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Morphologic changes and methodological issues in the rabbit experimental model for diaphragmatic hernia

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Summary. Fetal lung development may be impaired by some congenital anomalies or in utero events. Animal models are used to understand the pathophysiology of these diseases and explore therapeutic strategies. Our group has an interest in the prenatal management of congenital diaphragmatic hernia (CDH). Isolated CDH remains associated with a 30% mortality because of lung hypoplasia and pulmonary hypertension.

On day 23 of gestation (pseudoglandular stage) CDH was created in both ovarian-end fetuses (n=28) in 14 time-mated pregnant white rabbits (hybrid of Dendermonde and New-Zealand White). At term (day 30) all survived operated fetuses and size-matched controls were harvested. Fetuses/lungs were assigned randomly to formalin fixation either under pressure of 25 cm H_2O (CDH_{25} n=5; CTR_{25} n=5) or without (0 cm H_2O (CDH_0 n=7; CTR_0 n=7). Fetuses and lungs were first weighed, and then the lungs were processed for morphometry. Pulmonary development was evaluated by lung-to-body weight ratio (LBWR) and airway and vascular morphometry.

Surgical induction of CDH does reduce the LBWR to hypoplastic levels. The contralateral lung weight is 81% of what is expected, whereas the ipsilateral lung is only 46% of the normal. This was accompagnied by a loss of conducting airway generations, precisely, terminal bronchioles (TB), which were surrounded by less alveoli. The ipsilateral CDH lung demonstrated a thickened media in the peripheral arteries as well. As a result, in the severely hypoplastic ipsilateral lung, an airway fixation pressure of 25 H_2O has no significant effect on the morphometric indices. The contralateral lung has a normal amount of alveoli around a single TB, which also behave like alveoli of the normal lung, i.e. expand under pressure fixation.

The present study on severely hypoplastic lungs that never respirated, shows that in contrast to normal lungs, the morphometric indices are not significantly influenced by a difference in fixation pressure. Increasing fixation pressure seems to expand the lung only when sufficient alveolated parenchyma is present.

Key words: Prenatal, Lung, Morphometry

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Introduction

Fetal lung development is a very complex process that follows a well-orchestrated time schedule with a relatively high similarity throughout different species (Pringle, 1986). Normal lung development can be interrupted acutely by preterm birth, or be impaired by *in utero* events, e.g. by some fetal malformations or the presence of chronic oligohydramnios. Depending on the time point of the in utero insult, structural changes will consist of a reduced number of conducting airways and branching of the vessels, and/or reduction of parenchymal airways and changes in the airway-blood barrier. There might be quantitative as well as qualitative changes in the interstitial tissue, all the above leading to abnormal post-natal lung function. One lung development anomaly our group has been focusing on is congenital diaphragmatic hernia (CDH). CDH occurs in 1:2,500-5,000 births and is thought to arise in the embryologic period. Typically the diaphragmatic defect is unilateral, left sided and located dorsolateral. This allows herniation of viscera into the thorax, where they compete for space with the developing lungs. CDH-

lungs display variable degrees of lung hypoplasia. There is a lower number of generation of airways, markedly less and smaller alveoli, thickened alveolar walls and there is an increased amount of interstitial tissue (Areechon and Reid, 1963; Kitagawa et al., 1971; Askenazi and Perlman, 1979). 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-acinar arterioles (Geggel et al., 1985; Yamataka and Puri, 1997; Chinoy, 2002; Bargy et al., 2006). The lung that is to the side of the lesion is more affected than the contralateral one (Stolar and Dillon, 1990; Bargy et al., 2006). These changes become symptomatic immediately after birth, when neonates suffer from variable degrees of ventilatory insufficiency and pulmonary hypertension, with a mortality that still exceeds 30 % and significant morbidity in survivors (Lotze et al., 1994; Skari et al., 2000; Chinoy, 2003).

Several animal models have been developed to study this condition. In those models the lung development should ideally mimic that of humans, as well as the pathology of interest should be inducible before birth. Three types of models are frequently used in the study of CDH, i.e. a teratogenic model, either mice or rats (Kluth et al., 1990; Rajatapiti et al., 2006), or a surgical model, typically using fetal lambs or rabbits (Ohi et al., 1976; Lipsett et al., 1997; DiFiore et al., 1994; Fauza et al., 1994; Wu et al., 2000). More recently, genetic mice models demonstrating a defect of different genes, such as COUP-TFII, Fog2 and Wt1, have been described (Ackerman et al., 2005; You et al., 2005; Clugston et al., 2006). In such models, impaired lung development shows up in several parameters. Gross anatomical evaluation will show reduced lung weight and volume, either wet or dry (Fauza et al., 1994; DiFiore and Wilson, 1995; Wu et al., 2000). Since the fetal body weight is not, or is marginally affected, the LBWR is reduced (Wu et al., 2000). Microscopic changes are usually quantified by morphometric techniques, which can be applied to the airway, vascular or interstitial fraction of the lung (Wu et al., 2000; Luks et al., 2002; Davey et al., 2006). This is done after fixation of the lung, with or without use of pressure within the airways and/or vessels.

We, as well as others, have experimented with rabbits for a variety of fetal conditions, including CDH (Ohi et al., 1976; Fauza et al., 1994; Wu et al., 2000; Roubliova et al., 2004). Rabbits alveolize prior to birth so that, at term, rabbit lungs are in the terminal air sac stage (Pringle, 1986). In addition, rabbits are relatively inexpensive, non-seasonal in their mating habits, have a short gestational period and a large litter size. The only surgical disadvantage of this model may be the rather small size of the fetuses. For instance, fetal weight at 23 days is around 15 grams and term birth weight is around 35-45 grams. This makes fetal surgery or neonatal chronic experiments more difficult. However, short lasting experiments for documenting mechanical ventilation are also possible in this model (Flemmer et al., 2007).

