the study was to document the effects of immersion rather than pressure-immersion fixation, in experimental circumstances where fetuses were delivered prior to respiration. Those 0 cm $H_2O$ group fetuses had lung weights compatible with hypoplasia. The absence of pressure

maintains changes in morphometric measurements of both normal and CDH lungs visible. Normal lungs for instances have apparently thicker alveolar walls, or a reduced number of alveoli, but the linear intercept (related to alveolar size) remained unchanged. We were obviously most interested in to what happened with the differences between normal and hypoplastic lungs. For instance the absence of pressure has an effect on MTBD score, which is more obvious in normal than CDH lungs. The net effect in both groups is such that ipsilateral CDH lungs apparently do not show any higher MTBD anymore, when compared to normal lungs. However in contrast, immersed CDH lungs have a significantly higher linear intercept than normal lungs. While Lm is inversely related to alveolar size, it is not solely determined by the alveolar diameter. For the same size alveole, changes in alveolar wall or interstitial tissues, may still cause changes in Lm. The higher values of Lm in CDH fetuses probably coincide with smaller alveoli (in the absence of expansion) but also thicker alveolar walls, because at closer look, Lmw was borderline significant thicker in CDH lungs.

One could summarize the effects of fixation pressure on morphometry as follows. In normal lungs, who have a normal compliance, the surface filled with alveoli measurably increases under (fixation) pressure. The linear intercept (Lm), closely related to alveolar diameter, does not increase but there is thinning of alveolar walls (Lmw). CDH lungs are hypoplastic, predominantly on the ipsilateral side. Pressure fixation shows a decreased air space fraction (MTBD increases) because there are fewer alveoli. These lungs are also less compliant, hence may give less way to pressure induced changes (27). In CDH lungs changes in alveolar wall thickness or alveolar size are therefore less easy demonstrable. However, they are present, as in the absence of pressure, changes in linear intercept and alveolar wall tend to change, or are obviously changed. In immersed lungs, terminal bronchiolar density was even increased on the *contralateral* side, pointing at decreased number alveoli on that side as well. That effect however disappears under pressure, potentially because that less affected lung is a bit more compliant and/or that the structural changes are less dramatical.

In summary, in rabbits, surgical induction of DH induces bilateral lung hypoplasia, as measured by LBWR. The ipsilateral lung is more affected by this process than the contralateral one. Different fixation protocols make different changes in the lung structure obvious. For ipsilateral lungs, that are fixed without pressure, the unexpanded lung will show a significant increase in mean linear intercept, which is directly related to alveolar size and also influenced by eventual increases in alveolar wall thickness or other tissue changes. In contrast, fixation under pressure will disclose an increased terminal bronchiole density, hence lower number of alveoli, when comparing the same parameter to a much more expanding normal lung. In both fixation protocols the vessels are not perfused under pressure, hence it is no surprise that observations on an increased medial thickness are no different for both fixation protocols. Contralateral lungs are less affected, so that morphometric changes induced by pressure fixation hardly reach significance. Unexpanded ipsilateral lung however show significantly higher number of bronchioli (pointing at less alveoli). This was not so under pressure fixation. We speculate that the contralateral lung is probably more compliant than the ipsilateral one, therefore resists less to pressure fixation. When expanded under 25 cm $H_2O$, the airspace fraction probably expands to such an extent that MTBD values fall within the range of the normal.

In the rabbit surgical model for CDH, ipsilateral lungs which are only fixed by immersion can therefore be safely used for morphometric purposes, as long as they have not been ventilated. Using a panel of morphometric indices still allows disclosure of structural alterations. The contralateral lung can be used for other studies, such as molecular determinations, which are less dependent on the degree of lung collapse or expansion. However one must remain cautious as the severity of disease seems to be more on the side of the lesion, and therefore the alteration in the lung on the other side may be less .

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