The present study was designed to answer two questions that are key to successful use of this animal model, as well as interpretation of lung changes. First we aimed to document in detail the nature and regional distribution of morphologic alterations that are caused by surgically induced diaphragmatic hernia, and whether these were different on either side of the lesion. Second, we aimed to measure and understand the effect of different fixation protocols on morphometric indices. Human lungs (or from other large species) are studied following fixation by immersion in, and perfusion of, airways and/or vessels with formalin or another fixative, at a given pressure (e.g. 25 cm H_2O) avoiding local collapse of the lung (Cumming et al., 1971; Singhal et al., 1973; Pache et al., 1993). These precautions make comparison possible to morphometric measurements done by others who were using the same conditions. When one works with animals with relatively small pups at birth, airway cannulation and/or vascular catheterization becomes relatively complex, irreproducible, prone to errors and practically impossible. Therefore, most studies involving mice, rat and even rabbit lungs perform lung morphometry on non-perfused lungs (Doolin et al., 1995; De Paepe et al., 1999; Kuhn et al., 2000; Guarino et al., 2002). Other authors however have reported fixation of small animal lungs under pressure (Kitano et al., 1999; Rodrigues et al., 2002). Neonatal rabbit lungs weigh 0.5 to 1.5 gram in the period of interest and their perfusion is relatively cumbersome (Ohi et al., 1976; Fauza et al., 1994; Wu et al., 2000). Apart from the logistics of size aspects, we typically harvest the lungs prior to the first breath. This requires a relatively high opening pressure prior to perfusion with the fixative. *In utero*, the lung is filled with fluid, under a pulmonary pressure which is during breathing movements equal to, or, in apnoea, exceeding amniotic fluid pressure by 2.72 cm H_2O in physiologic circumstances (Hooper and Harding, 2001). Pressure should therefore exceed that value, and both 10 cm H_2O, 20 as well as 25 cm H_2O have been used (Kitano et al., 1999; Wu et al., 2000; Rodrigues et al., 2002).

Material and methods

Fourteen time-mated pregnant white rabbits (hybrid of Dendermonde and New-Zealand White) were transported to the animal facility a few days prior to first manipulation. 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 experiments were approved by the Ethics Committee for Animal Experimentation (Faculty of Medicine, Katholieke Universiteit Leuven). Fourteen does

underwent feto-maternal surgery under general anesthesia in sterile conditions.

Anaesthesia and surgical procedure

At 23 d of gestation (pseudoglandular stage; term=31 d) left-sided diaphragmatic hernia (DH) was induced, as described in detail elsewhere (Wu et al., 2000). Shortly, on the day of operation animals were premedicated with ketamin 50 mg/kg i.m. (Ketalin®; Apharmo, Arnhem, The Nederlands), promazinum hydrochloridium 5 mg/kg i.m. (Prazine®; Libamedi, Brussels, Belgium), and penicillin G 3000 000 IU i.m. Anaesthesia was maintained with 2-5% halothane in oxygen 1 L/min. Maternal heart rate and oxygen saturation were monitored with a pulse oximeter (Nellcor® N-20P; Nellcor Inc., Haasrode, Belgium).

The animals were placed in the supine position and the abdomen was shaved under continuous vacuum aspiration, disinfected with povidone iodine (Iso-Betadine®; Acta medica, Brussels, Belgium) and draped in a sterile fashion. The pregnant uterus was exposed through a lower midline laparotomy. Uterine interventions were performed with micro-instruments under an operating microscope (Carl Zeiss, Oberkochen, Germany; magnification x5-25). After determining the fetal position by gentle palpation, a 1 cm longitudinal incision was made on the anti-mesometrial side of the uterus. The membranes were fixed to the uterine wall with four 6-0 sutures (Prolene®; Ethicon, Dilbeek, Belgium). The left side of the fetal chest was exposed by gentle manipulation and fixed to the uterine wall with a single 6-0 suture. The diaphragm was exposed through a low left lateral thoracotomy using purpose-designed retractors and the membraneous part was opened with scissors. The thoracotomy was closed in one layer with 6-0 interrupted sutures.

After removing the stay suture, the fetus was gently manipulated back into the uterine cavity and the hysterotomy was closed with a running 6-0 Prolene suture. The uterus was replaced into the abdominal cavity and the abdomen was closed in layers with 3-0 polyglactin (Vicryl®; Ethicon) for the fascia and subcutaneous tissue and intracutaneous 2-0 nylon (Ethilon®; Ethicon) for the skin. Medroxyprogesterone acetate 4.5 mg i.m. (Depo-Provera®; Pharmacia-Upjohn, Puurs, Belgium) was given postoperatively for tocolysis. Preoperative daily care was resumed after the operation. We operated on the two ovarian-end fetuses because they are in general larger and that might decrease the risk for miscarriage, which in our experience is higher for cervical-end fetuses.

At term, the does were first euthanized and surviving fetuses were delivered by caesarean section at least 20 minutes later, to ensure all fetuses were dead and no respiration would take place. For each surviving ovarian-end DH fetus a size-matched control was harvested. In case two DH-fetuses survived within the same litter, the second fetus was processed such that organs could be snap-frozen for later molecular work, which was not part of this study.

In two out of the 14 does there were no surviving DH fetuses, which left 12 DH fetuses and an equal number of controls (CTR). Fetuses were assigned randomly to formalin fixation 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 and organs were weighed using a scale measuring accurately up to 0.001g (HF 2000; A&D Instruments, Haasrode, Belgium). Lungs assigned to the 0 cm H_2O group were first separated from the trachea, individually weighed and then immersed in 6% neutral buffered formalin solution for 24 hours. Lungs assigned to the 25 cm H_2O group were weighed, kept on the trachea, which was first cannulated for formalin perfusion under 25 cm H_2O during 24 hours. The pressure is set by keeping the level of formalin of the perfusion fluid container which is connected to the tracheal cannula, exactly at 25 cm above the fluid surface of the formalin bath, wherein the lungs are suspended once they have expanded. A pump permanently circulates formalin to the perfusion fluid container. The trachea is needed for cannulation and therefore lungs could not be separated from it. This precludes measurement of individual wet lung weights prior to fixation in the 25 cm H_2O group. For clarity, lung vessels were not perfused. Lungs were paraffin embedded and cut into 5 μm sections. As the fetal rabbit lung is rather small, a section through the entire lung was made (Gundersen et al., 1988; Roubliova et al., 2008). 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 (Roubliova et al., 2004).

Main outcome measures

Gross anatomy

Fetal body weight (FBW), fetal liver weight (FLW), left lung weight (LLW), right lung weight (RLW), total fetal lung weight (TLW) were measured in wet conditions. From FBW and lung weights the respective LBWR (Left LBWR, Right LBWR, (total) LBWR) were calculated. In humans a cut off of 0.015 resp. 0.0123 has been previously used to define pulmonary hypoplasia prior to resp. after 28 weeks (Areechon and Reid, 1963).

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 virtually divided in up to 20 random non-overlapping fields for morphometric study.

Airway morphometry

The parameters of interest were measured in the lung parenchyma focusing on the respiratory airways as shown in Figure 1A-C. Two special eyepieces were used, one with a grid, another with a ruler, as shown in Figure 1A and C, respectively (Weibel et al., 2007). We determined a number of morphologic indices, one that scores the parenchymal architecture, and others pointing to the alveoli (Lmw, Lm, Lma).

Mean terminal bronchiolar density (MTBD). 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 Figure 1A and C. For this parameter a grid of 10x10 squares was used.

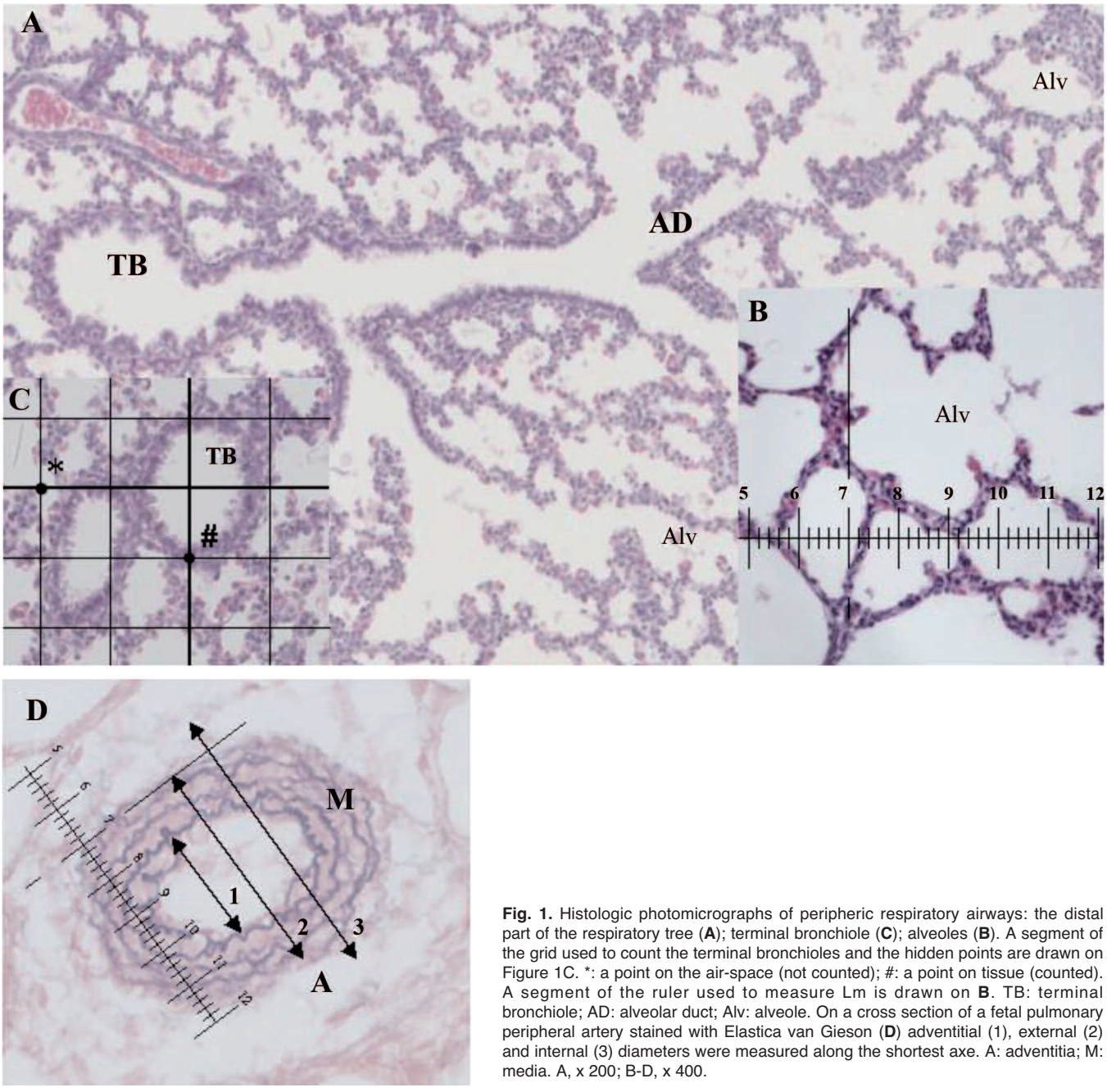

Fig. 1. Histologic photomicrographs of peripheric respiratory airways: the distal part of the respiratory tree (**A**); terminal bronchiole (**C**); alveoles (**B**). A segment of the grid used to count the terminal bronchioles and the hidden points are drawn on Figure 1C. *: a point on the air-space (not counted); #: a point on tissue (counted). A segment of the ruler used to measure Lm is drawn on **B**. TB: terminal bronchiole; AD: alveolar duct; Alv: alveole. On a cross section of a fetal pulmonary peripheral artery stained with Elastica van Gieson (**D**) adventitial (1), external (2) and internal (3) diameters were measured along the shortest axe. A: adventitia; M: media. A, x 200; B-D, x 400.

Only the bronchioles that are in the central 6x6 squares part of the grid were counted. All bronchioles crossing or even only touching the left and lower borders of this 6x6 squares were excluded. All bronchioles crossing or touching the right or upper borders were included. The sum of all bronchioles was first calculated, to obtain the average number per field, which then in turn 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 (Verbeken et al., 1994).

Mean wall transection length (Lmw). This is an index of the thickness of alveolar septa. To calculate this parameter we checked how many of 25 hidden points per field fall either on air-space or on tissue. This is shown in Figure 1A either for hidden points falling on air-space (*) or on tissue (#). From the total number of hidden points (not less than 500 per lung) we calculated the percentage points falling on tissue, which is used in the equation: Lmw = (Lm x % of tissue) / 100 (μm) (Dolnikoff et al., 1995).

Mean linear intercept (Lm), and mean linear intercept of parenchymal airspace (Lma). Lm is an index directly related but not equal to alveolar size. The number of cross-sections of an alveolar wall with half of the ruler (from point "7" till point "14") was counted, as shown in Figure 1C. Each alveolar wall was counted as two crossings. This was counted first in the horizontal direction, then in the vertical direction, turning the eyepiece over 90°. Two numbers per field are obtained this way, their average calculated and used in the equation: Lm = (0.57/average number of intercepts) x 1000 (μm). The constant coëfficient 0.57 has been calculated from the length of the ruler and the coefficient of shrinkage = 0.612. Lma is an index of size of the airspaces (Verbeken et al., 1992). To calculate this parameter we used the equation: Lma=Lm-Lmw.

Vascular morphometry

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 (Kay, 1983). These are believed to be the resistance arteries (Shehata et al., 1999). Their adventitial diameter (AD, μm), external diameter (ED, μm) and internal diameter (ID, μm) were measured along the shortest axis of the vessel (Figure 1D) (Luks et al., 2000; Shehata et al., 2000). 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) / ED x 100. These proportional parameters actually nullify the effects of vasodilatation, vasoconstriction and tissue shrinkage (Shehata et al., 2000).

Statistical analysis

Morphometric measurements were made 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 groups were determined by ANOVA (Tukey's test). We used GraphPadPrism version 4.0 (GraphPadPrism Software, San Diego California USA). P values less than 0.05 were considered as statistically significant.

Results

Gross anatomy, 0 cm H_2O

In the group of lungs fixed at 25 cm H_2O we needed the trachea for cannulation and maintenance of fixation pressure. For this reason, accurate weighing of wet left and right lungs prior to fixation was only possible in the 0 cm H_2O group (the gross anatomic findings between the two fixation pressure groups were comparable). These results are displayed in Figure 2. In the normal fetuses, the mean wet left lung weight was 70 % of the right one (left: 0.37±0.018 vs right: 0.53±0.026; p=0.0001). For CDH lungs, the difference between left and right was more pronounced, the left lung weighing 55% of the right one (left: 0.17±0.01 vs right: 0.31±0.018; p=0.0001) (Fig. 2a, b).

The same trend was observed for Lung-to-Body Weight Ratio (LBWR). In the normal fetuses, LLBWR was 70% of the RLBWR (left: 0.007±0.0007 vs. right: 0.01±0.001; p=0.01) whereas for CDH lungs the LLBWR was 53% of the RLBWR (left: 0.0037±0.0002 vs. right: 0.007±0.0004; p=0.0001) (Figure 2d,e; Table 1). In terms of having caused pulmonary hypoplasia by inducing a diaphragmatic defect, we compared LBWRs of fetuses with CDH to lungs of normal controls. LLBWR of the CDH lungs was 53% of that of normal (left CDH: 0.004±0.0002 vs. left CTR: 0.007±0.0007, p=0.0001), whereas RLBWR of the CDH lungs was 70% of that of normal (right CDH: 0.007±0.0004 vs. right CTR: 0.01±0.001; p=0.0001) (Figure 2d,e). These values are compatible with lung hypoplasia on both sides, although the ipsilateral lung is more hypoplastic than its contralateral counterpart (Areechon and Reid, 1963).

Airway and vascular morphometry

Morphometric indices were first studied at 25 cm H_2O (Table 1). We focused on the left normal lungs, because in left-sided CDH they are most affected. We compared morphometric measures of left control lungs to both the ipsi- and contralateral CDH lungs. Measurements in ipsilateral and contralateral CDH lungs were comparable, with the exception of MTBD scores. MTBD in the ipsilateral lung was 70% higher than in the contralateral one (p=0.01). When compared to normal littermates, alveolar size (Lm) and wall thickness (Lmw) were not significantly different. Overall, the ipsilateral

Table 1. Key outcome measures of ipsi- and contralateral lungs in fetuses with surgically induced diaphragmatic hernia at 23 days, assessed at term. Morphometry was done after pressure fixation (25 cm H_2O for 24 hours). Reference values are those of the left lungs of normal littermates (no significant differences left to right were present).

Outcome measures	normal fetuses	fetuses with left-sided induction of diaphragmatic hernia		P-values			Comment and interpretation
	left	Ipsilateral	Contralateral	ipsi/ contra	normal left/ ipsi	normal left/ contra	
Lm (mm)	82.0±6.2	79.1±6.0	84.2±4.0	0.54	0.75	0.76	Normal alveolar size, both sides
MTBD (br/mm^2)	2.4±0.2	4.4±0.5	2.7±0.3	0.02	0.003	0.11	Desalveolarisation left in addition to lesser generations (bilateral)
Lmw (mm)	26.4±1.7	39.5±9.5	39.4±7.5	0.21	0.58	0.19	Symmetrical increase in alveolar wall thickness
Lma (mm)	55.6±5.6	39.6±4.4	44.8±4.6	0.45	0.054	0.17	
%MT	29.9±0.8	39.6±0.7	32.5±1.0	0.001	0.0001	0.1	ipsilateral wall thickening
Wet lung weight (gr)	0.37±0.02	0.17±0.01	0.31±0.03	0.0001	0.0001	0.02	
Single LBWR (x10^{-3})	7.4±0.3	3.7±0.2	7.0±0.4	0.0001	0.0001	0.59	
Total LBWR (x10^{-3})	18.0±1.8	11.0±0.7		x10 exp -6			Ipsilateral parenchymal changes adding to bilateral missing generations of airways

All results are presented as mean±SEM. Lm: mean linear intercept; MTBD: mean terminal bronchiolar density; Lmw: mean wall transection length; Lma: mean linear intercept of the parenchymal airspaces; %MT: proportionate medial thickness; differences between groups were considered as significant at p<0.05

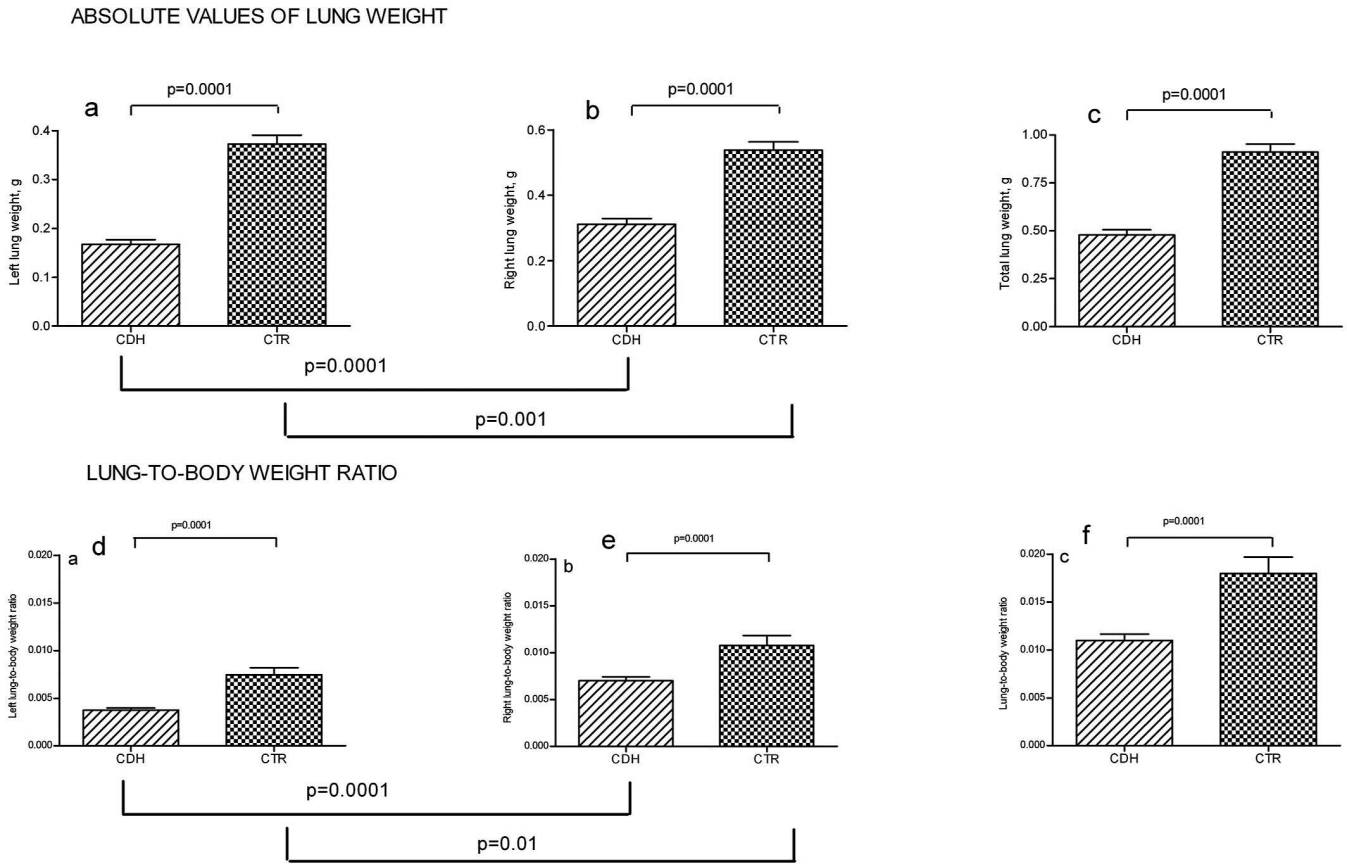

Fig. 2. Graphical display of gross anatomical findings: lung weights and Lung-to-Body Weight Ratios in CDH and CTR fetuses (0 cmH_2O; * p<0.05).

lung has twice the density of terminal bronchioles per mm^2 parenchymal tissue than that in normal littermates (ipsi: 4.4±0.5 vs. left CTR: 2.4±0.2; p=0.003). In other words, in the most severely affected ipsilateral CDH lung there are significantly less alveoli with significantly smaller alveolar spaces reflected by the mean linear intercept of the parenchymal airspaces (Lma) than in the left CTR lungs (ipsi: 39.6±4.4 vs. left CTR: 55.6±5.6; p=0.054). In contrast, right or contralateral CDH lungs had MTBD scores comparable to those of normal lungs. As to vascular changes, the medial thickness of peripheral pulmonary arteries was significantly larger in the ipsilateral CDH lungs than those of controls (ipsi: 39.6±0.7 vs. left CTR: 29.9±0.8 (p=0.0001) and those in the contralateral lungs (32.5±0.1; p=0.001).

Further, we compared the same key outcome measures in lungs fixed under different pressures. We first looked to the left normal lungs; fixation without pressure did not cause a significant decrease of Lm of normal left lungs, but resulted in a significantly higher MTBD (p=0.02) as well as Lmw (p=0.01), as shown in Table 2. In left CDH lungs, the morphometric indices were comparable between pressure and immersion fixation.

Looking at the "behavior" of the most important morphometric parameters when lungs are fixed under different pressures (Table 2), one can see that the trends in differences (CDH/CTR) for most if not all critical morphometric parameters are maintained (Fig. 3). Under both fixation protocols the medial thickness in CDH lungs was increased (Fig. 3g). Under pressure, ipsilateral DH lungs had twice as high a MTBD count when compared to normal. The difference in MTBD between CDH and normal lungs became smaller when omitting

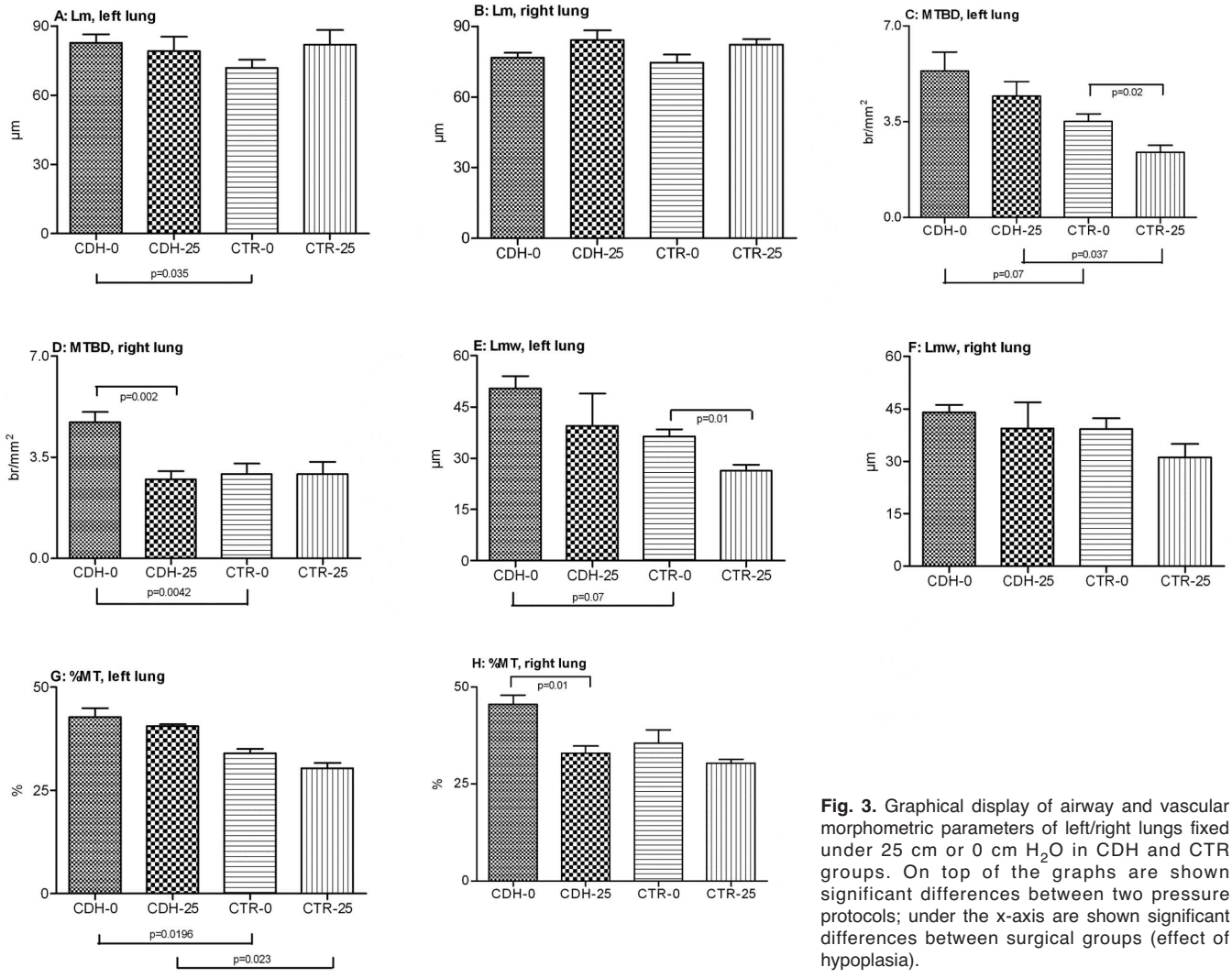

Fig. 3. Graphical display of airway and vascular morphometric parameters of left/right lungs fixed under 25 cm or 0 cm H_2O in CDH and CTR groups. On top of the graphs are shown significant differences between two pressure protocols; under the x-axis are shown significant differences between surgical groups (effect of hypoplasia).

fixation (no longer significant) (Fig. 3c).

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 cm H_2O fixation pressure (Fig. 3a,e). In right CDH lungs, which are apparently morphologically within normal range under a pressure protocol, immersion fixation now revealed a significant increase in MTBD (Fig. 3d). The Lm and Lmw were not significantly different under both fixation protocols (Fig.

Table 2. Key outcome measures in term lungs of normal fetuses or with diaphragmatic hernia, using 25 cm resp. 0 cm H_2O pressure at fixation for 24 hours.

	Left lung Normal fetus			Right, contralateral lung fetus with diaphragmatic hernia			Left, ipsilateral lung Fetus with diaphragmatic hernia		
Outcome measure	25 cm H_2O	p-value	0 cm H_2O	25 cm H_2O	p-value	0 cm H_2O	25 cm H_2O	p-value	0 cm H_2O
Lm	82.0±6.0	NS	72.0±4.0	84.2±4.0	NS	76.7±2.0	79.1±6.0	NS	83.0±3.5
Lma	55.6±5.6	0.01	35.5±4.1	44.8±4.6	0.03	32.6±1.2	39.6±4.4	NS	32.4±4.4
MTBD	2.4±0.2	0.02	3.5±0.3	2.7±0.3	0.002	4.7±0.4	4.4±0.5	NS	5.3±0.7
% decrease in MTBD without pressure (%)		120 %			180 %			130 %	
Lmw	26.4±1.7	0.01	36.4±2.1	39.4±7.5	NS	44.0±2.1	39.5±9.5	NS	50.4±3.7
%MT	29.9±0.8	NS	33.7±0.6	32.5±1.0	0.01	44.6±1.1	39.6±0.7	NS	42.0±1.1

All results are presented as mean±SEM. Lm: mean linear intercept; MTBD: mean terminal bronchiolar density; Lmw: mean wall transection length; %MT: proportionate medial thickness; differences between groups were considered as significant at p<0.05

Table 3. Expected and observed changes of lung volume and morphometric indices according to possible scenarios inducing lung hypoplasia.

EXPECTED		Lung volume	Lm	MTBD	Lma	Lmw	%MT
1) Miniature lung		↓	↓	=	↓	↓	↓
2a) Alveolar changes only with a normal airway tree							
Alv.size	N Alv/TB						
NL	NL	=	=	=			
NL	↑	↑	=	↓		np	
NL	↓	↓	=	↑			
↑	NL	↑	↑	↓			
↑	↑	↑	↑	↓		np	
↑	↓	np	↑	np			
↓	NL	↓	↓	↑			
↓	↑	np	↓	np		np	
↓	↓	↓	↓	↑			
2b) Alveolar changes associated with a loss of airway generations							
Alv.size	N Alv/TB						
NL	NL	↓	=	=			
NL	↑	np	=	↓		np	
NL	↓	np	=	↑			
↑	NL	np	↑	↓			
↑	↑	np	↑	↓		np	
↑	↓	np	↑	np			
↓	NL	np	↓	↑			
↓	↑	np	↓	np		np	
↓	↓	np	↓	↑			
3)Lobar, (sub)segmental amputations		↓	Gross changes in lung shape				
OBSERVED							
Contralateral lung		↓	=	=	=	=	=
Ipsilateral lung		↓	=	↑	(↓)	=	↑

NL: normal lung; np: not predictable; N Alv/TB: number of alveoli per tracheal bronchiole; "=": unchanged; ↑↓: increase/decrease.

3b,f).

Discussion

Surgical induction of diaphragmatic hernia does not only decrease the LBWR, but lowers it to hypoplastic levels. The contralateral lung weight is 81% of what is expected, whereas the ipsilateral lung is only 46% of the normal. This is in concordance with earlier experimental studies (Fauza et al., 1994; Doolin et al., 1995). The purpose of the present study was to further detail the anatomical changes experimental diaphragmatic hernia induces. To understand these, one must conceptualize the lung as a dichotomous bifurcating system of conducting airways (accompanied by their pulmonary arteries) ending each in a terminal bronchiole (TB), which in turn proceeds into a number of further dichotomous branching respiratory bronchioles and alveolar ducts bearing alveoli in their walls that belong to the gas exchanging airspaces (Verbeken et al., 1996).

For practical purposes and the understanding of the morphologic changes, we define the functional lung unit, or acinus, as that part of the alveolar parenchyma, which is supplied by one terminal bronchiole. For this study the architecture of the acinus is sufficiently quantified by two parameters: (1) Lm, which is a reflection of airspace or alveolar size; (2) MTBD: this value depends on a specific interaction of the number and size of the alveoli that are supplied by that TB.

When the anatomy of the lung is schematically described as above, hypoplasia can theoretically result from each of the following abnormal configurations of either the airway tree, the alveolar size or the number of respiratory bronchioles and alveoli per TB. The theoretical scenarios, and the respectively expected changes in Lm and MTBD are displayed in Table 3. At this point, Lmw (the alveolar wall), Lma and %MT are not considered necessarily to be related to the changes hypothesized below leading to hypoplasia. Hence, the latter indices cannot be predicted in view of one or another scenario, inducing hypoplasia.

1)Theoretically, the smaller, hypoplastic lung can be nothing more than a miniature version of the normal lung. In this scenario, the number of airways and the alveoli remain constant, but all of them are smaller than what is found in a normal lung.

2)Smaller lungs can also be the consequence of loss of one or more generations of conducting airways. When one peripheric generation is lost in a dichotomous branching system, the number of TBs more distally is halved. The effect of loss of generations on Lm and MTBD cannot be simply predicted. The alveolar size (hence the Lm value) and their number per TB (hence the MTBD value) may theoretically vary, even change in opposite directions. In other words, the decrease in lung dimensions will be the net effect of changes in Lm, MTBD and the number of TB present in the lung.

When the number of TBs is normal, the lung can only become smaller by a decrease in alveolar size. When there are less TB, and if the alveoli would increase in size, the net result on MTBD is dependent on the balance between the number of TB and the change in alveolar size.

3)A last scenario might be that the conducting airways and alveoli are normal in number and size, but that one or several branches of the conducting airways are not formed (or were lost or amputated at an early phase). This results in lesser respiratory units, but each of them morphologically identical to what is found in a normal lung. This means that in that case, Lm and MTBD would be normal. Very proximal amputations along the airway tree would result in gross anatomical changes or even abnormal shapes.

Thinking along the lines of the above scenarios, we try to position our experimental observations obtained in CDH fetuses. These point to conservation of Lm at normal values in both the contralateral and ipsilateral lung. MTBD follows a different pattern. MTBD remains normal in the contralateral lung. Given that there was a 25% weight loss of that lung as compared to normal lungs, this inherently means that there are less TB. These are surrounded by a normal amount of alveoli with roughly a normal size. However, in the ipsilateral lung MTBD increased by (4.40/2.65)= 66%. Also, there was significant weight loss of that lung. This means that there was not only a loss of conducting airway generations, but also that each TB is surrounded by less alveoli. These were apparently of a normal individual size (Lm is normal). In summary, the morphology of the contralateral lung is characterized by changes suggestive of loss of conducting airway generations, whereas the changes in the ipsilateral lung suggest additional loss of alveolization.

Changes in Lmw and %MT describe the local architecture of individual alveoles and, respectively, the peripheral branches of the pulmonary arterial tree along the terminal bronchioles. Both the contralateral and ipsilateral lung display an identical increase of Lmw of over 35% as compared to normal lungs. Though this appears an impressive number, this was not significant. Apparently, the extent of thickening of the alveolar walls is an independent process of that of more proximal airway changes (it is dangerous to assume that hypoplasia is not affecting the alveoli). In the severely affected ipsilateral lung, but not in the contralateral lung, the net alveolar dimensions (Lma) decreased by 30% (this nearly reached significance). As to vascular changes, the peripheric pulmonary arteries of the ipsilateral, yet not the contralateral, lung displayed a thicker media. Changes in Lmw and %MT most probably relate more to later changes during the maturation process, rather than being an early event affecting the conducting and respiratory airways.

When fixing normal lungs under a distension pressure of 25 cm H_2O, the alveolar size (Lma) is significantly increased (57%), whereas the alveolar wall (Lmw) thickness drops by 38%. This coincides with a decrease of MTBD by 46%. The net consequence is that

Lm (=Lma+Lmw) does not change significantly. The %MT is not influenced by the distension pressure. In our work we did not study in detail the morphologic basis of lung compliance changes, neither measured lung compliance. The relationship between pressure and compliance is very complex, but certainly present. Others as well as we have shown in other experiments that abnormally developed lungs in DH have a reduced compliance, and this coincides with thickened alveolar walls, increased interstitial tissue, as well as increased vascular muscularization (Rodrigues et al., 1998; Tannuri et al., 1998; Wu et al., 2000; Roubliova et al., 2004a; Flemmer 2007). Conversely, the administration of BM induces structural changes, such as thinning of the alveolar and vessel walls, and others have shown that this coincides with increased compliance (Tannuri et al., 1998; Rodrigues et al., 1998; Roubliova et al., 2009).

In the severely hypoplastic ipsilateral lung, an airway pressure of 25 H_2O has no significant effect on the morphometric indices. Lma increases only by 22% and Lmw decreases by 28%. Correspondingly, Lm decreases only by 5% due to the pressure. Accordingly, MTBD is not significantly influenced either. Apparently, alveolar septa are not markedly stretched, at least in ipsilateral lungs. The less affected contralateral lung reacts somewhere in between the above sketched two scenarios. These changes include a 37% increase in Lma, without a significant decrease of Lmw (12%) under pressure. As a consequence, the MTBD decreases. In other words, the contralateral lung has a normal amount of alveoli around a single TB, which also behave like alveoli of the normal lung, i.e. expand under pressure fixation.

Of clinical relevance is that the number of alveoli is dramatically reduced in the hypoplastic left lung. It can be roughly calculated from their weights that ipsilateral lungs have only half of the number alveoli of the contralateral lung. Apparently, the dramatic reduction of the alveolar parenchyma supplied per TB induces a higher resistance of individual airspaces to the distending pressure.

Conclusion

The present study on severely hypoplastic lungs that never respirated, shows that in contrast to normal lungs, the morphometric indices are not significantly influenced by a difference in fixation pressure. Increasing fixation pressure seems to expand the lung only when sufficient alveolated parenchyma is present. This is to a certain degree so in the less affected contralateral lung. Therefore we would suggest to use for morphologic studies in experimental diaphragmatic hernia, the ipsilateral, most hypoplastic lung. Whether pressure is used or not is less relevant for morphometric purposes. In other words, morphometry of the hypoplastic lung can be safely done as well at zero transpulmonary pressure.

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